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# Development of a diagnostic ELISA for the hepatitis B x-protein using monoclonal antibodies

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# DEVELOPMENT OF A DIAGNOSTIC ELISA FOR THE HEPATITIS B X-PROTEIN USING MONOCLONAL ANTIBODIES

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of

Master of Science in Medicine

Johannesburg, 2010

# DECLARATION

I, Bongiwe Mashinini, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……..

…….day of……..2010

# DEDICATION

This dissertation is dedicated to my parents, Mr & Mrs F.E. Mashinini, my grandparents, Mr & Mrs S. Zwane and my siblings Wezile, Simphiwe, Themba and Ntokozo Mashinini.

Thank you for believing in me and encouraging me every step of the way.

Thank you for always reminding me that God's plans for our lives are much greater than the plans that we have for ourselves.

Thank you for all the sacrifices that you have made in order for me to be where I am today. God has great plans for our family and may He keep you as we go through this journey called life, together.

The sky is the limit.

# ABSTRACT

The hepatitis B virus remains a major public health problem even after decades of its discovery. Horizontal transmission during early childhood is the predominant mode of transmission in highly endemic regions such as sub-Saharan Africa. Infection exhibits a wide spectrum of clinical manifestations, from an asymptomatic stage to severe liver disease which may result in hepatocellular carcinoma (HCC).

The HBV X protein (HBx) has been implicated in carcinogenesis, which often has a poor prognosis, consequently the use of highly specific monoclonal antibodies (mAbs) directed against HBx in an enzyme-linked immunosorbent assay (ELISA) could lead to early identification of HBV carriers at risk of developing liver cancer.

A variety of mixed hybridoma cell cultures secreting anti-HBx antibodies were cloned and sub-cloned by "limiting dilution". Clonal supernatants were assessed for anti-HBx antibody production by Indirect ELISA and Western/Immunoblotting. Monoclonal antibodies were then characterized according to their relative binding affinity (Indirect ELISA) and relative epitope specificity (Competitive ELISA). One of our monoclonal antibodies was found to bind to the same epitope on HBx as the commercial anti-HBx antibody and with the same high affinity.

In the developed Sandwich ELISA, our monoclonal antibody proved effective as the 'detecting' antibody when the commercial anti-HBx antibody was deployed as the

‘capture’ antibody. This Sandwich ELISA will be further developed in our laboratory with the object of applying it to patient sera.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 6xHis-HBx | 6x histidine-tagged HB x protein |
| AAG | Alpha-1-acid glycoprotein |
| Ab | Antibody(s) |
| AFP | Alpha-fetoprotein |
| ALT | Alanine aminotransferase |
| anti-HBc | Antibody to the HBV core antigen |
| anti-HBe | Antibody to the HBV e antigen |
| anti-HBs | Antibody to the HBV surface antigen |
| AS | Ammonium sulphate |
| AST | Aspartate aminotransferase |
| BSA | Bovine serum albumin |
| C | Core |
| CA | Commercial anti-HBx antibody |
| DCP | Desgamma carboxyprothrombin |
| DHBV | Duck hepatitis B virus |
| DNA | Deoxyribonucleic acid |
| $E_1$ | First elution |
| $E_2$ | Second elution |
| ECM | Extracellular matrix |
| ELISA | Enzyme-linked immunosorbent assay(s) |
| FCS | Foetal calf serum |

| | |
|---|---|
| FPLC | Fast Protein Liquid Chromatography |
| GP73 | Golgi protein 73 |
| GSHV | Ground squirrel hepatitis virus |
| HAT | Hypoxanthine-aminopterin-thymidine |
| HBcAg | Hepatitis B core antigen |
| HBeAg | Hepatitis B e antigen |
| HBsAg | Hepatitis B surface antigen |
| HBV | Hepatitis B virus |
| HBxAg/HBx | Hepatitis B x antigen |
| HDV | Hepatitis delta virus |
| HCC | Hepatocellular carcinoma |
| HRP | Horseradish peroxidase |
| HT | Hypoxanthine-thymidine |
| hTERT | Human telomerase reverse transcriptase |
| IFN | Interferon(s) |
| Ig | Immunoglobulin(s) |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IPTG | Isopropyl-β-D-thiogalactoside |
| kDa | KiloDalton(s) |
| LB | Luria-Bertani |
| mAb | Monoclonal antibody |
| MAPK | Mitogen-activated protein kinase |
| MW | Molecular weight |

| | |
|---|---|
| NER | Nucleotide excision repair |
| OPI | Oxaloacetate, pyruvate and insulin |
| ORF | Open reading frame |
| P | Polymerase |
| preC | Precore |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| PKC | Protein kinase C |
| PVDF | Polyvinylidene fluoride |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| RPMI | Rose Park Memorial Institute |
| SAS | Saturated ammonium sulphate |
| s.c. | Subcutaneously |
| SCID | Severe combined immunodeficiency disease |
| SDS-PAGE | Sodium dodecyl sulphate Polyacrylamide gel electrophoresis |
| SREBF 1 | Sterol regulatory element binding factor 1 |
| WHV | Woodchuck hepatitis virus |

# Chapter 1: INTRODUCTION

The hepatitis B virus (HBV) is classified in the *Hepadnaviridae* family. Infection-susceptible mammals include humans and chimpanzees, although mammals such as ground squirrels, woodchucks as well as a range of bird species are prone to infection by hepadnaviruses similar to HBV, namely: ground squirrel hepatitis virus (GSHV), woodchuck hepatitis virus (WHV) and duck hepatitis B virus (DHBV), respectively (Miller & Robinson, 1986; MacDonald, *et al*., 2000; Dandri *et al*., 2005). Interestingly, HBV has been found to have a common evolutionary origin with retroviruses, sharing the property of encoding a reverse transcriptase and replicating via an RNA-to-DNA step (Miller & Robinson, 1986; Strauss & Strauss, 2002). While safe and effective vaccines have been available since 1981, more than 350 million adults are chronically infected with HBV, of which approximately 90 % recover and develop protective antibodies against future infections (Wieland & Chisari, 2005; Wasley *et al*., 2008; http://www.hepb.org/hepb/statistics.htm).

HBV is highly endemic in Asia and Africa and it is estimated that one million people die each year from HBV and its complications (http://www.hepb.org/hepb/statistics.htm). Infection is further compounded, in Africa, by the contamination of staple crops through exposure to aflatoxin (Hainaut & Boyle, 2008). The virus is transmitted by the exchange of infected body fluids e.g. blood, vaginal secretions, semen and a case of transmission through saliva has been reported (van der Eijk *et al*., 2004; Hui *et al*., 2005; http://ww.cdc.gov/hepatitis/HBV/HBVfaq.htm#b1). Healthcare workers and individuals

living in close contact with an infected person are at a high risk of being infected (Wasley *et al*., 2008). All known hepadnaviruses are hepatotropic i.e. infect liver cells and can all cause hepatitis in their host (Strauss & Strauss, 2002; Wieland & Chisari, 2005). Once infected, initial symptoms may include fatigue, fever and mild nausea (http://www.cdc.gov/hepatitis/B/bFAQ.htm#symptoms). As the disease develops, jaundice and liver enlargement may ensue. While most patients recover and become immune to the virus, it is (clinically) estimated that 5 – 10 % of infected adults will develop chronic hepatitis and will be at high risk of developing hepatocellular carcinoma (Hwang *et al*., 2003).

## 1.1 DISCOVERY OF HBV

The path to the discovery of HBV began in 1965 when Dr Baruch Blumberg and a team of researchers observed a precipitin reaction between the sera of an Australian aborigine and a transfused haemophiliac from the United States of America (Blumberg, 2003). The lipoprotein responsible for the reaction, also known as the 'Australia antigen', was found to be present at a higher frequency in sera of hepatitis patients than in control subjects (McLachlan, 1991). This led the researchers to propose that this antigen may be involved in the occurrence of viral hepatitis (Prince, 1968). Through electron microscopy several particles were observed and, based on morphology, it was suggested a 42 nm Dane particle represented the infectious agent (Dane *et al*., 1970; Cossart, 1971). Further analysis of the structures allowed for biochemical and physical characterization of the components which then led to the discovery of the other antigens (McLachlan, 1991).

Dr. Blumberg was awarded the Nobel Prize in Medicine for his discovery of the hepatitis B virus in 1976 (http://www.hepb.org/about/blumberg.htm).

## 1.2 GENOMIC STRUCTURE OF HBV

The viral genome is a partially double-stranded circular molecule of approximately 3200 base pairs. The small genome comprises four overlapping open reading frames (ORFs) encoding the surface, core, polymerase and X proteins (Araki *et al*., 1989), which are essential for the viral life cycle (Blum *et al*., 1992) *(Figure 1.1).*

Translation of the S gene results in three surface proteins (small, middle and large) which only differ at the N termini (Imamura *et al*., 1987; Dalgleish & Weiss, 1999). The pre-core/core ORF contains two start codons coding for two overlapping proteins, the e (HBeAg) and core (HBcAg) antigens. The core protein is produced when translation begins at the second AUG start codon. Translation from the first start codon results in the formation of the pre-core protein which then undergoes post translational modification to yield the HBeAg (Roossinck & Siddiqui, 1987). The HBeAg has not been well characterized chemically but has been shown to play an important role in the interaction of the virus with the immune system (Alter *et al*., 1976; Neurath & Strick, 1977; Baumert *et al*., 2007). The DNA polymerase is encoded for by the P ORF and allows replication by reverse transcription (Anzola, 2004).

However, all known hepadnaviruses encode the X protein (HBx) (Reifenberg *et al*., 2002).

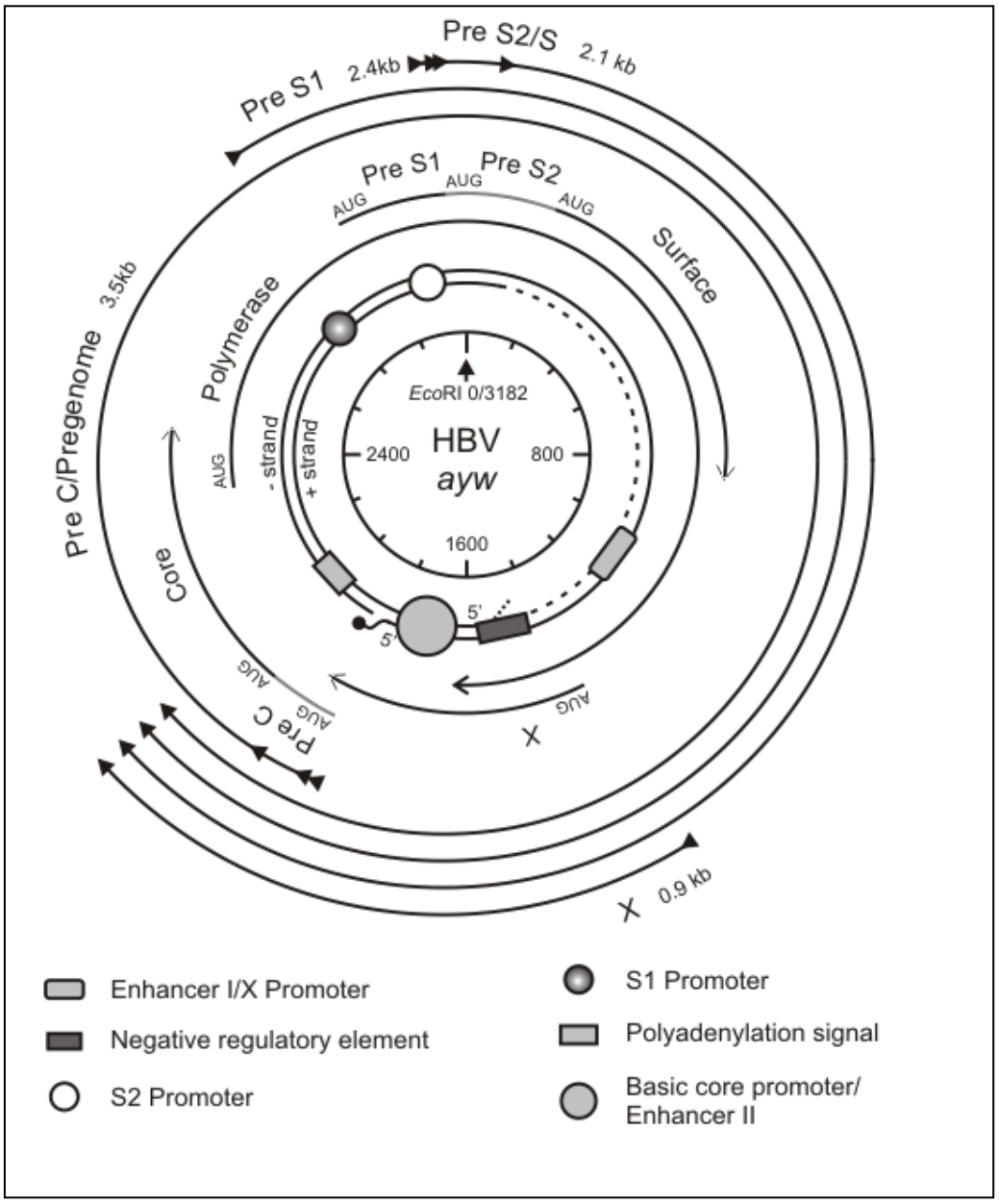



**Figure 1.1: A representation of the gene organization of the HBV genome.** HBV DNA was obtained from a genotype A virus (Arbuthnot *et al*., 2007). The HBV genome is a double-stranded circular DNA containing four open reading frames coding for polymerase (P), surface antigens (preS1, preS2 and S), precore (preC), core (C) and X. *cis*-elements that regulate HBV transcription are indicated by the circular and rectangular symbols.

Although its function has not been completely elucidated, this protein has been shown to be involved in carcinogenesis as it induces liver cancer in transgenic mice (Will, 1991; Blum *et al*., 1992). Of the HBV proteins, HBx is one of the most intensely studied.

## 1.3 HBV X PROTEIN

The hepatitis B virus *x* gene is the smallest, with a length of 465 nucleotides and is conserved among mammalian hepadnaviruses (Murakami, 1999). The structural gene first identified by Galibert *et al*. has the potential to produce a protein of between 145 – 154 amino acids (Galibert *et al*., 1982; Tiollas *et al*., 1985; Feitelson, 1986) that has been shown to require an enhancer element for maximal activity (Treinin & Laub, 1987; Dalgleish & Weiss, 1999). It has been generally reported that the *x* gene produces a ~ 17 kDa protein, but research at The Fox Chase Cancer Center showed that some sera from HBV-infected patients contained another polypeptide species of ~ 13 kDa in size (Feitelson & Clayton, 1990). Despite the various sizes of HBx reported, the ORF-encoded protein is ~ 17 kDa. The role of the HBx protein in the viral life cycle and in the pathogenesis of HBV infection has been studied extensively. The strict conservation of the X ORF strongly suggests that the protein plays an important role in the viral life cycle (Reifenberg *et al*., 2002).

A large portion of the current knowledge of HBV comes from the study of parallel animal hepatitis virus models (Marion *et al*., 1986; Lambert *et al*., 1991; Dandri *et al*., 2005). This is because difficulties, such as maintaining functional infected hepatocytes, arise

when propagating virus in tissue culture, making the system inappropriate for investigating the mechanism of infection (Gripon *et al.,* 1993; Sprinzl *et al.,* 2001). Infecting woodchucks with WHV has proved to be an outstanding model for researching HBV infection in humans (Chen *et al.,* 1993; Gouillat *et al.,* 1997; Tennant *et al.,* 2008). Studies in this model have demonstrated that HBx is required for the establishment of infection *in vivo* (Zoulim *et al.,* 1994; Seeger & Mason, 2000) but is dispensable for viral DNA synthesis in transfected tissue culture cells (Blum *et al.,* 1992; Seeger & Mason, 2000; Reifenberg *et al.,* 2002). HBx has been shown to trans-activate the transcription of a wide range of viral and cellular genes (Colgrove *et al.,* 1989). It has been speculated that the activation of the HBV enhancer by HBx may be required for viral replication (Shaul *et al.,* 1985; Spandau & Lee, 1988). The mechanism by which the activation occurs is unknown, but it is thought the protein may bind to or recruit other proteins to the enhancer, thus activating it (Spandau & Lee, 1988). While being dispensable *in vitro,* HBx supports viral DNA transcription through its functional similarity to nucleotide diphosphate kinases (De-Medina & Shaul, 1994).

Sequence homology among mammalian hepadnaviruses was used to subdivide HBx into regions, and it was found that the middle region is involved in signal transduction (Nijhara *et al.,* 2001). The highly conserved N-terminal region was found to have a negative regulatory function and is dispensable for trans-activation *(See Figure 1.2)* (Misra *et al.,* 2004). The carboxy-terminal plays a crucial role in the trans-activation function of HBx(Reddi *et al.,* 2003). Furthermore, transfecting animals with HBx truncated at the C-terminal causes them to be serologically negative for at least 24 weeks (Chen *et al.,* 1993).

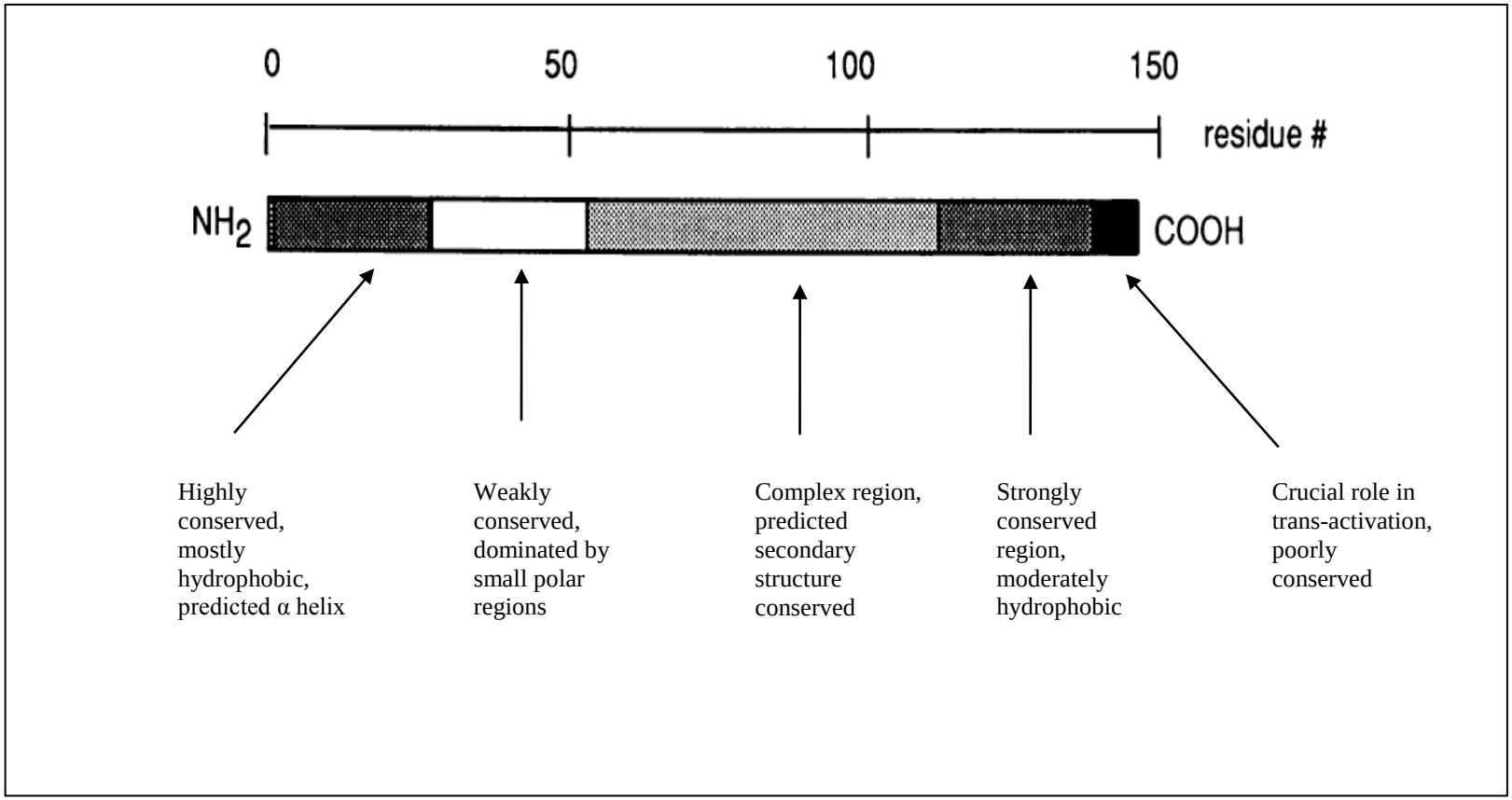



**Figure 1.2: Schematic representation of the HBV X ORF gene product showing the N and C termini** (Adapted from Colgrove *et al*., 1989; McLachlan, 1991). The amino acid positions are represented by the scale above.

HBx is rapidly degraded by a cellular 26S complex through ubiquitination, resulting in low intracellular levels of HBx (Kim *et al.*, 2008). It has also been shown that ubiquitination-independent proteolysis can occur (Hershko & Ciechanover, 1998; Orlowski & Wilk, 2003). *In vivo* HBx functions as both the substrate and inhibitor of the proteasome complex (Hu *et al.*, 1999). Kim *et al.* have shown that the middle and carboxy-terminal domains of HBx play a role in its degradation while the amino-terminal was shown to have little effect on the protein's stability (Kim *et al.*, 2008). In the same study, proteasome inhibitors hindered this degradation but also blocked the trans-activation function of HBx.

### 1.3.1 Localisation

Knowledge of hepatitis B antigens to date has been made possible through the development of sensitive radioimmunoassay and immunofluorescence techniques for their detection in serum and tissue, respectively (Berquist *et al.*, 1975). HBx variants have been found integrated in host chromosomes as well as being expressed in HCC tissues (Chen *et al.*, 2000). Many debates have arisen regarding the sub-cellular localisation of the X protein, but it is generally agreed that the protein predominantly localises in the nucleus (Su *et al.*, 1998; Henkler *et al.*, 2001); however, accumulation in the cytoplasm and association with cytoskeletal components has also been observed (Siddiqui *et al.*, 1987; Zentgraf *et al.*, 1990; Su *et al.*, 1998; Henkler *et al.*, 2001).

Cytoplasmic HBx is found with dispersed and sometimes punctate distribution (McLachlan, 1991). An extensive association of HBx with mitochondria has been

observed, but no association with the endoplasmic reticulum, lysosomes or the plasma membrane (Henkler *et al*., 2001). From the cytoplasm, HBx is transferred to the nucleus through interaction with trafficking proteins (Murakami *et al*., 2001). Interaction of HBx with a mitochondrial anion channel may cause the loss of mitochondrial membrane potential, thus inducing mitochondria-dependent cell death (Rahmani *et al*., 2000; Chami *et al.*, 2003). This was observed at high levels of HBx expression involving clumping and organelle aggregation leading to abnormal mitochondrial distribution (Henkler *et al*., 2001). Expression of HBx may also induce apoptosis which has, likewise, been associated with mitochondrial membrane alterations (Terradillos *et al*., 2002).

### 1.3.2 Pathways disrupted

HBx has been shown to induce HCC through a variety of mechanisms such as transcriptional activation (Murakami *et al*., 2001; Carretero *et al*., 2002), signalling cascades, DNA repair, p53 expression and telomere dynamics.

HBx is implicated in activating cell pathways such as the mitogen-activated protein kinase (MAPK) pathway which plays an important role in tumour promotion (Kekulé *et al*., 1993; reviewed in Arbuthnot *et al*., 2000). The NF-kB pathway may also undergo stimulation by HBx thus conferring resistance of virus-infected cells to anti-Fas killing (Pan *et al*., 2001). In a study by Lucito & Schneider, the X protein was able to achieve maximal activation of NF-kB independently of the protein kinase C (PKC) system (Lucito & Schneider, 1992). Furthermore, HBx disrupts the first step of nucleotide excision repair (NER) thus

preventing the recognition of DNA damage which contributes to carcinogenesis (Becker *et al*., 1998). p53, a tumour suppressor protein, normally causes cell cycle arrest in response to DNA damage (reviewed in Prives & Manfredi, 1993). It has been shown through affinity chromatography that, during infection, HBx directly binds p53 thus inhibiting it, further promoting carcinogenesis (Truant *et al*., 1995; Feitelson, 1998; Murakami *et al*., 2001).

Progressive shortening of telomeres has been shown to occur during hepatocellular carcinoma, compounding the normal telomere length loss occurring in normal somatic cells (Miura *et al*., 1997). This in turn leads to the activation of human telomerase reverse transcriptase (hTERT), the expression and activity of which has been shown to be upregulated by the HBx antigen (HBxAg) (Zhang *et al*., 2005). hTERT adds repeated telomere sequences onto the end of chromosomes, thus stabilizing telomere length in immortal cells (Tian *et al*., 2009). hTERT and HBxAg expression was observed in all cirrhotic tissues and 67.6 % and 76.5 % in tumour samples, respectively (Zhang *et al*., 2005). This relationship may be a useful marker for the early detection of disease progression in liver disease (Miura *et al*., 1997).

### 1.3.3 The X protein and disease progression

Hepatocellular carcinoma is a common tumour worldwide, typically developing in patients with chronic liver disease and cirrhosis (Chen *et al*., 2000; Lian *et al*., 2003). The development of fibrosis is an important factor in disease progression in HBV patients

(Norton *et al.,* 2004). Fibrosis is characterized by an enhanced synthesis of the extracellular matrix (ECM) attributable to the altered expression of its genes. HBx has been identified as a culprit, activating NF-kB, thus trans-activating the fibronectin promoter leading to excessive amounts of ECM (Norton *et al*., 2004).

HCC appears in the liver, arising from the hepatic parenchymal cells (Schafer & Sorrell, 1999). The molecular pathways of HBV-mediated HCC have not been well characterized, but in most HCCs, integration of the HBV DNA into the host genome occurs, thus inducing chromosome instability (Chen *et al.,* 2000). This then leads to insertional mutations which then activate endogenous genes such as those involved in cell proliferation (Pang *et al.,* 2006). Expression of viral proteins, such as HBx, then alters various pathways, leading to carcinogenesis.

HBx, in particular, plays an important role in the development of HBV-related HCC (Koike, 1995). HBx has been previously described as an activator of a variety of pathways such as the lipogenic pathway (Yamashita *et al.,* 2009). This activation is brought about indirectly through the activation of the MAPK pathway which, in turn, causes an upregulation of sterol regulatory element binding factor 1 (SREBF 1). Activation of this pathway has been identified as a key feature in the development of cancer and the poor prognosis of HCC (Yamashita *et al.,* 2009). HCC with elevated levels of SREBF1 was also found to correlate with a high mortality rate.

Research has also been conducted to identify the natural effectors of the HBV X protein. In HBx positive HepG2 cells, two groups identified the upregulated genes to be URG11

and URG4, respectively (Tufan *et al.,* 2002; Lian *et al.,* 2003). Both genes were found to accelerate tumour formation and stimulate hepatocellular growth. URG4 was weakly expressed in uninfected liver but the opposite was found in HBV-infected liver and HCC cells. Upregulation of URG11 also contributed to the production of larger tumours in SCID mice (Tufan *et al.,* 2002; Lian *et al.,* 2003).

Lian *et al.* also studied differentially expressed genes in HBx positive and negative cells (Lian *et al.,* 1999). The human homologue of *sui1* (*hu-sui1),* that encodes a translation initiation factor, was found to be suppressed in HBx positive HepG2 cells. *Hu-sui1* was expressed in non-tumour liver cells, thus appearing to be negatively regulating cell growth in HCCs.

It has been suggested that the ingestion of chemical carcinogens from food is required before HCC develops in the liver (Kasai *et al.,* 1996). One such carcinogen, aflatoxin, is an important environmental contaminant which contributes to the high incidence of HCC in Asia and Africa (Liu *et al.,* 2008). Alcohol consumption and underlying liver disease can also contribute to the increasing mortality rate of HCC patients (Marrero *et al.,* 2005). Usually, HCC has a poor prognosis; therefore, markers are required to determine disease progression, allowing for the treatment of patients before the disease becomes too severe.

In a review conducted by Wright *et al.*, a wide variety of serum markers was identified but were said to lack specificity and sensitivity (Wright *et al.,* 2007). They include, among others, HBx, alpha-fetoprotein (AFP), serum albumin, hepatocyte and insulin growth factors (Stuart *et al.,* 1996; Wright *et al.,* 2007). In several studies conducted in patients

with HBV-associated HCC, staining patterns of the HBx protein were analyzed in tumour and non-tumour liver sections (Wang *et al*., 1991; Pál *et al*., 2001; Zhu *et al*., 2006). HBx was found to be the most prevalent marker and its expression during chronic infection could help in determining progression to HCC. Liver tissue samples from HCC and chronic hepatitis patients were treated with an anti-HBx antibody by Hwang *et al.* in a study conducted in 2003. Reactive antigen was detected in 80% of HCC liver samples and 30% of chronic hepatitis liver samples (Zentgraf *et al*., 1990; Hwang *et al*., 2003). This evidence strongly suggests that HBx may be an important prognostic marker for the development of HCC.

Anti-HBx antibody was also found in significantly higher levels in the sera of HCC patients than in chronically infected patients and healthy individuals (Hwang *et al*., 2003; Zhang *et al*., 2006). The level of this antibody (Ab) has been found to be lower in acutely infected individuals, being only detected in 5 out of 17 patients (Abraham *et al*., 1989). This strongly suggests that both the qualitative and quantitative analyses of HBx are important in prognosis, and that the anti-HBx Ab might be used as a prognostic marker as well.

## 1.4 THE HEPATITIS B VIRUS (HBV) IN AFRICA

The world is divided into regions of high, intermediate and low HBV endemicity. Most countries in Africa have high endemicity, with chronic infection rates of 7 – 26 % (West and sub-Saharan Africa) and 6.5 – 7.5 % (Central and Southern Africa) (André, 2000).

Previously, Asia was also classified as an area of high endemicity, but this has changed due to the introduction of successful vaccination programme. China now remains the only country in this category, with a 7 – 20 % prevalence of HBV surface antigen (André, 2000). Approximately, more than 75 % of the world's HBV chronic carriers are located in Asia (Maynard, 1990).

The second highest proportion of chronic carriers is located in sub-Saharan Africa where financial resources and adequate healthcare systems are very limited (Maynard, 1990). In 1985 it was reported that 230 000 children would die due to long-term complications of HBV infection, out of a total of 19.4 million births in Africa (Maynard, 1990). In the period between 1998 to 2007, 2 077 cases of HBV infection were reported in South Africa, however these numbers do not represent true incidence due to under-reporting, among other reasons (Department of Health – South Africa, 2008).

The main mode of transmission in Asia and Africa is horizontal transmission, where virus is transmitted between children (under 5 years of age) especially in crèches and children homes (Toukan & the Middle East Regional Study Group, 1990). Young HBV carriers have a higher frequency of HBeAg positivity than older carriers, thus suggesting the younger carriers are a likely source of infection (Kiire, 1990; Toukan & the Middle East Regional Study Group, 1990). Living in large families, often sharing eating and cleaning utensils (may be source of infected serum and blood); intrafamilial contact due to cultural habits and a poor socioeconomic status are contributing factors to the prevalence of the HBV carrier state (Toukan *et al*., 1990).

It has been estimated that HBV infection is the cause of acute hepatitis in 40 % of hospitalized patients in Algeria (Nouasria *et al*., 1984). The HBV-related hepatitis delta virus (HDV) has also been implicated in the increased disease burden in Africa and the Middle East. Algeria, among other countries, reported increased levels of HDV to be associated with chronic liver disease (Toukan & the Middle East Regional Study Group, 1990). However, the prevalence rates of these two viruses were not found to parallel each other on a wider global scale (Toukan & the Middle East Regional Study Group, 1990).

The global control and prevention of HBV infection has involved the incorporation of HBV mass immunisation into the Expanded Programme of Immunisation. This is true for countries whose carrier rate exceed 2.5 % but is limited as the country needs to possess the financial means to purchase the vaccine (Maynard, 1990). The mode of transmission, correlating with age, needs to be taken into consideration as the high risk groups need first preference when vaccinating.

Universal immunisation began in 1995 in South Africa, giving infants three doses of vaccine at six, ten and fourteen weeks of age (François *et al*., 2008). This can prove difficult in most countries in Africa as most babies requiring vaccination only get introduced to the healthcare system at approximately three months due to being born at home and at rural areas (Maynard, 1990). This makes the rapid delivery of the first dose of vaccine very difficult.

With all this said on preventative measures, focus is also placed on efficient and effective diagnostic strategies in order to delay disease progression, thus aiding in the reduction of

the number of deaths occurring due to the hepatitis B virus.

The use of antibody technology, specifically monoclonal antibodies, in the detection of HBx would allow for the rapid and specific detection of this antigen in patient sera and liver samples. Early detection of which is crucial in the prognosis of HCC.

## 1.5 MONOCLONAL ANTIBODIES

Monoclonal antibodies are specialized glycoproteins, immunoglobulins (Igs), which are produced by B-lymphocytes *(See Figure 1.3)* (Milstein, 1980; Liddell & Cryer, 1991). These antibodies differ from antibodies in general, in that they are produced from a single clone of B lymphoid cells (tumours of an antibody-producing cell) (Mayer & Walker, 1987). B-lymphocytes are short-lived but can be 'immortalized' by fusion with a myeloma cell line to produce a hybridoma. Antibody-secreting hybridomas were first generated by Köhler & Milstein for which they were awarded the Nobel Prize. The development of monoclonal antibodies in biological research has, ever since, increased exponentially (Köhler & Milstein, 1975, 1976; Liddell & Cryer, 1991).

Previously, hybridomas derived from myelomas produced the Ig of the myeloma parent as well as the antigen-specific antibody of the spleen cell parent (Shulman *et al.*, 1978). Hybridomas making only the desired specific antibodies are obtained by fusing splenocytes with a tumour cell fusion partner that does not produce Ig itself. Cell lines

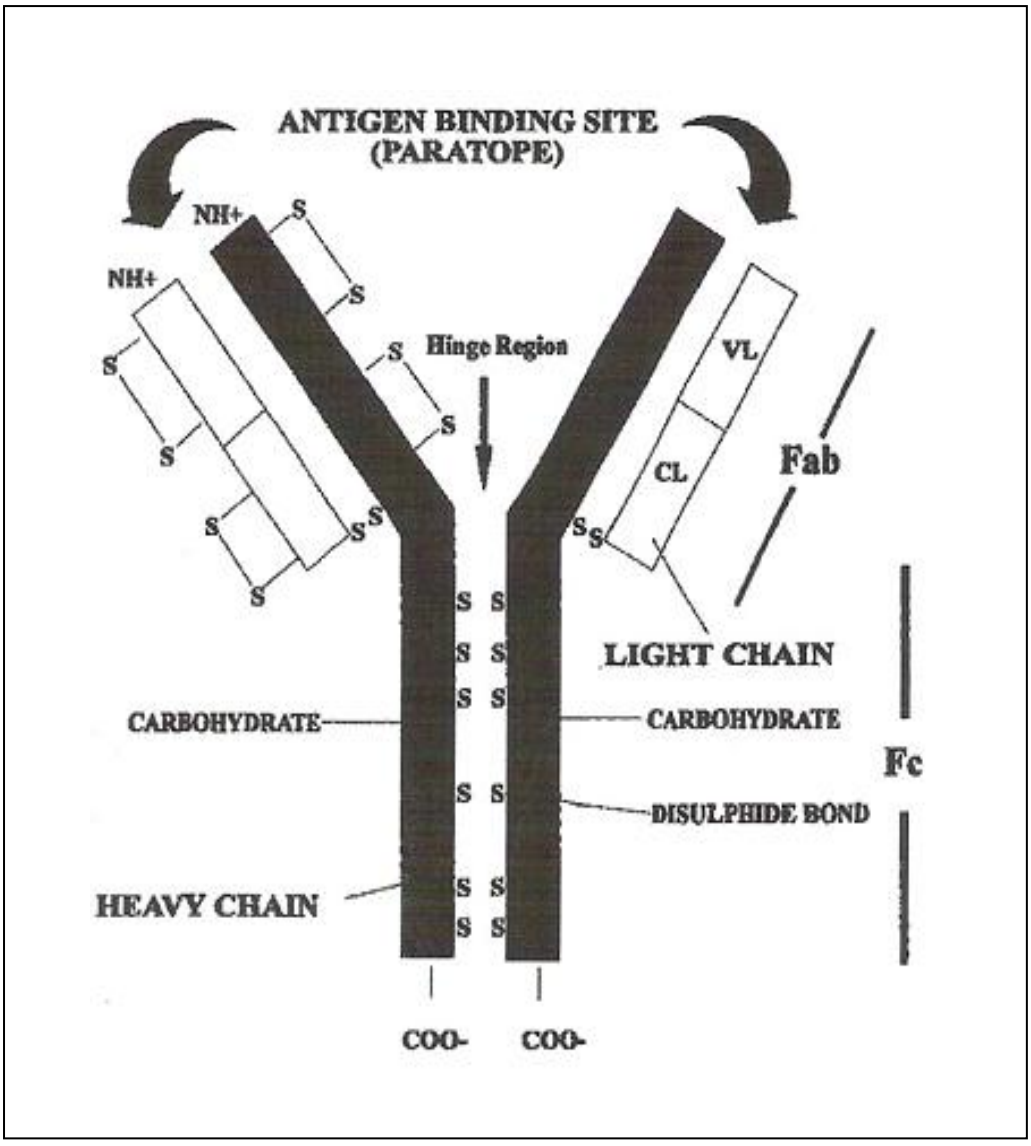



**Figure 1.3: A schematic diagram of the structural components of an immunoglobulin G (IgG) molecule** (Crowther, 1995). Antibodies utilized in this research were monoclonal antibodies. The paratope indicated by the black arrows is the site at which the antibody interacts with the antigen epitope. The Fc region is the portion of the antibody which the secondary antibody binds to in an ELISA or Immunoblot.

extensively used in research include: Sp2/O-Ag 14 and P3-X63-Ag8 (Shulman *et al.*, 1978; Kearney *et al.*, 1979), which were used in this research project.

The generation of Abs to HBx is bedevilled by weak immunogenicity of the viral protein (Park *et al.*, 2000; Pál *et al.*, 2003). However, HBxAg remains the most expressed antigen in late stage disease and its detection should, at least, have prognostic value (Pfaff *et al.*, 1987; Pál *et al.*, 2001).

## 1.6 DIAGNOSIS AND TREATMENT

### 1.6.1 Diagnosis

In diagnosing viral hepatitis, it must first be differentiated from other disorders causing similar symptoms; standard liver function tests (serum aminotransferases, bilirubin and alkaline phosphatase) are performed first (Beers *et al.*, 2006). Acute viral hepatitis is typically associated with alanine aminotransferase (ALT) levels higher than those of aspartate aminotransferase (AST); alcoholic hepatitis typically features higher AST levels (Matloff *et al.*, 1980). ALT and AST are transaminase enzymes which are associated with liver parenchymal cells and their serum concentration is elevated in acute liver damage (Huang *et al.*, 2006). Viral DNA detection by polymerase chain reaction (PCR) is the most accurate diagnostic tool for HBV (Balderas-Renteria *et al.*, 2002). However, the outcome of infection is determined by the interplay of viral replication and the host's immune system; therefore diagnosis, in clinical practice, involves the detection of viral protein

products and host-produced antibodies through serology (Tsitsilonis *et al*., 2004). ELISAs are used to detect these diagnostic markers (Hwang *et al*., 2003; Tsitsilonis *et al*., 2004).

The main hepatitis B-related viral markers are: HBV surface antigen (HBsAg), HBV e antigen (HBeAg), antibodies to HBsAg (anti-HBs), antibody to HBeAg (anti-HBe) and the antibody to the HBV core antigen (anti-HBc) (Keeffe *et al*., 2008). HBsAg is a diagnostic marker, which shows infectivity of the blood, and can be detected from the first week of infection (Zoulim *et al*., 1992). If viral replication is elevated, the HBeAg can also be detected in the serum, suggesting increased infectivity (Beers *et al*., 2006). This antigen is usually present at low levels and has been detected in 30 % of HBsAg positive patients (New Zealand National Eye Bank Quality Manual, 2001). HBeAg is considered a better marker of viral infection (as its level aids in the characterization of viral replication and infectivity) (Tsitsilonis *et al*., 2004). In approximately 90 % of acutely infected adults, both antigens are cleared from the system by an effective T cell response, and their corresponding antibodies are then produced (New Zealand National Eye Bank Quality Manual, 2001). Antibody levels persist in the blood even after recovery and their detection indicates past HBV infection and relative immunity (Beers *et al*., 2006). Anti-HBs is the best serological indicator of recovery (Raimondo *et al*., 2003). This Ab is only detectable in patients once they recover from infection, thereafter, having lifelong immunity from re-infection (New Zealand National Eye Bank Quality Manual, 2001; Wei *et al*., 2002*).*

Acute infection lasts approximately two to four weeks and does not require specific treatment because more than 90 % of adults spontaneously clear the infection (Gitlin, 1997; Rizzetto & Ciancio, 2008). On the other hand, perinataly acquired infection may

last for decades with ~ 5 % of patients progressing to cirrhosis (Rizzetto & Ciancio, 2008). Risk is further increased in HBeAg positive mothers (Plitt *et al*., 2007).

Persistence of HBsAg and HBeAg in serum for more than twenty weeks increases the risk of progressing to chronic infection (Gitlin, 1997). Anti-HBc appears at the onset of clinical illness and can sometimes be the only marker detected during HBsAg seroconversion when HBsAg levels are below the sensitivity threshold of diagnostic tests (Holland, 1994; Rizzetto & Ciancio, 2008). The Ab is mainly found as immunoglobulin M (IgM) in acute infection, subsequently switching to IgG, which dominates in chronic infection (Gitlin, 1997). Low levels of anti-HBc (IgM) have been associated with liver disease (Zoulim *et al*., 1992).

A 'chronic HBV carrier' refers to a person with persistent levels of HBsAg, testing positive for more than six months (Rosenthal, 2006). This type of infection occurs in ~ 10 % of acutely infected adults or if infection took place at birth or in infancy (New Zealand National Eye Bank Quality Manual, 2001). This type of infection may last a lifetime, duration correlating with the risk of developing cirrhosis and HCC (Rizzetto & Ciancio, 2008). The HBeAg and anti-HBc (IgG) are also detected in patient sera (Rizzetto & Ciancio, 2008). The presence of HBeAg suggests increased viral replication, therefore increased infectivity (Beers *et al*., 2006).

HBV DNA is detected in the serum of patients with active HBV infection (Keeffe *et al*., 2008). Its detection and quantification also play a role in diagnosis and may provide insight into the severity of disease and therefore its prognosis (Rizzetto & Ciancio, 2008).

In some cases, DNA detection may be the only indication of infection (occult infection) (Carman *et al.*, 1989). Carriers with active replication are at risk of developing cirrhosis, which often leads to HCC (Rizzetto & Ciancio, 2008). On the other hand, inactive carriers may ultimately clear the infection from the blood, but remain occult carriers of HBV in the liver (Rizzetto & Ciancio, 2008).

An interesting question to ask would be: "What role does the HBV X protein play in the diagnosis and prognosis of HCC?" Disease stage at the time of HCC diagnosis greatly affects the prognosis; clearly rapid, specific and sensitive diagnostic assays are required to minimise this problem (Bachtiar *et al.*, 2009).

N-glycan fingerprinting has recently been adapted as a tool for diagnosing HCC in cirrhotic patients (Liu *et al.*, 2007; Valerie *et al.*, 2009). The blood test, known as the GlycoHCCTest, measures the concentration and log ratio of two glycans associated with tumourogenesis. In the glycosylation pathway, sugar chains can be altered, depending on the physiological condition of the cell; therefore; the type of glycan produced reflects the physiological state of a cell (Valerie *et al.*, 2009). Branch alpha (1,3)-fucosylated triantennary glycan was found more abundant in HCC patients whereas the bisecting core alpha (1,6)-fucosylated biantennary glycan was elevated in cirrhotic patients. HCC has also been diagnosed by alpha-fetoprotein (AFP) analysis, through ultrasonography, computed tomography and histology (Valerie *et al.*, 2009). AFP is normally produced by the embryonic liver cells and is measured as a biomarker to detect a subset of tumours (França *et al.*, 2004). Studies have shown that a mild elevation of AFP occurs in patients with cirrhosis; therefore AFP analysis alone is not a reliable test for HCC diagnosis and

should be used in conjunction with other tests (Colli *et al.*, 2006). Apart from being expressed mildly in cirrhosis, AFP has been shown to be expressed at higher than normal levels in patients with high regenerative activity, without them even having HCC (Nomura *et al.*, 1989).

AFP analysis is limited as it cannot be used as the sole test for HCC and is not useful for its early detection due to its poor sensitivity (França *et al.*, 2004; Marrero & Lok, 2004). Instead, AFP levels are used to monitor treatment, as a decrease in AFP concentration correlates with a decrease in tumour growth (Yang *et al.*, 2008). When screening for HCC, blood AFP levels are tested every six months, with ultrasound performed on the liver at least once a year (http://www.hepb.org/professionals/hepb_and_liver_cancer.htm). HCC is diagnosed when AFP levels are greater than or equal to 500 ng/ml (Diamandis *et al.*, 2002).

Early detection of HCC is key in order to offer the possibility of curative treatment (Marrero & Lok, 2004), which this study aims to achieve through the use of the HBV X protein as the marker for the detection and screening of HCC.

### 1.6.2 Treatment

Patients with active infection (infection currently producing symptoms) are the most in need of treatment, which is primarily aimed at controlling HBV replication and inducing remission of liver disease (Mangia *et al.*, 2008). Through remission, cirrhosis and HCC

could be prevented (Liaw, 2005). One of the indicators for therapy is the persistence of HBeAg and HBV DNA for approximately six months as well as the elevation of aminotransferases (Hoofnagle & di Bisceglie, 1997). Treatment options for HCC are selected on the basis of the presence or absence of cirrhosis, the degree of hepatic deterioration and the number and size of tumour nodules (Colombo, 2001).

The woodchuck model has been useful in the discovery and development of antiviral drugs and the testing of novel forms of immunotherapy (Tennant *et al*., 2008). Interferon alpha was previously the only available therapy for the treatment of hepatitis B infection but has since been joined by other interferons (immune modulators) and nucleoside analogues (viral polymerase inhibitors) (Malik & Lee, 2000; Mangia *et al*., 2008). Interferons (IFNs) are species-specific proteins found in vertebrates which activate a number of signalling pathways when bound to the IFN alpha receptor (Wieland *et al*., 2000; Bekisz *et al*., 2004), thus terminating viral replication (Wong *et al*., 1993). Robek *et al*. found that IFN alpha requires the proteasome to mediate its antiviral effect (Robek *et al*., 2002). The inhibition of proteasome activity by HBx has been previously discussed and this may have great implications in IFN treatment.

Nucleoside analogues, such as lamivudine, entecavir and adefovir mainly target HBV polymerase thus inhibiting virus replication which could delay the onset of virus-associated cancer (Thomas *et al*., 2003). Lamivudine enters cells and is phosphorylated stepwise to its triphosphate metabolite where it acts as a strong inhibitor of HBV DNA polymerase (Rosenberg & Dienstag, 1999; Cheng, 2001). It is then incorporated at the HBV DNA terminus, inhibits HBV DNA synthesis, thus preventing HBV DNA elongation

(Cheng, 2001). Lamivudine has been reported to have a high incidence of resistance, as viral breakthrough occurred in some patients (Lai, 2003; Keeffe *et al.*, 2008). Resistance was reported in 66 % of patients on lamivudine treatment after four years (Mangia *et al.*, 2008).

Surgical resection and liver transplant have been identified as two curative treatments for HCC (Pang *et al.*, 2006). This unfortunately applies to a small percentage of patients only, whose tumours are identified early. On the other hand, systemic chemotherapy is widely used to treat inoperable HCC but has been shown to have a very low response rate (Colombo, 2001). Gene therapy, such as RNA interference, may offer new hope to patients with untreatable HCC (Cheng *et al.*, 2007; Wu *et al.*, 2007).

## 1.7 AIM OF RESEARCH PROJECT

Monoclonal antibodies have a wide variety of applications such as their potential use in therapeutics (Seeger & Mason, 2000); their use in the analysis of the genetic complexities of antigens (Howard *et al.*, 1979), but the aim of this research is to use them in the development of a diagnostic ELISA for the hepatitis B virus X protein.

HCC is a very aggressive cancer, especially in sub-Saharan Africa, where most people presenting with symptoms usually die within three months (Mendy & Walton, 2009). Hence clinical practice requires the continuous development of sensitive and rapid

diagnostic assays for HCC (Valerie *et al*., 2009). Early detection would allow for a better prognosis for the identified patient.

Detecting alpha fetoprotein has been one of the ways of surveying HCC, especially in cirrhotic patients, but has been shown to be insensitive for the early detection of HCC (Marrero & Lok, 2004). The role of other markers such as alpha-1-acid glycoprotein (AAG), glypican 3, golgi protein 73 (GP73) and desgamma carboxyprothrombin (DCP) has also been investigated in the diagnosis of HCC (Marrero & Lok, 2004; Bachtiar *et al*., 2009). These markers were found to lack at least some of the criteria required for an ideal marker of HCC, such as specificity, sensitivity, reproducibility and being inexpensive (Marrero & Lok, 2004; Mangia *et al*., 2008). The markers would have to be used in various combinations for the accurate diagnosis of HCC.

HBx is a good marker as it is not found in the human body, unlike other markers, unless HBV infection has occurred. Its concentration in serum also been observed to increase as the patient progresses from chronic hepatitis to HCC (Hwang *et al*., 2003). Thus, not only is it a good marker for diagnosis but for HCC prognosis as well.

At present, no standard, large-scale routine laboratory assays are available for the quantitative determination of serum HBxAg content (Pál *et al*., 2005). ELISAs are currently ranked amongst the most sensitive laboratory methods which allow for the rapid, easy, reliable and accurate detection of both antigens and antibodies (Tsitsilonis *et al*., 2004). Specifically, Sandwich ELISAs have been used in a variety of applications and are generally used for large-scale routine laboratory measurements of a number of other

antigens (Salgame *et al*., 1997; Pál *et al*., 2005). Typically the design of Sandwich ELISAs entails the use of two monoclonal antibodies that differ in epitope specificity (i.e. recognise different epitopes). This however is not critical as a Sandwich ELISA may be designed with two mAbs that recognise the same epitope on the condition that they do not interfere with each other. In this research project the Sandwich ELISA was developed using the latter approach.

To our knowledge, there is currently no commercially available ELISA kit available for the assay of the HBx protein. Therefore our aim was to develop a new detection system for epitopes on the HBx protein.

# Chapter 2: MATERIALS AND METHODS

## 2.1 HBx preparation and purification

Recombinant 6xHis-HBx protein preparation and purification was carried out following the method in Capovilla (2003). Recombinant pEt15B clones were transformed into *E. coli* strain BL21 and transformants selected on agar plates containing 100 µg/ml ampicillin and 35 µg/ml chloramphenicol. One colony of BL21/PE15X was inoculated in 50 ml Luria-Bertani (LB) Broth (Appendix B1.) containing 50 µl ampicillin and incubated overnight at 37 ºC. This culture was divided in two 25 ml aliquots and each added to 375 ml fresh LB broth containing 350 µl ampicillin. The culture was agitated at 37 ºC for approximately 1 hour 30 minutes to stimulate log phase growth, where after it was induced with 1 ml of a 200 mg/ml isopropyl-β-D-thiogalactoside (IPTG, Roche Diagnostics) stock solution in 50 % ethanol for 4 hours at 37 ºC with vigorous agitation. Induced cells were collected by centrifugation at 3000 rpm for 5 minutes.

The supernatant was discarded and the pellet re-suspended in 100 ml lysis buffer (Appendix B1.). Suspensions were snap-frozen and thawed in a water bath at 37 ºC. While on ice, the cell suspensions were sonicated gently (5 cycles: 30 second pulse, 30 second break). The lysate was then centrifuged at 8000 rpm for 10 minutes and the supernatant discarded. Pellet was re-suspended in 10 – 15 ml re-suspension buffer (Appendix B1.) which contained an 8M concentration of urea. The mixture was then

centrifuged at 8000 rpm for 10 minutes and the supernatant collected for affinity purification

Protein purification was effected by affinity binding of tagged histidines for $Ni^{++}$ which had been chelated to cross-linked sepharose 6 B (Sigma). The steps involved in HBx purification are diagrammatically illustrated overleaf *(Figure 2.1).*

## 2.2 Immunisation of Balb/c Mice

### 2.2.1 Animal Ethics Clearance

The applicable AEC number was 2005/14/3.

### 2.2.2 Synopsis of procedure

Recombinant 6xHis-HBx derivatives coupled to bovine serum albumin (BSA) using benzidine, gluteraldehyde and carbodiimide (Coligan *et al*., 1992) were obtained from Dr Wolfgang Prinz. (Coupling of poly-His-HBx was considered necessary to increase the immunogenicity of HBx).

For the primary immunisation, Balb/c mice were injected subcutaneously (s.c.) in an emulsion of coupled HBx-derivative in phosphate buffered saline (PBS)/Freund's

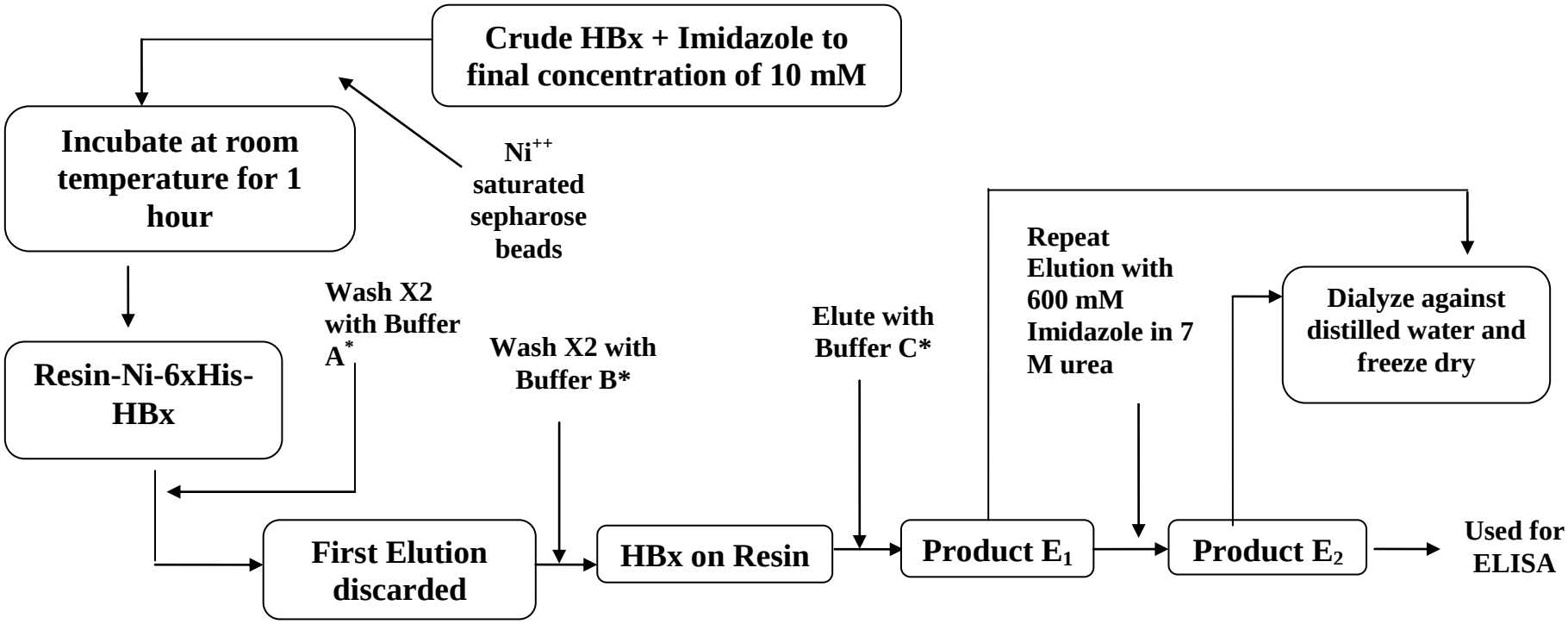



**Figure 2.1: Flow diagram: Purification of HBx.** All purification steps were carried out at room temperature. His-tagged protein can be eluted at concentrations of 500 mM and above of imidazole. Subsequent to purification, Product $E_1$ (purified HBx from the first elution) was used in Western Blotting and Product $E_2$ (purified HBx from the second elution) was used in ELISAs. Dialysis was performed to concentrate the protein. Purified 6xHis-HBx was then electrophoresed using SDS-PAGE to determine the presence of the protein, followed by densitometry to determine the protein's concentration.

complete adjuvant (400 μg per mouse). A booster of the same amount with Freund's incomplete adjuvant was administered 4 weeks later (also s.c). The final booster in PBS was administered intraperitonially 3 weeks after the 1st booster. Mice were sacrificed 5 days later, with the spleen providing the cells for fusion.

## 2.3 Cell Culture

Hybridoma cells were maintained in culture in RPMI (Appendix B2.) supplemented with 10 % heat-inactivated foetal calf serum (FCS, Delta Bioproducts). In the case of a cell fusion, medium was further supplemented with the selective medium, HAT (hypoxanthine-aminopterin-thymidine) (Appendix B2.) and cells were maintained in this medium for 2 weeks. Medium lacking aminopterin (RPMI/10 % FCS, HT) (Appendix B2.) was then substituted for another week. Incubation occurred in a 37 °C/5 % $CO_2$ incubator at 95 % relative humidity.

### 2.3.1 Cell fusion

Myeloma cell lines used in the fusions were either the Sp2/0-Ag14 (Shulman *et al*, 1978) or the P3-X63-Ag8.653 (Kearney *et al*, 1979) line. To ensure that myeloma cells were in the log phase of growth, cells were diluted 1 in 5 with RPMI/10 % FCS two days prior to their use in the cell fusion technique.

To obtain splenocytes, a spleen, obtained from an immunised Balb/c mouse, was disrupted

by gentle agitation through a stainless steel wire mesh. The cell suspension was then transferred to a sterile centrifuge tube, topped up to 30 ml with RPMI and centrifuged in a Beckman TJ-6 centrifuge for 10 minutes at 1000 rpm. Supernatant liquid was then discarded and 5 ml erythrocyte lysis buffer (0.14 M $NH_4Cl$) was then added to the pellet. Pellet was dislodged by gentle flicking and allowed to stand for approximately 5 minutes. Forty millilitres RPMI was then added to remove lysis buffer and the cell suspension was re-centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded and washed once with 40 ml RPMI. The splenocyte pellet was then re-suspended in 20 ml RPMI/10 % FCS and a trypan blue exclusion cell count was performed using a haemocytometer (Neubauer, Germany). "Feeder cells" were obtained in the same way as above, using a spleen from an unimmunised mouse.

Log-phase myeloma cells were washed in RPMI and centrifuged for 10 minutes at 1000 rpm. They were re-suspended in 20 ml RPMI/10% FCS for a cell count. Cell count was performed as above. Following the cell counts, the splenocytes (from an unimmunised mouse) and myelomas were diluted appropriately, and mixed such that their ratio was 5:1, respectively. The cell mixture was centrifuged for 10 minutes at 1000 rpm and the supernatant discarded.

Cell fusion took place at 37 °C. One millilitre of polyethylene glycol (50 % PEG 1500, Roche) was added dropwise to the pellet with gentle shaking during 1 minute. One millilitre of RPMI was then added and the mixture allowed to stand for 1 minute. Then 2 ml of RPMI were added and the cell suspension was allowed to stand for 2 minutes. The sequence of adding 2 ml aliquots of RPMI at 2 minute intervals was repeated 3 times.

Thereafter, the cell suspension was topped up to 30 ml with RPMI/10% FCS and centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded and the cell pellet re-suspended in "selection medium" (RPMI/10 % FCS, HAT). The "feeder cell" pellet was also re-suspended in the "selection medium" and added to the fused cell mixture such that the ratio of fused splenocytes to "feeders" was 1:1.

Seven drops (approximately 0.5 ml) of the mixed cell suspension, containing $3 – 5 \times 10^5$ fused splenocytes and an equal amount of "feeders", were added to each well of a 48-well tissue culture plate. Plates were then placed in a 37 °C/5 % $CO_2$ incubator at 95 % relative humidity and monitored for 1 to 2 weeks by observing medium colour change, as well as microscopic examination. Wells that were positive for growth (clones of hybridomas) were tested for HBx recognition by Indirect ELISA and Western Blotting (Immunoblotting).

### 2.3.2 "Limiting dilution" of anti-HBx positive cell cultures

This was performed by the standard methodology (Liddell & Cryer, 1991). Briefly, the selected hybridomas were plated out into 48-well plates at an average density of 0.5 cells per well, together with approximately 500 000 "feeder" cells obtained from spleens of unimmunised mice. Cells were cultured in RPMI/10 % FCS containing OPI (oxaloacetate-pyruvate-insulin) (Harlow & Lane, 1988) (Appendix B2.). Plates were incubated under the same conditions as previously mentioned for approximately 7 to 14 days. Wells that had turned yellow in colour and were positive for clonal growth were then tested for anti-HBx

antibody production by Indirect ELISA and Western blotting (Immunoblotting).

To ensure monoclonality, the “limiting dilution” procedure was repeated twice.

## 2.4 Electrophoresis and Blotting techniques

### 2.4.1 Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were separated using one-dimensional SDS-PAGE, employing the mini-gel system (Hoefer), with a 4 % stacking gel and 15 % running gel (Appendix B3.). Electrophoresis was carried out at a constant 10 mA per gel thorough the stacking gel and 15 mA per gel through the running gel for approximately 2 ½ hours. Gels were either stained in Coomassie Brilliant Blue R-250 (Merck, Appendix B3.) overnight then destained (Destaining solution I and II, Appendix B3.), or subjected to Western blotting.

In some cases where sensitivity was imperative, silver staining was used. Gel was fixed by rocking in Solution A (Appendix B3.) for one hour. The solution was discarded and the gel washed 3 times with 50 % ethanol (for 20 minutes). The gel was treated in Solution B (Appendix B3.) for 1 minute and the solution then discarded. MilliQ water was used to wash the gel 3 times (20 seconds each) then stained in Solution C (Appendix B3.) for 30 minutes. The gel was removed from the solution and washed in MilliQ water as above. The gel was then developed in Solution D (Appendix B3.) until bands of interest were

observed. The reaction was stopped by washing the gel in Solution E (Appendix B3.) for 20 minutes, followed by gentle washing in a large volume of distilled water for 30 to 60 minutes. The image was captured on the Gel-Doc system and analysed.

### 2.4.2 Western Blotting

Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon Millipore) using the small transfer unit (TE 22, Hoefer). The stacking gel from the SDS-PAGE gel was discarded and the running gel equilibrated for up to 5 minutes in transfer buffer (Appendix B4.). The PVDF membrane was initially soaked in 100 % methanol for a few seconds then equilibrated in transfer buffer for approximately 15 minutes. Sponges and filter paper to be used were also equilibrated prior to assembly of the sandwich cassette assembled. Transfer was effected overnight at a constant voltage of 14V for a maximum of 16 hours.

## 2.5 Immunoassay

### 2.5.1 Immunoblot

Following transfer, the PVDF membrane was cut into strips corresponding to the lanes on the SDS-PAGE gel. Membrane strips were then incubated in a protein blocking solution (Appendix B4.) for 1 hour at room temperature with gentle agitation. The membrane strips

were then washed 3 times (5 minutes each) in washing solution (Appendix B4.) and then incubated in various dilutions of clonal supernatant solution for 2 hours at room temperature with gentle agitation. After washing as described previously, membrane strips were incubated in a 1 in 1000 dilution of secondary antibody (HRP(horseradish peroxidase )-linked anti-mouse Ig, DAKO) in blocking solution. Strips were then washed again for 15 minutes before being exposed to freshly prepared substrate (visualization) solution (Appendix B4.).

### 2.5.2 Visualisation by Chemiluminescence

Chemiluminescence involves the detection of horseradish peroxidase, an enzyme that catalyses the oxidation of luminol (substrate) using hydrogen peroxide (oxidizing agent), through the production of light (Harper & Murphy, 1991). This enzyme is conjugated onto the secondary antibody and the light emitted can be imaged on photographic film and used to identify and quantitate the protein of interest (Bronstein *et al*, 1992).

A kit substrate mixture (Pierce) was used. Exposure was effected with an X-ray cassette (PL-C type, Kodak) and X-ray film (Fuji Medical). Different exposure times (2 to 30 minutes) were tested, until optimal signal was obtained. Following exposure, the X-ray film was developed (X-ray Manual Developer & Replenisher solution, CAT # 9X23018) until the desired band intensity was obtained. The X-ray film was then washed in distilled water, placed in fixer (X-ray Manual Fixer & Replenisher solution, CAT # 9X23013) for 30 seconds and washed again.

## 2.6 Enzyme-linked Immunosorbent Assay (ELISA)

### 2.6.1 Indirect ELISA

Recombinant 6xHis-HBx, diluted in coating buffer (10 µg/ml, Appendix B5.), was used to coat a 96-well ELISA plate (NUNC "maxisorp"); 100 µl was dispensed into each well and incubated at 37 °C for 2 hours or overnight at room temperature. Following incubation, the coating solution was discarded and the plate was washed three times with washing solution (Appendix B5.). The plate was then dried by inverting it and tapping onto paper towel. Possible remaining sites were blocked with the addition of 150 µl per well of blocking buffer (Appendix B5.) and incubated for 1 hour at room temperature with gentle agitation. The blocking solution was then discarded and the plate was dried as before.

One hundred microlitres clonal supernatant or RPMI/10% FCS (control) was added to duplicate wells together with 50 µl blocking buffer and incubated at room temperature for 1 hour with gentle agitation. Plates were then washed and dried as before. Polyclonal rabbit anti-mouse Ig/HRP (DAKO, P 0260) was diluted 1 in 1000 PBS (Appendix B5.); 100 µl of the horseradish peroxidase-conjugated secondary antibody solution was then added to each well and incubated for 1 hour at room temperature with gentle agitation. After washing as before, the plate was also washed once with 0.1 M citrate buffer (Appendix B5.)

One hundred microlitres of freshly prepared substrate solution (Appendix B5.) was then

added to each well for colour development. Following incubation for 30 to 60 minutes, the reaction was stopped by the addition of 1 M sulphuric acid (100 µl per well). Optical density at 490 nm was read in a BIORAD ELISA reader.

### 2.6.2 Competitive ELISA

The same methodology was employed as described in 2.6.1, with the following modification: instead on 100 µl of clonal supernatant of one mAb being added, 50 µl of two separate mAbs was added.

### 2.6.3 Sandwich ELISA

One hundred microlitres "capture" antibody was added at various dilutions (neat, 1 in 10, 1 in 100 and 1 in 1000) to a 96-well ELISA plate (NUNC "maxisorp") and incubated at room temperature overnight. Coating solution was then discarded and the plate was washed with washing solution (Appendix B5.) and dried by inversion and tapping on paper towel. Wells were then blocked by the addition of 150 µl of blocking buffer (Appendix B5.) to each well. Blocking took place for 1 hour at room temperature with gentle agitation. Blocking solution was then discarded and the plate was dried as before.

One hundred micolitres of HBx in PBS (Appendix B5.), was added to each well and incubated for 1 hour at room temperature with gentle agitation. After washing, as before,

100 µl of "detecting" antibody was then added at a concentration of 1 in 10, 1 in 100, 1 in 1000 for 1 hour at room temperature with gentle agitation; washing and drying as before.

Detection, using the rabbit anti mouse Ig/HRP and substrate solution, was performed as described previously in 2.6.1.

## 2.7 Monoclonal antibody purification

Antibody purification was carried out using the ammonium sulphate precipitation method (Liddell & Cryer, 1991), followed by an affinity gel selection procedure, as illustrated in *Figure 2.2* overleaf:

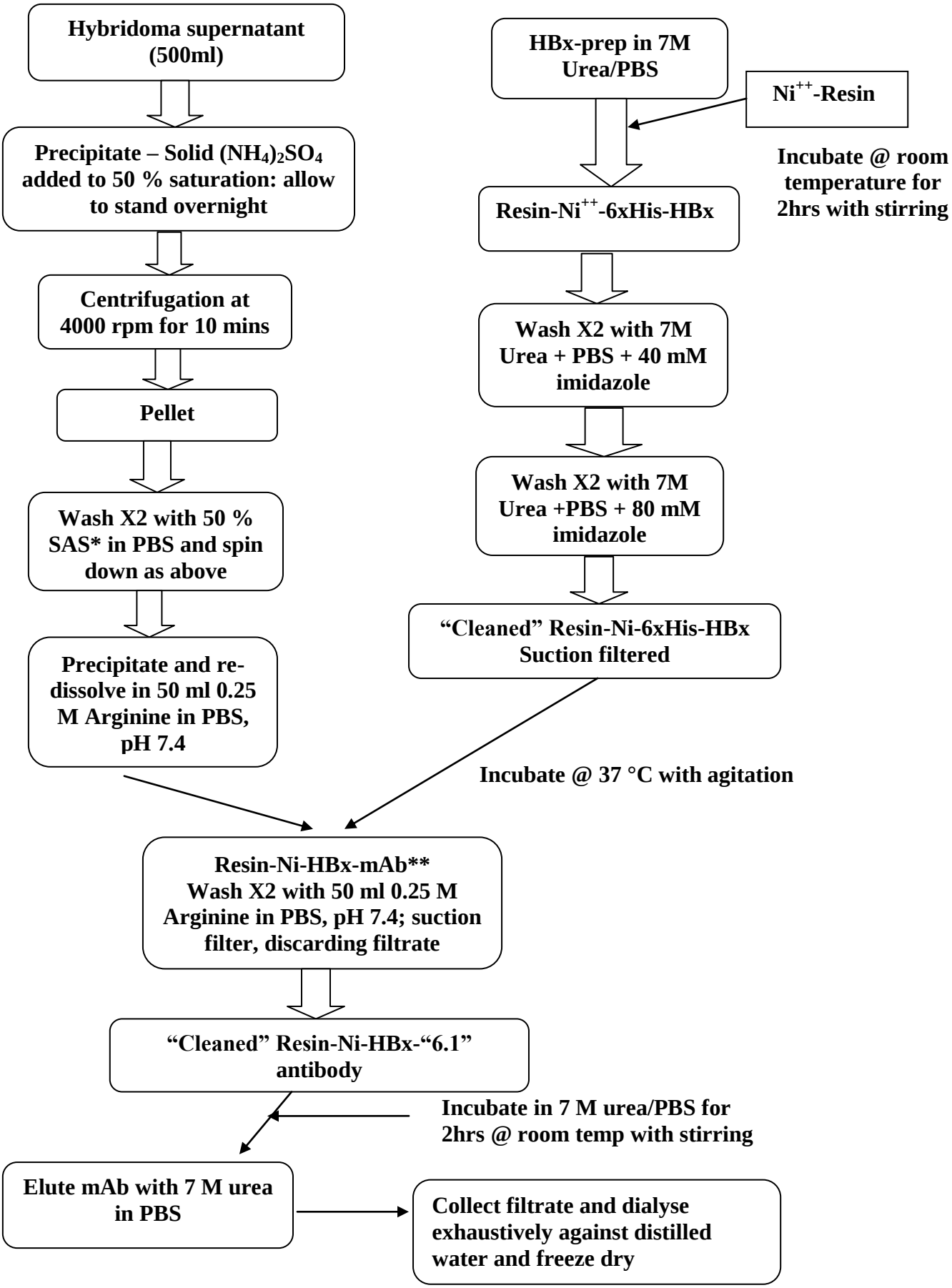

Hybridoma supernatant (500ml)
Precipitate – Solid $(NH_4)_2SO_4$ added to 50 % saturation: allow to stand overnight
Centrifugation at 4000 rpm for 10 mins
Pellet
Wash X2 with 50 % SAS* in PBS and spin down as above
Precipitate and re-dissolve in 50 ml 0.25 M Arginine in PBS, pH 7.4
HBx-prep in 7M Urea/PBS
$Ni^{++}$-Resin
Incubate @ room temperature for 2hrs with stirring
Resin-$Ni^{++}$-6xHis-HBx
Wash X2 with 7M Urea + PBS + 40 mM imidazole
Wash X2 with 7M Urea +PBS + 80 mM imidazole
"Cleaned" Resin-Ni-6xHis-HBx Suction filtered
Incubate @ 37 °C with agitation
Resin-Ni-HBx-mAb** Wash X2 with 50 ml 0.25 M Arginine in PBS, pH 7.4; suction filter, discarding filtrate
"Cleaned" Resin-Ni-HBx-"6.1" antibody
Incubate in 7 M urea/PBS for 2hrs @ room temp with stirring
Elute mAb with 7 M urea in PBS
Collect filtrate and dialyse exhaustively against distilled water and freeze dry

**Figure 2.2**: **Flow diagram: Monoclonal antibody purification.** A hybridoma clone was grown to exhaustion to obtain large amounts of monoclonal antibody for the supernatant. Purification steps, unless otherwise indicated, were carried out at room temperature. Glass containers were used to minimise the loss of antibody due to non-specific adsorption to plastic surfaces. The purified mAb sample was then electrophoresed to determine the presence of the antibody and its concentration was obtained by densitometry. (* SAS – saturated ammonium sulphate.

# Chapter 3: RESULTS AND DISCUSSION

## 3.1 HBx preparation and purification

Products from the first ($E_1$) and second ($E_2$) eluates obtained during the purification process were electrophoresed in a 15 % SDS PAGE gel (Appendix B3.) and stained with Coomassie stain (Appendix B3.) *(Figure 3.1)*. The HBx band in each lane was found in the expected position of approximately 19 kDa (6xHis-HBx). It is evident that product $E_1$ had contaminating proteins; using densitometry, it was found to have an HBx concentration of 0.68 mg/ml. Product $E_2$ had barely visible contamination and had an HBx concentration of 0.48 mg/ml, hence the routine use of this protein product when performing various ELISA tests. Product $E_1$ was routinely used in Western blots as this method is less sensitive than ELISAs and, therefore, requires a higher concentration of antigen. Purified HBx was subsequently stored in PBS/7M urea.

## 3.2 Generation of hybridoma cells producing anti-HBx antibodies

### 3.2.1 The Immunogenicity of HBx

The production of anti-HBx Abs is bedevilled by the poor immunogenicity of HBx. This is partially due to the 'physico-chemical' feature of the protein, its secondary and tertiary

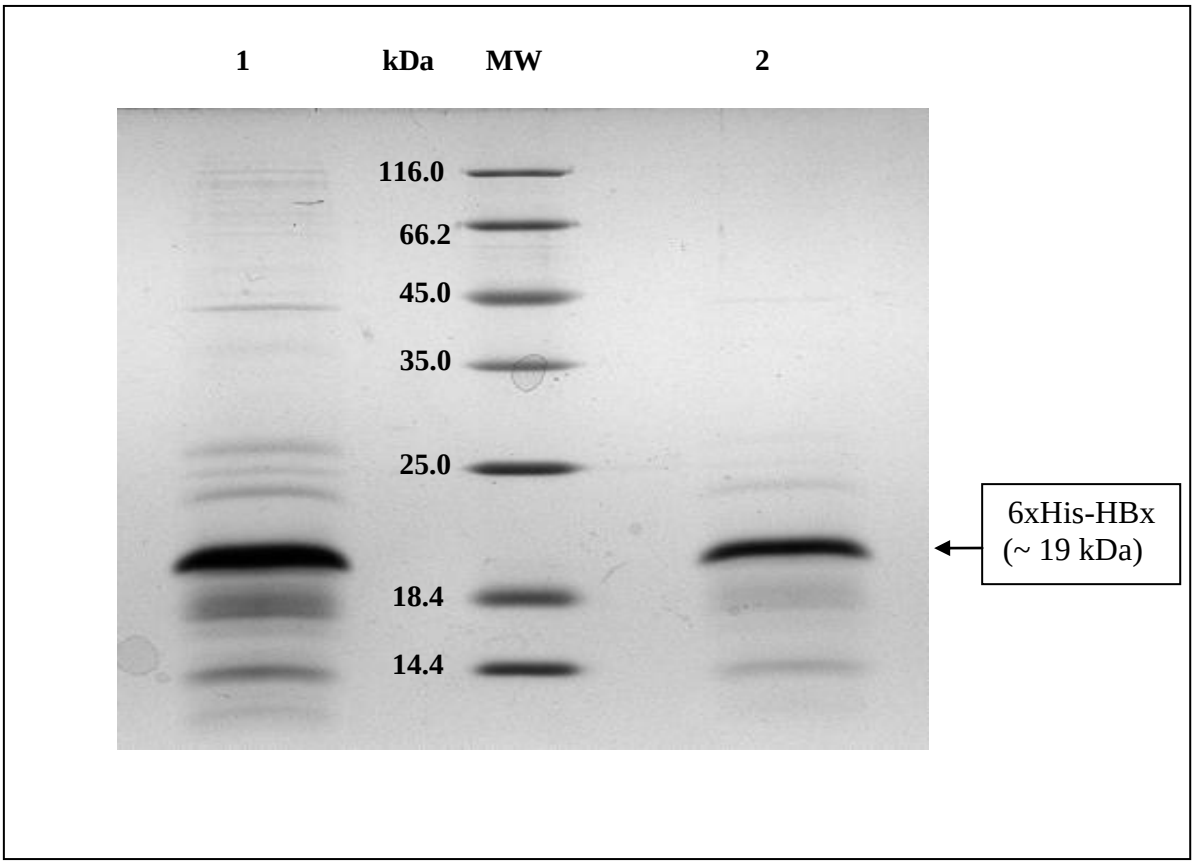



**Figure 3.1: Coomassie stained 15 % SDS PAGE gel.** MW: Molecular weight marker (116.0: β-galactosidase, 66.2: Bovine serum albumin, 45.0: Ovalbumin, 35.0: Lactate dehydrogenase, 25.0: REase Bsp981, β-lactoglobulin, 14.4: Lysozyme). Lanes 1 and 2 were loaded with product $E_1$ and product E2, respectively.

structures and its highly hydrophobic amino acids, rendering it insoluble in water (Pál *et al.*, 2003; Pál *et al.*, 2005). This has resulted in the inconsistency of results obtained when monitoring HBxAg expression in liver tissue (Doria *et al.*, 1995; Park *et al.*, 2000). Patients acutely infected with HBV were found to have relatively weak anti-HBx reactivity when compared to chronically infected patients (Abraham *et al.*, 1989).

In our study we attempted to overcome this by coupling HBx to larger carrier proteins. The deployment of recombinant HBx (with a 6xHis tag) on its own in immunisation resulted in only one usable mAb ("6.1"). Other research groups have reported a similar lack of success (Park *et al.*, 2000). On the other hand, HBsAg has been found to display excellent immunogenicity (Roohi *et al.*, 2005).

The HBx protein has been reported to have a variety of antigenic determinants recognized by anti-HBx Abs, with a major domain in close proximity to the C-terminal of HBx (Stemler *et al.*, 1990). Pal *et al.* found it necessary to determine the fine epitope structure recognized by a mAb they had developed, for its use in immunoserological assays (Pál *et al.*, 2003). Through computer analysis and mass spectroscopy they identified three sequences predicted to be highly immunogenic. These were amino acids (22 – 31), (110 – 114) and (14 – 26). Nonetheless, these sequences failed to be recognised by their antibody. Mapping of epitopes, in future, would be a useful approach in studying their relevance and reactivity with antibodies developed.

### 3.2.2 Cell fusion

Hybridoma cells were generated using splenocytes of mice immunised with 6-His-HBx coupled to BSA carrier via benzidine, glutaraldehyde or carbodiimide. This was considered necessary to augment the immunogenicity of the HBx protein.

The "selection medium" for hybridoma growth contained the appropriate concentration of "HAT" (Hypoxanthine-aminopterin-thymidine). The success of the procedure resides in the ability of the hybridoma cells to use the "salvage" pathway for purine and pyrimidine synthesis, while the *de novo* pathway is blocked (Campbell, 1984; Liddell & Cryer, 1991). (Aminopterin blocks the *de novo* pathway, while the salvage pathway relies on the presence of enzymes thymidine kinase (TK) and hypoxanthine (guanine) phosphoribosyl transferase (H(G)PRT). Cells lacking these enzymes will die in "HAT" medium because both pathways for the production of DNA precursors are blocked. Nearly all rodent cell lines employed in cell fusion are devoid of HGPRT. Post fusion, only myeloma-splenocyte hybrids will survive.

Within 10 to 14 days post fusion, wells were examined for clonal growth. Hybridomas, if present, had grown sufficiently and deemed to have secreted enough detectable antibody into the culture supernatant. A typical mouse hybridoma, approximately two weeks after fusion, is shown in *Figure 3.2* (Campbell, 1984). Indirect ELISA was then performed to screen for hybridomas secreting anti-HBx Ab.

Numerous fusions were performed but the results reported are of only the successful

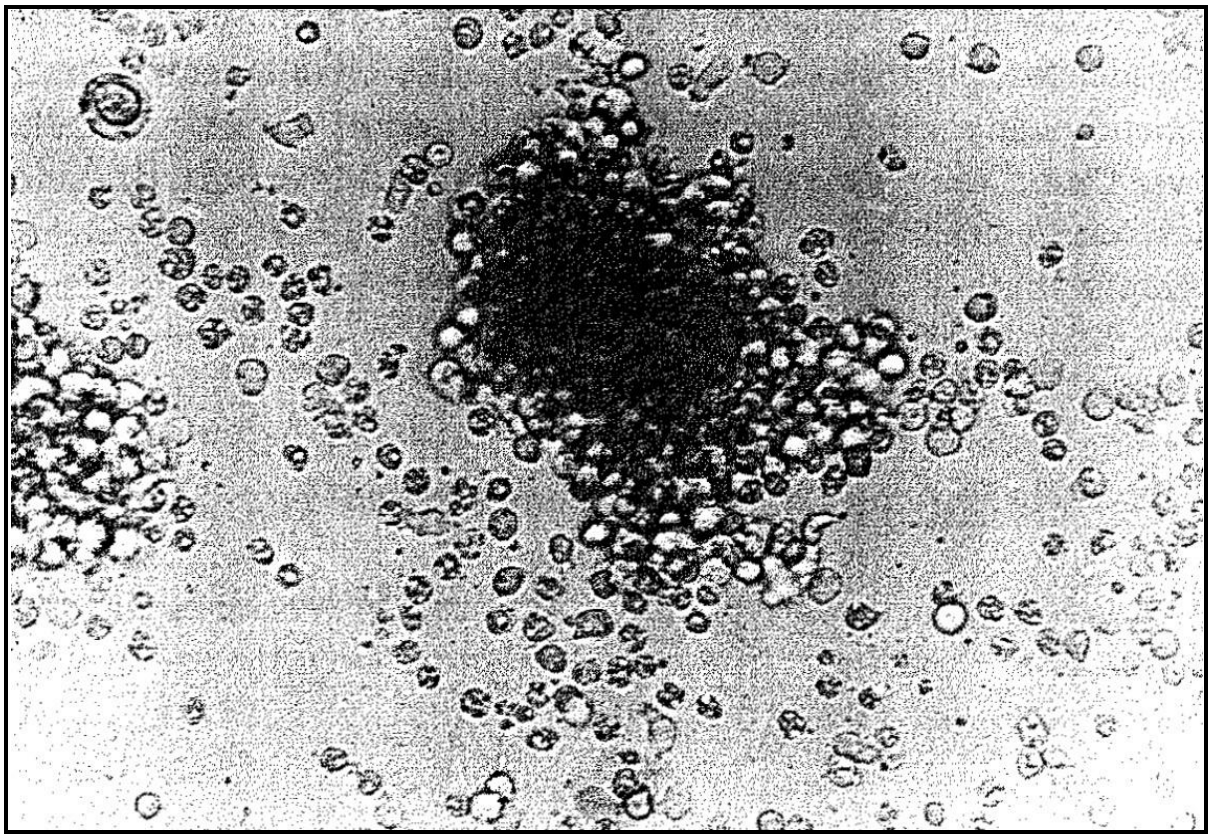


**Figure 3.2: A typical mouse hybridoma two weeks after fusion** (Campbell, 1984).

fusions that continued to produce anti-HBx Abs even after cloning and sub-cloning by "limiting dilution". Up to the time of the commencement of this dissertation, we found four clones (additional to the previously-mentioned "6.1") positive for anti-HBx Ab production *(Figure 3.3)*. On ELISA-testing, absorbance values ranged from 0.24 to 0.67, but only clonal supernatants with absorbance values above 0.50 were selected for cloning and sub-cloning by "limiting dilution". These absorbance values were compared to those of our previously developed mAb (termed "6.1"), which had a value in excess of 2.00. Only one "clonal mixture" was cloned at a time while the remaining ones were frozen (Appendix B2.) in liquid nitrogen to be cloned at a later stage.

## 3.3 Cloning by "Limiting dilution"

"Limiting dilution" aims to isolate hybridomas capable of synthesizing and secreting the mAb of choice from all other irrelevant hybridomas. Cloning involves the selected growth of cell colonies until it can be adduced that a colony has been derived from a single cell. Progeny cells are then said to be monoclonal. mAbs derived from such cells exhibit reactivity with a single determinant on an antigen (Liddell & Cryer, 1991).

Approximately, 10 to 14 days post cloning, clonal supernatants that had turned yellow in colour were examined for clonal growth. Just as with the cell fusion, these hybridomas had grown sufficiently and considered to have secreted enough detectable antibody into the culture supernatant. Clonal supernatants were then subjected to a screening process, a flow diagram of which is shown in *Figure 3.4*.

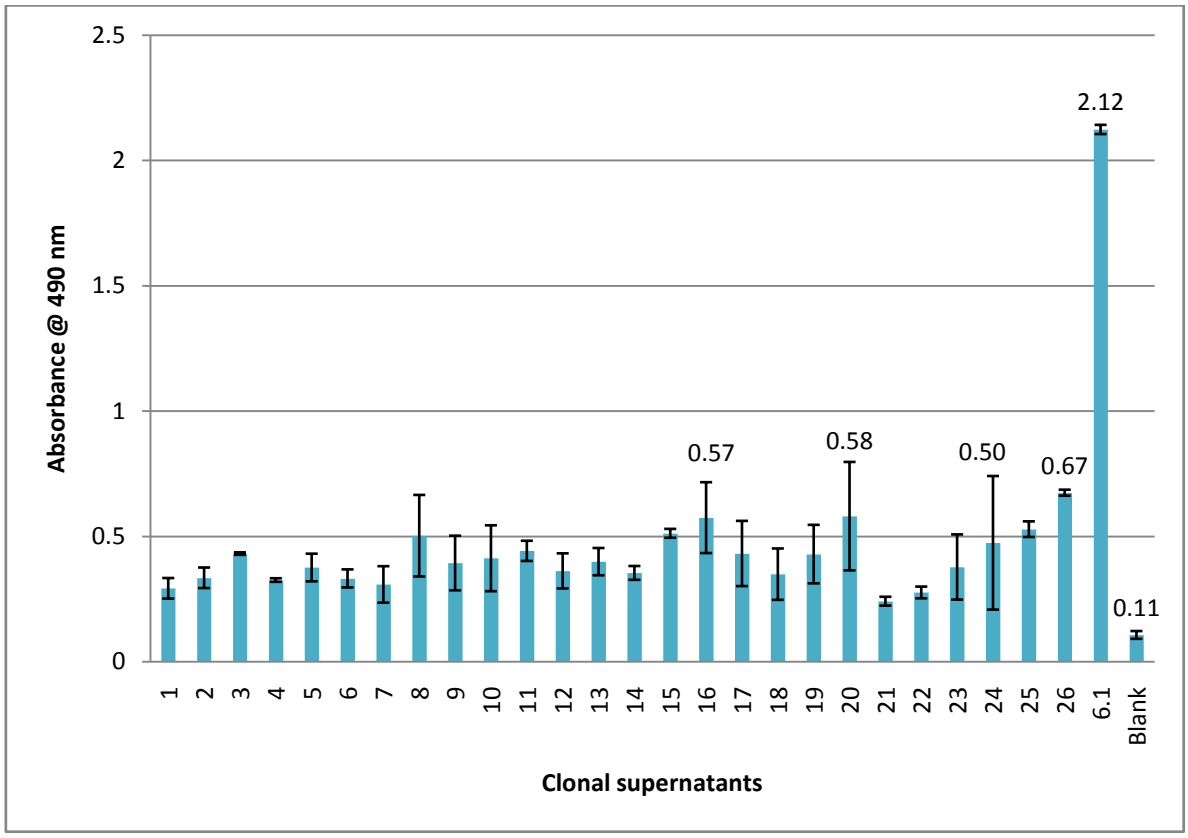



**Figure 3.3: Indirect ELISA following fusion.** Absorbance values of clonal supernatants (labelled 1 to 26) were compared to "6.1" (positive control), four of which had absorbance values greater than 0.50. Clonal supernatants were utilized undiluted and clonal supernatant "6.1" at a 1/100 dilution.

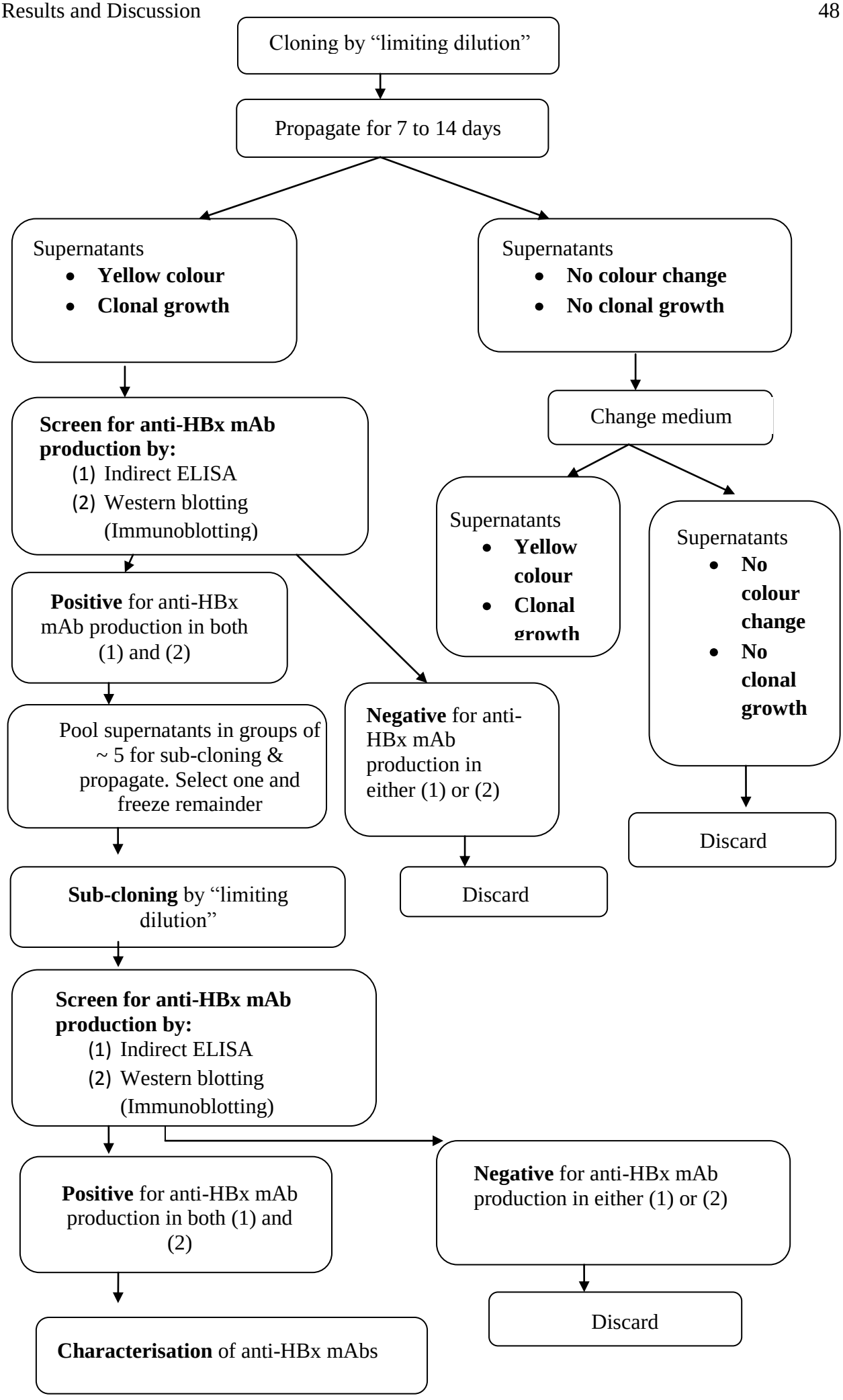

Cloning by "limiting dilution"
Propagate for 7 to 14 days
Supernatants
Yellow colour
Clonal growth
Supernatants
No colour change
No clonal growth
Screen for anti-HBx mAb production by:
(1) Indirect ELISA
(2) Western blotting (Immunoblotting)
Change medium
Supernatants
Yellow colour
Clonal growth
Supernatants
No colour change
No clonal growth
Positive for anti-HBx mAb production in both (1) and (2)
Negative for anti-HBx mAb production in either (1) or (2)
Discard
Pool supernatants in groups of ~ 5 for sub-cloning & propagate. Select one and freeze remainder
Discard
Sub-cloning by "limiting dilution"
Screen for anti-HBx mAb production by:
(1) Indirect ELISA
(2) Western blotting (Immunoblotting)
Positive for anti-HBx mAb production in both (1) and (2)
Negative for anti-HBx mAb production in either (1) or (2)
Discard
Characterisation of anti-HBx mAbs

**Figure 3.4: Flow diagram – Screening for anti-HBx antibodies in culture supernatants.** A positive result in the Indirect ELISA was deemed to pertain when the culture supernatant had an absorbance of 0.50 and above. A negative result was deemed to pertain when the absorbance was less than 0.50.

### 3.3.1 Screening of anti-HBx Abs in culture supernatants by Indirect ELISA

Clonal supernatants were tested for anti-HBx Ab production by the Indirect ELISA method. Absorbance values of clonal supernatants from a "successful" "limiting dilution" are shown in *Figure 3.5*. The culture wells corresponding to absorbance values of ± 2.00 were expanded and subjected to a second "limiting dilution".

A typical decrease of absorbance values after initial testing by Indirect ELISA is shown in *Figure 3.6 a and 3.6 b*. Chromosomal loss may occur in the early stages of cloning, thus possibly causing a loss of immunoglobulin secretion (Liddell & Cryer, 1991). 'Maxi-producers' are referred to as hybrids that produce a high percentage of antibodies as compared to 'mini-producers'. 'Maxi-producers' are more likely to die after a number of generations (Mayer & Walker, 1987). Thus, it is advisable to maintain the parent cell line in culture while cloning and sub-cloning.

It is generally maintained that two "limiting dilutions" suffice to produce a "fully monoclonal antibody producing hybridoma" (Campbell, 1984). Clonal supernatants shown in *Figure 3.5* were pooled and sub-cloned by a second "limiting dilution". Absorbance values of clonal supernatants following the second "limiting dilution" are shown in *Figure 3.7*. Twenty seven supernatants, out of a total of thirty six tested, had absorbance values above 0.50. Absorbance values close to a value of 2.00 or above compared well with our positive control ("6.1"). Pooled clonal supernatants from the first

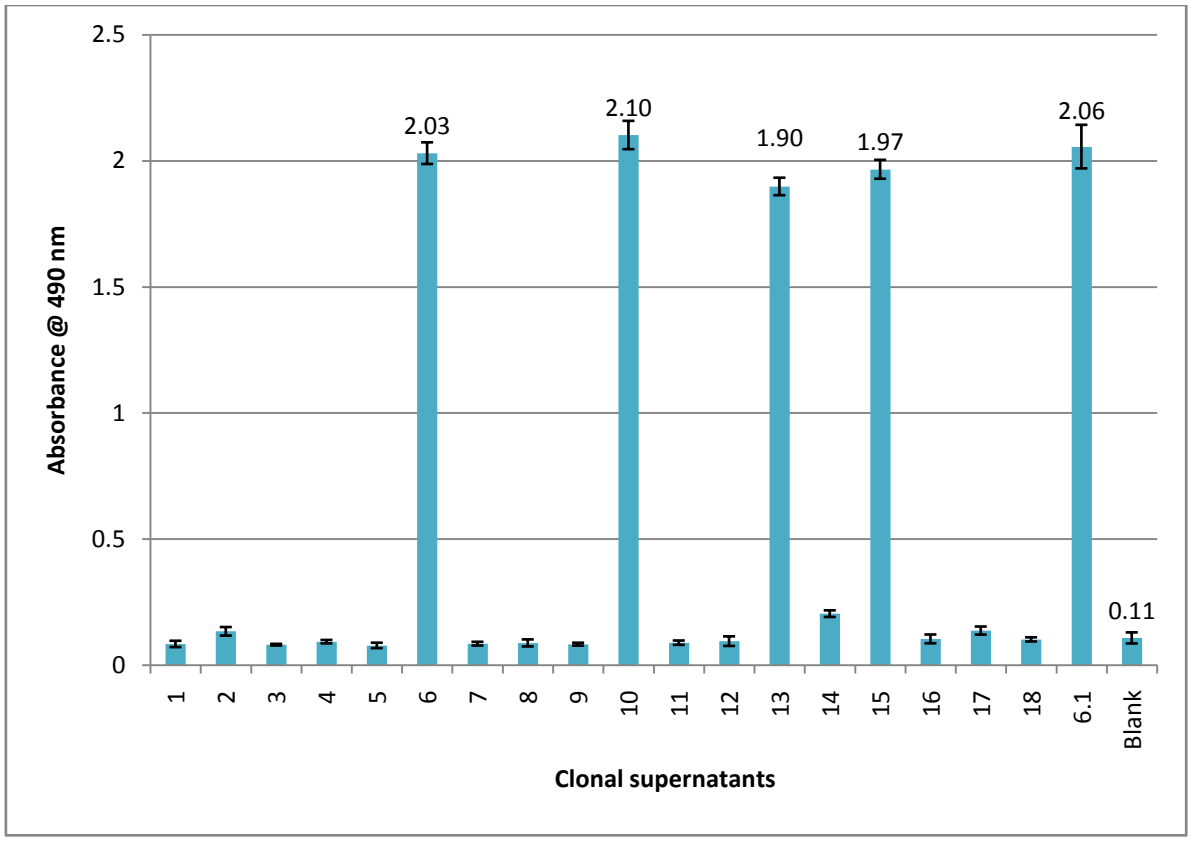



**Figure 3.5**: **Indirect ELISA following cloning by "limiting dilution".** Clonal supernatants were compared to "6.1" (positive control), four of which had absorbance values greater than 0.50. Clonal supernatants were utilized undiluted and clonal supernatant "6.1" at a 1/100 dilution.

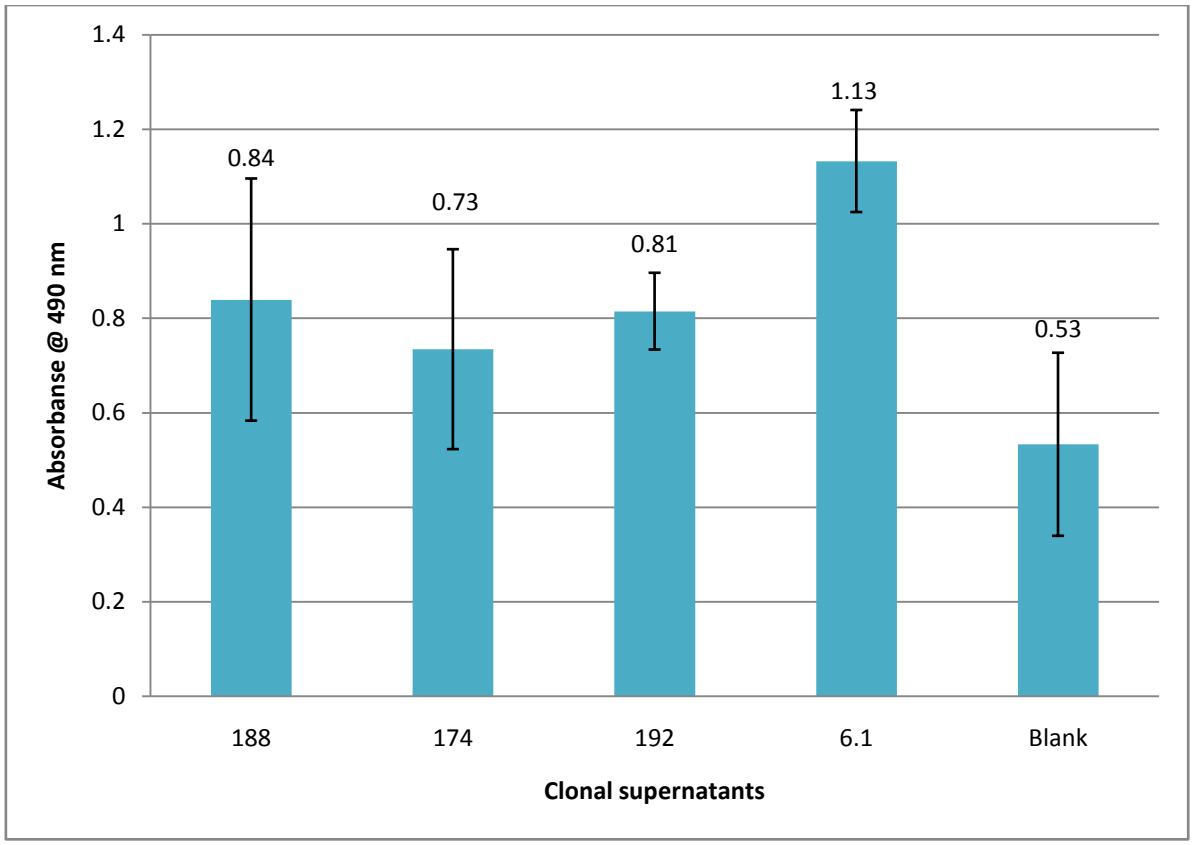
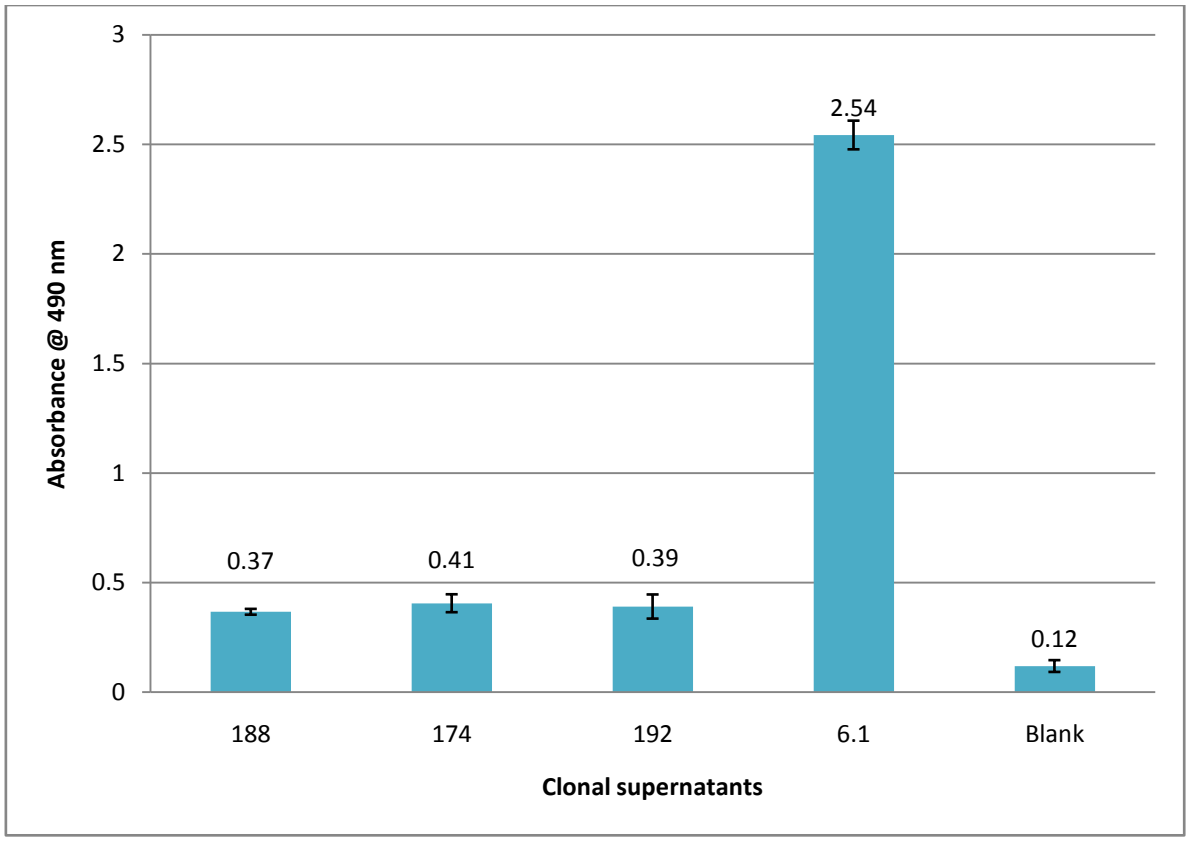



**Figure 3.6: a) An example of an Indirect ELISA of clonal supernatants 22 days after fusion.** All supernatants had absorbance values greater than 0.50. (Supernatants were not from the same fusion as the ones in ***Figure 3.3***). Clonal supernatants were utilized undiluted. Clonal supernatant "6.1" was used as a positive control at a 1/1000 dilution.




**b) – An example of an Indirect ELISA of clonal supernatants 29 days after fusion.** All supernatants had absorbance values below 0.50. Clonal supernatants were utilized undiluted. Clonal supernatant "6.1" was used as a positive control at a 1/10 dilution.

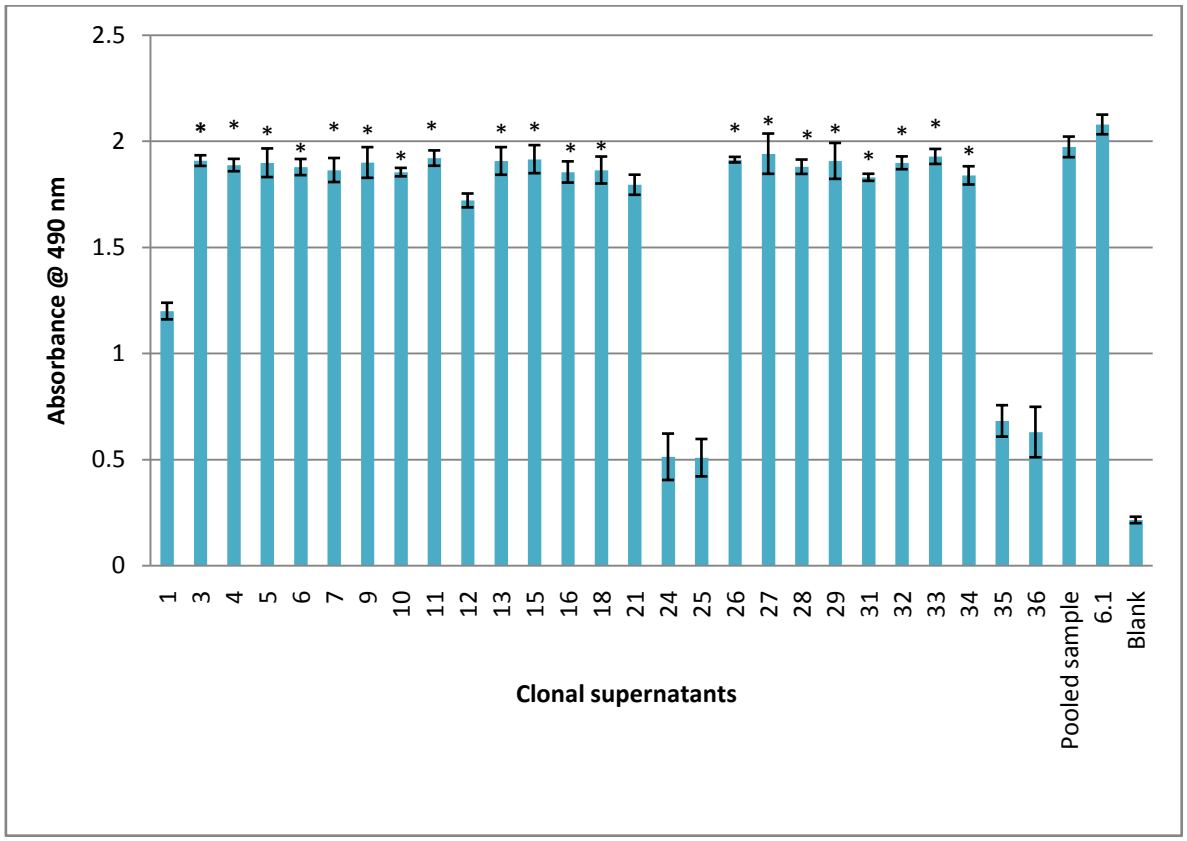



**Figure 3.7: Indirect ELISA following sub-cloning by "limiting dilution".** Twenty seven samples had absorbance values greater than 0.50, with twenty* having absorbance values close to 2.00. Pooled sample: clonal supernatants from the first "limiting dilution" that had absorbance values greater than 0.50 (supernatants 6, 10, 13 and 15 in ***Figure 3.5***). For ELISAs and Immunoblotting, clonal supernatants and the pooled sample were utilized undiluted.

"limiting dilution" were also tested along with second "limiting dilution" supernatants; the pooled sample had a comparable absorbance value.

One of the criteria in this research project was that mAbs produced had to give a positive result in both the Indirect ELISA and in Immunoblotting. Characterisation of the mAbs could then take place once this had been established.

### 3.3.2 Screening of anti-HBx Abs in culture supernatant by Immunoblotting

Clonal supernatants that were positive and had high absorbance values when tested by Indirect ELISA were subjected to Immunoblotting.

The pooled sample of clonal supernatants from the first "limiting dilution" was tested for its ability to bind to HBx fixed on PVDF membrane. At a dilution of 1/10, the pooled hybridoma supernatant sample failed to show reactivity with HBx *(Figure 3.8 a)*. This remained so, even after a thirty minute exposure.

Our previously developed mAb ("6.1") and the commercial anti-HBx Ab were used as positive controls *(Figure 3.8 b, 3.8 c* and *3.8 d)*. A strong signal was observed from the commercial anti-HBx Ab at a 1/5000 dilution after five minutes and our mAb "6.1" showed reactivity after twenty minutes. Not all the bands of the protein marker were clearly visible on the blot after development but bands representing 6xHis-HBx indicated on *Figures 3.8 c* and *3.8 d* correlate with the position of HBx on a Coomassie stained gel in

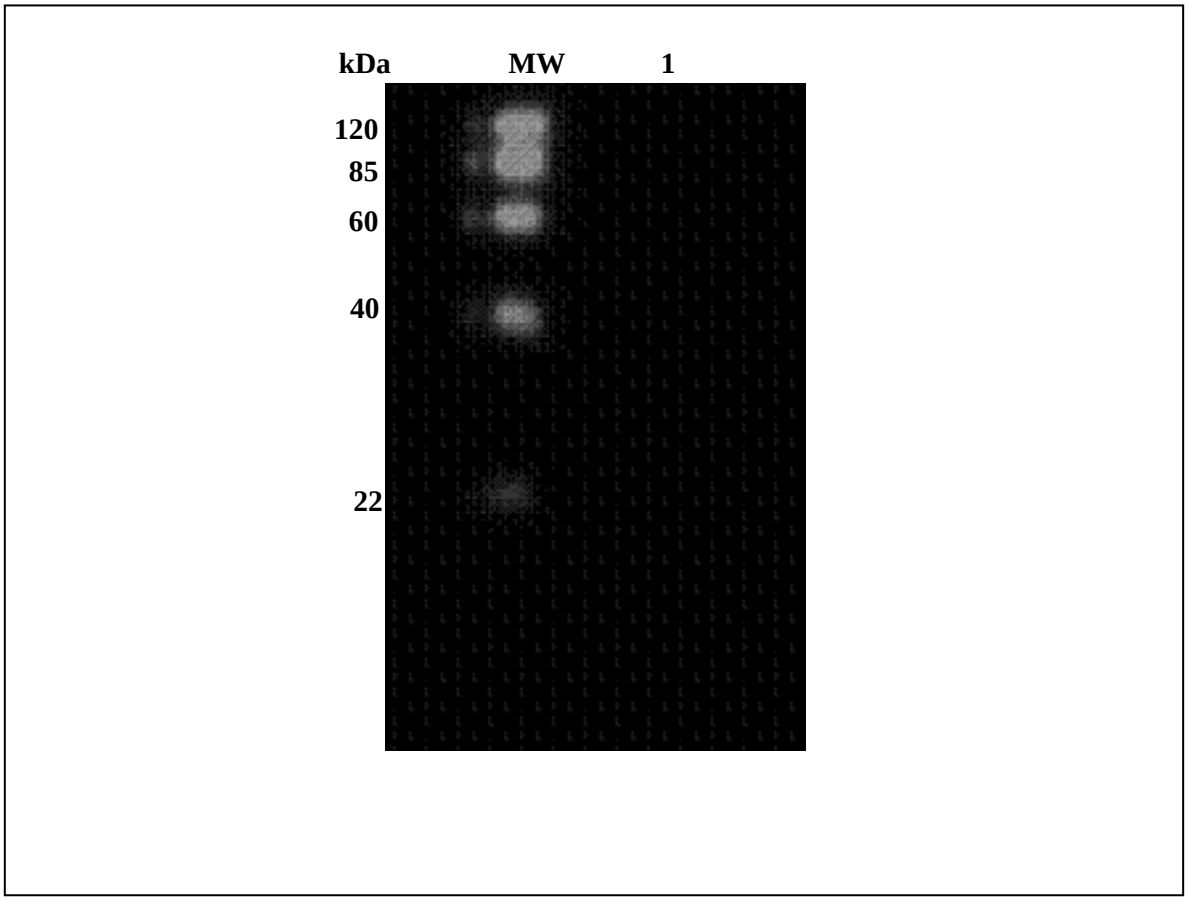
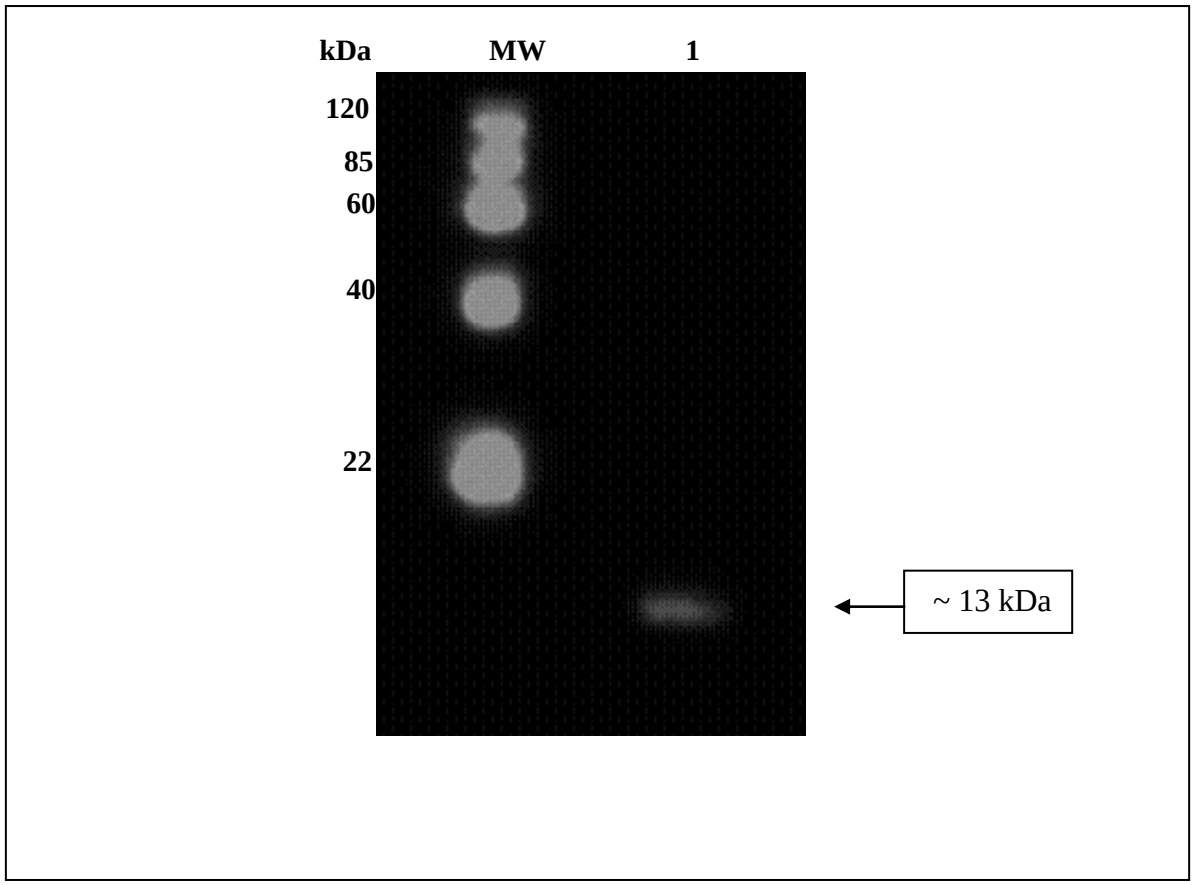
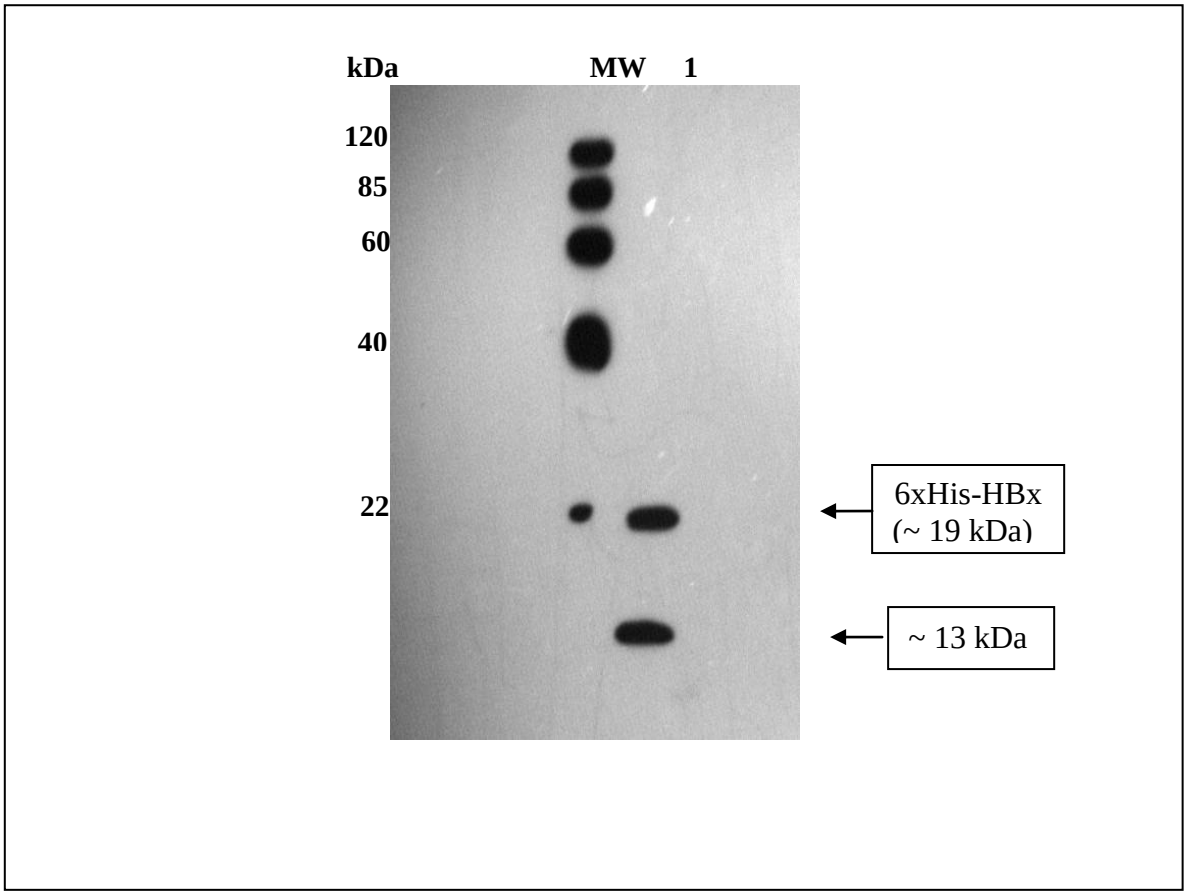


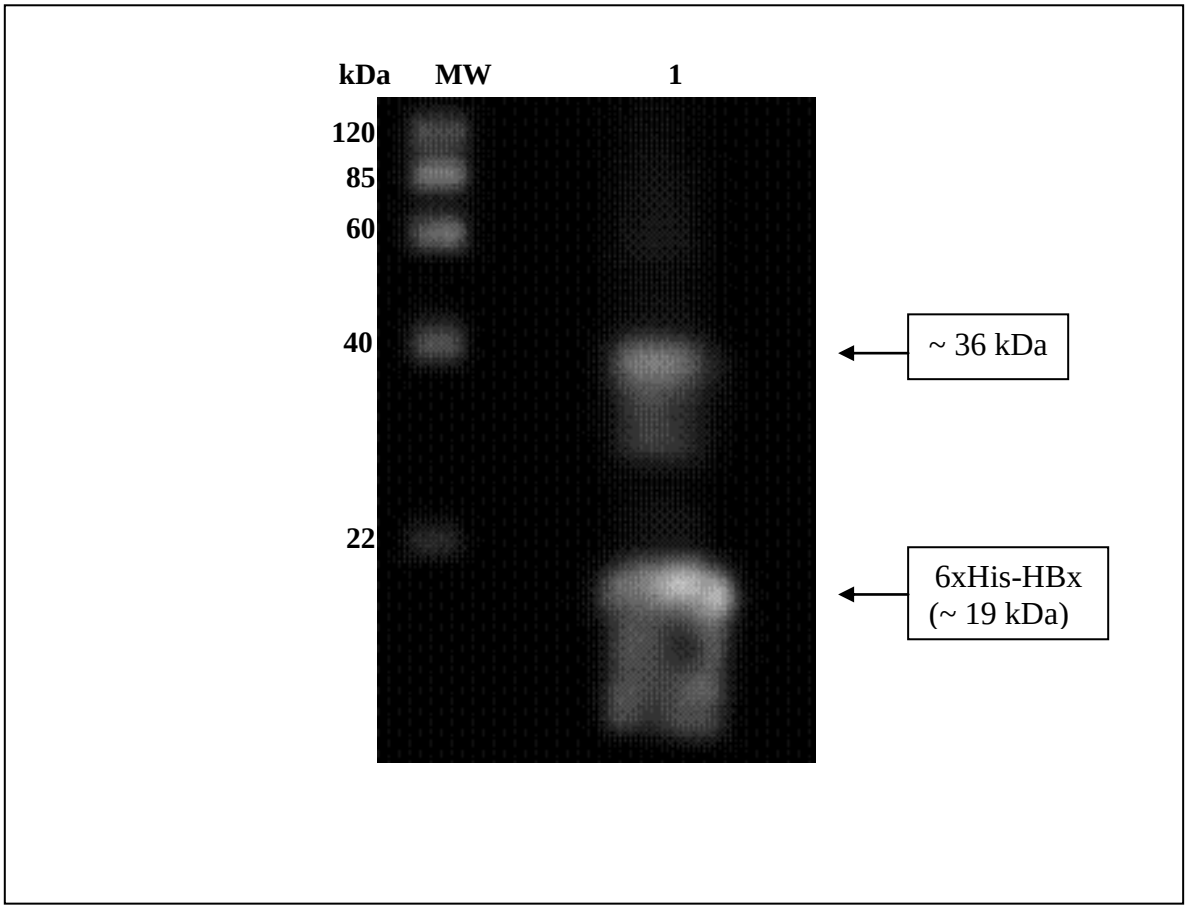

**Figure 3.8: a) Immunoblot of pooled hybridoma supernatants.** MW (Molecular weight): IgG protein marker. Lane 1 was loaded with HBx (0.68 mg/ml) and treated with a 1/10 dilution of a pooled hybridoma sample. Exposure time for signal development was thirty minutes.




**b) Immunoblot of the "6.1" supernatant.** MW: IgG protein marker. Lane 1 was loaded with HBx (0.68 mg/ml) and treated with a 1/100 dilution of the clonal supernatant, "6.1". Exposure time for signal development was thirty minutes.




**c) Immunoblot of the "6.1" supernatant.** MW: IgG protein marker. Lane 1 was loaded with HBx (0.68 mg/ml) and treated with a 1/50 dilution of the clonal supernatant, "6.1". Exposure time for signal development was ten minutes.




**d) Immunoblot of the commercial HBV anti-HBx antibody.** MW: IgG protein marker. Lane 1 was loaded with HBx (0.68 mg/ml) and treated with a 1/5000 dilution (of a 1 mg/ml stock solution) of the commercial HBV anti-HBx Ab. Exposure time for development of signal was five minutes.

relation to the protein marker *(Figure 3.1)*.

[One band was observed in the immunoblot of the clonal supernatant, "6.1" *(Figure 3.8 b)* but did not correspond to the approximate molecular weight of 6xHis-HBx (~ 19 kDa). The band was found below 19 kDa and suggests a breakdown product. A similar result was observed in an immunoblot of clonal supernatant "6.1" from a separate experiment *(Figure 3.8 c)*. In this blot both the ~ 19 kDa band and the suggested breakdown product were observed. Two bands were observed in the immunoblot with the commercial anti-HBx Ab *(Figure 3.8 d)*. Even at a 1/10 000 dilution additional bands were still observed (data not shown). Bands found below the position of HBx suggest HBx breakdown products, while the band above ~ 19 kDa suggests a dimer of the 17 kDa HBx protein (~ 36 kDa).

Our findings can be explained by results obtained by Feitelson & Clayton (Feitelson & Clayton, 1990). When conducting a study to screen for HBxAg reactive material in HBV-infected individuals, they identified an approximately 17 kDa and an approximately 13 kDa band in some patients' sera (Feitelson & Clayton, 1990). They found that cleavage of the 17 kDa HBxAg may result in the appearance of the 13 kDa HBxAg. This has also been reported in other findings (McLachlan, 1991); cleavage of HBc antigen gave rise to HBe antigen (Feitelson & Clayton, 1990). The 17 kDa HBxAg was, however, the dominant polypeptide. Feitelson & Clayton also reported on the presence of an approximately 36 kDa polypeptide species in several sera, which it claimed to be consistent with a dimer of the 17 kDa HBxAg (Feitelson & Clayton, 1990).

Some of our blots failed to yield a visible HBx protein band. One reason may be that antigens electrophoresed in gels containing SDS will be denatured, leading to failure to recognize their respective epitopes (Campbell, 1984)].

Since the pooled sample only yielded a positive result in the Indirect ELISA, "6.1" and the commercial anti-HBx antibody were characterised and paired in the Sandwich ELISA.

## 3.4 Characterisation of monoclonal antibodies

Procedures used in the characterisation of monoclonal antibodies vary widely with their intended use. It is highly unlikely that a single mAb developed will have all the required characteristics; therefore it is advisable to screen a large panel of mAbs (Campbell, 1984).

In this study, antibodies were characterised according to their binding affinities and relative epitope specificity. These characteristics are important in the application of the antibody, even more so when more than one mAb is used in the same assay. In the development of a Sandwich ELISA, it is imperative to obtain mAbs with non-overlapping epitope specificity.

### 3.4.1 Relative binding affinity

Affinity has been described as "the strength of interaction between an antibody and an antigen in a given system" (Liddell & Cryer, 1991). Antibodies with high binding

affinities are essential in immunoassays as they have to endure vigorous washing procedures and still remain attached to the target antigen/antibody. Clonal supernatants eliciting a strong positive result whilst having a low antibody level are likely to have a high binding affinity (Campbell, 1984).

Determination of binding affinity was carried out using the Indirect ELISA *(See Figure 3.7).* The high absorbance obtained in the Indirect ELISA suggests that the respective mAbs were able to bind to the HBx protein with a high binding affinity. Absorbance values obtained compared well with those of the commercial anti-HBx Ab *(Figure 3.9*) and those of serial dilutions of our developed Ab ("6.1"), shown in *Figure 3.10*.

### 3.4.2 Relative epitope specificity

Relative epitope specificity is an important aspect of epitope analysis. Its relevance comes into play when determining the intended use of the monoclonal antibody. For instance, when dealing with more than one mAb in an assay, it is very important to take the combination of the mAbs to be used into consideration, so as to reduce cross-reactivity. Determination of the relative epitope specificity of the mAbs was carried out using the Competitive ELISA, a necessary procedure, in order to determine whether antibodies are directed at the same or different epitopes.

The results obtained from a competitive ELISA of our previously developed mAb ("6.1") and the commercial anti-HBx Ab are shown in *Figure 3.11 a*. Our previously developed

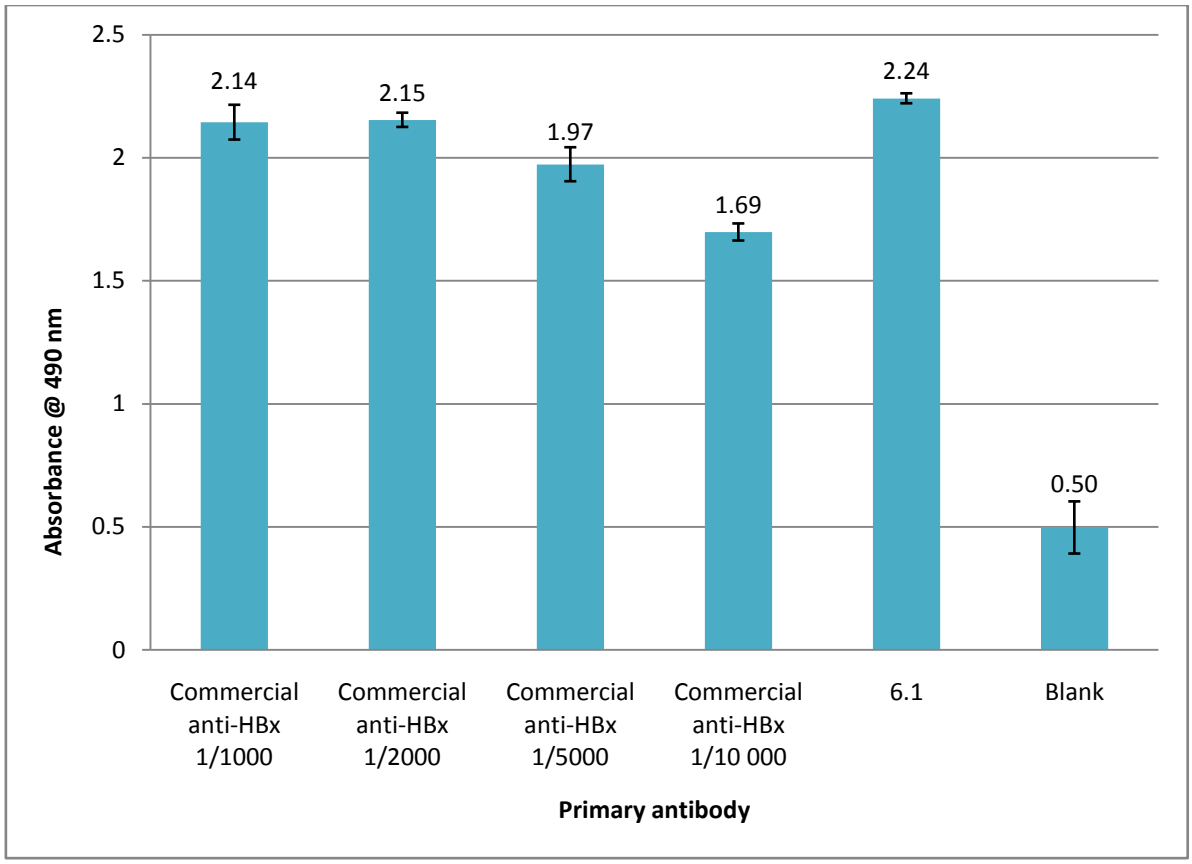



**Figure 3.9: Absorbance values of the commercial anti-HBx antibody at increasing dilutions.** Quantitative analysis was carried out using the Indirect ELISA. "6.1" was obtained from a clonal supernatant and deployed undiluted.

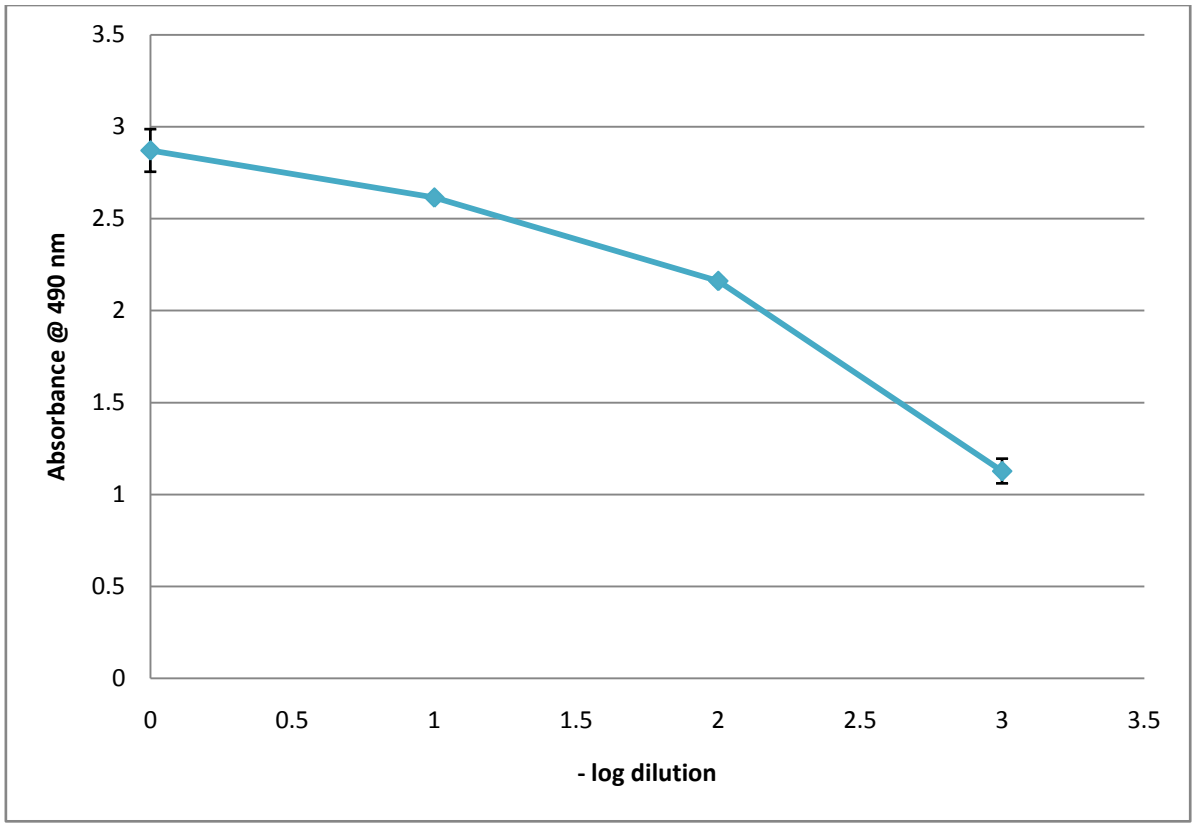



**Figure 3.10: Serial dilutions of the developed antibody "6.1".** Quantitative analysis was carried out using the Indirect ELISA. The HBV X protein had a concentration of 0.48 mg/ml.

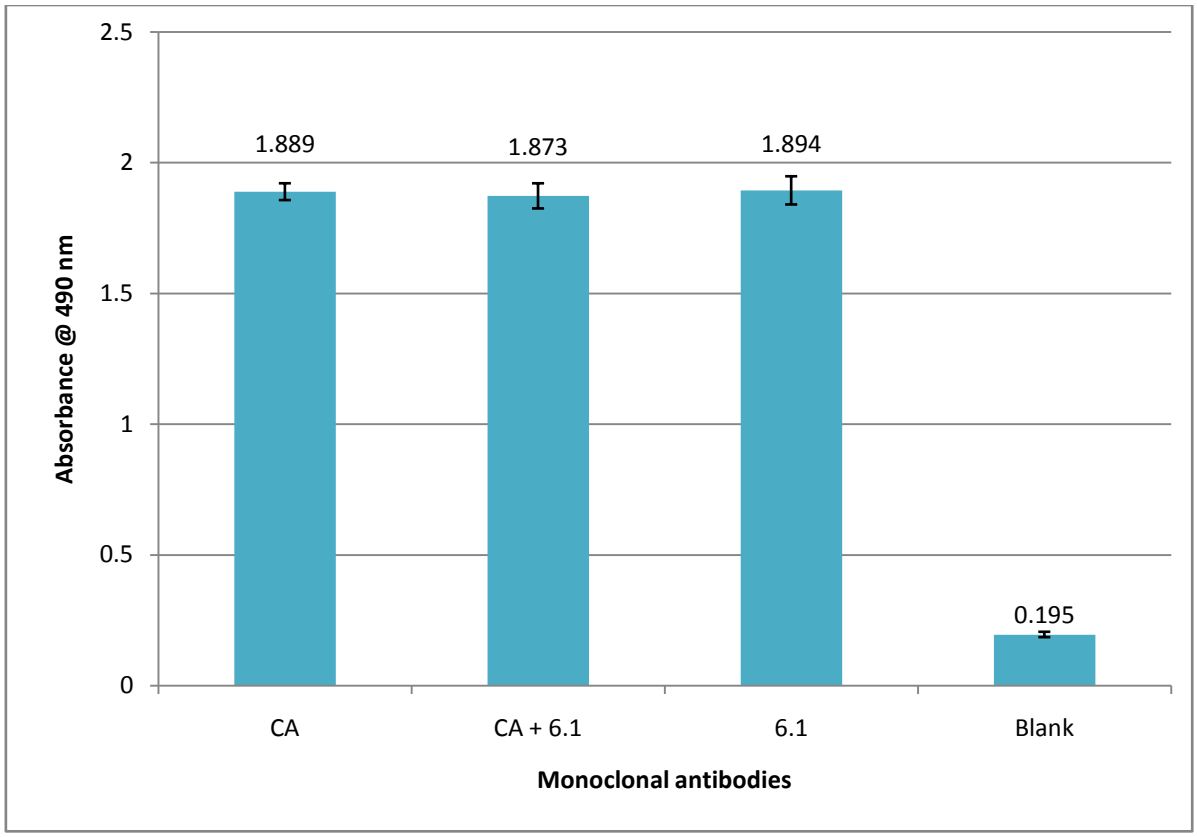



**Figure 3.11: a) Competitive ELISA of "6.1" and CA to assess relative epitope specificity.** 6.1: monoclonal antibody "6.1" and CA: commercial anti-HBx Ab. When antibodies were added together, 50 µl of each mAb was added, making a total of 100 µl primary antibody added, the same total volume used when tested singly. "6.1" was used undiluted and the commercial antibody was used at a 1/1000 dilution of a 1 mg/ml stock solution.

mAb ("6.1") was also competed with clonal supernatants from the first "limiting dilution" of a subsequent fusion (6, 10, 13 and 15), the results being shown in *Figure 3.11 b* and *Figures C1* to *C9* (Appendix C). The absorbance values of some combined mAbs were similar to when they were added individually. This was true for "6.1" and CA (commercial anti-HBx Ab), "mAb1" & "mAb2", "mAb2" & "mAb4", "mAb3" & "mAb4", "mAb1" & "6.1" and "mAb1" & "mAb4" *(Figures 3.11 a, C1, C2, C3, C4* and *C9)*. These results were indicative of competition between the respective antibodies. This then translates into them sharing the same epitope and/or their binding sites too close together for them to bind independently of each other.

On the other hand, the absorbance values for the combination of "mAb4" & "6.1", "mAb2" & "6.1", "mAb2" & "mAb3", "mAb3" & "6.1" and "mAb1" & "mAb3" were greater than of those added individually *(Figure 3.11 b* and *Figures C5, C6, C7* and C8 (Appendix C)). According to Kuffner *et al*. (Kuffner *et al*., 1988), this is indicative of absence of competition between the antibodies, showing their binding to different epitopes (Kuffner *et al*., 1988); however a degree of overlapping epitope specificity may be possible.

Other methods used to identify whether mAbs recognize the same epitope on an antigen include FPLC (Fast Protein Liquid Chromatography) Superose 12 gel filtration (Karande *et al*., 1987), a competition assay where the binding of a labelled antibody to an antigen is blocked by a second antibody (Stähli *et al*., 1983) and a previously developed screening ELISA (Alsenz & Loos, 1988). The screening assay is rapid, simple and requires no labelled antigen or antibody. The antigen is pre-incubated with a mAb and then added to a

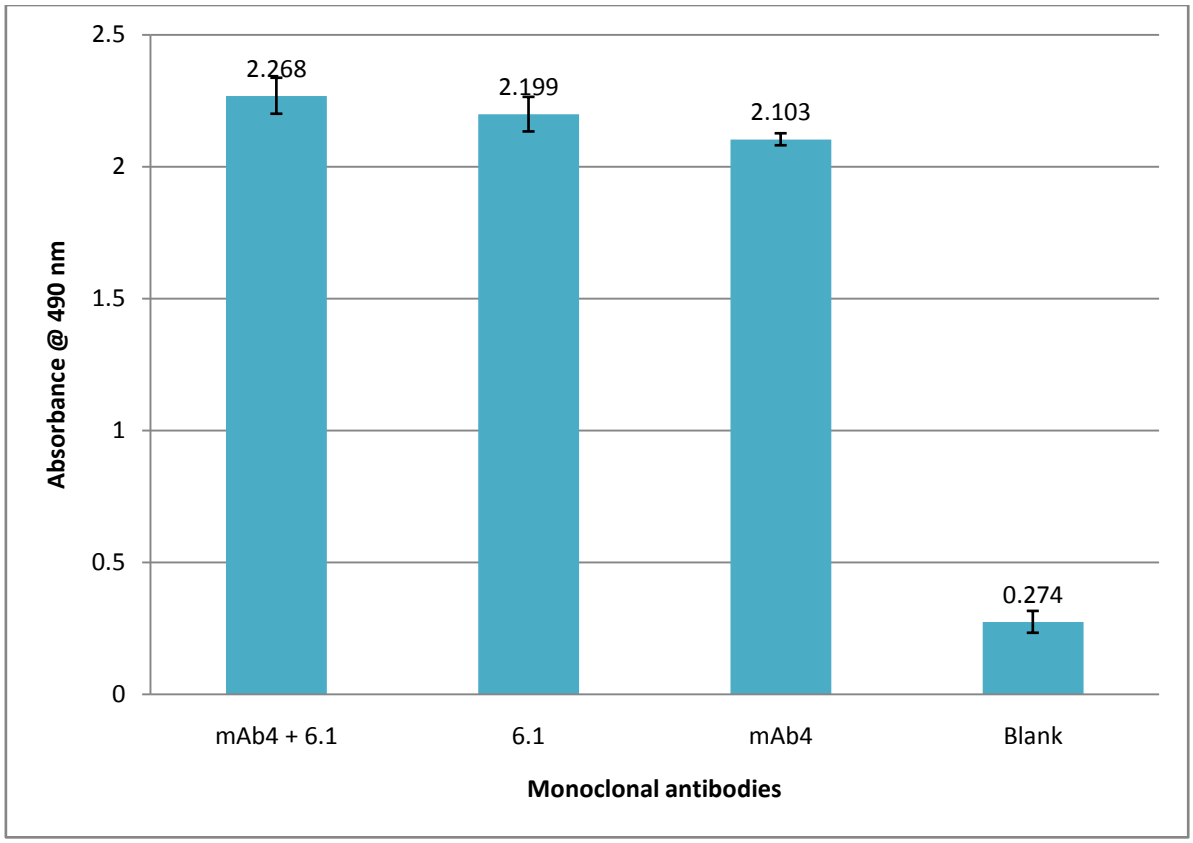



**Figure 3.11: b) Competitive ELISA of "6.1" and mAb4 to assess relative epitope specificity.** 6.1: monoclonal antibody "6.1" and "mAb4": clonal supernatant from the first "limiting dilution". When antibodies were added together, 50 µl of each mAb was added, making a total of 100 µl primary antibody added, the same total volume used when tested singly. Both "mAb4" and "6.1" were used undiluted.

microtitre plate coated with a second mAb. The principle behind the screening ELISA is based on the theory that the antigen pre-incubated with a mAb will not be recognized by a 'capture' antibody if the first mAb has the same epitope specificity as the second mAb added.

Not all the clonal supernatants were found having the same epitope specificity. Some bound to the same epitope while others bound to mutually exclusive epitopes. For the purpose of this research, the binding of antibodies to different epitopes was not crucial, as long as their combination allowed for efficient functioning in a Sandwich ELISA.

## 3.5 Antibody purification

Purification of antibodies was necessary to rid the clonal supernatants of contaminating γ-globulins from the foetal calf serum so as to allow for optimal binding of HBX-specific mAb to the ELISA plate in the Sandwich ELISA.

The first step in antibody purification was precipitation by ammonium sulphate (AS) precipitation. This method was chosen because of its general applicability and that it is far less expensive than Protein A purification (for mouse and human IgG). Up to the time of commencing this report, only our previously developed mAb ("6.1") was purified, by the method specified in the Materials and Methods (section 2.7). It was paired with the commercial anti-HBx Ab in the Sandwich ELISA.

## 3.6 Design of the Sandwich ELISA

Two antibodies are necessary for a Sandwich ELISA, one as the 'capture' Ab and the other as the 'detecting' Ab. The 'capture' Ab, which may or may not be monoclonal, is bound to the solid phase, and then antigen is added followed by the 'detecting' Ab which may or may not bind to the antigen on another site (Campbell, 1984). In this study, both "6.1" and the commercial anti-HBx Ab were tested as the 'capture' or 'detecting' Ab. Results of which are discussed in section 3.6.1.

### 3.6.1 Optimisation of the Sandwich ELISA

The following parameters were tested in the Sandwich ELISA: sequence of the 'capture' and 'detecting' Ab, blocking buffer, coating buffer, concentrations of the HBV X protein, and the concentrations of the secondary antibody and substrate.

Development of this type of ELISA requires the careful selection of a complementary pair of mAbs. A schematic representation of this assay is shown in *Figure 3.12*. Some mAbs have been reported to function better as 'detecting' antibodies than as 'capture' antibodies (Kuffner *et al.*, 1988). This could be caused by partial "denaturation" of antibodies when adsorbed to the solid matrix (microtitre plate), rendering them ineffective for antigen capture (Kuffner *et al.*, 1988).

When the commercial anti-HBx Ab was used as the 'capture' Ab, "6.1" worked well as the

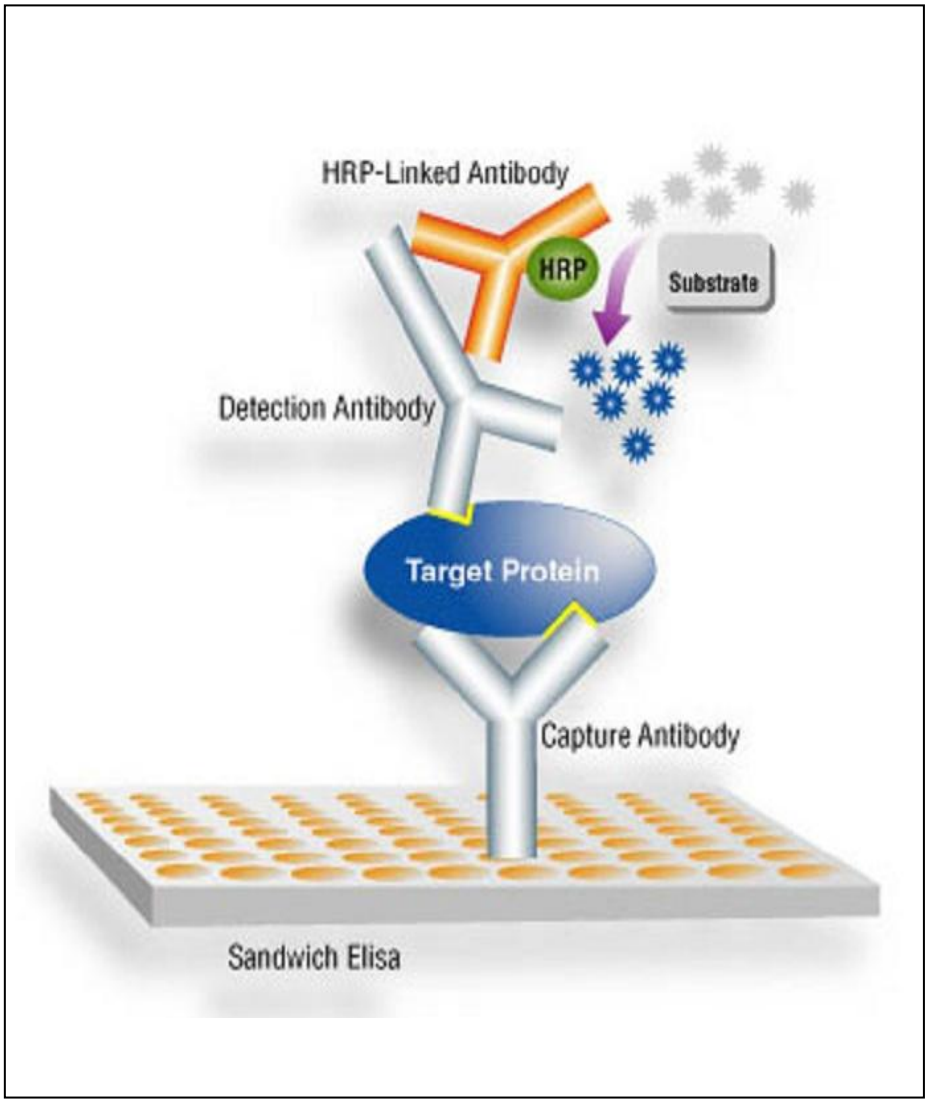



**Figure 3.12: A schematic diagram of a Sandwich ELISA.**

(newenglandbiolabs.de/…/cst_elisa_gross.jpg)

'detecting' Ab *(Figure 3.13 a).* A good result was also obtained when the commercial anti-HBx Ab was the antibody in both positions. When the roles of these two antibodies were reversed, the result was less satisfactory *(Figure 3.13 b).* Obtaining 'capture' and 'detecting' antibodies that work well together allows for the efficient binding and quantification of the target antigen.

The coating buffer used to prepare the 'capture' Ab for coating may also have an effect. Secondary antibody has the ability to bind to both the 'capture' and 'detecting' Abs but in this assay, through the careful selection of buffer, it should only detect the 'detecting' antibody and not the 'capture' Ab. Therefore, the absorbance obtained should only reflect the amount of 'detecting' antibody bound to antigen and not the amount of 'capture' Ab.

The absorbance values obtained when phosphate buffered saline (PBS) was used as the coating buffer are shown in *Figure 3.14 a.* An absorbance value of approximately 1.85 was obtained when "6.1" was added as the 'detecting' Ab. Likewise, when RPMI/10 % FCS (blank) was added instead of the 'detecting' Ab, a similar absorbance value was obtained. Hence, the secondary antibody added bound to the Fc region of the 'capture' Ab. With bicarbonate as the coating buffer *(Figure 3.14 b),* a very low absorbance was obtained when RPMI/10 % FCS (blank) was added in place of the 'detecting' Ab. When "6.1" was added as the 'detecting' Ab, a final absorbance value of greater than 1.00 was obtained and an absorbance values of 0.04 when RPMI/10 % FCS (blank) was added, indicating that the secondary antibody was detecting "6.1" and not the 'capture' Ab. Bicarbonate was, therefore, chosen as the coating buffer in our Sandwich ELISA.

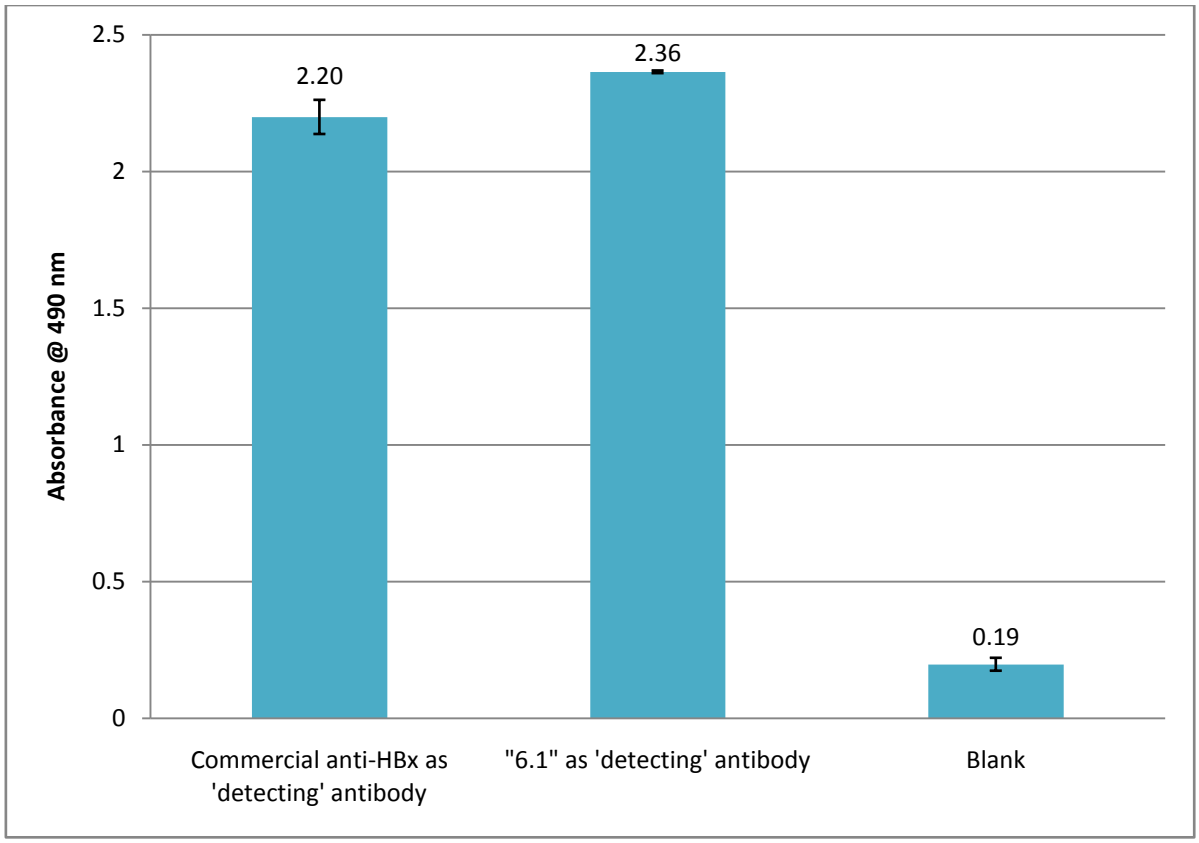
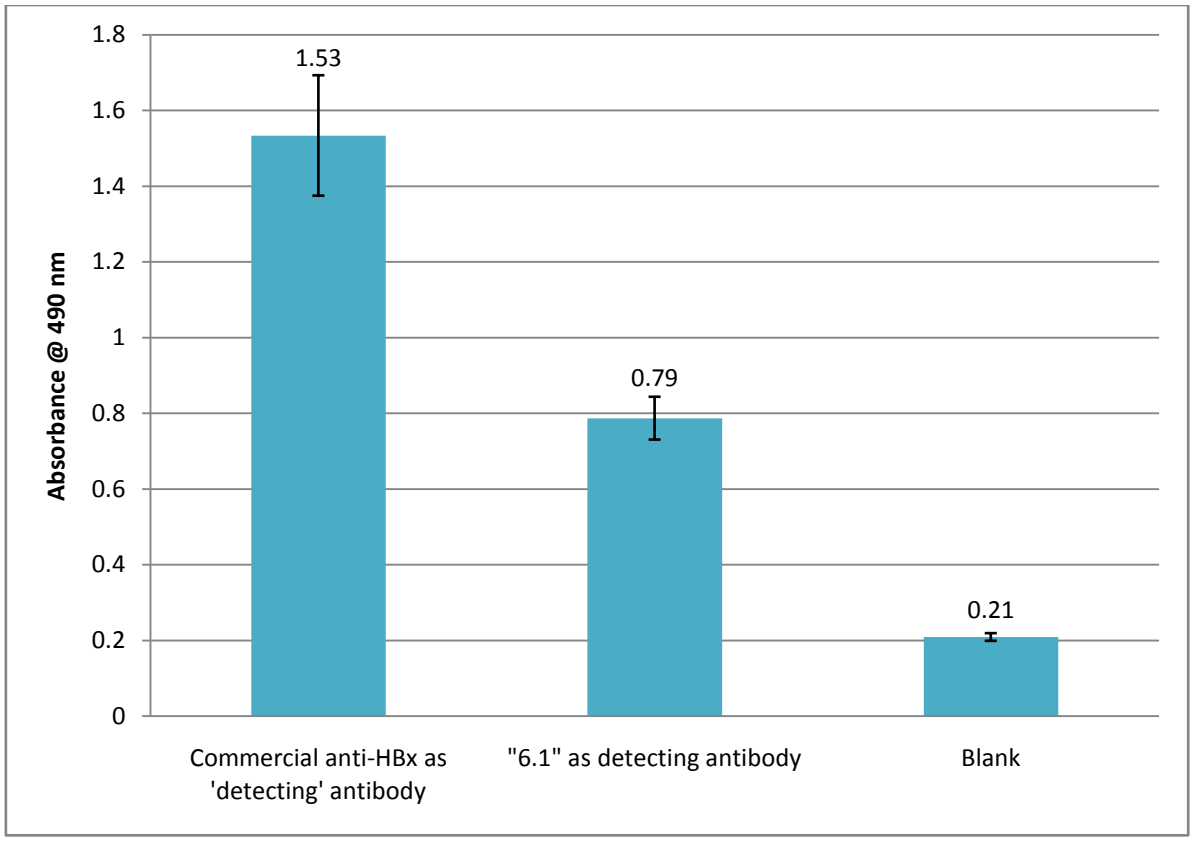



**Figure 3.13: a) Sandwich ELISA to select a preferred antibody for the 'detecting' position.** The commercial anti-HBx Ab was used as the 'capture' Ab at a 1/200 dilution (of a 1 mg/ml stock solution). Our previously developed Ab, "6.1", was used at a 1/100 dilution of a 3 mg/ml stock solution.




**b) Sandwich ELISA to select a preferred antibody for the 'detecting' position.** Our previously developed Ab ("6.1") was used as the 'capture' Ab at a 1/100 dilution of a 3 mg/ml stock solution. The commercial anti-HBx Ab was deployed at a 1/200 dilution (of a 1 mg/ml stock solution).

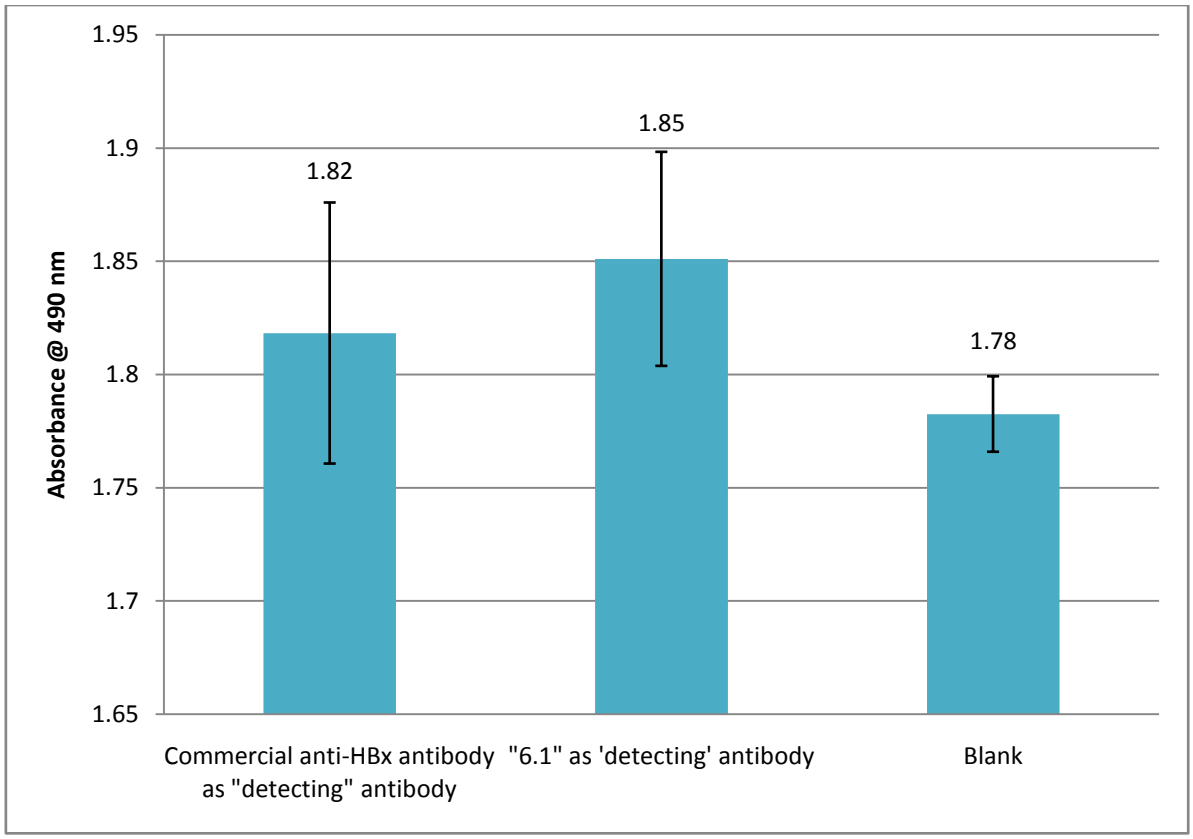
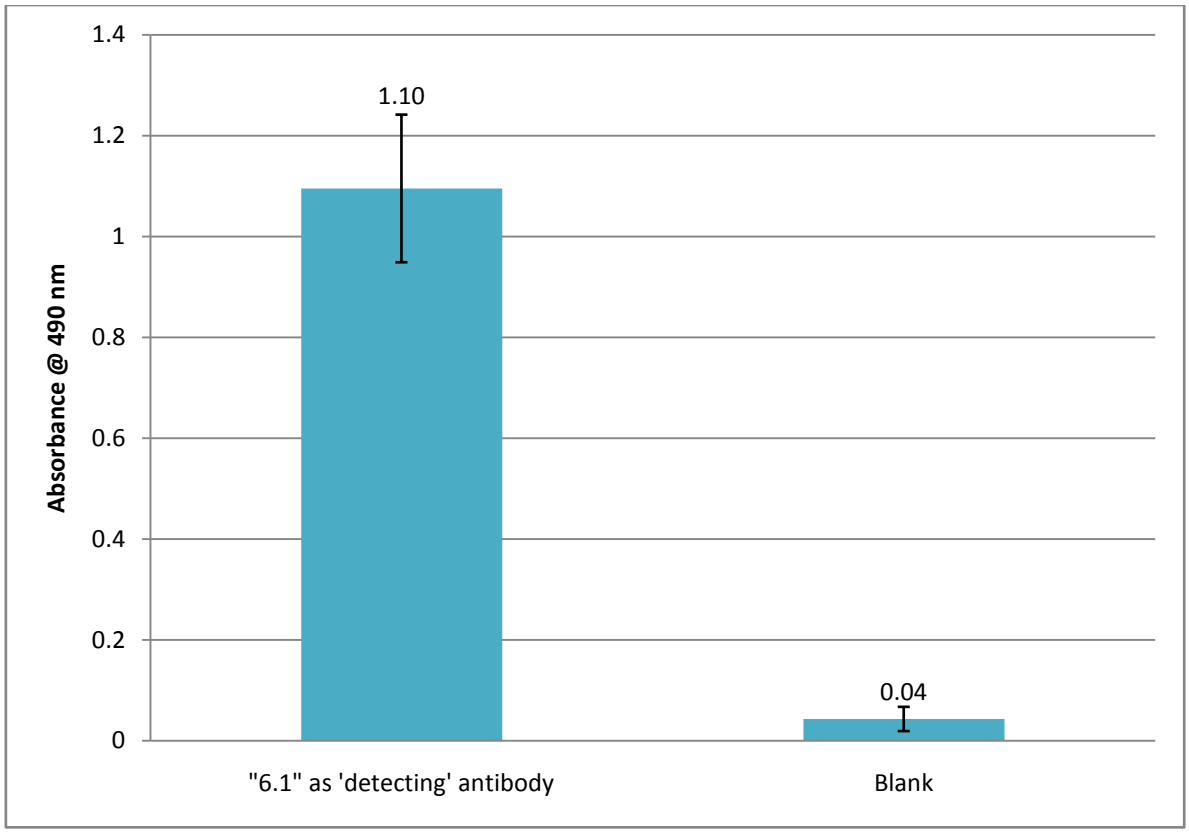



**Figure 3.14: a) Sandwich ELISA with PBS used as the coating buffer.** The commercial antibody was used as the 'capture' Ab at a 1/200 dilution (of a 1 mg/ml stock solution) and "6.1" as the 'detecting' antibody at a 1/100 dilution of a 3 mg/ml stock solution. The high absorbance value obtained for the blank showed that secondary antibody was detecting the 'capture' Ab.




**b) Sandwich ELISA with bicarbonate buffer used as the coating buffer.** The commercial antibody was used as the ‘capture’ Ab at a 1/200 dilution (of a 1 mg/ml stock solution) and “6.1” as the ‘detecting’ Ab at a 1/100 dilution of a 3 mg/ml stock solution. The low absorbance value obtained for the blank showed that secondary antibody was not detecting the ‘capture’ Ab.

When using solid-phase assays, blocking of possible remaining binding sites on the microtitre plate (to prevent non-specific adsorption of proteins) is essential (Campbell, 1984). Commonly used blocking agents include BSA, non-fat milk powder and gelatine (Campbell, 1984). In this study, 2 % BSA, 2 % non-fat milk powder and RPMI/10 % FCS were tested in determining the optimal blocking agent in the Sandwich ELISA (data not shown). In all cases, BSA yielded the lowest blank absorbance values, followed by RPMI/10 % FCS, while 2 % non-fat milk powder gave rise to the highest blank absorbance values, therefore, 2 % BSA was used as the blocking agent in our Sandwich ELISA.

A high blank absorbance is indicative of the secondary antibody detecting the 'capture' Ab as no 'detecting' Ab is added. On the other hand, a low blank absorbance value indicates that the secondary antibody is not binding to the 'capture' Ab.

The absorbance values obtained with antigen at various dilutions are shown in *Figure 3.15*. A straight-line relationship between the absorbance and the log of the concentration is well-established in ELISAs. It clearly applies in our instance. In our Sandwich ELISA, HBx was used at a concentration of 0.68 mg/ml. In our laboratory, this assay is currently being adapted to detect HBx in serum.

The concentration of the 'capture' Ab used in our assay was somewhat in excess of the putative binding capacity of the ELISA plate plastic. The commercial anti-HBx Ab was used as the 'capture' Ab, at a 1/200 dilution of a 1 mg/ml stock solution, while "6.1" was used at a 1/100 dilution of 3 mg/ml stock solution as the 'detecting' Ab.

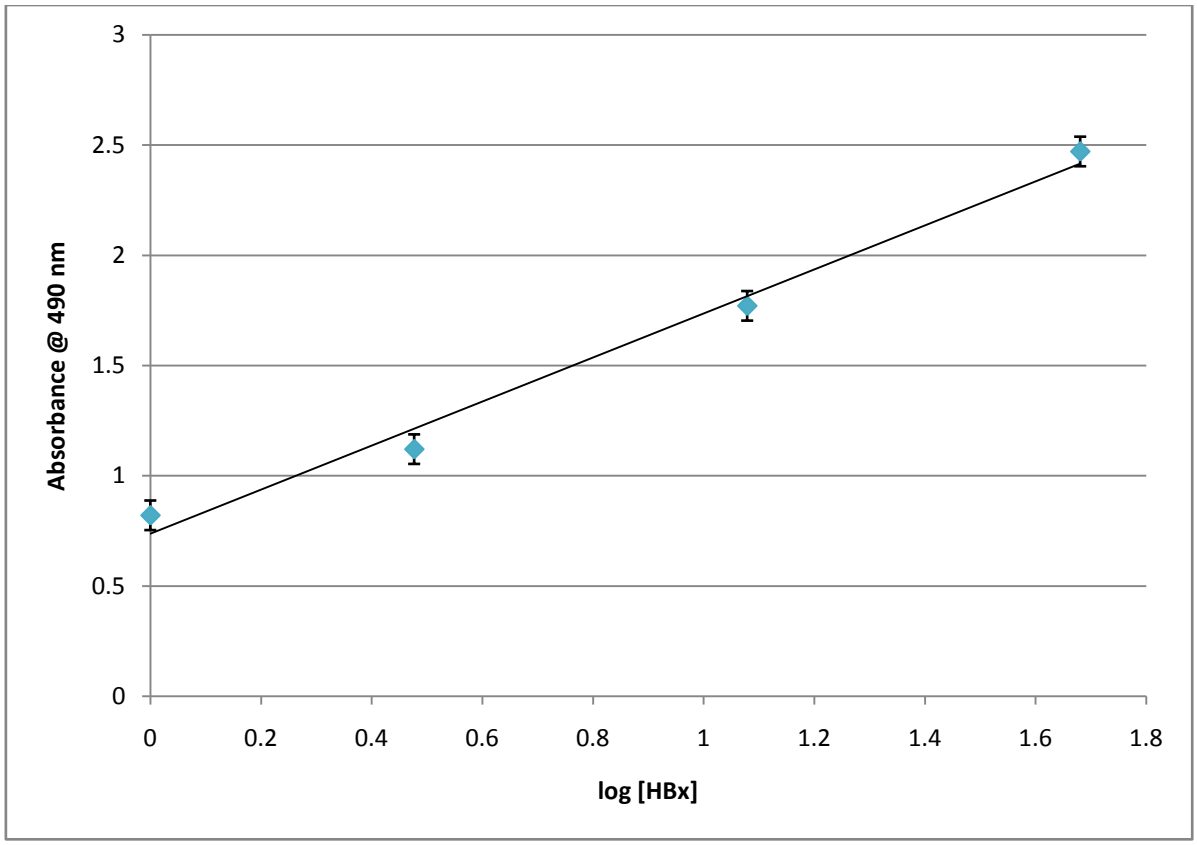



**Figure 3.15: Sandwich ELISA with antigen at various dilutions.** The commercial antibody was used as the 'capture' Ab at a 1/200 dilution of a 1 mg/ml stock solution and the previously developed antibody, "6.1", was used as the 'detecting' Ab at a 1/100 dilution (of a 3 mg/ml stock solution). Graph shows 'best fit' of experimental points.

The concentration of the secondary antibody and substrate were kept standard, following the protocol for the Indirect ELISA (see Materials and Methods, 2.6.1).

Coupling the 'detecting' antibody to biotin has been reported to produce an enhanced signal as compared to the simpler HRP-labelled antibody method (van Gijlswijk *et al.*, 1996). The 'detecting' Ab in this study was not labelled in any way; instead a HRP-labelled secondary antibody was employed to detect the 'detecting' antibody. Biotin-avidin detection systems are also reported as being more sensitive than simple HRP systems. Some researchers have also reported the use of polyclonal or multiple mAbs as the 'capture' Ab (Tsitsilonis *et al.*, 2004). Polyclonal antibodies recognizing various epitopes on a particular antigen have been reported to eliminate non-specific cross-reaction and increase the overall sensitivity of the assay, but long-term reproducibility cannot be guaranteed.

# Chapter 4: CONCLUSION

We have successfully developed a Sandwich ELISA for HBx using a commercial anti-HBx antibody and an "in house" monoclonal antibody. Through Indirect ELISA and Competitive ELISA we revealed that our mAb "6.1" and the commercial anti-HBx bound to HBx with a high affinity and recognizing the same or closely-related epitope/s on HBx. Both mAbs also showed good reactivity with HBx on immunoblots. Our other hybridomas cloned by "limiting dilution" produced mAbs directed against HBx, with a binding affinity comparable to our mAb "6.1" (as evident on Indirect ELISA), but failed to react with HBx in immunoblots. PAGE under non-denaturing conditions might prove otherwise.

Since commencing this dissertation, three more monoclonal antibodies were generated in our laboratory. We are planning to deploy these in a diagnostic ELISA testing for HBx in patient sera.

We are confident of developing a Sandwich ELISA applicable to patient sera which would allow for the early identification of carriers at risk of developing hepatocellular carcinoma. Since a commercial ELISA for the detection of the HBx protein in body fluids is not, to our knowledge, currently available, our proposed Sandwich ELISA would be of significant value in the evaluation of the disease status.

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# APPENDIX A: ETHICS APPROVAL

**STRICTLY CONFIDENTIAL**

**UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG**

**ANIMAL ETHICS SCREENING COMMITTEE**

**CLEARANCE CERTIFICATE NO:**

| 2005 | 14 | 3 |
|---|---|---|

**APPLICANT:** W Prinz

**DEPARTMENT:** Molecular Medicine & Haematology

**PROJECT TITLE:** Production of hybridoma clones secreting monoclonal antibodies against the X-Protein of the Hepatitis B Virus

| Species | Number | Expiry Date |
|---|---|---|
| Mice | 24 | Feb 2007 |
| | | |

i) **Approval** is hereby given for the experiment described in the above application. Subject to clarification of;
- the precise duties of the co-workers
- discussing with CAS their role in the study

The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

SIGNED D. A. Gray DATE: 1st March 2005
(Chairman: Animal Ethics Screening Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1)(c) of the Veterinary and Para-veterinary Professions Act (19 of 1982)

SIGNED _____ DATE: 1st March 2005
(Registered Veterinarian)

NOTE: First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available, and the procedures required by the CAS for the carrying out of experiments.

Feb 5
May 4
June 5
Aug 4
Dec 3

# APPENDIX B: SOLUTIONS AND CHEMICALS

## *B1. HBx/Protein preparation*

### Luria-Bertani (LB) Broth

- 10 g Tryptone
- 5 g Yeast extract
- 10 g NaCl
    - Make up to 1 litre with distilled water;
    - Autoclave;
    - Store at 4 ºC

### Lysis Buffer

- 20 mM $PO_4$, pH 7.8
- 2mM β-mercaptoethanol
- 1 % Nonidet P.40
- 1mM EDTA
- 100mM KCl

### Re-suspension Buffer

- 20 mM $PO_4$, pH 7.8
- 2mM β-mercaptoethanol
- 8 M Urea

- 10 mM Imidazole

## Buffer A

- 20 mM Na-phosphate, pH 7.5
- 8 M Urea
- 2 mM β-mercaptoethanol
- 10 mM Imidazole

## Buffer B

- 20 mM Na-phosphate, pH 7.5
- 8 M Urea
- 2 mM β-mercaptoethanol
- 60 mM Imidazole

## Buffer C

- 20 mM Na-phosphate, pH 7.5
- 7 M Urea
- 2 mM β-mercaptoethanol
- 600 mM Imidazole

## *B2. Tissue culture*

### RPMI

- 32.88 g 1640 RPMI (Gibco)
- 6.9 g HEPES (Sigma)
- 6.3 g $NaHCO_3$
- 0.2 g Penicillin G (Sigma)
- 0.33 g Streptomycin (Sigma)
- 315 mg Glutamine (Sigma)
    - Dissolve gently in 3150 ml distilled water using a magnetic stirrer;
    - Sterilize by filtration through a 22 µm filter (Osmonics Inc) and two prefilters (Millipore Corporation);
    - Place bottles containing filtered RPMI in a 37 °C incubator overnight to check for sterility.

### RPMI/10 % FCS

- 100 ml heat-inactivated foetal calf serum
- 900 ml RPMI
    - Store at 4°C.

### HAT and HT stock solution

- Purchased from GIBCO

### OPI stock solution (100x)

- 1.5 g Oxaloacetate
- 500 mg Pyruvate
- 82 mg (2000 IU) Insulin
    - Make up to 100 ml with distilled water

### Freezing Medium

- 28 ml RPMI (without FCS)
- 17 ml FCS
- 5 ml DMSO

## *B3. Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

### Monomer solution

- 60 g acrylamide
- 1.6 g bisacrylamide
    - Make up to 200 ml with distilled water;
    - Store for up to 3 months at 4°C in the dark.

### 4 X Running gel buffer (1.5 M Tris-Cl, pH 8.8)

- 36.3 g Tris
    - Add 150ml with distilled water;

- Adjust pH with HCl;
- Make up to 200 ml with distilled water;
- Store for up to 3 months at 4°C in the dark.

## 4 X Stacking Gel Buffer (0.5 M Tris-Cl, pH 6.8)

- 3.0 g Tris
  - Add 40 ml distilled water;
  - Adjust pH with HCl;
  - Make up to 50 ml with distilled water;
  - Store for up to 3 months at 4°C in the dark.

**Table 1:** The ratios of mixtures for a 15 % running gel (for 4 gels)

| **Mixtures** | |
|---|---|
| Monomer solution | 22.5 ml |
| 4 X Running gel buffer | 11.25 ml |
| 10 % SDS | 450 µl |
| Distilled water | 10.65 ml |
| Ammonium persulphate | 300 µl |
| TEMED* | 25 µl |

* N,N,N,N – Tetramethyl-ethylenediamine

**Table 2:** The ratios of mixtures for a 4 % stacking gel (for 4 gels)

| **Mixtures** | |
|---|---|
| Monomer solution | 4 ml |
| 4 X Stacking gel buffer | 7.5 ml |
| 10 % SDS | 300 µl |
| Distilled water | 18 ml |
| Ammonium persulphate | 210 µl |
| TEMED* | 22.5 µl |

* N,N,N,N – Tetramethyl-ethylenediamine

## 10 % SDS

- 10 g SDS
    - Make up to 100 ml with distilled water;
    - Store for up to 6 months at room temperature.

## 10 % Ammonium persulphate (Initiator)

- 0.1 g ammonium persulphate
    - Make up to 1.0 ml with distilled water;
    - Use fresh; do not store.

## 2 X Loading buffer (pH 6.8)

- 2.5 ml 4 X stacking gel buffer
- 4 ml 10 % SDS

- 2 ml glycerol or 2.3 ml 87 % glycerol
- 2.0 mg bromophenol blue
- 0.31 g dithiothreitol (DTT)
    - Make up to 10 ml with distilled water;
    - Store for up to 6 months in 0.5 ml aliquats at – 20 °C.

## Running buffer

- 3.03g Tris
- 14.4 glycine
- 1 g SDS
    - Make up to 1 litre with distilled water
    - Store at room temperature for up to 1 month.

## Coomassie stain

- 0.5 g Coomassie Blue R-250
- 250 ml isopropanol
- 100 ml acetic acid
    - Make up to 1 litre with distilled water;
    - Store at room temperature for up to 6 months.

## Destaining solution I

- 400 ml methanol
- 70 ml acetic acid

- Make up to 1 litre with distilled water;
- Store at room temperature indefinitely.

## Destaining solution II

- 50 ml methanol
- 70 ml acetic acid
    - Make up to 1 litre with distilled water;
    - Store at room temperature indefinitely.

## Silver stain

## Solution A

- 150 ml ethanol
- 36 ml glacial acetic acid
- 150 µl 37 % formaldehyde
    - Make up to 300 ml with MilliQ water.

## Solution B

- 30 mg sodium thiosulfate
    - Make up to 300 ml with MilliQ water.

## Solution C

- 300 mg silver nitrate
    - Make up to 300 ml with MilliQ water;

- Make up fresh.

## Solution D

- 9 g sodium carbonate
- 150 µl 37 % formaldehyde
- 6 ml Solution B
    - Make up to 300 ml with MilliQ water.

## Solution E

- 2.8 g EDTA
    - Make up to 300 ml with MilliQ water.

### *B4. Western/Immunoblotting*

## Transfer buffer

- 7.58 g Tris
- 36.03 g glycine
- 2.5 g SDS
    - Measure pH (8.2 – 8.4) but do not adjust;
    - Make up to 2 litres with distilled water;
    - Add 500 ml methanol;
    - Store at 4 º C.

## Tris buffered saline (TBS), pH 7.4

- 3.63 g Tris
- 13.5 g NaCl
    - Make up to 1400 ml with distilled water;
    - Measure and adjust pH to 7.4 with HCl;
    - Make up to 1500ml with distilled water;

## Protein blocking solution

- 25 g non-fat milk powder
    - Make up to 500 ml with TBS;
    - Add 5 ml of a 10 % sodium azide stock solution;
    - Store at 4 º C.

## Washing solution

- 1 g non-fat milk powder
    - Make up to 1 litre with TBS;
    - Add 10 ml of a 10 % sodium azide stock solution.

## Antibody incubation solution

- 2 g non-fat milk powder
- 1 ml of a 10 % Tween-20 stock solution
    - Make up to 200 ml with TBS;
    - Add 2 ml of a 10 % sodium azide stock solution.

## Substrate solution

- Kit substrate mixture (Pierce)
- 1:1 ratio of luminol and hydrogen peroxide
    - Make up fresh in a foil covered container;
    - Discard after use.

### *B5. Enzyme-linked immunosorbent assay (ELISA)*

## Coating buffer (pH 9.6)

- 1.59 g $Na_2CO_3$
- 2.93 g $NaHCO_3$
- 0.05 % sodium azide
- 0.1 % Tween-20 (pH 9.6)
    - Make up to 1 litre with distilled water.

## Washing solution

- 0.9 % NaCl
- 0.1 % Tween-20
    - Make up to 1 litre with distilled water.

## Dilution/blocking buffer

- 0.05 M Tris (pH 7.4)
- 2 % Bovine serum albumin (BSA, Sigma)

- 0.1 % Tween-20
- 0.02 % sodium azide
    - Make up to 250 ml with distilled water.

## Citrate buffer (0.1M, pH 5)

- 7.3 g citric acid·$H_2O$
- 9.5 g $Na_2HPO_4$ or
- 11.8 g $Na_2HPO_4·2H_2O$ or
- 20 g $Na_2HPO_4·12H_2O$
    - Make up to 1 litre with distilled water.

## Substrate solution

- 16 mg o-phenylenediamine dihydochloride (Sigma)
    - Make up to 30 ml with 0.1 M Citrate buffer;
    - Add 18 µl 30 % $H_2O_2$.

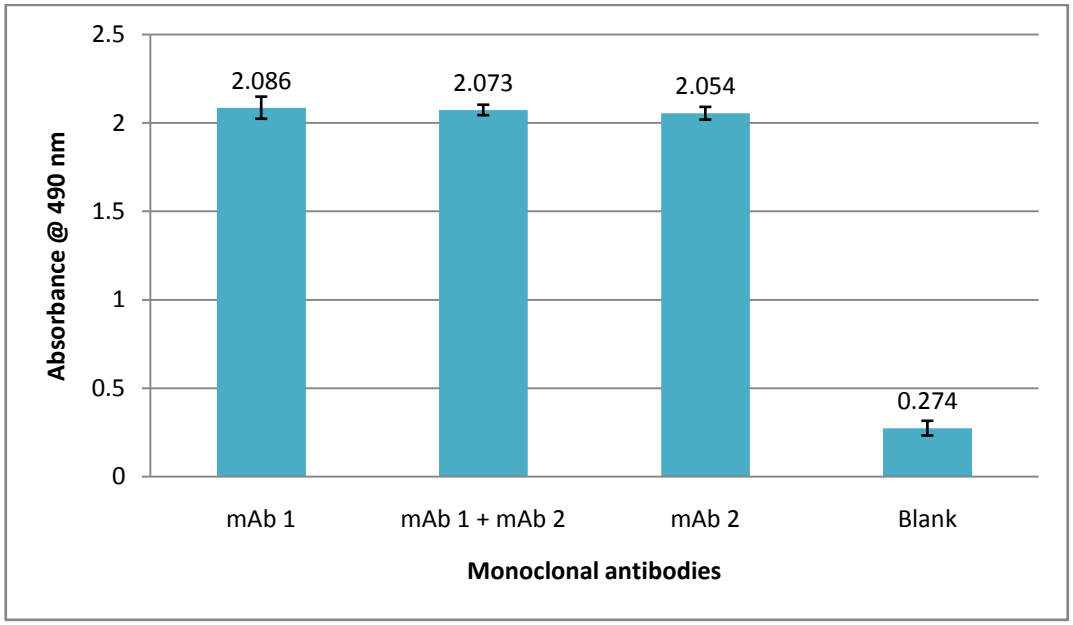
## Phosphate buffered saline (PBS), 10X

- 90 g NaCl
- 2.1 g KCl
- 2 g $KH_2PO_4$
- 12 g $Na_2H\ PO_4$
    - Make up to 1 litre with distilled water;
    - Store at room temperature.

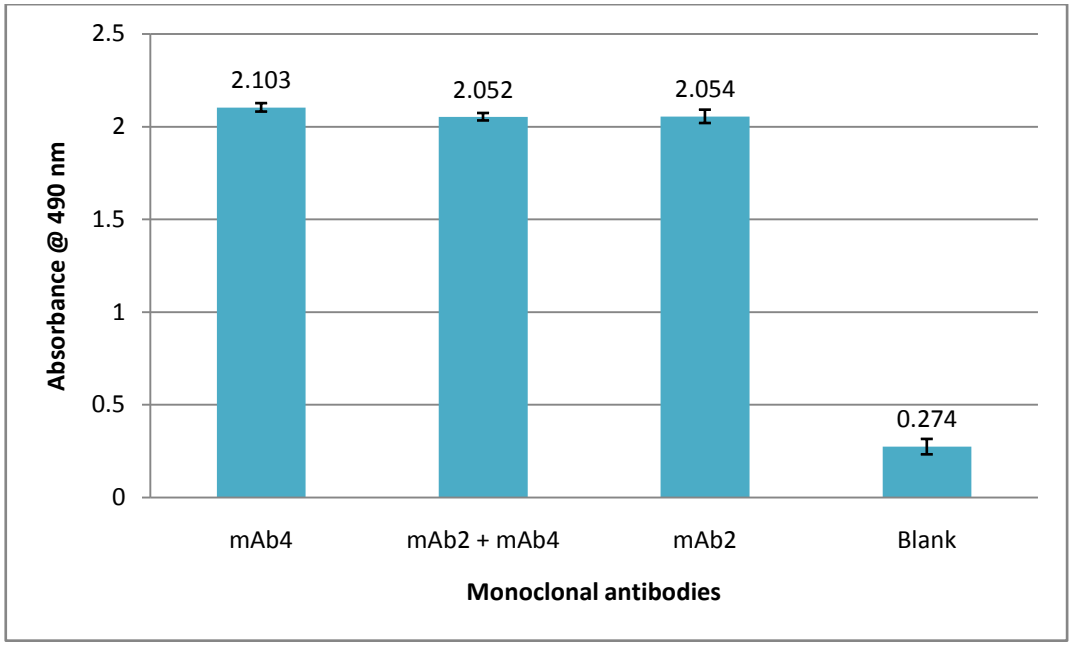
# APPENDIX C: FIGURES

**Figures C: Competitive ELISA to assess relative epitope specificity.** 6.1: monoclonal antibody "6.1", mAb1 – mAb4: different clonal supernatants from the first "limiting dilution". When antibodies were added together, 50 µl of each mAb was added, making a total of 100 µl primary antibody added, the same total volume used when tested singly.

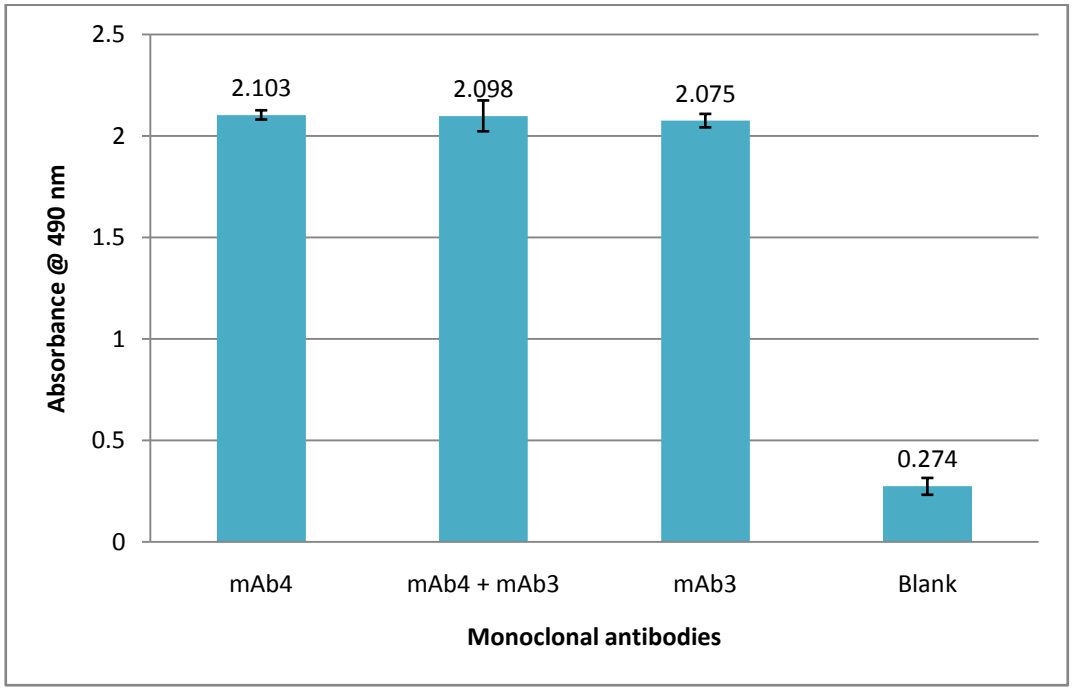
**C1**



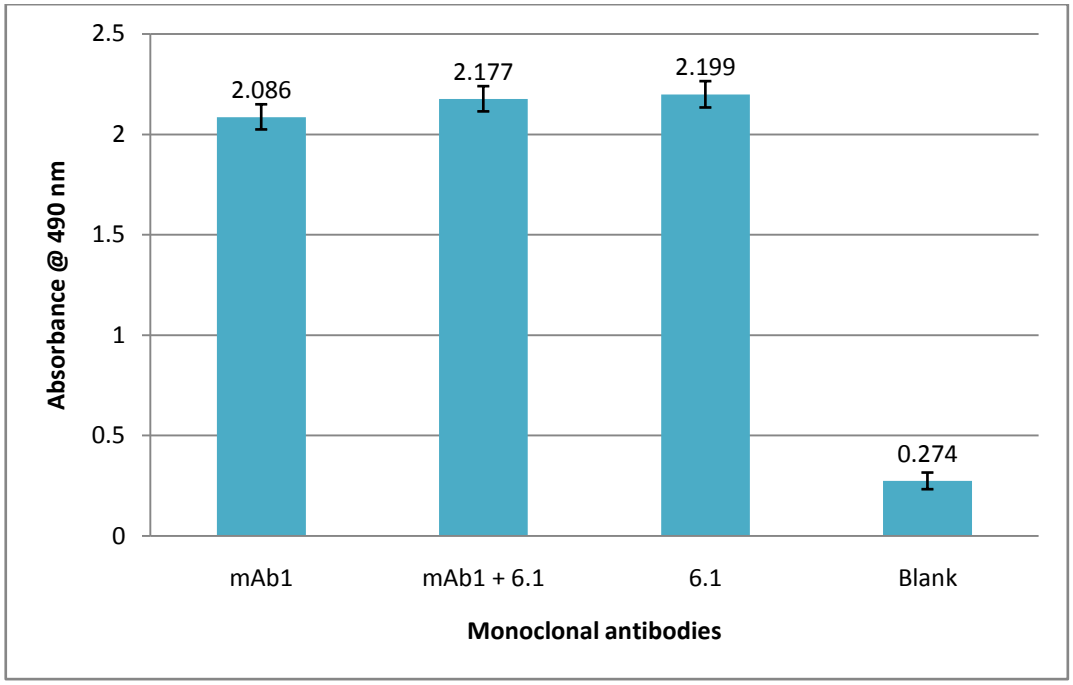
**C2**


2.5
2
1.5
1
0.5
0
Absorbance @ 490 nm
2.103
2.052
2.054
0.274
mAb4
mAb2 + mAb4
mAb2
Blank
Monoclonal antibodies

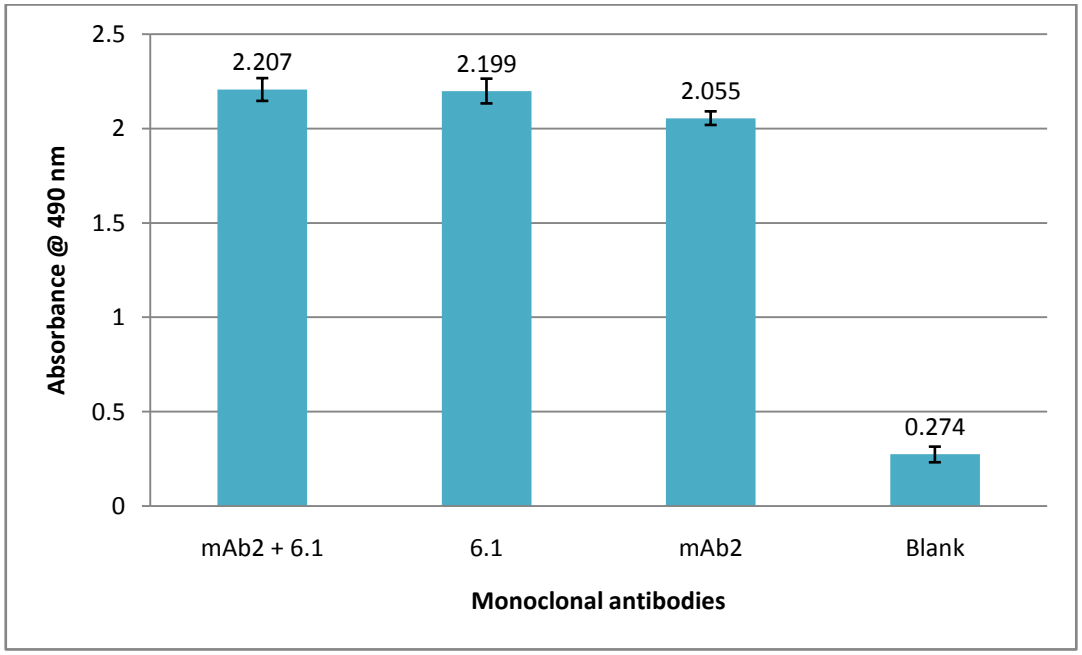
**C3**


2.5
2
1.5
1
0.5
0
Absorbance @ 490 nm
2.103
2.098
2.075
0.274
mAb4
mAb4 + mAb3
mAb3
Blank
Monoclonal antibodies

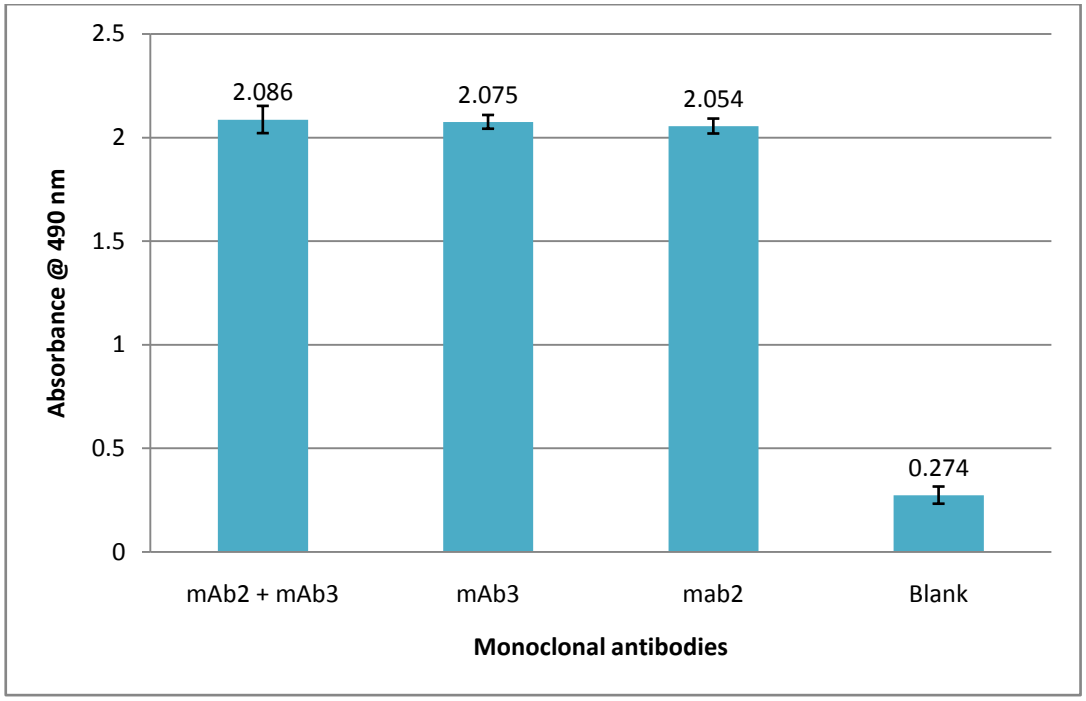
**C4**


2.5
2
1.5
1
0.5
0
Absorbance @ 490 nm
2.086
2.177
2.199
0.274
mAb1
mAb1 + 6.1
6.1
Blank
Monoclonal antibodies

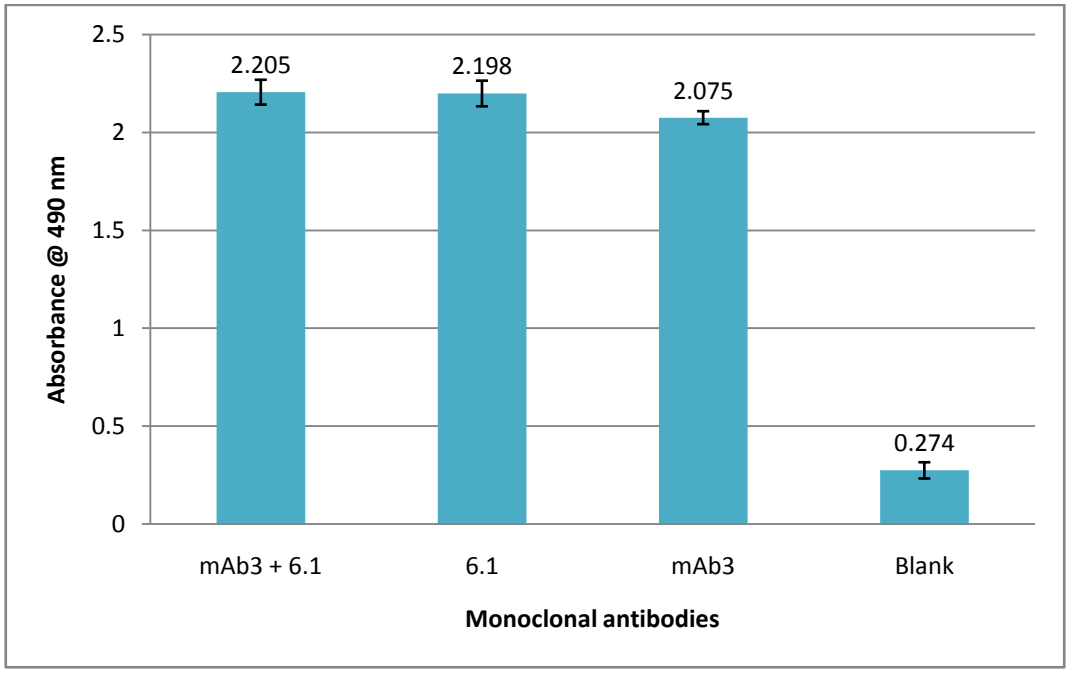
**C5**


2.5
2
1.5
1
0.5
0
Absorbance @ 490 nm
2.207
2.199
2.055
0.274
mAb2 + 6.1
6.1
mAb2
Blank
Monoclonal antibodies

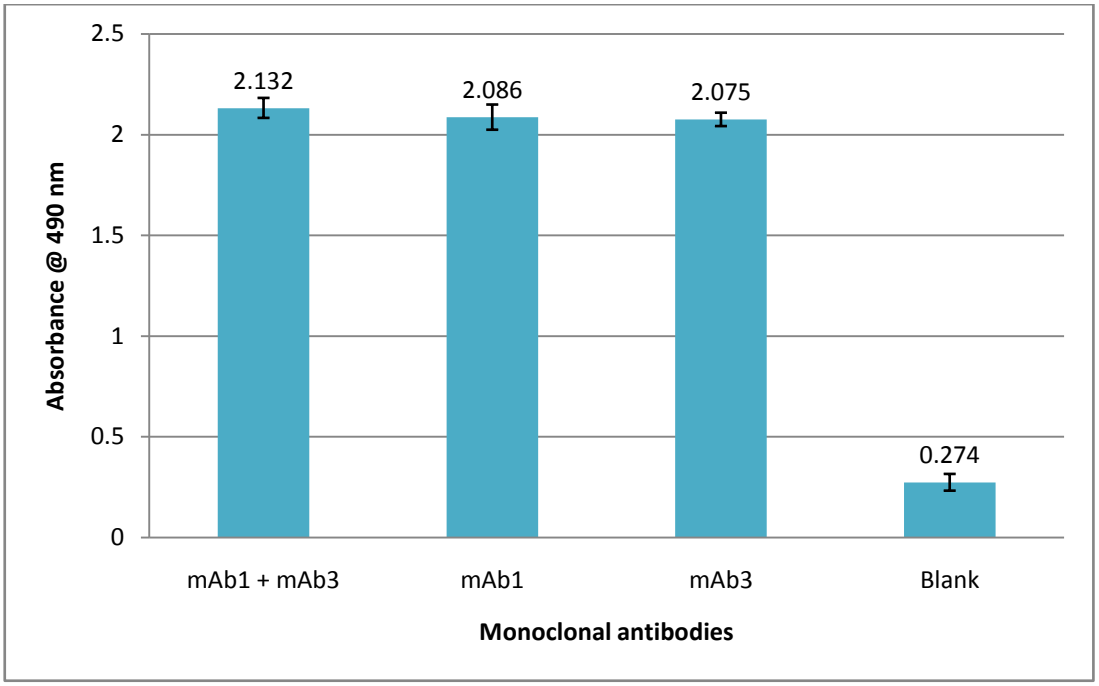
**C6**


2.5
2
1.5
1
0.5
0
Absorbance @ 490 nm
2.086
2.075
2.054
0.274
mAb2 + mAb3
mAb3
mab2
Blank
Monoclonal antibodies

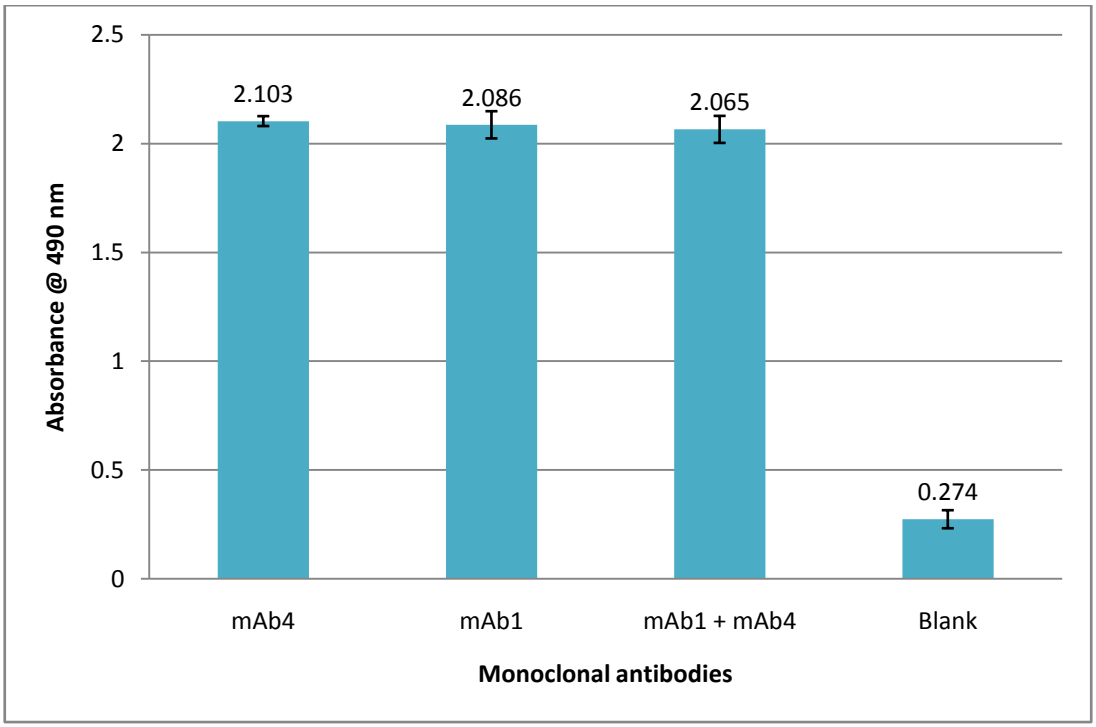
**C7**


2.5
2
1.5
1
0.5
0
Absorbance @ 490 nm
2.205
2.198
2.075
0.274
mAb3 + 6.1
6.1
mAb3
Blank
Monoclonal antibodies

**C8**


2.5
2
1.5
1
0.5
0
Absorbance @ 490 nm
2.132
2.086
2.075
0.274
mAb1 + mAb3
mAb1
mAb3
Blank
Monoclonal antibodies

**C9**


2.5
2
1.5
1
0.5
0
Absorbance @ 490 nm
2.103
2.086
2.065
0.274
mAb4
mAb1
mAb1 + mAb4
Blank
Monoclonal antibodies