THE DESIGN AND APPLICATION OF A REAL-TIME PCR ASSAY TO ASSESS rcDNA AND cccDNA PRODUCED BY HBV DURING INFECTION

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DECLARATION

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

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Kristie Michelle Bloom

_____ day of _____ 2010

PUBLICATIONS AND PRESENTATIONS

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is endemic to sub-Saharan Africa, and despite the availability of anti-viral agents, there is currently no cure. This double stranded DNA virus is hepatotropic, and active viral replication results in two genomic equivalents, the relaxed circular DNA (rcDNA) and covalently closed circular DNA (cccDNA). The virion encapsulated rcDNA contains a partially synthesised positive DNA stand and a gap region within the negative strand. After infection of hepatocytes, the rcDNA is repaired in the nucleus to form cccDNA. An important objective of HBV therapy is the elimination of cccDNA, as its persistence within hepatocytes has been attributed to chronic HBV infection. Therefore a reliable assay for this replication intermediate is crucial. The objective of this study was to develop a method based on real-time PCR to detect and quantify HBV cccDNA. PCR primers which flank the rcDNA gap were designed to amplify cccDNA whilst primers flanking the pre-S1 region quantify total HBV DNA. Viral DNA was extracted from HepG2.2.15 cells, along with serum and livers from HBV transgenic mice. According to this assay, cccDNA was readily detectable in transgenic mouse livers, but was present at low concentrations in serum samples. The intrahepatic HBV DNA profile of transgenic mice was found to be 40% cccDNA to 60% rcDNA. In HepG2.2.15 cells, only 2% of HBV DNA was cccDNA whilst the majority was in the form of rcDNA. These results were validated using non-radioactive Southern blot hybridisation. Additionally, it was established that although RNAi-based effecters inhibit HBV replication, established cccDNA pools were not eliminated. Real-time PCR provides a convenient platform for HBV cccDNA detection as it allows for the rapid simultaneous amplification and quantification of a specific DNA target through either non-specific or specific DNA detection chemistries. In conclusion, this HBV qPCR assay should enable improved monitoring of patients' responses to antiviral therapy.

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1.   ALT   -   Alanine aminotransferase

2.   Anti-HBc   -   Hepatitis B core antibody

3.   Anti-HBe   -   Hepatitis B e antibody

4.   Anti-HBs   -   Hepatitis B surface antibody

5.   cccDNA   -   covalently closed circular DNA

6.   cccR   -   cccDNA region

7.   CMV   -   Cytomegalovirus

8.   CP   -   Crossing Point

9.   DIG   -   Digoxygenin

10.   DMEM   -   Dulbecco's modified eagles medium

11.   DNA   -   Deoxyribonucleic Acid

12.   dNTP   -   Deoxynucleotide triphosphate

13.   DR1   -   Direct Repeat 1

14.   DR2   -   Direct Repeat 2

15.   dsDNA   -   double stranded DNA

16.   DTT   -   Dithiothreitol

17.   *E.coli*   -   Escherichia coli

18.   ELISA   -   Enzyme-linked immunosorbant assay

19.   FCS   -   Fetal calf serum

20.   FRET   -   Fluorescence resonance energy transfer

21.   GAPDH   -   Glyceraldehydes-3-phosphate dehydrogenase

22.   HBV   -   Hepatitis B virus

23.	HBcAg	-	Hepatitis B core antigen
24.	HBeAg	-	Hepatitis B e antigen
25.	HBsAg	-	Hepatitis B surface antigen
26.	HBx	-	Hepatitis B virus X protein
27.	HCC	-	Hepatocellular carcinoma
28.	HDV	-	Hepatitis delta virus
29.	HIV	-	Human immunodeficiency virus
30.	IARC	-	International Agency for Research on Cancer
31.	IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
32.	kb	-	kilobase
33.	MCS	-	Multiple cloning site
34.	mRNA	-	Messenger RNA
35.	miRNA	-	micro RNA
36.	ng	-	nanogram
37.	nt	-	nucleotide
38.	ORF	-	Open reading frame
39.	PBS	-	Phosphate buffered saline
40.	PCR	-	Polymerase chain reaction
41.	pgRNA	-	pregenomic RNA
42.	qPCR	-	Real-time quantitative PCR
43.	rcDNA	-	relaxed circular DNA
44.	rcR	-	rcDNA region
45.	RNA	-	Ribonucleic acid
46.	RNAi	-	RNA interference
47.	rpm	-	revolutions per minute
48.	RT-PCR	-	Reverse transcription PCR

49.	shRNA	-	short hairpin RNA
50.	siRNA	-	small interference RNA
51.	ssDNA	-	single stranded DNA
52.	St6gal1	-	Beta-galactoside alpha-2,6-sialyltransferase 1
53.	*Taq*	-	*Thermus aquaticus*
54.	Tm	-	Melting temperature
55.	WHO	-	World Health Organisation
56.	X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1 – INTRODUCTION

1.1 Hepatitis B virus biology

1.1.1 Pathogenesis and chronic HBV

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, is an enveloped partially double-stranded DNA virus that predominantly infects hepatocytes, resulting in acute or chronic infection (Seeger and Mason, 2000). Acute infections are readily cleared by the host's immune system, in response to antigenic epitopes presented on the viral envelope or the surface of infected hepatocytes (Jung and Pape, 2002). Chronic infections, however, increase the risk of developing hepatocellular carcinomas (HCC) and cirrhosis, decades after initial infection (Kao and Chen, 2002; Lai et al., 2003; Seeger and Mason, 2000). It is estimated that over 350 million people are chronically infected with HBV, which is responsible for between 60 to 80% of all HCC incidences worldwide (reviewed by Lavanchy, 2004). Furthermore, the World Health Organisation (WHO) International Agency for Research on Cancer (IARC) has declared chronic HBV infection carcinogenic to humans (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. and International Agency for Research on Cancer., 1994).

The probability of an acute infection developing into chronic HBV is dependent on both cytolytic and non-cytolytic immune responses. Children and immunocompromised adults are more likely to develop chronic infections as their immune systems are immature or depleted (Fattovich et al., 2008; Seeger and Mason, 2000). There are four, well

characterised phases of disease progression associated with HBV infection (reviewed by Custer et al., 2004; Fattovich et al., 2008; Kao and Chen, 2002; Lai et al., 2003). These include: 1) active viral replication and immune tolerance, 2) immune clearance and symptomatic hepatitis, 3) inactive carrier with low level replication, and 4) reactivation or occult infection. The first phase, active viral replication and immune tolerance, is associated with high levels of serum HBV DNA, the prevalence of the HBV e antigen (HBeAg), and normal serum alanine aminotransferase (ALT) concentrations, correlating with typical liver histology and no defining symptoms. The immune clearance and symptomatic hepatitis phase presents as mild to severe hepatitis caused by immune-mediated inflammation and fibrosis. This is a result of immune activation and subsequent HBeAg seroconversion. The third phase is associated with the clearance of serum HBV DNA, normal ALT concentrations, and the presence of anti-HBe. Acute infections are readily cleared after this stage, however if seroconversion is not achieved and viral replication persists, the infection is regarded as chronic. The development of chronic HBV requires the administration of anti-viral therapies to assist in viral clearance. The final stage, reactivation or occult infection, is dependent on the persistence of covalently closed circular DNA (cccDNA), which can remain dormant in the nucleus of hepatocytes, thus evading the immune response. Reactivation may occur as a consequence of terminating anti-HBV treatment, HBV escape mutants, superinfection with hepatitis delta virus (HDV), or conflicting therapies associated with immune suppression or co-infections (Feitelson, 1998; Lai et al., 2003). Although chronic HBV can be clinically monitored, there is no existing cure. Consequently, the most efficient means of preventing HBV infection is through immunisation.

A proficient plasma-derived HBV surface antigen (HBsAg) vaccine has been available since 1981, following which a more universal recombinant DNA vaccine was established (Franceschi and Raza, 2008; Kao and Chen, 2002). As 90-95% of vaccinated individuals

are efficiently protected against HBV infection (Margolis, 1998), the WHO has embarked on a global campaign to introduce the vaccine into national infant immunisation schemes, focussing specifically on endemic countries (Franceschi and Raza, 2008). Despite the availability of a vaccine, HBV still remains endemic to sub-Saharan Africa, with a chronic infectivity rate of over 8% accounting for a large incidence of clinically observed cirrhosis and HCC (Parkin, 2006). Horizontal transmission of HBV is considered to predominate in Africa, where infection prevalence rates of up to 98% have been observed in some rural areas of South Africa (Custer et al., 2004). Furthermore, the risk of developing HCC is exacerbated by the exposure of HBV infected individuals to the carcinogenic aflatoxin, as well as Human Immunodeficiency Virus (HIV) co-infection (Hainaut and Boyle, 2008). The probability of HIV/HBV co-infection is escalating in sub-Saharan Africa as both diseases remain hyper-endemic to the area (Hoffmann and Thio, 2007). As acquired immunodeficiency syndrome (AIDS) can lead to the reactivation of HBV viral replication, chronic HBV progresses more rapidly, thus increasing the likelihood of developing HCC (Hoffmann and Thio, 2007). A high incidence of occult HBV infection has also been observed in HIV positive patients who have not yet progressed to AIDS (Firnhaber et al., 2009; Hoffmann and Thio, 2007). As chronic HBV-related HCC escalates, the mortality rate is expected to dramatically increase, further burdening countries with limited medical and economic standings (Hainaut and Boyle, 2008). Accordingly, new sensitive HBV diagnostic techniques and advancements in therapeutics are unquestionably required to improve chronic HBV patient management.

1.1.2   HBV DNA species and the viral genome

There are two major forms of HBV DNA identified during infection, namely relaxed circular DNA (rcDNA) and cccDNA. rcDNA, which is partially double-stranded, is encapsulated

within circulating virions (Robinson et al., 1974; Summers et al., 1975). This form of HBV DNA was primarily isolated from Dane particles, otherwise referred to as circulating viral particles or virions, recovered from the serum and liver of chronic HBV patients (Dane et al., 1970; Huang et al., 1972). Figure 1.1 illustrates the HBV genome organisation as well as the rcDNA conformation. rcDNA consists of an incompletely synthesised positive DNA stand, which is capped at the 5' end by a short RNA oligomer (Summers and Mason, 1982; Will et al., 1987). A gap region, formed by the association of the 5' end with the polymerase protein, is found in the negative strand (Gerlich and Robinson, 1980). This gap region is flanked by two 11 nucleotide repetitive sequences, Direct Repeat 1 (DR1) and Direct Repeat 2 (DR2), situated on both the positive and negative strands (Will et al., 1987). These repeats act as important recognition sites during viral replication. The HBV polymerase is inherently required during replication, for reverse transcription of the pregenomic RNA (pgRNA), thus directing the synthesis of new rcDNA (Hirschman et al., 1971; Kaplan et al., 1973; Robinson and Greenman, 1974; Summers and Mason, 1982). rcDNA acts as the precursor for the second HBV DNA construct, the cccDNA. In the nucleus of hepatocytes, the partially synthesised positive DNA strand of the rcDNA is repaired; generating episomal cccDNA (Summers and Mason, 1982; Tuttleman et al., 1986). The cccDNA is the fundamental viral transcription intermediate required for replication.

HBV viral replication is dependent on the transcriptional regulation of the cccDNA core promoter, which drives the production of viral subgenomic RNAs required for new virion assembly. cccDNA may be epigenetically regulated through the acetylation status of DNA coupled histones, and as a result, it is capable of forming minichromosomes (Bock et al., 2001; Newbold et al., 1995; Pollicino et al., 2006). This allows the virus to remain dormant in the nucleus of hepatocytes, often resulting in occult HBV infection (reviewed by Hu, 2002; Raimondo et al., 2007). The HBV genome is approximately 3.2 kilobases (kb) in length, and

comprises four overlapping open reading frames (ORFs) which encode the *precore/core*, *HBx*, and two *surface* or *envelope* (*pre-S1* and *pre-S2/S*) genes (reviewed by Moolla et al., 2002; Nassal, 2008; Robinson, 1977; Seeger and Mason, 2000) as illustrated in Figure 1.1. Gene expression is regulated by four individual promoter sequences and two enhancer elements. Transcription from the *precore/core* ORF yields the 3.5 kb pgRNA which is translated to core and polymerase proteins. Core proteins or HBV core antigens (HBcAg) form the viral capsid. HBV polymerase initiates reverse transcription of the pgRNA to produce rcDNA. The *precore* transcript is also transcribed from the *precore/core* ORF and is translated to produce precore protein which is post-translationally modified and secreted as HBeAg. Like the *core* gene, the *surface* or *envelope* ORF includes two upstream reading frames, the pre-S1 and pre-S2 regions. A 2.4 kb mRNA is transcribed from the pre-S1 ORF and yields the large surface protein (L). The pre-S2 ORF is transcribed to produce a 2.1 kb mRNA from which the middle (M) and major (S) surface proteins are translated (Heermann et al., 1984). These proteins can be identified immunologically as HBsAg and are essential in the formation of new viral envelopes. The *HBx* ORF encodes the transactivator protein HBx. The function of HBx is still elusive however it is thought to be important in maintaining viral replication in hepatocytes, and facilitate the integration of HBV DNA into the host genome (Feitelson, 1998; Kao and Chen, 2002; Robinson, 1977).   Overall, the transcriptional regulation of cccDNA drives the transcription of subgenomic viral RNAs and pgRNA, which in turn increases the production of new viral proteins leading to increased viral replication and HBV viremia.
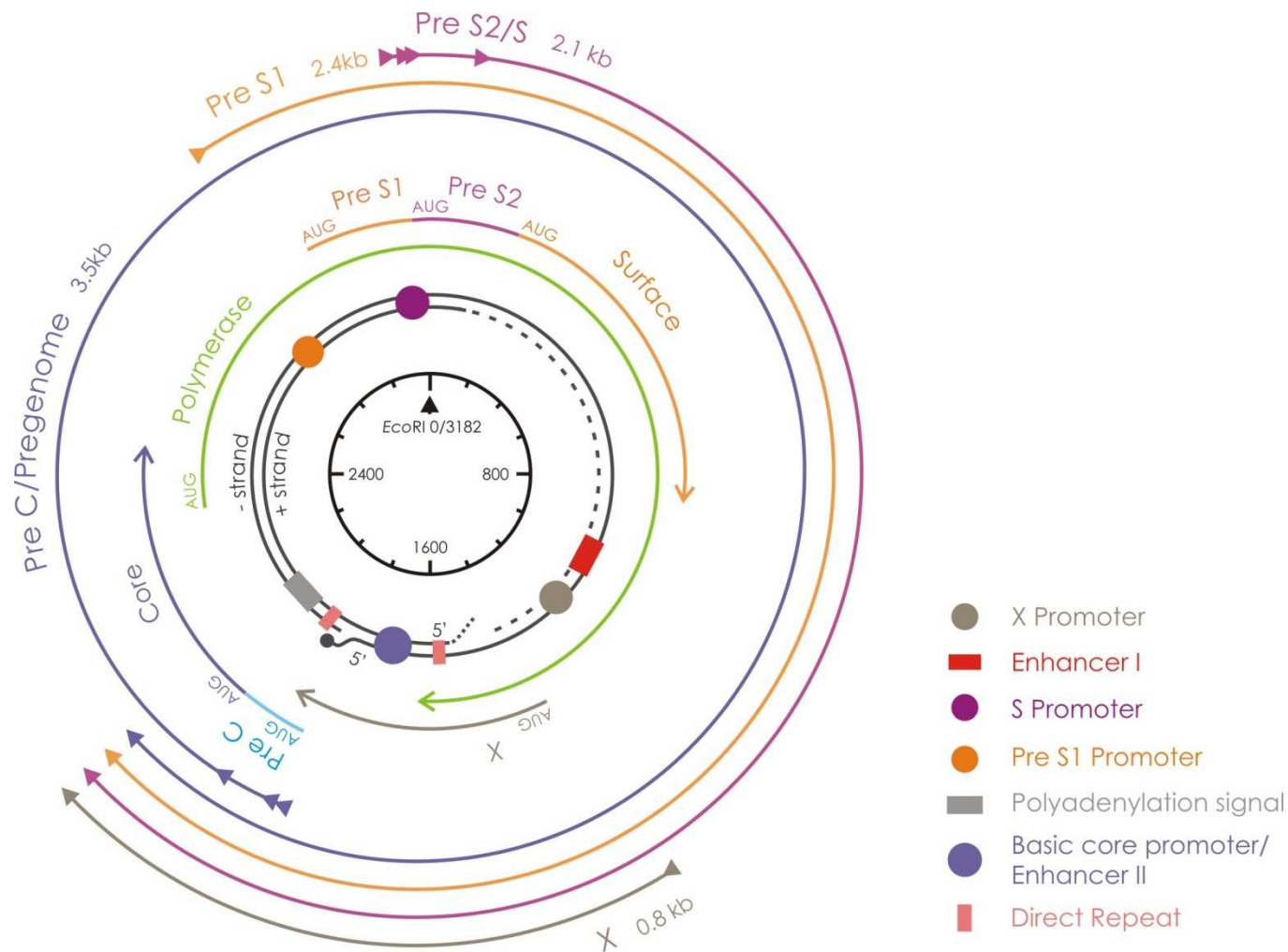
Figure 1.1: Genomic organisation of HBV. The rcDNA positive strand is only partially synthesised whilst the negative strand contains a gap region flanked by DR1 and DR2. The viral genome comprises of a *precore/core*, *pre-S1*, *pre-S2/S* and *HBx* ORFs. Transcription of the genome yields pgRNA and various subgenomic mRNAs which are transcribed into core, surface and X proteins.

1.1.3   Viral infection and replication

Circulating HBV virions denote the infectious particles associated with HBV transmission. Each virion contains an rcDNA genome, which is encapsidated within an icosahedral core or nucleocapsid, and subsequently enclosed inside a lipid envelope.   The capsid comprises dimerised HBV core proteins and the envelope incorporates the L, M and S surface proteins (Blum et al., 1989; Nassal, 2008). HBV is primarily hepatotropic, however the virus has been isolated from peripheral blood mononuclear cells (Neurath et al., 1992; Trippler et al., 1999). Figure 1.2 outlines hepatocyte infection and the viral replication strategy of HBV, which has been studied extensively over the past three decades (Blum et al., 1989; Nassal, 2008; Seeger and Mason, 2000; Summers and Mason, 1982; Will et al., 1987).

Initially the viral envelope attaches to the cellular membrane of hepatocytes and the capsid is internalised most likely through clathrin-mediated endocytosis (Cooper and Shaul, 2006). The pre-S1 and pre-S2 regions of the surface proteins have been shown to be central in viral attachment and entry; however this process is still not fully understood (Glebe and Urban, 2007; Neurath et al., 1986; Neurath et al., 1992).   Once the capsid has been released into the cytoplasm of the hepatocyte, it locates to the nuclear pore complex on the membrane surface of the nucleus.   The core dissociates releasing the rcDNA into the nucleus where it is repaired, resulting in cccDNA.   The establishment of this proviral DNA entails the removal of the polymerase and RNA oligomer from the 5' ends of the negative and positive strands respectively, followed by positive strand synthesis and the ligation of the 3' and 5' ends flanking the gap region (Blum et al., 1989).   Intact episomal cccDNA is the transcriptional intermediate required for HBV replication and virion assembly.
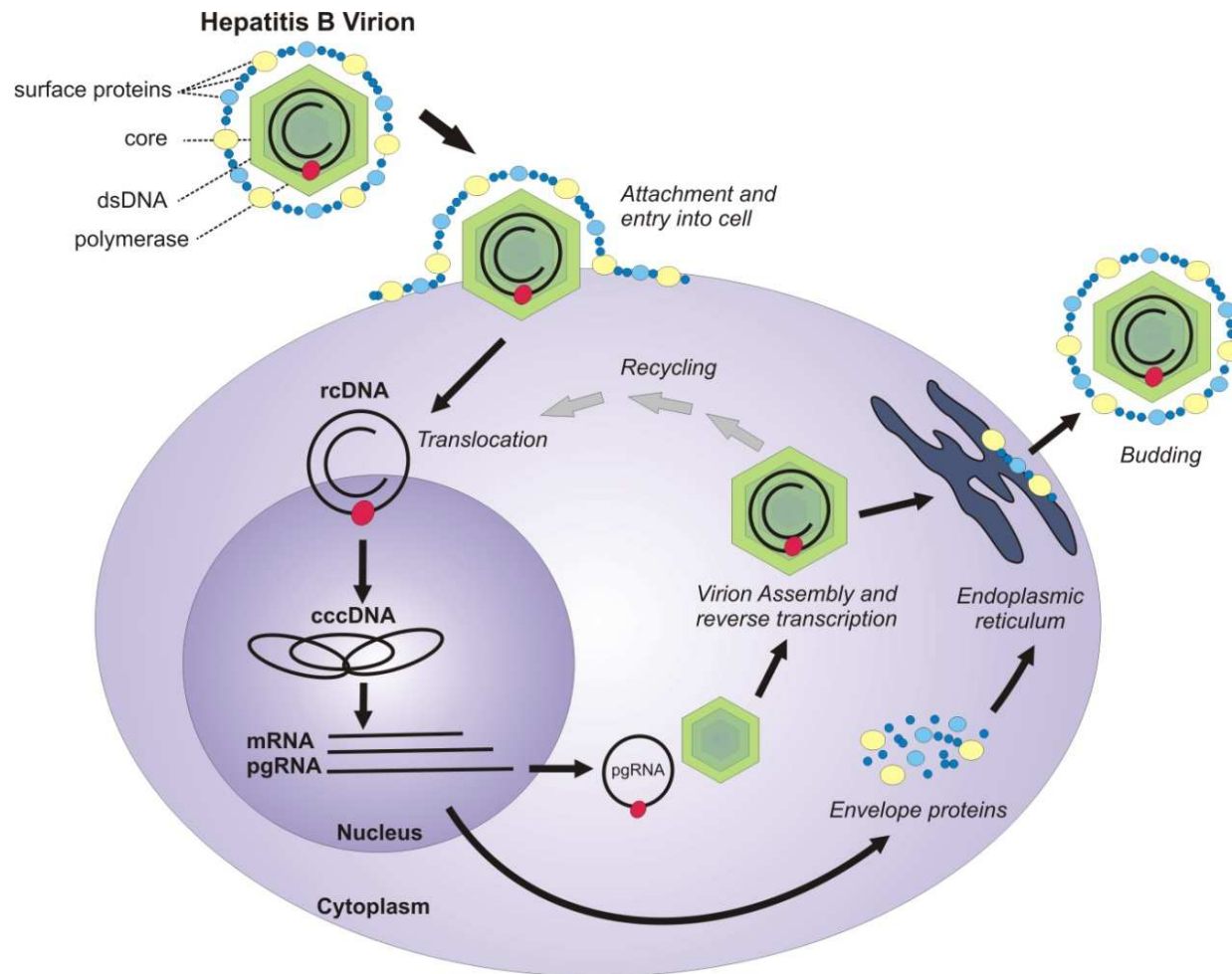
Figure 1.2: Hepatitis B virus replication cycle. In the cytoplasm the viral genome is released from the capsid and translocates to the nucleus. rcDNA is then repaired to form cccDNA from which pgRNA as well as various subgenomic mRNAs are transcribed. The mRNAs are translated into proteins required for virion assembly whilst the pgRNA associates with polymerase and the new core proteins. Within the capsid, the pgRNA is reverse transcribed to form rcDNA. Inside the endoplasmic reticulum, surface proteins surround the virion allowing the virus to bud out of the cell.

The cccDNA is transcribed, with the aid of host transcription factors, yielding various subgenomic mRNAs and pgRNA (Blum et al., 1989). The subgenomic RNAs include the two *surface* mRNAs and the *HBx* transcript which are exported to the cytoplasm and translated. pgRNA is transcribed from the negative strand of cccDNA, resulting in a greater than genome length RNA template required for rcDNA synthesis (as previously described). The pgRNA is also exported to the cytoplasm, where it is translated into core particles and the viral polymerase. Association of the pgRNA with a viral polymerase and core protein dimers drives nucleocapsid formation (Summers and Mason, 1982). Each core protein is divided into two domains, the core domain and the protamine domain. The core domain facilitates capsid assembly whilst the protamine domain binds the pgRNA/polymerase complex (Hatton et al., 1992). The polymerase protein, like core, is also organised into two domains. The first, a terminal protein domain, facilitates pgRNA packaging and DNA priming whilst the second, a carboxy-terminal domain, has reverse transcriptase and RNaseH activity (reviewed by Seeger and Mason, 2000). Once the pgRNA and polymerase have been successfully encapsidated the RNA genome undergoes reverse transcription.

The complex strategy of HBV reverse transcription has been extensively reviewed (Beck and Nassal, 2007; Nassal, 2008; Seeger and Mason, 2000; Will et al., 1987) and will only be briefly described. During core protein encapsulation, the polymerase associates with an epsilon (ε) structure, created by a stem-loop present on the 5' end of the pgRNA. This initiates the transcription of a 3 to 4 base DNA oligonucleotide that binds to proximal DR1 located at the pgRNAs 3' end. This initiates reverse transcription of the negative DNA strand. During this transcription, the pgRNA is degraded by the polymerase's inherent RNaseH activity. A residual 5' capped oligonucleotide, not degraded after negative strand production, primes the initial stage of positive DNA synthesis. This oligonucleotide contains a DR1 coding region that corresponds to the DR2 sequence present at the 3' end of the newly synthesised negative

strand. Only a small portion of the positive strand is assembled as transcription is halted when the 5' terminal end of the negative DNA strand is reached. To complete the transcription of this strand, the small positive oligonucleotide undergoes a template switch, based on the presence of a terminal redundancy at the negative strand's 5' and 3' ends. The positive strand is able to switch to the 3' end of the negative strand and resume elongation.   This results in the establishment of the rcDNA genome. The viral nucleocapsid containing the newly synthesised rcDNA either infects the same hepatocyte, expanding the nuclear cccDNA pool, or associates with surface proteins and buds out of the cell as a complete virion.   These new virions go on to infect other hepatocytes and increase serum viraemia.

1.1.4   Current therapeutics

There is a limited number of registered anti-HBV therapeutic agents designed to treat chronic HBV infections, but several potential agents are currently undergoing clinical evaluation (Hilleman, 2003).   HBV therapeutics can be broadly divided into two major categories, immunomodulators and anti-viral agents (Hilleman, 2003; Lai et al., 2003). Immunomodulators, including interferon-α, -β, and pegylated-interferon-α, act against HBV infected cells by aiding the adaptive immune response in eliminating infected hepatocytes. Interferon therapy has been successfully used to treat chronic HBV infections (Wong et al., 1993), however its efficacy is based on certain predictors of response such as HBV viraemia, serum ALT levels, host immune response, and viral mutations (Hilleman, 2003). Despite its use for over two decades (Seeger and Mason, 2000), the anti-HBV efficacy of interferon is considered to be low and is contraindicated by co-infection with HIV (Krastev, 2006).   Anti-viral agents on the other hand are more specific inhibitors of HBV as they directly target the virus or elements of its life cycle.

Two important classes of anti-viral agents are chemotherapeutics and gene therapies. The nucleotide and nucleoside analogues Lamivudine, Adefovir dipivoxil, Entecavir, Telbivudine, and Tenofovir are licensed chemotherapeutics that are used to treat HBV infections (European Association For The Study Of The Liver, 2009; Krastev, 2006). They act by inhibiting viral reverse transcriptase and polymerase which in turn halts HBV replication. Lamivudine, the first licensed therapy against chronic HBV, has shown significant anti-HBV efficacy, specifically in reducing HBV DNA levels, inhibiting new cccDNA establishment, and reversing liver damage (Dienstag et al., 2003; Lai et al., 1998; Yuen et al., 2005). However long term Lamivudine monotherapy results in escape mutants, particularly the YMDD motif mutation found within the viral polymerase, leading to reactivation of HBV (reviewed by Fischer et al., 2001). Adefovir dipivoxil and Entecavir have both shown increased efficacy against HBV in comparison to Lamivudine (Hilleman, 2003; Krastev, 2006). Additionally these agents inhibit HBV mutant strains, established during prolonged Lamivudine therapy (Lee et al., 2009; Ono-Nita et al., 1999). Telbivudine and Tenofovir have recently become available for chronic HBV treatment (Delaney et al., 2006; Osborn, 2009), and they also exhibit activity against HIV (Fung et al., 2002; Low et al., 2009) which may be useful for treating HBV/HIV co-infections. Although nucleotide and nucleoside analogues have proven efficacy against HBV they are unable to eradicate established cccDNA reservoirs. Furthermore, long term treatment is mandatory as cessation of therapy often results in HBV reactivation or viral mutations. Anti-viral gene therapies, including RNA interference (RNAi) effecters, antisense compounds, ribozymes and aptamers, have shown promise in the fight against persistent infectious diseases (Schreier, 1994; Uprichard, 2005), particularly chronic HBV (Arbuthnot et al., 2005; Hilleman, 2003).

RNAi is a naturally occurring RNA-dependant gene silencing mechanism (Agrawal et al., 2003; Fire et al., 1998; reviewed by Hammond et al., 2001), which has more recently been developed for therapeutic applications (reviewed by Aagaard and Rossi, 2007). Many RNAi effecters

expressing short hairpin RNAs (shRNA), short interfering RNAs (siRNA) and microRNA (miRNA) shuttles have been shown to efficiently knock down HBV replication (Arbuthnot et al., 2005; Arbuthnot and Thompson, 2008; Carmona et al., 2006; Chattopadhyay et al., 2009; Ely et al., 2008; Kayhan et al., 2007; Konishi et al., 2003; Li et al., 2007; Liu et al., 2004; Wu et al., 2007). This, in turn, successfully impedes reverse transcription and virion assembly. The fundamental challenge associated with gene therapy is the facilitation of exogenous nucleic acid transfer into infected cells, primarily through chemical modifications and delivery vectors (reviewed by Chen et al., 2008). Despite the availability of diverse immunomodulators, chemotherapeutics and gene therapies, there is no cure for chronic HBV and total eradication of the virus is rarely accomplished.

1.1.5 Persistence of cccDNA

The persistence of episomal cccDNA represents a major hurdle in the treatment of chronic HBV, as established infections are refractory to inhibition by approved anti-viral therapies. This is problematic as cccDNA determines the replicative activity of the virus, transcribing all pgRNA and viral mRNA from its genome. As cccDNA is epigenetically regulated (Pollicino et al., 2006) it is able to remain dormant in the nucleus of hepatocytes, establishing a latent or occult infection (Mulrooney-Cousins and Michalak, 2007; Rehermann et al., 1996). Consequently, monitoring HBV clearance during anti-viral drug regimes remains a challenge. Although markers of infection may indicate eradication of the virus, the persistence of cccDNA often results in the re-emergence of infection after cessation of therapy (Levrero et al., 2009). To maintain this stable infection, cccDNA relies on the host for minichromosome formation and viral gene transcription.

Throughout infection, a single hepatocyte is capable of retaining multiple copies of cccDNA, generally ranging between 1 and 50 molecules per cell (Newbold et al., 1995; Wong et al., 2004; Zhang et al., 2003), but up to 173 replicates have been clinically observed (Chen et al., 2004). As each cell preserves its own viral pool, the number of cccDNA replicates fluctuates between hepatocytes. There are three major factors involved in sustaining the cccDNA viral pool, the immune-mediated, virological, and epigenetic factors (reviewed by Levrero et al., 2009). Immune-mediated factors contribute to the dilution and/or clearance of cccDNA. This includes non-cytolytic cytokines, which block viral replication and new cccDNA formation (Guidotti et al., 1999; Thimme et al., 2003) and cytotoxic T lymphocytes, which destroy infected hepatocytes (Fisicaro et al., 2009; Thimme et al., 2003). Constant cellular proliferation additionally dilutes the cccDNA pool during consecutive mitotic divisions (Mason et al., 2007; Summers et al., 2003). Despite this, immune-mediated clearance of cccDNA remains inefficient during chronic HBV infection. Virological factors include HBV infection, replication and integration. Viral mutagenesis is the most important virological factor associated with cccDNA persistence, as escape mutants facilitate re-infection. Host immunity and anti-viral therapies further exacerbate the rate of mutation as a result of selection pressure (reviewed by Locarnini, 2005). Finally, epigenetic factors facilitate organisation of cccDNA into minichromosomes, regulating transcription of the viral genome. Nuclear histone and non-histone proteins associate with the cccDNA to create a chromatin-like structure (Bock et al., 2001; Newbold et al., 1995). This minichromosome is transcriptionally regulated, based on the acetylation and methylation status of key histones (Pollicino et al., 2006). HBV chromatin remodelling results in the recruitment of host transcription factors (Levrero et al., 2009), ultimately permitting viral replication. Despite the available anti-viral therapies and host immunological responses, cccDNA is able to persist as a minichromosome indefinitely. Chronic HBV infections therefore require close clinical monitoring, to detect viral escape mutants and HBV reactivation, accompanied by personalised anti-viral therapeutic regimes.

1.1.6   Detecting HBV infection

Multiple serological and intrahepatic markers of HBV infection are detectable during acute and chronic hepatitis. Furthermore, antigen seroconversion and fluctuating viral DNA levels help characterise the phases of chronic infection and disease progression (Custer et al., 2004; Fattovich et al., 2008; Kao and Chen, 2002; Lai et al., 2003). In 1965, Blumberg and colleagues described the first HBV serological marker, the Australia antigen (Blumberg et al., 1965), now more commonly referred to as the HBsAg. Following this discovery, several viral antigens and antibodies have been isolated including HBcAg, HBeAg, and Anti-HBs, Anti-HBc and Anti-HBe. Viral antigens are primarily isolated from circulating viral particles found in the serum of infected individuals. As the viral particles encapsulate rcDNA, both serological and molecular diagnostic tools may be used to detect infection with HBV.

Serological diagnostics include solid-phase immunoassays, enzyme linked immunosorbent assays (ELISA), branched DNA (bDNA) assays, and electron microscopy, whilst molecular diagnostics incorporate radioimmunoassay and DNA hybridisations, polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qPCR) (reviewed by Chernesky and Mahony, 1984; Custer et al., 2004; Evans, 2009; Krastev, 2006; Landry et al., 1989; Lok, 2000). Figure 1.3 briefly outlines the basic principles of the HBV ELISA, bDNA assay, Southern blot hybridisation, and qPCR. The most universally exploited HBV antigen/antibody serological analysis is the ELISA. This technique has been used since the early 1980's to detect core, surface and e-antigens (Chernesky and Mahony, 1984; Landry et al., 1989), as well as to establish HBV seroconversion in order to track the progression of chronic carrier states. The HBsAg is most frequently measured as it is indicative of chronic infection and viral replication. However this viral antigen is capable of producing subviral

particles, coincidently used in the first HBV vaccines (McAleer et al., 1984; McAuliffe et al., 1980), which are essentially non-infectious, micelle-like viral envelopes. Consequently the HBsAg ELISA may be detecting subviral particles and not infectious virions, which makes it difficult to gauge the level of HBV DNA infection.

Distinguishing between the two forms of HBV DNA is critical for identifying occult infections, chronic disease progression, and determining the efficacy of anti-HBV therapies. The rcDNA form of the virus, confined within the HBV capsid, is readily found circulating throughout the blood and the cytoplasm of hepatocytes. The cccDNA on the other hand is predominantly located within the nucleus of infected hepatocytes. In extreme circumstances cccDNA may be isolated from the sera or peripheral blood mononucleocytes, however this is generally only true for chronic HBV patients with early stage liver damage (Chen et al., 2004) or transgenic animal models. Radioimmunoassays, bDNA assays, and DNA hybridisations are used to detect viral DNA from serum samples and liver biopsies. HBV bDNA, used mainly to quantify serological viral loads, amplifies a chemiluminescence signal, enabling detection of low copy sequences (Hendricks et al., 1995; Urdea et al., 1991). Southern blot hybridisations are frequently used to distinguish rcDNA and cccDNA from replicative intermediates and single stranded products by observing variations in their relative migration patterns (Brechot et al., 1981; Miller and Robinson, 1984). The precise quantification of viral DNA from Southern blot hybridisation is complicated and often imprecise. Therefore, neither ELISA nor Southern blot is able to precisely resolve the level of HBV infection. Advancements in PCR molecular techniques, however, permit the direct detection of both serological and intrahepatic HBV DNA allowing accurate quantification of viral DNA.
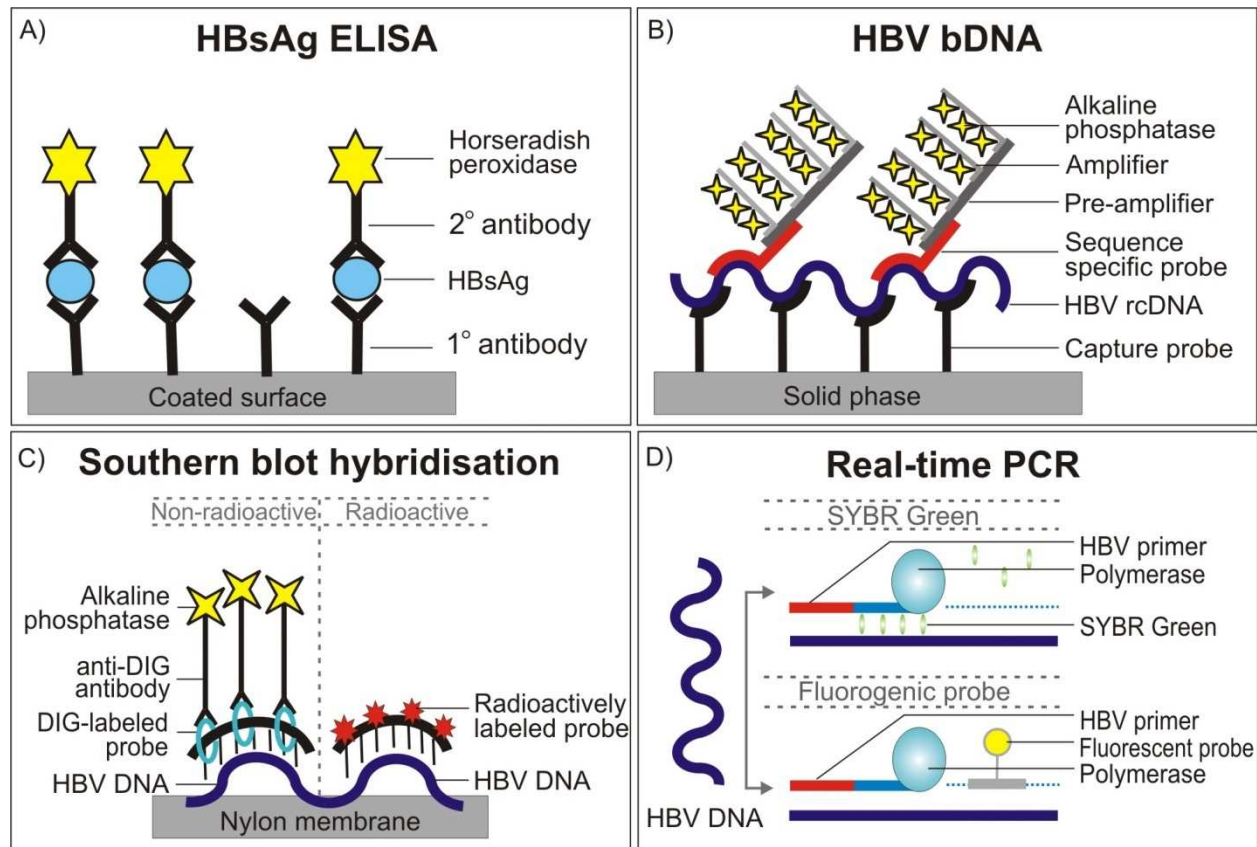
Figure 1.3: A brief overview of the most frequently used HBV detection and quantification platforms. A) A primary antibody binds and fixes the HBV surface antigen to the ELISA plate. A secondary antibody, conjugated to horseradish peroxidase, then binds the antigen. Signal emission and HBsAg quantification is determined spectrophotometrically. B) DNA is immobilised on a solid phase by capture probes. A sequence specific probe then binds to serum isolated rcDNA. Subsequently, the pre-amplifier binds to the sequence specific probe and the amplifier, which contains multiple alkaline phosphatase binding sites, then attaches to the pre-amplifier. Alkaline phosphatase is detected by chemiluminescence. C) HBV DNA is UV cross-linked to a nylon membrane. Sequence specific probes are labeled with radioactive isotopes, such as γ^{32}P-ATP, or non-radioactive immunohistochemical markers, like digoxigenin (DIG). Radioactive probes are detected using X-ray film or a phosphoimager. Non-radioactive probes require conjugation of specific antibodies as well as alkaline phosphatase for chemiluminescence detection. D) HBV sequence specific primers bind to the viral DNA. Polymerase extends the primer sequence, incorporating either non-specific or specific fluorescent probes. SYBR Green binds to double stranded DNA during elongation, where as fluorescent probes bind upstream of the primer. Detection and quantification is performed by measuring wavelength specific fluorescence emission

Conventional PCR and RT-PCR are better candidates for viral DNA and pgRNA quantification, as they are less laborious than Southern blotting, have higher specificity, and the DNA can be detected without using radioactive probes. Conventional PCR with HBV primers allows for the amplification and subsequent detection of total HBV DNA.   This can be successfully exploited to determine serological viral loads, which predominantly consists of rcDNA. The COBAS HBV Amplicor Monitor (Roche Diagnostics) is the most extensively used commercially available HBV PCR quantification assay (Kessler et al., 1998a; Kessler et al., 1998b; Lopez et al., 2002; Valsamakis, 2007).   This competitive PCR, based on the co-amplification of a known concentration of target DNA, has been shown to be more sensitive than ELISA's and branched DNA technologies (Pawlotsky et al., 2000), however it still requires an immunological detection platform to quantify HBV viral loads. The PCR is unable to discriminate between rcDNA and cccDNA efficiently, making it difficult to monitor intrahepatic HBV. Nested PCR has been shown to successfully amplify cccDNA alone, however radioimmunoassay are still required for quantification (Zhang et al., 2003). RT-PCR on the other hand, entails the reverse transcription of pgRNA into complementary DNA before amplification. By measuring pgRNA levels, one can determine the replicative efficacy of the virus and determine if anti-viral therapies, aimed at inhibiting viral transcription and translation, are effective (Chou et al., 2005). But, as it is an RNA based assay, it is unable to amplify HBV DNA and thus cannot quantify rcDNA or cccDNA. The subsequent development of qPCR, has, however, revolutionised viral DNA detection.

The scientific advancement of PCR technologies has facilitated the real-time amplification, detection and analysis of viral DNA in a single reaction. qPCR allows the rapid quantification of HBV viral loads using fluorescence based chemistries. As it is a single amplification and detection procedure, the likelihood of contamination is minimised when compared to conventional PCR, and no immunoassays are necessary. Real-time PCR has been available since the early 1990s (Higuchi et al., 1992) and has been developed to routinely screen for

bacterial and fungal diseases, viral infections, and human diseases (reviewed by Evans, 2009; Mackay et al., 2002). Detection and quantification is based on fluorogenic probes, designed to bind either non-specifically to double stranded DNA, or specifically to regions of single stranded DNA. Numerous HBV qPCR assays have been developed to quantify serological viral loads using SYBR-green (Brechtbuehl et al., 2001), Fluorescence resonance energy transfer (FRET) probes (Aliyu et al., 2004), and TaqMan probes (Abe et al., 1999; Allice et al., 2007; Cheng et al., 2007). Furthermore, intrahepatic cccDNA can be isolated and measured by qPCR (He et al., 2002; Jun-Bin et al., 2003; Singh et al., 2004; Wong et al., 2004). The clinical monitoring of serological and intrahepatic cccDNA would provide insights into disease progression, HBV reactivation, and occult infections. Considering that cccDNA is at the heart of HBV replication, is able to form minichromosomes, and is involved in viral integration, it only seems rational to measure cccDNA when monitoring a patient's response to therapy, or when designing new anti-HBV therapeutic agents. The advances in HBV qPCR allow for the accurate quantification of both serological and intrahepatic viral loads as well as the discrimination between rcDNA and cccDNA.

1.2 The biochemistry of conventional and real-time PCR

PCR is regarded as the 'gold standard' for routine clinical detection and diagnosis of many viral pathological agents (Evans, 2009). The development of PCR is accredited to Kary Mullis who, with the help of his colleagues, first used this technique to diagnose sickle-cell anaemia (Mullis et al., 1986; Mullis and Faloona, 1987; Saiki et al., 1985). PCR is based on the exponential amplification of an *in vitro* DNA target through repetitive temperature gradient cycles allowing end-point quantification. The biochemistry and methodology of conventional PCR have been extensively described (Arnheim and Erlich, 1992; Evans, 2009; Schutzbank and Stern, 1993; Xu

and Larzul, 1991). The amplification of target DNA is an enzymatic reaction which requires oligonucleotide primers, thermostable DNA polymerase with an associated buffer, deoxyribonucleoside triphosphates (dNTPs), magnesium ions, and the DNA template. The oligonucleotide primers are specifically designed to flank the DNA target allowing subsequent amplification of that single region. The lengths of the primers confer DNA binding specificity which is critical to prevent non-specific DNA amplification. PCR is performed in an automated thermocycler permitting the step-wise amplification of target DNA.

Automated PCR includes the cyclic amplification and synthesis of new DNA strands through denaturation, annealing, and elongation steps. Denaturation is performed at high temperatures, between 94 – 96 ℃, which facilitates the activatio n of the polymerase and the disassociation of double-stranded DNA. Following denaturation, the temperature is decreased to allow annealing or hybridisation of the oligonucleotide primers to the target DNA. Optimal annealing is dependent on the melting temperature (Tm) of the individual primers, which is calculated based on primer length, GC content, and complementarity of the primer to the template DNA. Higher annealing temperatures improve specificity but reduce the end-point yield.   Subsequently, the polymerase extends from the oligonucleotide primers, amplifying the target DNA sequence. This elongation step is preferentially performed at 72℃. Each newly synthesised DNA strand then acts as a template for the next reaction cycle, exponentially amplifying the original DNA. As automated PCR is a closed system reaction, only end-point or relative quantification can be conducted, which is dependent on the simultaneous amplification of a housekeeping gene. Therefore conventional PCR is considered semi-quantitative.   These limitations have encouraged the development, adaptation and enhancement of conventional PCR to permit the rapid, accurate and sensitive quantification of DNA products during exponential amplification.

Real-time PCR (qPCR) enables the absolute quantification of specific DNA products using fluorescent reporter systems (Heid et al., 1996; Higuchi et al., 1993). Like conventional PCR, the biochemistry and methodology of qPCR has been comprehensively discussed (reviewed by Evans, 2009; Kubista et al., 2006; Mackay et al., 2002; Wilhelm and Pingoud, 2003). Real-time quantification allows the measurement of initial target DNA as it undergoes amplification past the threshold or crossing point (CP), as well as its exponential accumulation throughout PCR cycling. Detection of amplification is achieved using either fluorescent DNA-binding compounds or specific fluorescent oligonucleotide probes. Non-specific chemistries include intercalating agents such as ethidium bromide and SYBR® green DNA binding dyes, which are incorporated into the newly synthesised dsDNA during elongation. The fluorescence emission signals generated by these non-specific chemistries are dependent on the length and number of dsDNA templates, and not on sequence specificity. Fluorophore labelled oligonucleotide probes on the other hand, are designed to bind exclusively to target sequences. There are several fluorescent and FRET probes currently used during qPCR analysis. As these primers and probes are designed to bind specifically to target DNA sequences, they only emit a signal when they are bound to their complementary sequence and therefore they are far more precise than non-specific dyes. As different fluorophores emit at diverse wavelengths, oligonucleotide probes and primers harbouring distinct fluorophores can be used to detect and quantify numerous DNA sequences through a procedure referred to as multiplexing. This is particularly appealing for the detection and diagnosis of viral infections as multiple pathological agents can be investigated during a single qPCR.

The LightCycler™ 2.0 is a real-time PCR thermocycler, manufactured by Roche (Germany), which contains a single thermal chamber that is able to accommodate up to thirty two samples in a reaction (Wittwer et al., 1997). It employs a blue-light emitting diode for fluorescence excitation and photodiodes for emission detection. Glass capillaries hold the samples which

are placed in a circular carousel for thermal cycling. The practical design of the thermal chamber and the multi-wavelength detection platforms result in exceptionally rapid PCR cycling which can be multiplexed to detect numerous DNA templates (Betzl, 2000). The LightCycler™ software is used to construct real-time standard curves, permitting absolute quantification of DNA, as well as melting curves, to determine specificity (reviewed by Evans, 2009; Kubista et al., 2006). Standard curves are created by generating a logarithmic (log) serial dilution series, containing known amounts of target DNA, which are then amplified during PCR. These standards are included in all qPCR reactions as internal controls to prevent inter-assay discrepancies. Melting curves, determined by the first negative derivative of fluorescence versus temperature, establish amplification specificity. A melting curve assists in identifying non-specific products, or single base changes and polymorphisms. The precise and rapid amplification properties of the LightCycler™ 2.0 make it a practical and proficient instrument for viral DNA quantification.

1.3 Aims

Based on the need for an efficient routine HBV cccDNA and rcDNA quantification assay, this thesis examines the significance of designing a real-time quantitative PCR method, specifically for acquisition on the LightCycler™ 2.0, and applying it to routine laboratory diagnostics. As there is virtually complete sequence homology between rcDNA and cccDNA, a set of qPCR primers will be designed to amplify cccDNA alone. As rcDNA contains an incomplete positive DNA strand and a gap region located between DR1 and DR2 (Figure 1.1), cccDNA primers will flank both repeats, allowing comprehensive amplification of cccDNA alone. A second primer set will be created to amplify both rcDNA and cccDNA, based on the *pre-S1* ORF which is common to both HBV genomes. This will allow the total viral DNA as well as each HBV DNA

species to be accurately quantified. By subtracting cccDNA quantification from total HBV DNA, the number of rcDNA copies can be determined.

Real-time PCR fluorescence reporter systems based on non-specific DNA intercalating chemistries, using SYBR® Green I, will be used for detection of amplification. HBV samples, acquired from the HepG2.2.15 cell line and hepatitis B transgenic mice, will be exploited for viral DNA quantification. The HBV qPCR assay will be validated using Southern blot hybridisation to determine the ability of each technique to detect and quantify HBV DNA. The efficiency of RNAi-based anti-viral agents will be assessed using the HBV qPCR assay. Both cccDNA and rcDNA will be measured to determine whether RNAi is able to knock down these viral genomes and abolish HBV replication.

CHAPTER 2 – MATERIALS AND METHODS

2.1 HBV plasmids and cell lines

2.1.1 pCH-9/3091 and pHBV1.3x

Replication-competent HBV plasmids, pCH-9/3091 (Nassal et al., 1990), and pHBV 1.3 ×, alternatively designated pCR-HBVA1 1.3 × (Mufamadi, 2008) were used to design HBV DNA primers and act as standard positive controls. Both plasmids are greater than genome length constructs, which are capable of replicating when transfected *in vitro* or *in vivo*. The HBV plasmid maps are shown in Appendix 5.1.

2.1.2 Plasmid construction and cloning of pTZ-HBVcccR and pTZ-HBVrcR

The pCH-9/3091 plasmid consists of an HBV DNA coding region that is 1.3 times the length of HBV DNA. It therefore encodes a full HBV genome; with certain sequences, such as the *pre-core* region, repeated. The first *pre-core* sequence is located immediately downstream of the CMV promoter, which drives replication. The second *pre-core* region is positioned after the X protein coding region. pHBV 1.3 × is similar to pCH-9/3091 in that it, too, is a replication competent plasmid which is 1.3 times the length of HBV DNA. However the repeated sequence is the X protein coding region, with a slightly truncated *pre-core* repeat located directly after the second *X* gene (Appendix 5.1). The use of pCH-9/3091 or pHBV 1.3 × to create a standard curve is therefore problematic as two sequences will be amplified during PCR. To overcome this, plasmids containing a single copy of the HBV *pre-core* and X protein regions (cccR) and

pre-S1 region (rcR) were constructed to aid in HBV quantification. Figure 2.1 outlines the process of pTZ-HBVcccR and pTZ-HBVrcR cloning as well as the regions of the HBV genome chosen for plasmid construction and qPCR analysis.

Design of primers for amplification of ccc-region (cccR) and rc/ccc-region (rcR)

Primers were designed, based on the sequence of pCH-9/3091 and pHBV 1.3×, to flank the cccR and rcR. The cccR denotes a portion of the *HBx* region, *pre-core* and *core* region whilst the rcR consists of the *pre-S1* region. Primer analysis and validation was performed using the Net Primer analysis software (PREMIER Biosoft International) and the multiple alignment tool, BLAST (NCBI). Oligonucleotide primers were synthesised by Inqaba Biotec (Pretoria, Gauteng, South Africa) using standard phosphoramidite chemistry. A 421 bp region of the cccR was amplified from pHBV 1.3× using the following primer set: cccR-F (3935) – 5' CTG CCG TAC CGT CCG ACC A 3' (forward), cccR-R (4348) – 5' CGA ATT CGG TCA ATG TCC ATG CCC CAA A 3' (reverse). The same primer set amplified a 416 bp region of pCH-9/3091 between nucleotides 2779 and 3196. A 390 bp region of the pre-S1 gene within pHBV 1.3×, was amplified using the following forward and reverse primers: rcR-F (1901) – 5' GAC AAA GGG ATT AAA CCT TAT TAT CCT GA 3' (forward), and rcR-R (2284) – 5' CGA ATT CCC TCC GTG TGG GGG AGT GAA 3' (reverse). In pCH-9/3091 the rcR primer set amplifies a 348 bp region between base pairs 784 and 1132. Both the cccR and the rcR reverse primers contain a single *EcoR*I restriction site (GAATTC) at their 5' ends.
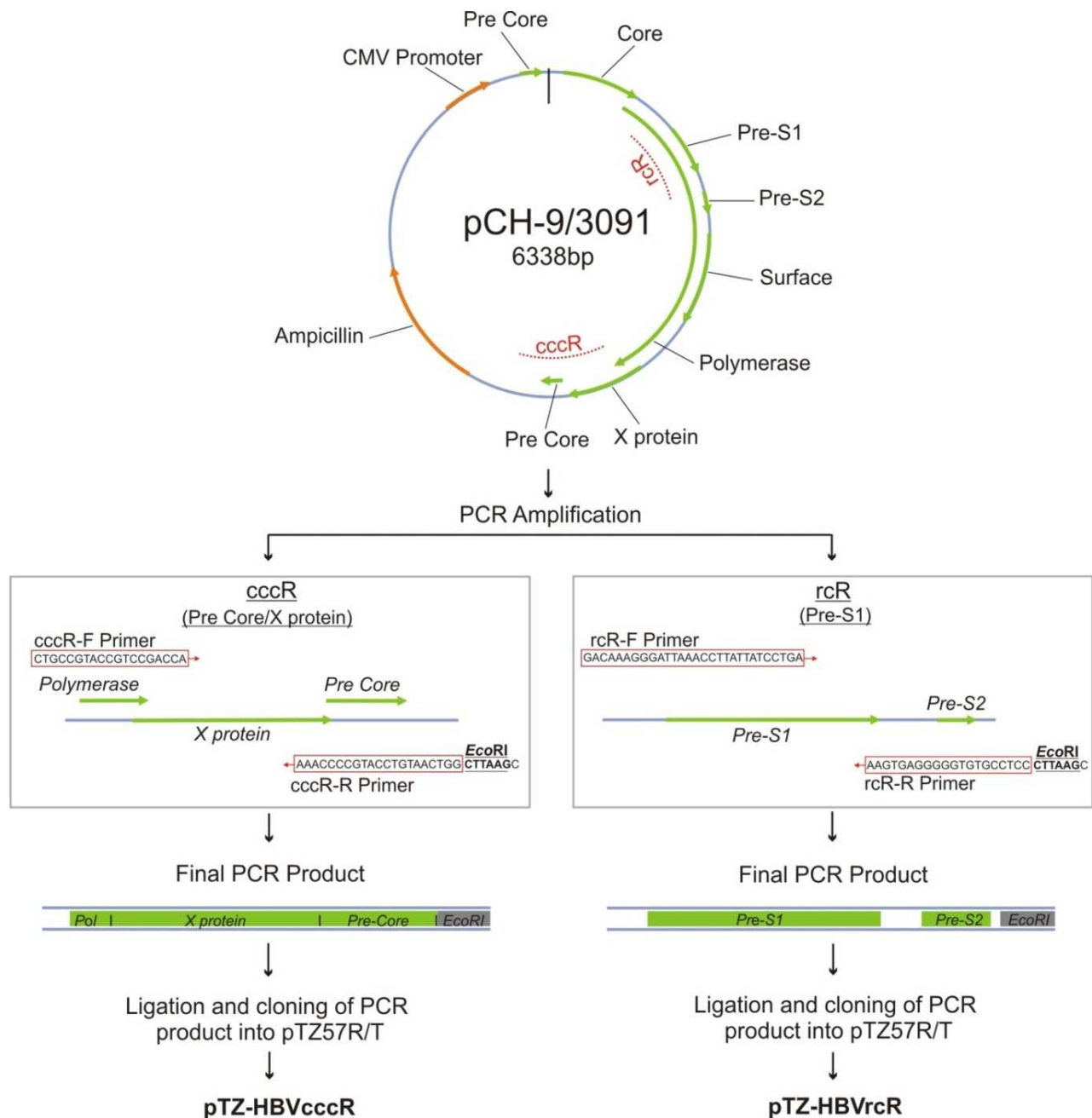
Figure 2.1: Overview of HBV plasmid construction and cloning. Primers were designed to amplify the 2779-3196 bp ccc-region (cccR) and 784-1132 bp rc/ccc-region (rcR) of pCH-9/3091. The cccR included the pre-core, X protein, and polymerase coding sequences. The rcR contained the *Pre-S1* and *Pre-S2* regions. An *Eco*RI restriction site was incorporated during amplification. Final PCR products were ligated into the multiple cloning site of pTZ57R/T, and transformed in chemically competent DH5α *Escherichia coli*.

PCR amplification and gel electrophoresis of cccR and rcR

The HBV plasmids, pCH-9/3091 and pHBV 1.3× were used as a templates for cccR and rcR amplification. The PCR system for cccR amplification included 120 mM Tris-HCL (pH 8.6), 100 mM HCL, 0.1% Tween-20, 1mM DTT, 10% glycerol, 3 mM $MgCl_2$, 0.625 U KAPATaq (Kapa Biosystems, Observatory, Cape Town, South Africa), 0.4 mM of each dNTP, 15 pmol cccR-F, 15 pmol cccR-R, and 254 ng of pHBV 1.3× or pCH-9/3091, to a final volume of 25 µl in a 0.5 ml PCR grade microcentrifuge tube (Axygen Scientific). The rcR PCR system components were the same as those used for the cccR amplification except for the substitution of the cccR forward and reverse primers with 15 pmol rcR-F and 15 pmol rcR-R. The PCRs were performed in an Eppendorf Mastercycler gradient thermocycler (Eppendorf, NY, USA) under the following conditions: initial denaturation at 95℃ for 2 min; 25 cycles of 95℃ for 30 s, 58℃ for 30 s, and 72℃ for 30 s; final elongation at 72℃ for 5 min and cooling at 4℃. Samples were analysed using agarose gel electrophoresis. PCR products were combined with 5 µl of 6× loading dye (0.125% bromophenol blue; 30% glycerol; 6× Tris-borate-EDTA (TBE) buffer [Appendix 5.2.1.]) and loaded into a 2% agarose gel containing 0.0025% Ethidium Bromide. An O'GeneRuler (Fermentas, MD, USA) was used as a molecular weight marker. The products were subjected to electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer containing 40 mM Tris acetate and 1 mM EDTA (Appendix 5.2.1). Band migration patterns were evaluated under UV transillumination, using the GelLogic 200 imaging system (Kodak, NY, USA) and the Kodak 1D image analysis software (Kodak).

Second round PCR amplification of the HBV plasmid amplicons

Agarose gel bands containing the DNA regions corresponding to the cccR and rcR were visualised on a UV transilluminator (UVP, CA, USA) and excised with a sterile surgical blade. The DNA was extracted from the gel and purified using the MinElute® Gel Extraction Kit (Qiagen, Hilden, Germany). The cccR and rcR DNA fragments were eluted in 10 µl of best

quality water (Sabax, Adcock Ingram, Gauteng, South Africa). The agarose gel extracted DNA products were amplified after a second round of PCR. The same PCR components including cycling conditions and gel electrophoresis (as described in section 2.1.2.2) were used, with the substitution of the HBV plasmid with the cccR DNA or rcR DNA. The second round PCR products were used for cloning into the T/A cloning vector pTZ57R/T (Fermentas, Maryland, USA).

Ligation of cccDNA region and rc/cccDNA region into pTZ57R/T

Two plasmids were generated by inserting the cccR or the rcR DNA into a pTZ57R/T vector (Fermentas, MD, USA). The InsTAclone™ PCR Cloning Kit ligation reaction was set up as follows: 0.165 µg of pTZ57R/T, 4 µl of 5× Ligation buffer, 7.5 U of T4 DNA ligase and 0.1 µg of either cccR DNA PCR product or rcR DNA PCR product, to a final volume of 20 µl in a 1.5 ml microcentrifuge tube. Ligation reactions were incubated at 18°C for 1 hour and 30 minutes. Nine microlitre aliquots were gently mixed with 100 µl of chemically competent DH5α *Escherichia coli* (*E. coli*) (Appendix 5.2.2).   The reaction mixture was incubated on ice for 25 minutes, heat shocked at 42°C for 90 seconds and finally rescued for 2 minutes on ice. The transformed *E. coli* were plated onto Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml), 40 µl (20 mg/ml) X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside in dimethyl-formamide), and 8 µl (100 mg/ml) IPTG (isopropyl-beta-D-thiogalactopyranoside). LB plates were incubated at 37°C overnight, followed by 10 minute refrigeration at 4°C to enhance the colouring of the pTZ57T/R blue colonies. Five white colonies from each plate (i.e. five cccR ligation colonies and five rcR ligation colonies) were picked and individually incubated in sterile capped tubes containing 4 ml of LB media (100 µg/ml ampicillin). The tubes were incubated in a shaking incubator (Labcon, Gauteng, South Africa) at 37°C and 200 rpm overnight. The pTZ57R/T plasmid containing the cccR DNA is hereafter referred to as pTZ-HBVcccR and the pTZ57R/T plasmid containing the rcR DNA is referred to as pTZ-HBVrcR.

Plasmid DNA extraction and restriction digestion analysis

Cloned plasmids were extracted from the DH5α *E. coli* bacterial cultures using the High Pure Plasmid Isolation Kit (Roche, Germany) [Appendix 5.2.3]. The purified plasmids were eluted into a 1.5 ml microcentrifuge tube (Axygen Scientific) with 100 µl of best quality water. The concentration and purity of the extracted plasmids was determined by measuring their relative absorbance's with the NanoDrop® ND-1000 UV-Vis spectrophotometry (NanoDrop Technologies, DE, USA). To determine if the cloned plasmids contain the cccR or rcR inserts, two separate restriction digestions were performed (Appendix 5.2.3). The first was with *Eco*RI whilst the second was an *Xba*I/*Bam*HI double digestion. Both restriction digests were incubated at 37℃ for 1 hour, followed by gel electrophoresis analysis. DNA bands were observed under UV light in the GelLogic 200 imaging system with the 1D image analysis software. Plasmid extractions were stored at -20℃.

Sequencing

The pTZ-HBVcccR and pTZ-HBVrcR plasmids were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Briefly, 100 ng template DNA (pTZ-HBVcccR or pTZ-HBVrcR plasmid) was mixed with 1× BigDye sequencing buffer (80 mM Tris-HCL (pH 9.0), 2 mM $MgCl_2$) (Applied Biosystems), 1× Ready Reaction Premix (Applied Biosystems), and 3.2 pmol M13 forward primer, in a final volume of 10 µl. Template DNA was amplified by conventional PCR in an Eppendorf Mastercycler gradient thermocycler (Eppendorf, NY, USA) under the following conditions: initial denaturation at 96℃ for 5 minu tes followed by 50 cycles of 96℃ for 30 sec, 50℃ for 10 sec and 60℃ for 4 min. Following fina l elongation samples were cooled to 4℃. PCR extension products were purified by ethanol precipitation and sequenced by automated cycle sequencing. The sequence of pTZ-HBVcccR is shown in Appendix 5.1.3.

2.1.3 HepG2.2.15 cell culture

HepG2.2.15 cells, the human hepatoma cell line HepG2, containing a stably integrated HBV genome (Sells et al., 1987), was used to quantify total HBV DNA and cccDNA generated using a cell culture model system. Cells were cultured in 75 cm^3 flasks using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, USA) supplemented with 10% fetal calf serum (FCS), penicillin (100 000 U/ml), and streptomycin (100 000 µg/ml). Cells were maintained in a humidified incubator at 37°C and 5% CO_2 until confluent. The cell culture supernatant was removed and stored at -20°C. Adherent cells were g ently washed with 20 ml 1% Phosphate Buffered Saline (PBS). Cells were harvested and stored in 1000 µl of 1% PBS at -20°C.

2.2 HBV DNA extraction

2.2.1 HBV transgenic mice

Total DNA from the liver

A male HBV transgenic mouse (Marion, 2003) was sacrificed and the whole liver harvested. Viral DNA was extracted from 50 mg of homogenised liver tissue, using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) tissue spin protocol (Appendix 5.2.4), following the manufacturer's instructions. Ethanol precipitation and column binding was performed using 230 µl 100% ethanol to improve viral DNA extraction. The viral DNA was eluted in 200 µl of best quality water. The concentration of the elution was determined spectrophotometrically using the NanoDrop® ND-1000 with the NanoDrop ND-1000 3.3 software. Samples were stored at -70°C.

Total DNA from the serum

The blood was set aside to clot for 1 hour at 4°C i n 1.5 ml microcentrifuge tubes. Samples were then centrifuged for 6 minutes at 3000 rpm in an Eppendorf MiniSpin microcentrifuge, rotor F45-12-11, GE 010 (Brinkmann [Eppendorf], New Haven, CT, USA). The serum was aspirated and transferred to a clean 1.5 ml microcentrifuge tube, followed by centrifugation for an additional 6 minutes at 3000 rpm in the Eppendorf MiniSpin (Brinkmann) to remove residual blood cells. The QIAamp® DNA mini kit blood spin protocol was used to extract viral DNA from 20 µl of serum, however carrier DNA was excluded from the extraction. The HBV DNA was eluted into a 1.5 ml microcentrifuge with 100 µl of best quality water and analysed spectrophotometrically (Appendix 5.2.5). Serum HBV DNA extractions were stored at -20°C.

DNA extraction from whole virions in serum

HBV transgenic mouse serum was prepared, as explained in the previous extraction step, and treated with two different DNases to destroy all unprotected DNA (genomic DNA and naked cccDNA) leaving only whole HBV virions. Twenty four microlitres of HBV transgenic mouse serum was incubated with either 4 µl of gDNA wipe out buffer (Qiagen, Hilden, Germany) for 6 minutes at 42°C, or with 10 U DNase (Promega, WI, U SA), 3 µl 10× buffer (Promega) and 1.5 µl best quality water for 30 minutes at 37°C. The HBV rcDNA, within the whole virions, was extracted using the QIAamp® DNA mini kit blood spin protocol (without carrier DNA) and eluted in 60 µl best quality water. Whole virion extractions were analysed using qPCR.

2.2.2   HepG2.2.15 cell line

Total HBV DNA was extracted from 200 µl of cell culture supernatant using the QIAamp® DNA Mini Kit blood spin protocol, and 200 µl of HepG2.2.15 cells using the QIAamp® DNA Mini Kit

tissue spin protocol. Extractions were according to the manufacturer's instructions with one adjustment: ethanol precipitation and column binding was performed using 230 µl 100% ethanol, instead of 200 ul, as recommended in the protocol. This was found to improve viral DNA extraction. DNA was eluted into a 1.5 ml microcentrifuge tube with 200 µl of best quality water. DNA samples were analysed (Appendix 5.2.5) and extractions were stored at -20℃.

2.3 Mouse genomic DNA extraction and detection

2.3.1   Extraction of DNA from mouse liver

Mouse genomic DNA was extracted from a normal mouse liver using the tissue protocol from the QIAamp® DNA mini kit. Mouse genomic DNA was eluted in 200 µl of best quality water and analysed (Appendix 5.2.5) before storing at -20℃.

2.3.2   Primer design for amplification of *St6gal1* mouse liver housekeeping gene

The *beta galactoside alpha 2,6 sialyltransferase 1* (*St6gal1*) gene was selected as a mouse liver housekeeping gene (Mouse Genome informatics accession number: 108470, NCBI accession number: NM145933) as it is located in the liver as a single copy gene. The forward and reverse *St6gal*1 primer set was: St6gal1-F – 5' GCT TCC CAG AAG ATC CGT TCT CAA GGG G 3' (forward) and St6gal1-R – 5' AGG GCT GCC TCT CAG CGC ACC CCT G 3' (reverse). These primers amplify a 303 base pair fragment on chromosome 16 of the mouse genome (NCBI accession number NT 039624). Primers were analysed using the Net Primer online software (PREMIER   Biosoft   International)   and   the   BLAST   multiple   alignment   tool   (NCBI).

Oligonucleotide primers were synthesised by standard phosphoramidite chemistry (Inqaba Biotec). Primers were stored at -20℃.

2.4 HBV detection and quantification by Southern blot hybridisation

2.4.1   Design and synthesis of digoxigenin (DIG)-labelled HBV probe

An HBV DNA probe was synthesised using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Germany). The probe was designed to span a 256 bp region of the *pre-S1* gene within the HBV, using the following primer set: HBV DNA-F – 5' TTA TTA TCC AGA ACA TGT AGT TAA TCA TTA CTT CC 3' (forward) and HBV DNA-R – 5' TTG ATG GGA TTG AAG TCC CAA TCT GGA TT 3' (reverse). The DIG labelled probe reaction mix included 5 µl of 10× PCR buffer, 5 µl of PCR DIG probe synthesis mix, 15 pmol of forward and reverse primer, 2.625 U of Enzyme mix, and 58 ng of pCH-9/3091 to a final volume of 50 µl with best quality water.   An unlabelled control was included to indicate whether the PCR was successful. This control incorporated all of the components of the DIG labelled probe, except the PCR Dig probe synthesis mix was substituted with 5 µl of dNTP stock solution (200 µM of each dNTP). The thermocycling parameters were a 95℃ hot start for 2 minutes followed by 30 cycles of 95℃, 60℃ and 72℃ (each for 30 seconds). Final elongat ion was performed at 72℃ for 7 minutes and cooling at 4℃.   The efficacy of the DIG labell ing reaction was determined by gel electrophoresis. A 5 µl aliquot of the DIG labelled and DIG unlabelled PCR products were subjected to electrophoresis on a 1.5% agarose gel (0.0025% Ethidium Bromide) in 1× TAE (Appendix 5.2.1) for 30 minutes at 100 V.   A 10 µl aliquot of O'GeneRuler DNA mix was run simultaneously to determine whether DIG was successfully incorporated into the labelled probe,

indicated by a shift in molecular weight. Band migration was observed under UV light in the GelLogic 200 imaging system with the 1D image analysis software. DIG labelled probe was stored at - 20°C.

2.4.2   Southern blot hybridisation

Concentrated HBV DNA extractions and pCH-9/3091 standards were subjected to endonuclease digestion (see section 2.4.4) prior to gel electrophoresis and alkaline capillary transfer. A known dilution series of pCH-9/3091, ranging from 116 ng to 0.11325 ng, was included as a positive control, and later used as a standard reference for quantification. Mouse genomic DNA was included as a negative control. Twenty-five microlitre aliquots were mixed with 6× loading dye (Appendix 5.2.1) prior to gel loading. Electrophoresis was performed using a 1% agarose gel (0.00025% Ethidium Bromide) in 1× TAE (Appendix 5.2.1). O'GeneRuler DNA ladder mix was used as a DNA molecular weight marker. DNA migration was visualised under UV light using the GelLogic 200 imaging system. Additionally, the migratory distance of the DNA ladder (in millimetres) from the well, was measured.

Once HBV DNA samples had migrated sufficiently, the gel was trimmed of excess lanes and subjected to alkaline transfer using capillary action (Brechot et al., 1981; Southern, 1975). Before transfer, the DNA in the gel was denatured and neutralised. Two 15 minute washes in 100 ml of 0.5 N NaOH; 1.5 M NaCl solution were used to denature the DNA, whilst neutralisation was performed using a single 30 minute wash in 100 ml of neutralising solution (1 M Tris, 3 M NaCl, pH 7.0). Alkaline capillary transfer was performed using 20× SSC (Sodium chloride sodium citrate buffer)(Appendix 5.2.1). The DNA was transferred overnight by capillary action onto a positively charged Hybond-N+ nylon membrane (GE Healthcare, NJ, USA). After

transfer the membrane was UV crosslinked for 2 minutes at 2000 J and baked at 80°C for 1 hour.   After baking, the membrane was stored at room temperature until probe hybridization.

Probe hybridisation and detection was adapted from the Roche molecular Biochemicals, DIG Application Manual for filter Hybridization (Roche Diagnostics, Germany).   Briefly, 25 ml of DIG Easy Hyb buffer (Roche) was heated to 42°C.   A 20 ml aliquot was added to a hybridisation cylinder containing the nylon membrane, and incubated for 30 minutes at 42°C in a rotating oven.   During the pre-hybridisation step, a 5 µl aliquot of DIG-labelled HBV probe was denatured at 95°C for 5 minutes, and mixed with 500 µl of pre-heated DIG Easy Hyb.   The remaining 4.5 ml of the pre-hybridisation buffer was later added to the probe.   The DIG Easy Hyb buffer was removed from the membrane and the 5.5 ml aliquot of probe and DIG Easy Hyb mix, was added. The Southern blot was allowed to hybridise overnight, at 42°C.   Excess probe was removed by washing the membrane as follows: a 20 minute low stringency wash (1% SDS, 5× SSC) performed at room temperature, followed by two 15 minute high stringency washes (1% SDS, 1× SSC) at 42°C.   The Southern blot membrane was remo ved from the hybridisation cylinder and rinsed with 200 ml of 1× washing buffer (0.1 M Malic acid, 0.15 M Sodium chloride, pH 7.5, 0.03% Tween) for 2 minutes. The membrane was blocked for 30 minutes in 200 ml of 5 % blocking buffer (5 g low-fat milk powder in 100 ml 1% PBS) followed by antibody binding (1 µl of Anti-Digoxigenin-alkaline phosphatase Fab fragment (Roche Diagnostics, Germany) in 40 ml of 1% blocking buffer) for 30 minutes.   Unbound antibody was washed off using four washes of 200 ml 1× washing buffer, each for 15 minutes, at room temperature.   Finally, to prepare for chemiluminescence detection, the membrane was washed with 40 ml of detection buffer (0.1 M Tris.HCl, 0.1 M sodium chloride, pH 9.5) for 5 minutes.

Chemiluminescence detection was performed by spreading the substrate for alkaline phosphatase, CPD-Star (Roche Diagnostics, Germany), evenly across the membrane.   A

transparency film was placed on top of the membrane to aid in the removal of air bubbles and excess CPD-Star. The Southern blot was placed in a developing cassette and under red light, Fuji RX film (Fuji, Japan) was inserted on top of the membrane. The film was exposed for between 5 to 20 minutes following which it was developed and fixed. Analysis was performed using image analysis software.

2.4.3   HBV DNA quantification using image analysis software

The Southern blot film was captured as a JPEG image using the GelLogic 200 imaging system with the 1D image analysis software. This image file was imported into the Java-based image processing software, ImageJ version 1.41 (Abramoff, 2004) and Southern blot hybridisation bands were quantified, using the pCH-9/3091 dilution series as internal standards. The bands were quantified by subtracting the background noise, inverting the image and determining the integrated density of each band (using the ImageJ software).

2.4.4   Enzymatic digestions to improve HBV migration patterns

_Hin_dIII and _Eco_RI digestions

To ensure that the migration of HBV DNA was not hindered by genomic DNA during Southern blot hybridisation, HBV DNA extractions and pCH-9/3091 samples were subjected to _Hin_dIII restriction digestion. As both murine and human genomic DNA contains multiple _Hin_dIII restriction sites within their genomes, the DNA will be digested. This restriction site is absent from the HBV genome, therefore _Hin_dIII will not affect the HBV DNA. Before digestion, the concentration of total DNA was determined by NanoDrop® ND-1000 UV-Vis spectrophotometry, following which the digestion mix was prepared as follows: 1U of _Hin_dIII (Fermentas, MD, USA) per microgram of DNA and 1× Buffer R (Fermentas, MD, USA). Samples were incubated at

37℃ for 1 hour and 15 minutes. The MinElute® Gel Extraction Kit was used to remove the enzyme, degraded DNA, and residual buffer R. Samples were eluted in 50 µl of best quality water. A second digestion, using *Eco*RI to linearise the HBV DNA, was performed with a 25 µl aliquot of the *Hin*dIII elution. The digestion mix included 1 U *Eco*RI (Fermentas, MD, USA) per µg of DNA, and 1× *Eco*RI buffer (Fermentas, MD, USA). Samples were incubated at 37℃ for 1 hour, followed by heat inactivation at 65℃. Dige sted samples were stored at -20℃.

Plasmid-Safe™ATP-dependent DNase treatment for the isolation of cccDNA

Plasmid-safe™ ATP-dependent DNase (Epicentre Biotechnologies, WI, USA) was used to degrade genomic DNA, rcDNA, HBV dsDNA and HBV ssDNA within the HBV transgenic mouse liver and HepG2.2.15 cell extracts. The samples were digested in a solution containing the following components: 25 mM ATP, 2.5µl Reaction buffer (330 mM Tris-acetate (pH 7.8), 660 mM potassium acetate, 100 mM magnesium acetate, and 5.0 mM dithiothreitol (DTT), 5 U Plasmid-safe™ DNase (Epicentre Biotechnologies), within a total volume of 50 µl in a 1.5 ml microcentrifuge tube. The samples were incubated at 37℃ for 30 minutes, and analysed by means of Southern blot hybridisation and qPCR.

2.4.5   Ethanol precipitation to increase HBV DNA concentrations

HBV DNA was concentrated using alcohol (isopropanol and ethanol) precipitation with resuspension in a small volume after *Hin*dIII digestion. This was to aid in HBV DNA detection during Southern blot hybridisation. Briefly, 10 µl of 3 M sodium acetate was gently mixed into 100 µl of the HBV DNA elution, followed by 100 µl of 100% isopropanol, in a 1.5 ml microcentrifuge tube. Elutions were subjected to centrifugation at 12 000 × *g*, for 20 minutes at 4℃. The supernatant was discarded and the pellet was gently washed in 50 µl of 70% ethanol, by spinning the ethanol through the pellet for 2 minutes at 10 000 × *g*. This was repeated three

times.   The supernatant was removed and the pellet was air dried at 40°C until all residual

ethanol had evaporated.   Finally the DNA pellet was resuspended in 50 µl of best quality water.

2.5   Design of total HBV DNA and cccDNA qPCR primers

2.5.1   HBV genomes and primer synthesis

The cccDNA and total HBV DNA primers were designed using the HBV subtype A1 complete

genome sequence (NCBI accession number AY738139), as well as the HBV plasmids pCH-

9/3091 and pHBV 1.3× (described in section 2.1.1).   The 3' ends of the primers were designed

to have complete sequence complementarity to the target for efficient primer binding and

amplification.   Primer designs were analysed and validated using Net Primer (PREMIER

Biosoft   International),   and   nucleotide   basic   local   alignment   search   tool   (BLAST).

Oligonucleotide   primers   were   synthesised   using   standard   phosphoramidite   chemistry

(Integrated DNA Technologies, Coralville, IA, USA and Inqaba Biotec).

2.5.2   Total HBV DNA (rc/cccDNA) primer set

DNA primers used to detect total HBV DNA were designed to amplify the *pre-S1* region within

the HBV genome.   The primer set includes: rc/cccDNA-F – 5' TTA TTA TCC AGA ACA TGT

AGT TAA TCA TTA CTT CC 3' (forward) and rc/cccDNA-R – 5' TTG ATG GGA TTG AAG TCC

CAA TCT GGA TT 3' (reverse).   The primers bind to HBV subtype A1 (AY738139) between

nucleotides 2705 to 2971, resulting in a 266 bp product.   In pCH-9/3091, the primer sets

amplifies a 256 bp region, from nucleotides 798 to 1054. In the pHBV 1.3× plasmid, the primers amplify between base pairs 1918 and 2184, which results in a 266 bp PCR product.

2.5.3   HBV cccDNA primer set

The cccDNA primers were designed to flank Direct repeat 1 and 2, which in turn will only amplify the repaired cccDNA form of the virus as the rcDNA includes a gap region within the 5' end of the negative DNA stand at this point.   The cccDNA primers amplify a portion of the core promoter region and the *X* gene. The sequences of the HBV cccDNA primer set used for both standard PCR and real-time PCR include: cccDNA-F – 5' TTC TCA TCT GCC GGT CCG TGT 3' (forward), and cccDNA-R – 5' TGG GAC ATG TAC AAG AGA TGA TTA GGC 3' (reverse). The cccDNA primers amplify between the nucleotide base positions 1560 and 1859 of the HBV subtype A1 sequence (AY738139), 2842 and 3115 on pCH-9/3091, and lastly between 774 and 1072 on pHBV 1.3×. Therefore cccDNA amplification should result in a 273 bp and 298 bp amplicons in pCH-9/3091 and pHBV 1.3× respectivley.   However as both the HBV plasmids include a greater than genome length transcript, they have two core promoter regions. Consequently they have an additional cccDNA binding site.   The pHBV 1.3× plasmid has additional recognition sites for both cccDNA forward and reverse primers between bases 3994 and 4268. Only a second HBV cccDNA-R binding site occurs in pCH-9/3091 at base position 6297. As there is no complementary cccDNA forward primer binding site present, only a single PCR strand is amplified during PCR. The cccDNA primer binding regions found in pCH-9/3091 and pHBV 1.3× are illustrated in Figure 2.2.   As a result of the multiple cccDNA primer binding sites, absolute quantification of cccDNA was performed using the pTZ-HBVcccR plasmid, as described in section 2.1.2.   PCR amplification of the HBV A1 sequence produces a 299 bp fragment, whilst amplification of pTZ-HBVcccR results in a 298 bp PCR product.
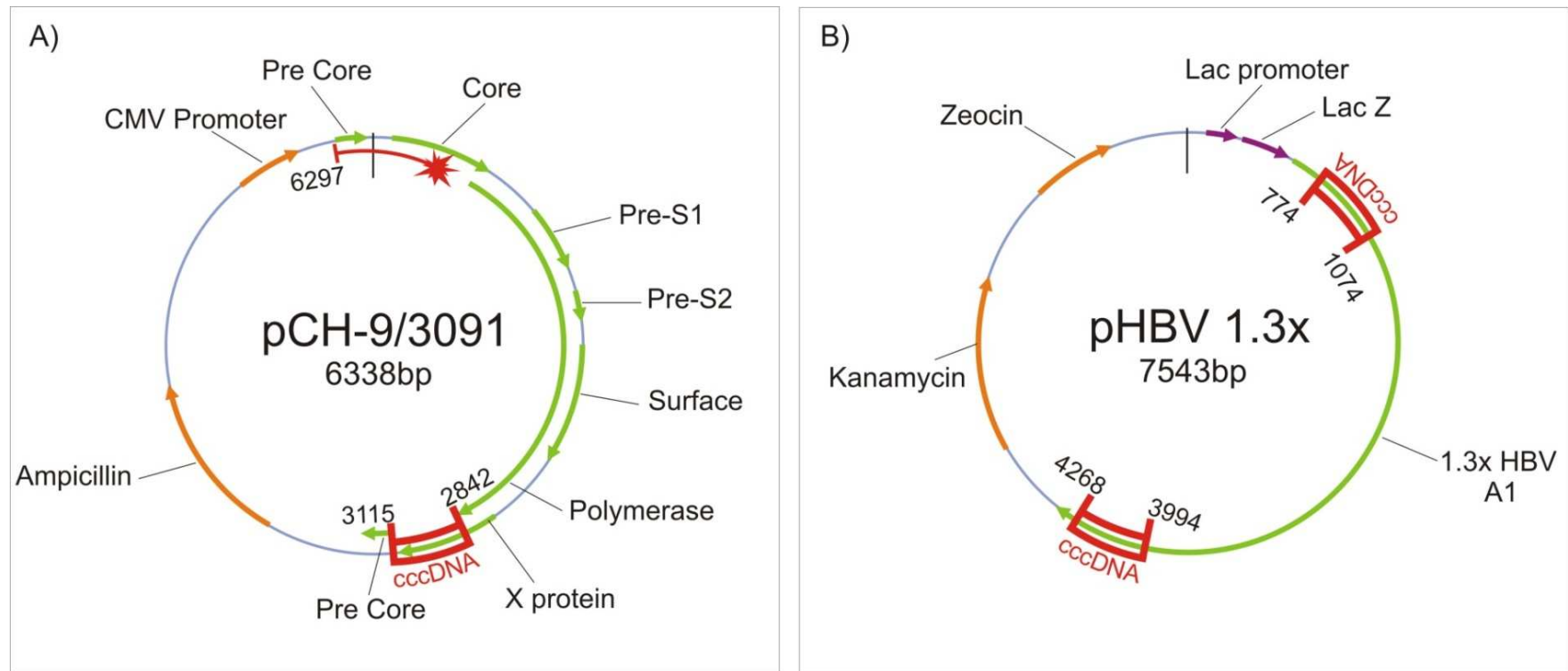
Figure 2.2: HBV plasmid primer binding positions for the amplification of cccDNA. **A)** The cccDNA primer set efficiently amplifies a single region of pCH-9/3091, from nucleotides 2842 to 3115 (273 bp). This amplicon corresponds to the X and pre-core protein coding regions. A second reverse primer binding site occurs at nucleotide base position 6297, however the region amplified does not correspond to the correct HBV coding region. Instead it amplifies the pre-core and core gene sequences which are not flanked by the cccDNA forward primer. **B)** In pHBV 1.3×, there are two cccDNA amplification regions found between nucleotide positions 774-1074 (298 bp) and 3994-4268 (274 bp). Both these regions correspond to the *HBx* and *pre-core* ORFs. It is difficult to perform absolute quantification with either of these plasmids, as a single round of PCR would create two different amplification products. The first pHBV 1.3× cccDNA amplicon, located between nucleotide positions 774-1074 was inserted into pTZ-HBVcccR.

2.6 Qualitative analysis of HBV primers by conventional PCR and gel electrophoresis

HBV cccDNA and total HBV DNA primer sets were evaluated against the two HBV DNA plasmids, pTZ-HBVcccR and pCH-9/3091. The PCR system for cccDNA detection included 0.5 U GoTaq® DNA polymerase (Promega, Madison, WI, USA), 15 pmol cccDNA-F, 15 pmol cccDNA-R, $1\times$ GoTaq® Flexi-Buffer (2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP)(Promega), and 0.1 ng pTZ-HBVcccR to a final volume of 25 µl. The same PCR system used for cccDNA amplification was used for total HBV DNA detection with the substitution of the cccDNA primer set with the rc/cccDNA forward and reverse primers, and pTZ-HBVcccR with pCH 9/3091, at the same concentrations. Negative controls were included using the same PCR reaction components but with the absence of HBV plasmid DNA. All conventional PCR runs were performed in an Eppendorf Mastercycler gradient thermocycler. Cycle parameters included initial denaturation at 95℃ for 2 min followed by 25 cycles at 95℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s, and fin al extension at 72℃ for 5 min. PCR products were analysed by agarose gel electrophoresis. Ten microlitre aliquots were mixed with $6\times$ Loading dye (Appendix 5.2.1), and 10 µl of O'GeneRuler was loaded separately as a DNA ladder. cccDNA amplification products were loaded into a 2% agarose gel containing 0.0025% Ethidium Bromide whilst the PCR products from the total HBV DNA amplification were loaded into a 1.5% Agarose gel (0.0025% Ethidium Bromide). The samples were subjected to electrophoresis for 90 min at 130 V in $1\times$TAE buffer (Appendix 5.2.1). Gels were analysed using the GelLogic 200 imaging system and visualised under UV light for DNA band migration with the Kodak 1D image analysis software.

2.7 Quantification of total HBV DNA and cccDNA by Lightcycler™ PCR

2.7.1   Total HBV DNA (rc/cccDNA) and cccDNA Real-time PCR standards

Eight-fold log serial dilutions of pTZ-HBVcccR, pTZ-HBVrc/cccR, and pCH-9/3901 were created. Triplicates of plasmid serial dilutions were amplified by real-time qPCR using the Lightcycler™ 2.0 thermocycler, and analysed using the Lightcycler ™ software version 4.0 (Roche Diagnostics, Germany). cccDNA amplification and quantification was performed using the pTZ-HBVcccR construct as the standard. The rc/cccDNA-F and rc/cccDNA-R primers were used to quantify total HBV DNA, through the amplification of both the pTZ-HBVrcR and pCH-9/3901 as plasmid standards.   The Lightcycler™ PCR mix for the quantification of cccDNA or total HBV DNA included 4 µl LightCycler® FastStart DNA Master^{plus} SYBR Green I (Roche Diagnostics, Germany), 15 pmol forward primer (either cccDNA-F or rc/cccDNA-F), 15 pmol reverse primer (either ccDNA-R or rc/cccDNA-R), and 5 µl of a log serial dilution (10^2 - 10^7) of DNA template (either pTZ-HBVcccR, pTZ-HBVrcR, or pCH-9/3901), to a final volume of 20 µl in a Lightcycler™ glass capillary (Roche Diagnostics, Germany), under sterile conditions. Capillaries were centrifuged (in cooled metal capillary adaptors) for 30 seconds at 700 × *g*. Capillaries were loaded into the Lightcycler carousel and the samples were amplified under the following conditions: hot start at 95℃ for 10 min; 50 PCR cycles of 95℃ for 10 s, 60℃ for 10 s and 72℃ for 10 s with a single SYBR® Green signal acquisition at this point. Melting curve analysis was performed after the PCR step by heating at 95℃ for 0 s, cooling to 65℃ for 1 min, and raising the temperature to 95℃ at a ramp of 0. 1℃/s with continuous SYBR® Green signal acquisition.   Standard curves were constructed for each of the dilution series samples, in triplicate, based on their relative crossing points (CP). Each log dilution had a particular threshold CT, or CP, which corresponded to the cycle at which the sample first enters

exponential amplification, and the fluorescence emitted is greater than that of the background fluorescence. The CP points were plotted against the known DNA concentrations provided by the dilution series to create a linear standard curve. Melting curve analysis was performed to assess the specificity of the amplification reaction and to monitor for contamination. A minimum detection limit was assigned for cccDNA quantification and total HBV DNA quantification based on the standard curves. The plasmid concentrations in each dilution were converted to plasmid copy numbers using the following equation:

$$\text{Number of copies} = (\text{mass} \times 6.022 \times 10^{23}) / (\text{length} \times 1 \times 10^9 \times 650)$$

Where the mass is the amount of plasmid in nanograms and length is the size of the plasmid in base pairs. This calculation is based on the assumption that a single base pair has a molecular weight of 650 Da. The derivation of the above equation is from first principles, as described below.

Mw^{dsDNA} = length x 650

Presuming the average weight of a base pair (bp) is 650 da, the molecular weight of double standed DNA (Mw^{dsDNA}) is it's length (size of the DNA in base pairs) multipled my the mass of a single mole of bp (650 g)

Mw^{dsDNA} = n / m

Additionally, from the molar mass equation, the Mw^{dsDNA} is equivalent to the number of moles (n) per gram (m)

molecules / gram = (moles / gram) x (6.0221415 x 10^{23} molecules/mole)

The number of molecules per gram of DNA can be calculated by multiplying the Mw^{dsDNA} (moles per gram) by Avogadro's number (molecules per mole)

Number of copies = (mass x 6.022x10^{23}) / (length x 1x10^{9} x 650)

Substitute the Mw^{dsDNA} with the first equation, convert grams to nanograms (ng), and multiply by the amount of DNA (ng) per sample to determine the number of moleules or copy number

2.7.2   Lightcycler™ quantification of HBV DNA from HepG2.2.15 cell culture and HBV transgenic mouse

DNA extracted from HBV transgenic mouse samples and HepG2.2.15 cell samples (described in section 2.2) were analysed by qPCR.   All samples were evaluated in triplicate using the cccDNA primer set and the rc/cccDNA primer sets. During HBV DNA quantification, mouse genomic DNA and water blanks were included as negative controls. Concurrently, standard

dilutions of pTZ-HBVcccR were run as positive controls for cccDNA quantification, whilst pTZ-HBVrcR and pCH-9/3901 were run as positive controls for total HBV DNA quantification. The qPCR components for cccDNA, total HBV DNA and *St6gal1* detection included 4 µl LightCycler® FastStart DNA Master^plus SYBR Green I, 15 pmol forward primer (either cccDNA-F, rc/cccDNA-F, or St6gal1-F), 15 pmol reverse primer (either ccDNA-R, rc/cccDNA-R or St6gal1-R), and 5 µl of DNA sample (either HBV mouse liver, HBV mouse serum, HepG2.2.15 cells, HepG2.2.15 media supernatant, mouse genomic DNA, pTZ-HBVcccR, pTZ-HBVrcR, or pCH-9/3901) to a final volume of 20 µl in a glass Lightcycler™ capillary. PCR cycling conditions were the same as those described in section 2.7.1. To quantify rcDNA, the total number of cccDNA copies/µl was subtracted from total HBV DNA copies/µl, to give the exact amount of cccDNA and rcDNA in each sample.

2.8 Determining HBV RNAi-knockdown efficiency and monitoring of transgene delivery through psiCHECK2.2

2.8.1. Murine hydrodynamic tail injection

The knockdown efficiency of RNAi-based, anti-HBV gene silencing was assessed using the HBV qPCR assay. Murine hydrodynamic tail vein injections (Liu et al., 1999) were used to administer an HBV replication-competent plasmid (Yang et al., 2002), linear cassettes, and miR-expression plasmids. The anti-HBV sequences used were miR- expression plasmids containing U6 shRNA5 (Carmona et al., 2006) or miR-122/5 (Ely et al., 2008), and linear cassettes comprising Lin-122/5 or ITR-122/5 (Chattopadhyay et al., 2009). A transgene delivery control plasmid, psiCHECK2.2 (Ely et al., 2008), was included in each hydrodynamic injection to

monitor liver specific transgene delivery.   The plasmid DNA solution used for assessing RNAi-knockdown efficiency included: 5 µg pCH-9/3091, 5 µg RNAi expression cassette (pU6 shRNA 5, pCMV miR-122/5, Lin-122/5, or ITR-122/5), and 5 µg psiCHECK2.2.   A mock (containing 5 µg pCH-9/3091, 5 µg pCI-neo backbone, and 5 µg psiCHECK2.2) as well as a negative control (containing only 5 µg psiCHECK2.2) were also included.

2.8.2.   Quantification of total HBV DNA and RNA from mouse hydrodynamic injection serum and liver samples

To determine the effects of RNAi and linear cassettes on HBV circulating virions and intrahepatic HBV DNA and RNA levels, total DNA and RNA was extracted from mouse serum and liver samples respectively. Murine blood samples were collected by means of retro-orbital bleeding, at days 3 and 5 post injection. Total DNA was isolated from   30 µl of serum using the QIAmp® DNA Blood Mini Kit as previously described in section 2.2.1. Serum HBV DNA was quantified using real-time qPCR, as depicted in section 2.7.2. Total RNA was extracted from 100 mg of homogenised mouse liver according to the guanidine single-step RNA isolation from cultured cells and tissues (Chomczynski, 1996) as shown in Appendix 5.2.6, followed by reverse transcription of the RNA to cDNA using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Appendix 5.2.7). The qPCR components for HBV cDNA detection included 4µl LightCycler® FastStart DNA Master[plus] SYBR Green I, 15 pmol forward primer (rc/cccDNA-F), 15 pmol reverse primer (rc/cccDNA-R), and 5 µl HBV mouse liver, to a final volume of 20 µl with best quality water in a glass Lightcycler™ capillary. HBV RNA was quantified relative to murine *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA using the following primers: GAPDH-forward 5'- TTC ACC ACC ATG GAG AAG GC -3' and GAPDH-reverse 5'- GGC ATG GAC TGT GGT CAT GA -3' (Song E et. al, 2005). Negative controls included water blanks and RNA samples not subjected

to reverse transcription. Thermocycling parameters were the same as those depicted in section 2.7.2.   Intrahepatic DNA was isolated from 50 mg of homogenised mouse liver as described in section 2.2.1, and analysed by qPCR as described in section 2.7.2.

2.8.3. *Renilla* **luciferase real-time qPCR standards**

The *Renilla* luciferase gene, present in psiCHECK2.2, was chosen as a qPCR amplification target for monitoring the efficacy of transgene delivery after hydrodynamic injection. The *Renilla* luciferase primers, Rluc-F (1031-1057): 5' AAA TCA TCT TTG TGG GCC ACG ACT GG 3' (forward) and Rluc-R (1263-1290): 5' GAA CTC CTC AGG CTC CAG TTT CCG CAT 3' (reverse), amplify a 260 bp region of the psiCHECK2.2 plasmid. Primer designs were analysed and validated using Net Primer (PREMIER Biosoft International) and synthesised by standard phosphoramidite chemistry (Inqaba Biotec).

A nine fold log serial dilution of psiCHECK2.2 was used to create a *Renilla* luciferase real-time qPCR standard curve. The Lightcycler™ PCR components and thermocycling conditions, using the *Renilla* luciferase primer set, were the same as those described in section 2.7.1.   To determine whether the standard curve constructed was of high-quality, the efficiency and linear regression error scores were analysed. The minimum detection limit was set at 85 copies of *Renilla* luciferase.

2.9 **Multiple Alignments**

To determine whether the total HBV DNA and cccDNA primer sets are universal, a multiple alignment was performed using the HBV A1 genotype from Uganda (NCBI accession number:

AY934772), Gambia (NCBI accession number: AY934764), and Germany (NCBI accession number: AY738139) (Appendix 5.3). The multiple alignments were evaluated using the AlignX software from the Vector NTI suite 9.0.0 (Invitrogen, MD, USA).

2.10   Statistical Analysis

All Southern blot and qPCR quantification was performed in triplicate, for each sample type, condition, and/or treatment performed, unless otherwise stated. Data is represented as the mean ± the standard error of the mean (SEM). Two-tailed paired and unpaired Student's t tests were performed using GraphPad Prism version 4.00 (GraphPad software, San Diego, California, USA). P values of < 0.05 were regarded as statistically significant.

CHAPTER 3 – RESULTS

3.1 Real-time qPCR is capable of independently quantifying total HBV DNA and cccDNA

The two major forms of HBV DNA, the rcDNA and cccDNA, share extensive sequence homology, differing only by the incomplete nature of rcDNA's positive strand and gap region. To differentiate between these two DNAs during PCR analysis, HBV cccDNA primers were designed to exclusively amplify the cccDNA form of the virus. Total HBV DNA (rc/cccDNA) primer set amplifies a portion of the *polymerase* and *pre-S1* region, which is common to both HBV DNAs. Figure 3.1 highlights fundamental differences between the two forms of HBV DNA, as well as the orientation of both primer sets. cccDNA primers flank the Direct Repeats (He et al., 2002), which encompass the 5' gap region of the HBV DNA negative strand. As this gap region is present in rcDNA, but repaired in cccDNA, the primers only efficiently amplify cccDNA. Any PCR amplification of the rcDNA gap region should result in truncated amplicons. Additionally, the cccDNA forward primer was designed to bind 30 bases upstream of DR2, which coincides with the 5' RNA end of the HBV rcDNA positive strand. This inhibits forward primer binding and exponential amplification of truncated PCR products. Using these primer sets to create cccDNA and total HBV DNA standard curves, HBV DNA can be quantified from various sources by qPCR.
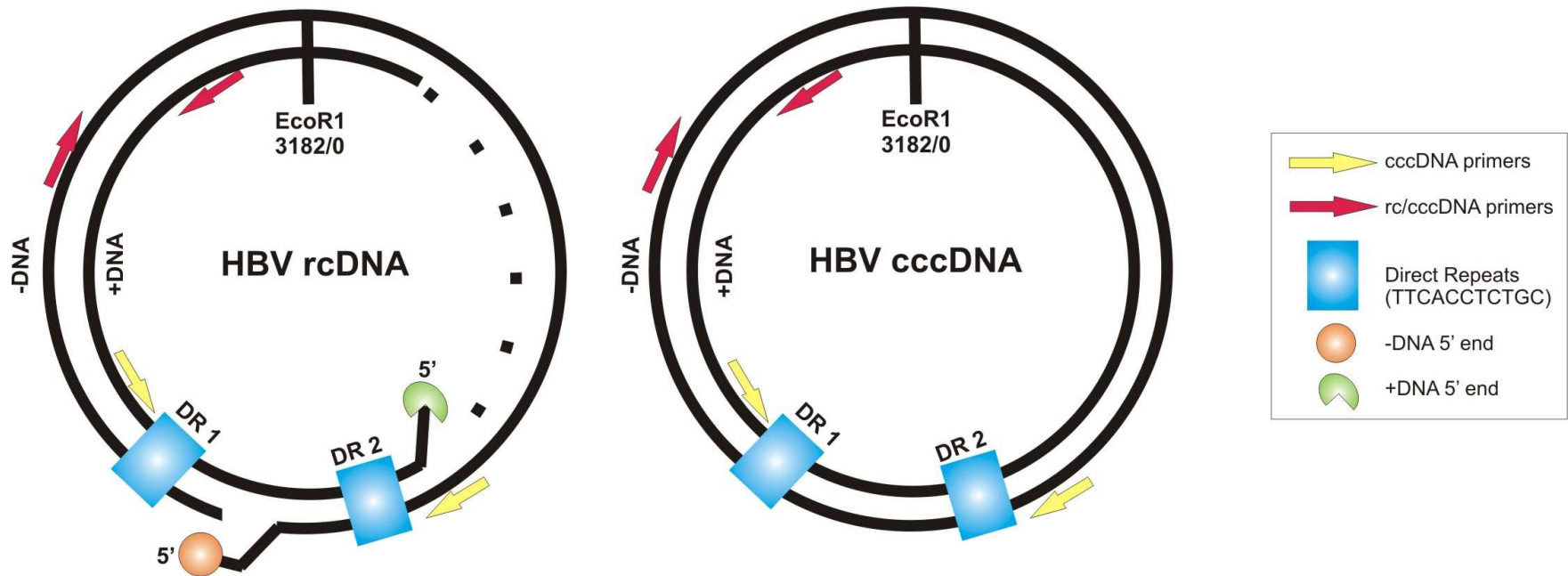
Figure 3.1: Schematic illustration of HBV rcDNA and cccDNA including specific qPCR primer binding sites. DR1 and DR2 are found in both the rcDNA and cccDNA forms of HBV, as indicated in blue. In rcDNA, DR1 and DR2 flank the 5' negative strand gap region which has subsequently been repaired in the cccDNA species. Additionally the positive DNA strand is only partially synthesised in the rcDNA but is complete in cccDNA. The cccDNA primers, shown in yellow, flank the Direct Repeats, which encompasses the gap region. The total HBV DNA (rc/cccDNA) primers, shown in red, are found further upstream and amplify sequences of the *polymerase* and *pre-S1* ORFs of HBV.

3.1.1   pTZ-HBVcccR contains a single copy of the HBV pre-core region

Replication-competent HBV plasmids contain a greater than genome length version of viral DNA, allowing pgRNA to be transcribed. As a result of this, both pCH-9/3091 and pHBV 1.3× contain two copies of the HBV *pre-core* ORF, and consequently two cccDNA primer binding regions. As neither pCH-9/3091 nor pHBV 1.3x could be used to accurately determine cccDNA copy number, a pTZ57T/R based plasmid clone, containing a single copy of the cccR, was constructed. An rcR plasmid was also constructed as a total HBV DNA control (Figure 2.1). The cccR and rcR regions were amplified from pCH-9/3091 and pHBV 1.3×.   Initially conventional PCR amplification of the cccR resulted in additional bands. This was as a result of the primers circularising the greater than genome length HBV transcript, caused by the presence of two HBV core regions. Therefore, bands corresponding to the correct cccR and rcR amplicon sizes, were excised, extracted, and re-amplified.   This resulted in a single amplification product which was cloned into pTZ57T/R. To confirm the presence of the cccR and rcR inserts, restriction digests were performed.

Two independent restriction digests were used to determine if the cccR and rcR inserts were successfully cloned into pTZ57T/R, as well as to determine their orientations (Figures 3.2 and 3.3). The first restriction digest, a double digestion including *Xba*I and *Bam*HI, was used to confirm the presence of the inserts within the selected clones. Both *Xba*I and *Bam*HI restriction sites directly flank the pTZ57T/R MCS. A double digestion of pTZ-HBVcccR and pTZ-HBVrcR resulted in DNA bands of 425 bp and 395 bp, corresponding to the cccR and rcR respectively. This confirms the presence of the inserts within the plasmids. A second restriction digestion, with *Eco*RI, was used to indicate whether the inserts were in a positive orientation. This is attributed to an *Eco*RI restriction site within pTZ57R/T plasmid, at base position 615, which

precedes the multiple cloning site. A second *Eco*RI restriction site was incorporated into the 5'

ends of the cccR and rcR reverse primers. If the HBV DNA insert is in the positive orientation,

an *Eco*RI restriction digest will, theoretically, result in a cccR and rcR insert of 450 bp and 419

bp respectively. However if a transcript of 39 bp or less is observed, a negative orientation is

assumed.

As shown, in Figure 3.3, all the clones were found to contain either the cccR or rcR insert,

however mostly in the negative orientation. Although the majority of the clones were in the

negative orientation, this does not affect real-time PCR. Therefore pTZ-HBVcccR clone iii (lane

14) and pTZ-HBVrcR clone number 1 (lane 17) were selected for the real-time PCR assays.

The pTZ-HBVcccR clone was found to have an elution concentration of 162.2 ng/µl whilst the

pTZ-HBVrcR clone has a final concentration of 147.6 ng/µl. To further validate the pTZ-

HBVcccR construct, the plasmid was sequenced (Appendix 5.1.3). The pTZ-HBVcccR plasmid

was used to construct a cccDNA real-time qPCR standard curve.   This construct is essential

for the accurate quantification of cccDNA during real-time PCR, as it contains only one copy of

the X protein and pre-core coding regions, unlike the greater than genome length plasmids

pCH-9/3091 and pHBV 1.3×.
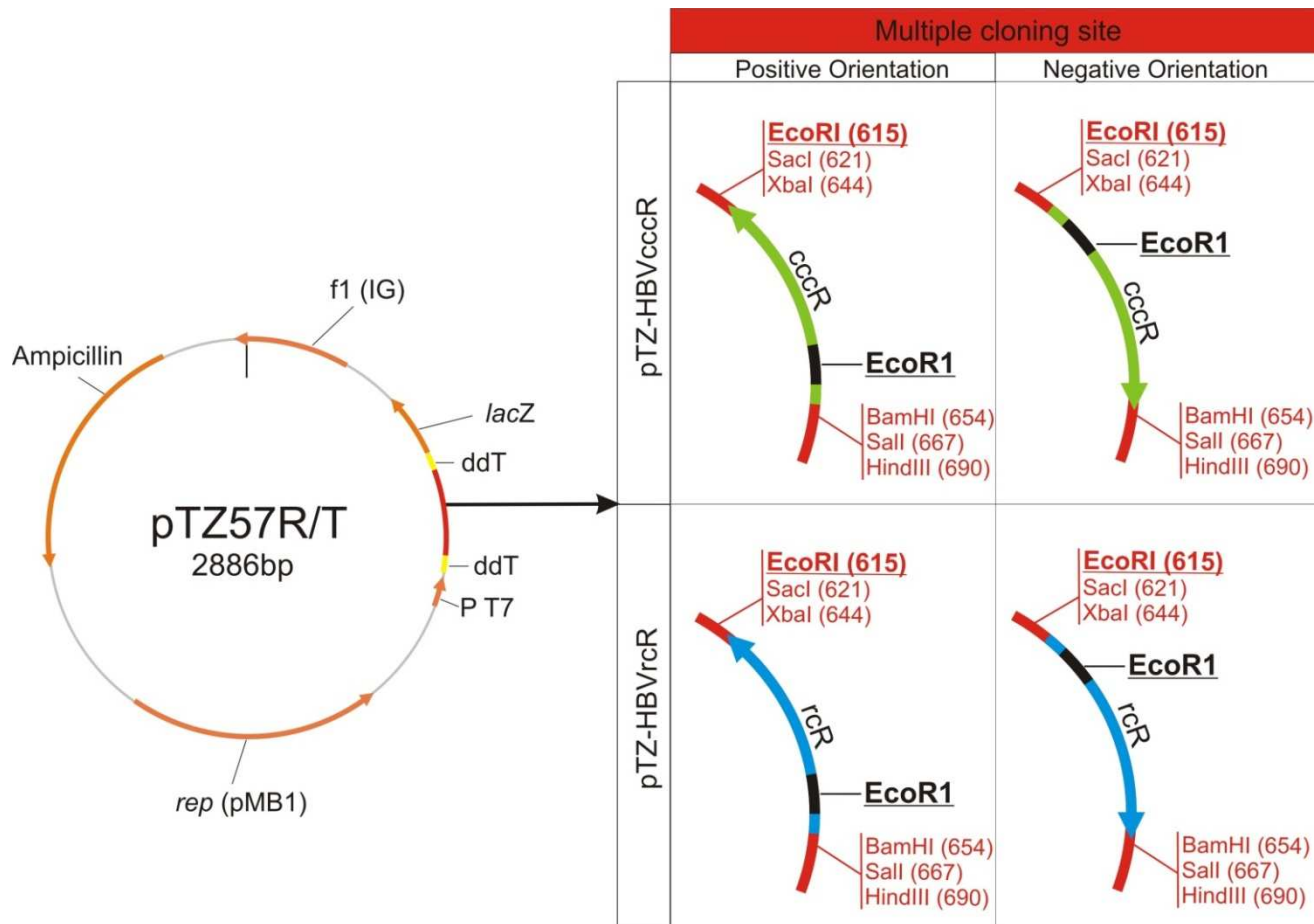
Figure 3.2: Positive and negative orientations of pTZ-HBVcccR and pTZ-HBVrcR. The cccR and rcR PCR products were cloned into pTZ57R/T. ORFs transcribed in the same direction as the T7 promoter (P T7) are in the positive orientation. A single inherent *Eco*RI site flanks the MCS whilst a second restriction site is generated during PCR amplification of the cccR or rcR ORFs. Orientation is resolved, by fragment size, subsequent to restriction digestion.
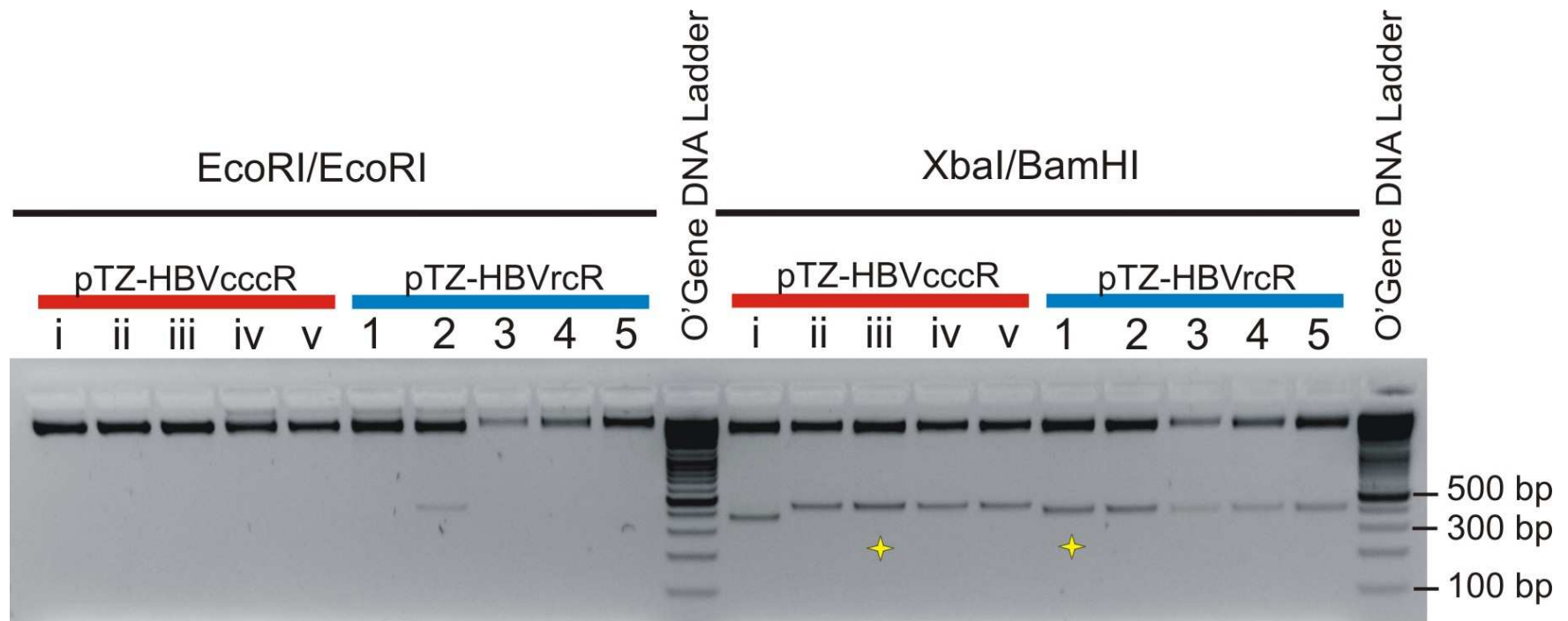
Figure 3.3: Restriction digest of pTZ-HBVcccR and pTZ-HBVrcR clones. Five pTZ-HBVcccR clones (i, ii, iii, iv, v) and five pTZ-HBVrcR clones (1, 2, 3, 4, 5) were chosen for restriction digest screening. All plasmids are positive for *Xbal*/*Bam*HI restriction digestion, based on the presence of a 450 bp band in pTZ-HBVcccR clones and a 419 bp band in pTZ-HBVrcR clones. A truncated product was observed for pTZ-HBVcccR clone i, which was subsequently eliminated from plasmid selection. The only clone positive for *Eco*RI double digestion is pTZ-HBVrcR clone 2, indicating the pre-S1 insertion is in the positive orientation. All remaining clones are in the negative orientation. Two clones, pTZ-HBVcccR iii and pTZ-HBVrc 1, were selected for qPCR analysis.

3.1.2   Primers designed for qPCR efficiently amplify HBV plasmid DNA using conventional PCR

Conventional PCR amplification together with agarose gel electrophoresis was used to determine the efficacy of both HBV primer sets. pTZ-HBVcccR and pCH-9/3091 acted as templates for cccDNA and total HBV DNA primers respectively. As shown in Figure 3.4, single amplicons were produced during both PCR assays, indicating that the primers bind exclusively to their designed targets. The post-PCR electrophoretic bands observed for pTZ-HBVcccR and pCH-9/3091 correlate with predicted amplicon sizes. cccDNA primers result in a band of approximately 300 bp, which equates to the 298 bp pTZ-HBVcccR target amplicon (Figure 3.4a), whilst total HBV DNA primers amplify a 256 bp region of pCH-9/3091, observed between the 200 and 300 bp molecular weight markers (Figure 3.4b). Primer-dimers, less than 100 bp in length, were observed at a very low intensity. Therefore, HBV qPCR primers efficiently amplify target DNA sequences. In addition to the PCR assays, a multiple alignment of various HBV A1 genotypes and pTZ-HBV cccR were assayed for efficient cccDNA primer binding. As shown in Figures 3.5 and 3.6, HBV A1 isolates and plasmid vectors have sufficient primer complementarity, specifically at the 3' ends, to amplify a range of HBV target sequences. HBV plasmid vectors were subsequently used to create qPCR standard curves.
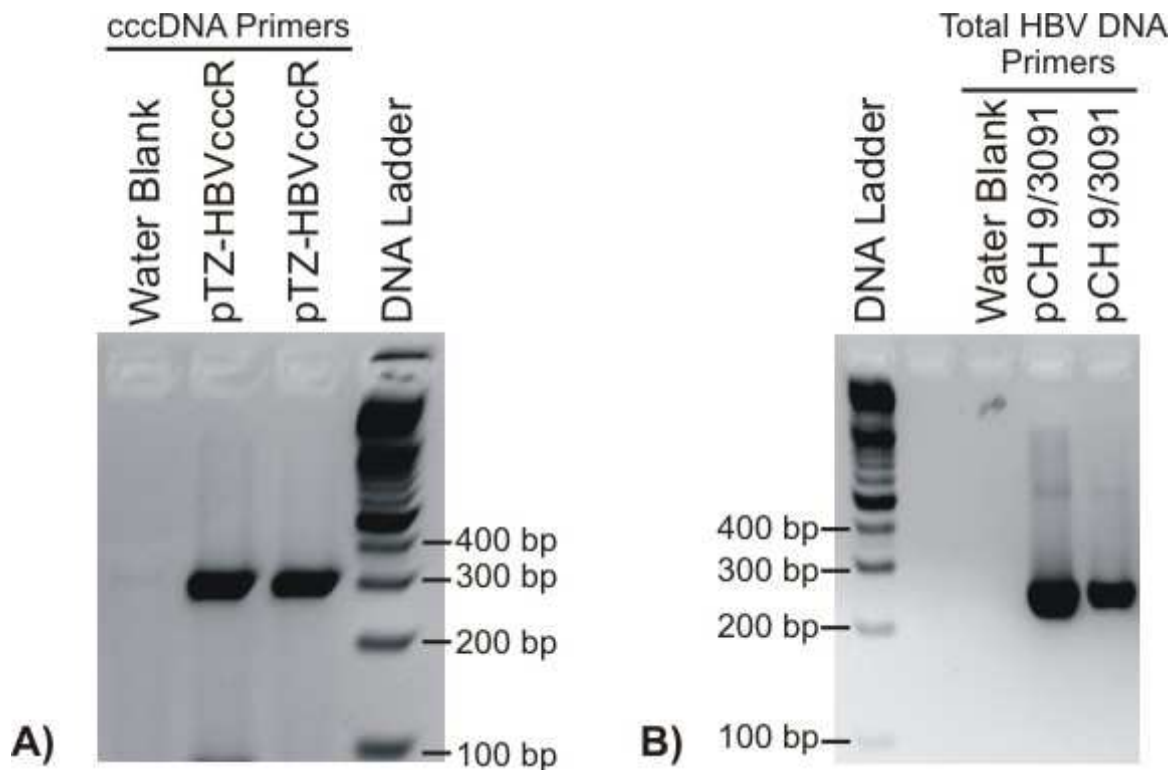
Figure 3.4: HBV qPCR primers amplify target sequences. Conventional PCR and agarose gel electrophoresis confirms single amplification products for both cccDNA and total HBV DNA primer sets. A) pTZ-HBVcccR was used as a cccDNA template. PCR amplification results in a 298 bp amplicon. B) Total HBV DNA primers solely amplify a 256 bp region of the pre-S1 ORF in pCH-9/3091. Water blanks were included during both PCR assays as negative controls.

```
   cccDNA -F     (1) ---------TTCTCATCTGCCGGTCCGTGT--------------------
HBV A1 Uganda (AY934772) (1551) GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGC
HBV A1 Gambia (AY934764) (1551) GTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC
HBV A1 Germany (AY738139) (1551) GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGC
      pCH-9/3091 (2833) GTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC
       pHBV 1.3x (3985) GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGC
     pTZ-HBVcccR  (668) GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTCC
       Consensus      GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGC
```

Figure 3.5: cccDNA forward primer has extensive sequence complementarity. All three HBV A1 sub-genotypes show high complementarity between the various HBV plasmid sequences, specifically pTZ-HBVcccR. The blue highlighted regions indicate highly complementary regions whilst the yellow highlights indicate completely complementary sequences. A single polymorphism (T to A) is common to both the HBV A1 Gambian strain and pCH-9/3091.

```
        cccDNA-R       (1) ----------------GCCTAATCATCTCTTGTACATGTCCCA-----
  HBV A1 Uganda (AY934772)  (1786) GCAACTTTTTCACCTCTGCCTAATCATCTCTTGTACATGTCCCACTGTT
  HBV A1 Gambia (AY934764)  (1786) GCAACTTTTTCACCTCTGCCTAATCATCTCTTGTACATGTCCCACTCTT
 HBV A1 Germany (AY738139)  (1786) GCAACTTTTTCACCTCTGCCTAATCATCTCTTGTACATGTCCCACTGTT
            pCH-9/3091  (3099) CAACTTTTT-CACCTCTGCCTAATCATCTCTTGTTCATGTCCTACTGTTC
             pHBV 1.3x  (4251) CAACTTTTTTCACCTCTGCCTAATCATCTCTTGTACATGTCCCACTGTTC
          pTZ-HBVcccR   (1034) CAACTTTTTTCACCTCTGCCTAATCATCTCTTGTACATGTCCTACTGTTC
             Consensus        GCGCACCAGCACCATGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTA
```

Figure 3.6: Multiple alignment of cccDNA reverse primer.   There is extensive sequence complementarity, specifically at the 3' end of the cccDNA-R primer to effectively amplify all three HBV A1 subgenotypes and viral plasmids. Blue highlighted sequences indicate highly complementary regions whilst yellow highlighted sequences indicate complete complementarity. Two polymorphisms, A to T and C to T, are present in pCH-9/3091 whilst pTZ-HBVcccR contains a single base change at nucleotide position 1077 (C to T). Although pTZ-HBVcccR does not show complete sequence complementarity at the 5' end, this does not affect PCR amplification efficiency.

3.1.3   Construction of Real-time qPCR standard curves for HBV DNA quantification

Real-time standard curves, essential for the accurate quantification of viral DNA, were created for cccDNA and total HBV DNA. Eight-fold log serial dilutions of pTZ-HBVcccR and pCH-9/3091 were used for the construction of HBV standard curves (Figure 3.7). pTZ-HBVcccR concentrations ranged from 0.33 ng/µl to 3.3×10^{-8} ng/µl, whilst pCH-9/3091 concentrations ranged from 5.8 ng/µl to 5.833×10^{-7} ng/µl. Water blanks and mouse genomic DNA were included as negative PCR and HBV controls respectively. To construct these curves the CP values were plotted against their relative log concentrations. Standard curves and internal controls were used during HBV qPCR analysis to accurately quantify both forms of viral DNA. Although it is useful to describe initial DNA quantities as nanogram concentrations, for HBV DNA analysis the number of DNA copies would provide a more practical representation of viral infection. In particular, the number of HBV DNA copies per hepatocyte is useful when describing the degree of HBV infection.

Plasmid DNA concentrations can be mathematically adjusted to represent the initial number of DNA copies per sample, as described in section 2.7.1. Therefore, standard curves were constructed to represent the CP values versus DNA copy number. By using DNA copy numbers instead of concentrations, the number of HBV DNA copies per sample can be accurately determined. The cccDNA standard curve is able to detect up to 9.24×10^7 copies/µl with an efficiency of 2.0 (100%) and error rate of 0.0229, whereas the total HBV DNA standard curve detects up to 8.48×10^8 copies/µl with an efficiency of 1.86 (93%) and error rate of 0.0255. PCR efficiency is an important factor to consider during absolute quantification as it reveals whether exponential amplification is achieved throughout analysis. Non-optimal

reagents or cycling conditions can affect this resulting in inaccurate quantification, specifically at higher CP values. Efficiency normally ranges between 1.5 and 1.9, with a maximum value of 2.0 (Wilhelm and Pingoud, 2003). Both the cccDNA and total HBV DNA standard curves have a high degree of efficiency indicating optimal qPCR conditions.

Minimum detection limits were established for HBV qPCR assays, as it became difficult to determine the differences between primer dimer formation and single amplification products at very low concentrations of plasmid DNA. Additionally, by including these limits in subsequent qPCRs, the detection of false-positives was prevented. Total HBV DNA and cccDNA qPCR analyses had minimum detection limits of 84 and 10 copies respectively. The ability of this assay to detect very low levels of cccDNA is imperative, as this form of HBV DNA is often present at clinically undetectable levels during implementation of therapeutic regimens or occult infections. It is also important that cccDNA primers do not amplify rcDNA as this would result in an inaccurate resolution of the HBV DNA profile.
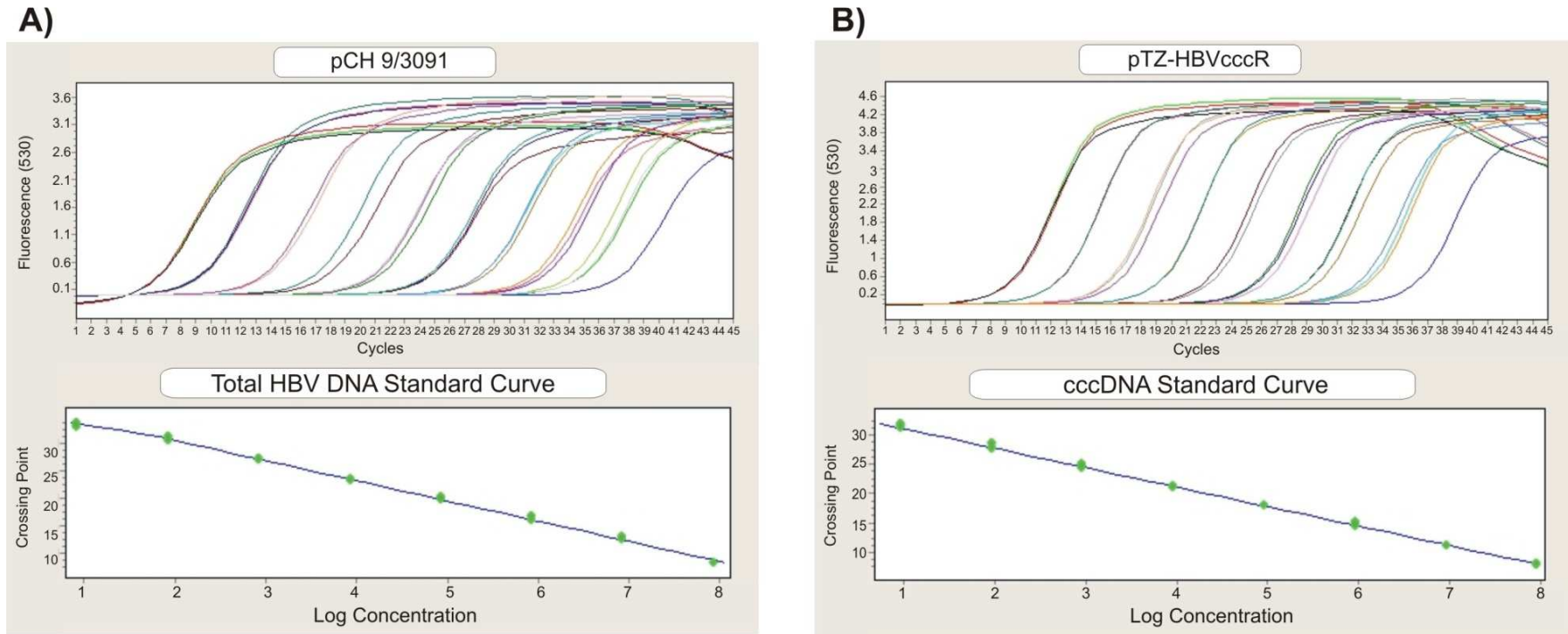
Figure 3.7: Real time PCR amplification and standard curves for pCH-9/3091 and pTZ-HBVcccR dilution series. Standard curves for total HBV DNA (rc/cccDNA) and cccDNA were constructed using the HBV greater than genome length plasmid, pCH-9/3091 (A) and cloned plasmid, pTZ-HBVcccR (B). Each standard curve was constructed from an 8-fold log serial dilution of the representative plasmid (n=3). High efficiencies, of 2.0 and 1.86, were observed for both the cccDNA and total HBV DNA respectively.

3.1.4   cccDNA primers do not amplify across the gap region of rcDNA

As both the cccDNA and rcDNA forms of HBV DNA share complete sequence homology, the precise amplification of cccDNA relies on the rcDNA gap region halting polymerase activity during qPCR. To ensure that cccDNA primers do not amplify rcDNA non-specifically three separate qPCRs were performed. The first with an HBV Eurohep standard (WHO international standard), the second using whole virion extracts, and the third to assess the need for viral DNA extraction. Figure 3.8 shows the amplification and melting curve analysis obtained with the Eurohep standard, confirming that cccDNA primers do not amplify rcDNA, whereas total HBV DNA primers do. Eurohep melting peaks observed during cccDNA primer amplification correspond only to the water blank, indicating no HBV amplification. Total HBV DNA primers on the other hand amplify the Eurohep standard, and display melting peaks equivalent to the pCH-9/3091 positive control. To corroborate these results, whole virions were isolated from HBV transgenic mouse serum before DNA extraction and qPCR analysis. As whole virions enclose only rcDNA, any circulating cccDNA will be excluded from the extraction during virion isolation. cccDNA was not detected above the minimum detection limit in serum virion isolated samples (Figure 3.9) whereas rcDNA was readily quantified (by subtracting the cccDNA from total HBV DNA). Furthermore, to illustrate that cccDNA was not merely absent from these samples, DNA extraction was found to be a prerequisite when quantifying both forms of HBV DNA.

A)

B)
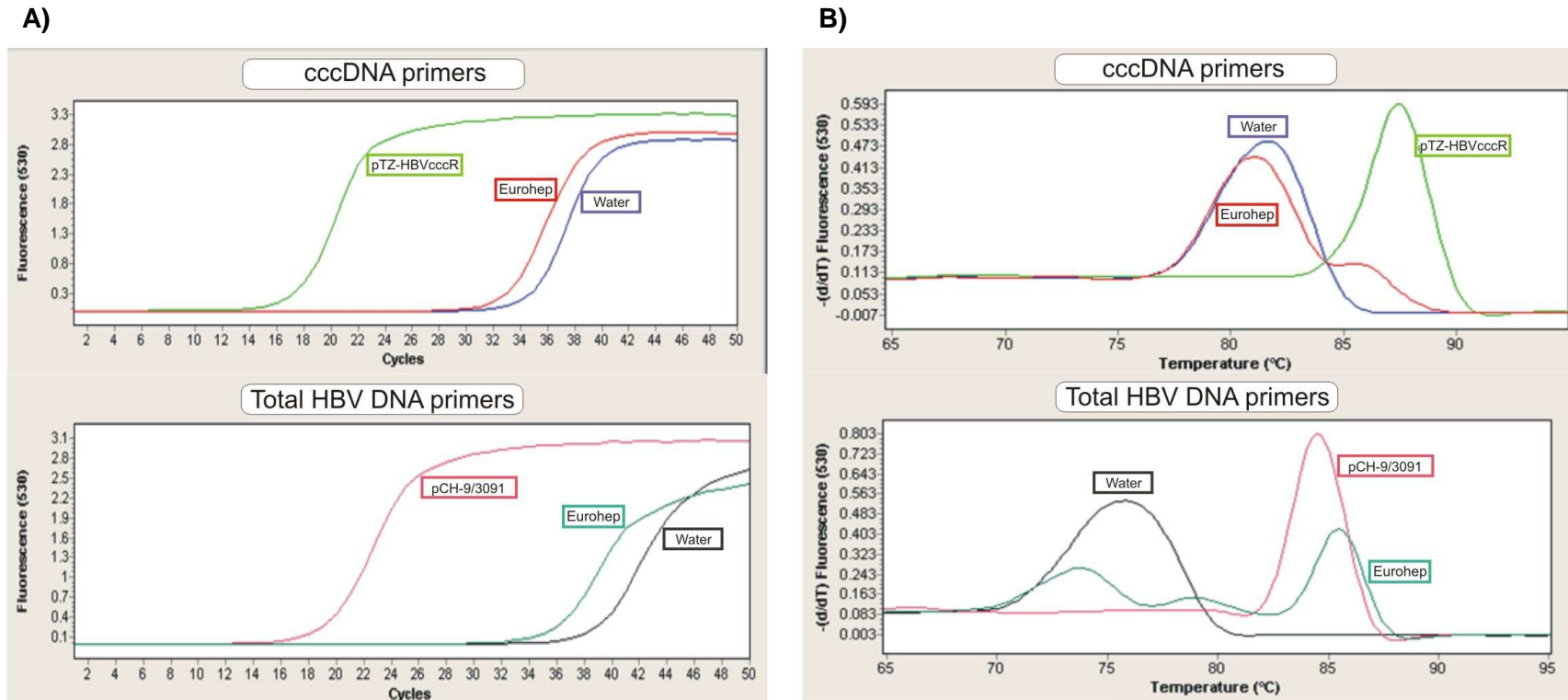
Figure 3.8: Eurohep standard qPCR assay amplification and melting curve analysis. A) Amplification curves of Eurohep standards with cccDNA and total HBV DNA primer sets. cccDNA primers are unable to amplify the rcDNA present in the Eurohep HBV standard, compared to pTZ-HBVcccR which was used as a positive control. The total HBV DNA primers amplify the Eurohep standard as well as the pCH-9/3091 positive control. B) Melting curve analysis indicates cccDNA primers do not amplify rcDNA, as Eurohep and water primer dimers are observed together at 81°C, when using cccDNA primers. qPCR with t otal HBV DNA primers resulted in amplification of the pCH-9/3091 positive control and the Eurohep HBV standard at 84°C and 85°C respectively. Primer dimers are present in both the water blank and to some extent in the Eurohep standard between 73 - 76°C.

Whole virion extraction
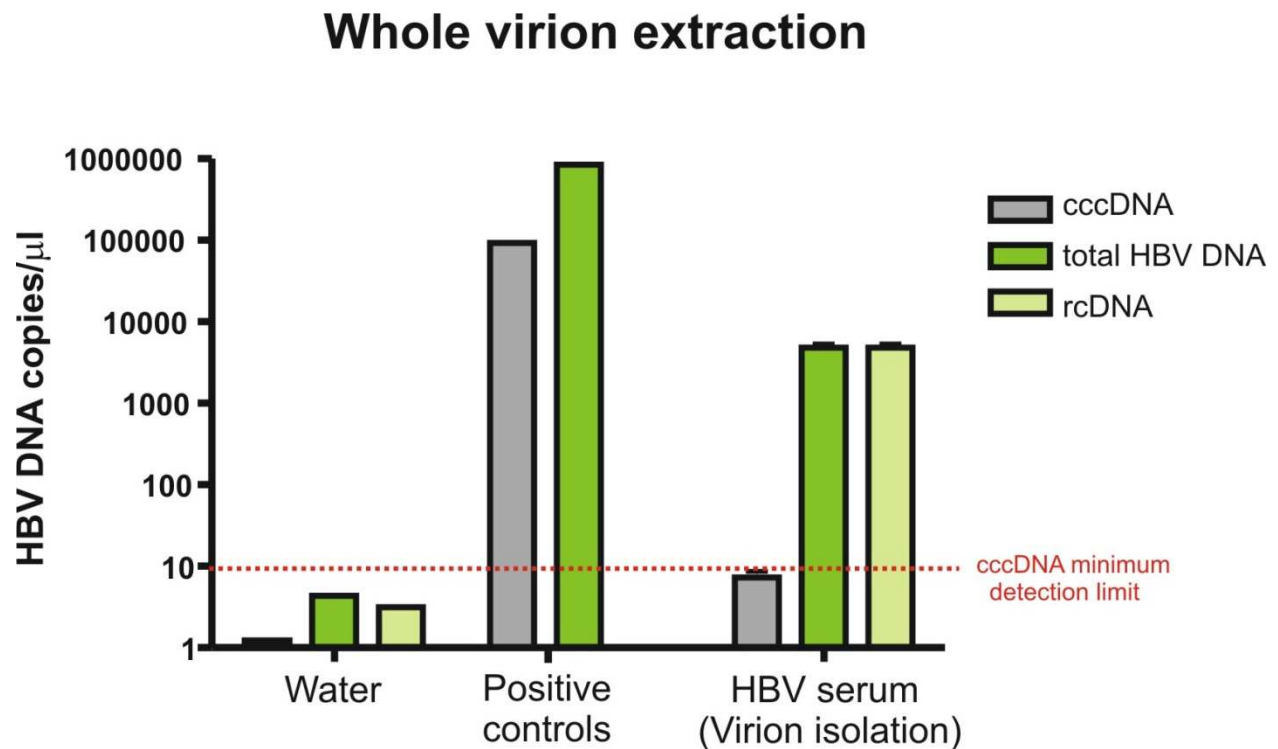
Figure 3.9: cccDNA primers do not amplify rcDNA during qPCR. HBV transgenic mouse serum rcDNA, confined within whole virions, was analysed by real-time qPCR. cccDNA and total HBV DNA primers were used to quantify both forms of HBV DNA. pTZ-HBVcccR and pCH-9/3091 acted as positive controls and internal standards. The HBV serum samples contain a total HBV DNA average of 4.800×10^3 copies/µl. The minimum detection limit for cccDNA quantification (as illustrated on the graph) is 10 copies; however HBV serum contained an average of only 7.29 copies/µl of cccDNA which is most likely an indicator of primer dimers. Consequently, rcDNA was found to be the predominant form of HBV DNA at an average of 4.792×10^3 copies/µl. Error bars indicate SEM of n=4.

To accurately quantify total HBV DNA loads, encapsulated rcDNA requires a DNA extraction step to free the viral genome and allow it to be amplified by qPCR. Serum cccDNA on the other hand, circulates as 'naked' DNA and can be amplified from samples without DNA extraction. To determine whether cccDNA primers amplify rcDNA and if DNA extraction protocols are essential for total HBV DNA quantification, HepG2.2.15 cell supernatants were analysed by qPCR. HepG2.2.15 cells contain a stably integrated HBV genome. As such, they constantly produce new HBV virions. Table 3.1 displays the mean cccDNA and total HBV DNA copy numbers observed. Log serial dilutions of pTZ-HBVcccR and pCH-9/3091 were included as internal standards. Figure 3.10 shows that DNA extraction is a critical process required for total HBV DNA quantification as non-extracted HepG2.2.15 supernatants are unable to amplify rcDNA, and thus underestimate the amount of total viral DNA present in the sample. Extracted HepG2.2.15 supernatants on the other hand, show that rcDNA is the predominant form of viral DNA. This is to be expected as newly synthesised virions harbouring rcDNA are released into the supernatant. Furthermore this qPCR shows that cccDNA primers do not amplify rcDNA, as there is no statistical difference between the amount of cccDNA present in the non-extracted supernatants versus total HBV DNA (p-value = 0.7922), however there is a significant difference between the amounts of cccDNA and total HBV DNA in the extracted sample (p-value = 0.0016), indicating that rcDNA is present and efficiently amplified. Although cccDNA was isolated from the HepG2.2.15 *in vitro* culture system, this is only clinically observed during hepatocellular death and liver damage associated with chronic HBV infection. As this qPCR assay allows for the efficient amplification of both forms of viral DNA, the HBV DNA profiles of *in vitro* and *in vivo* samples can be analysed. Overall, viral DNA extraction is essential for the accurate measurement of HBV viral loads, specifically in serum samples where circulating viral particles predominate.

Table 3.1: Quantification of HBV DNA from HepG2.215 cell culture

HepG2.2.15 Supernatant	HBV DNA copies/µl*(±SEM)		*p-value***
	cccDNA	*Total HBV DNA*	
Extracted	1650 (±12)	29367 (±1129)	0.0016**
Non-Extracted	739 (±48)	749 (±72)	0.7922

mean value (n=3)
*** statistical significance < 0.05 (5%)*
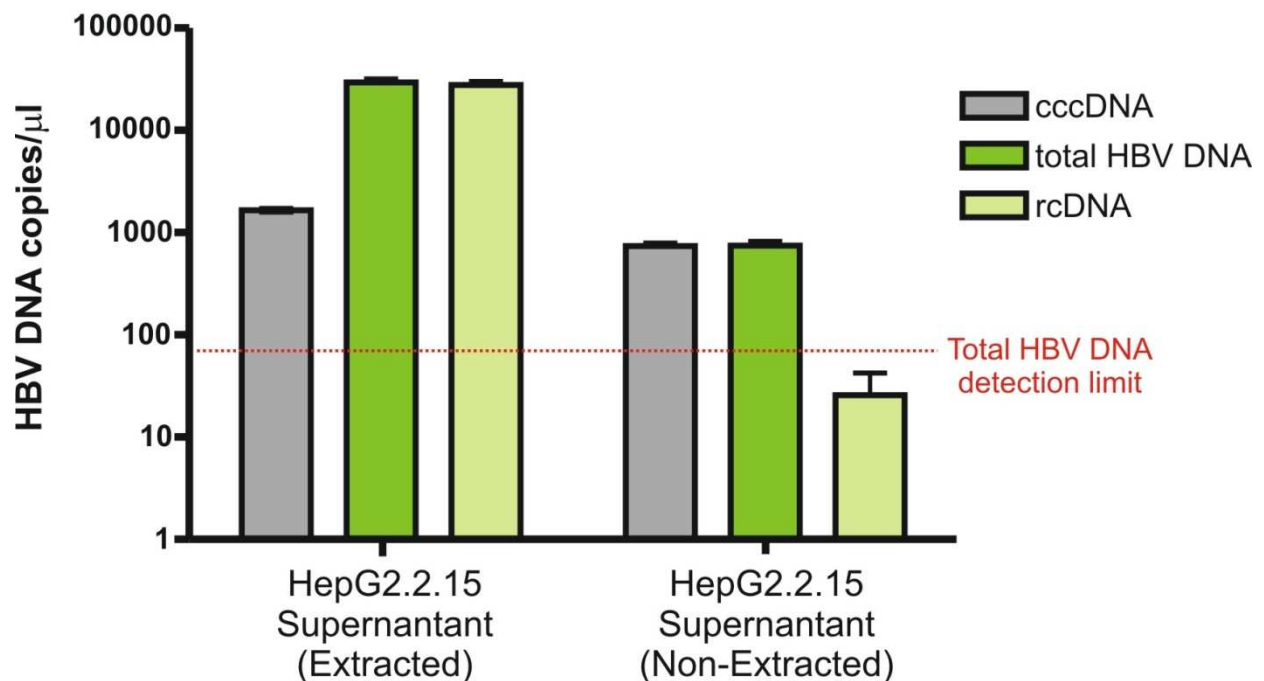
Figure 3.10: DNA extraction is a prerequisite for HBV qPCR analysis. HepG2.2.15 supernatants contain both cccDNA and rcDNA forms of HBV. Extracted samples contain an average of 29367 copies of total HBV DNA of which only 6% is cccDNA. Non-extracted samples contain 749 copies of total HBV DNA comprising mainly cccDNA (99%). Only extracted samples were able to quantify rcDNA bellow the detection limit. Samples analysed in triplicate and error bars indicate SEM.

3.1.5   Absolute quantification of total HBV DNA and cccDNA from *in vitro* and *in vivo* HBV model systems

The HBV DNA profiles of HepG2.2.15 cell culture systems and HBV transgenic mice were evaluated using Lightcycler™-based qPCR. Absolute quantification of HBV DNA from HepG2.2.15 cells and culture supernatants as well as HBV transgenic mouse liver and serum samples, was achieved through the simultaneous analysis of HBV plasmid standards as positive controls and internal references. Three different log serial dilutions of pTZ-HBVcccR and pCH-9/3091 were used to establish cccDNA and total HBV DNA copy numbers respectively. Water blanks and mouse genomic DNA samples (191.6 ng/µl), were included as negative controls. As previously described (Section 3.1.3) the minimum detection limit for cccDNA quantification is 10 copies compared to total HBV DNA with 84 copies. Preliminary qPCR of HBV transgenic mouse liver displayed large amounts of HBV DNA which were difficult to accurately quantify. Therefore all liver samples were diluted 1:2 prior to qPCR, but quantification values were adjusted accordingly during post-PCR analysis.

During each PCR reaction, melting curve analysis was performed to assess specificity of amplification. Accurate qPCR quantification was confirmed with melting curves of 84℃ for total HBV DNA and 87℃ for cccDNA. The *in vitro* and *in vivo* HBV DNA concentrations obtained by qPCR are shown in Table 3.2. HBV primers were incapable of amplifying non-transgenic mouse genomic DNA, demonstrating that any co-precipitated genomic DNA did not affect HBV quantification. Although transgenic mouse genomic DNA contains two integrated copies of the HBV viral genome within in each hepatocyte, amplification of these integrated transcripts accounts for, at most, only 1% of total HBV DNA and 2.5% of cccDNA detected by qPCR. The HepG2.2.15 cell extracts show the highest total HBV DNA quantities followed by HBV

transgenic mouse liver, HepG2.2.15 supernatant, and lastly HBV transgenic mouse serum. Transgenic mouse liver has the greatest proportion of cccDNA, followed by HepG2.2.15 cells. This is to be expected as cccDNA resides in the nucleus of hepatocytes. rcDNA remains the most prominent form of HBV DNA in all samples, indicating active replication. Although rcDNA predominates in HepG2.2.15 cell and supernatant samples, transgenic mouse livers were found to contain a high percentage of cccDNA, indicating that rcDNA may be recycled and translocate to the nucleus of murine hepatocytes (illustrated in Figure 1.2). Figures 3.11 and 3.12 display the HBV DNA profiles of transgenic mice and HepG2.2.15 samples. HBV transgenic mouse liver consists of 40% cccDNA and 60% rcDNA whilst serum contains only 3% cccDNA and 97% rcDNA. HepG2.2.15 cell cultures predominantly contain rcDNA, at 98% in cell extracts and 100% in supernatants. Despite the vast differences in HBV DNA profiles, both the transgenic mice and HepG2.2.15 cell cultures harbour similar amounts of cccDNA. HepG2.2.15 cells however, produce and release vast quantities of rcDNA compared to the mouse model.

This HBV qPCR assay results in the sensitive detection of both rcDNA and cccDNA species, demonstrating its capacity for monitoring HBV DNA. Although qPCR is widely used for viral DNA quantification, Southern blot hybridisation is considered the 'gold standard' for HBV DNA detection and analysis. Therefore HBV qPCR results were validated using non-radioactive Southern blot hybridisation.

Table 3.2: Absolute quantification of HBV DNA through Lightcycler™ qPCR

	HBV DNA copies per assay ($\pm$SEM)		
	cccDNA	*Total HBV DNA*	*rcDNA**
pTZ-HBVcccR	9.25×10^4	-	-
pCH-9/3091	-	8.48×10^5	-
HBV Transgenic Mouse Liver**	9.90×10^5 ($\pm 1.36 \times 10^5$)	2.46×10^6 ($\pm 8.33 \times 10^4$)	1.47×10^6 ($\pm 2.05 \times 10^5$)
HBV Transgenic Mouse Serum**	227 ($\pm$16)	6637 ($\pm$451)	6410 ($\pm$463)
HepG2.2.15 Cells**	1.34×10^5 ($\pm 4.10 \times 10^3$)	8.83×10^6 ($\pm 5.57 \times 10^4$)	8.70×10^6 ($\pm 5.30 \times 10^4$)
HepG2.2.15 Supernatant**	478 ($\pm$6)	2.36×10^5 ($\pm 3.28 \times 10^4$)	2.36×10^5 ($\pm 3.29 \times 10^4$)

*Determined by subtracting cccDNA from total HBV DNA
**mean values (n=3)

HBV Transgenic Mouse qPCR
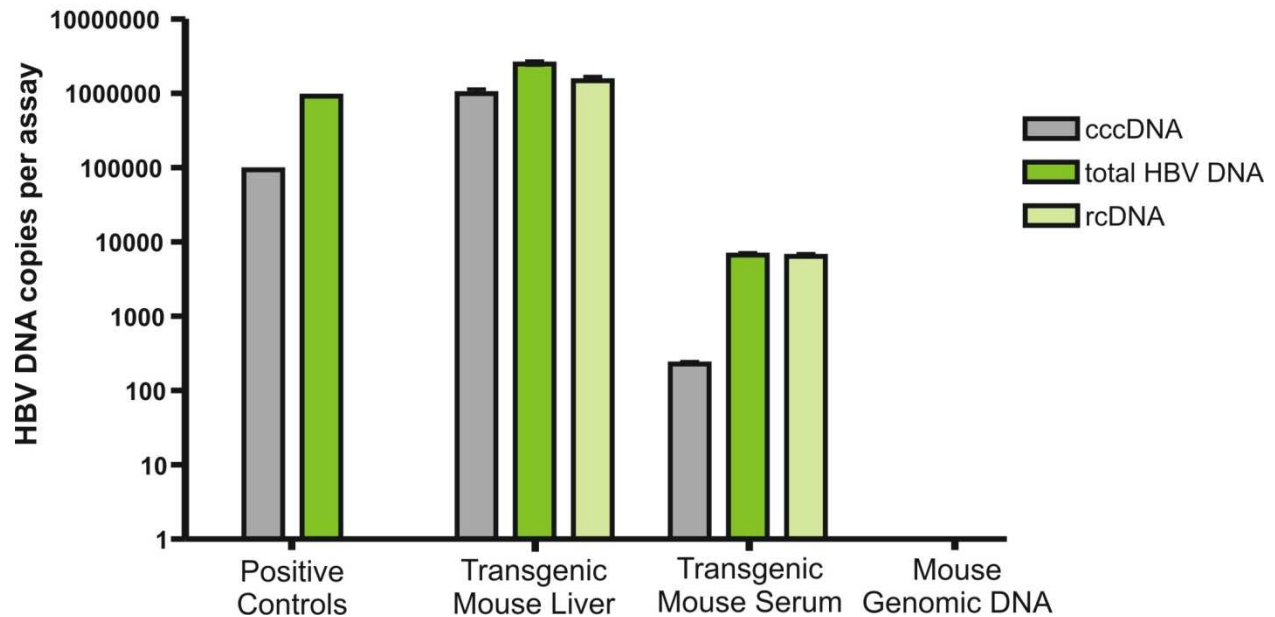
Figure 3.11: HBV DNA profiles of transgenic mice. Total HBV DNA and cccDNA quantification was performed on DNA extracted from the livers and serum of HBV transgenic mice. pTZ-HBVcccR and pCH-9/3091 were included as positive controls and standard references. Transgenic liver samples contained an average of 2.46×10^6 copies of HBV DNA, of which 40% was cccDNA. Considerably less viral DNA was isolated from serum, where cccDNA only constitutes 3% of the 6637 copies of total HBV DNA. No HBV DNA was detected in mouse genomic DNA samples. Error bars indicate the SEM (n = 3).

HepG2.2.15 cell culture qPCR
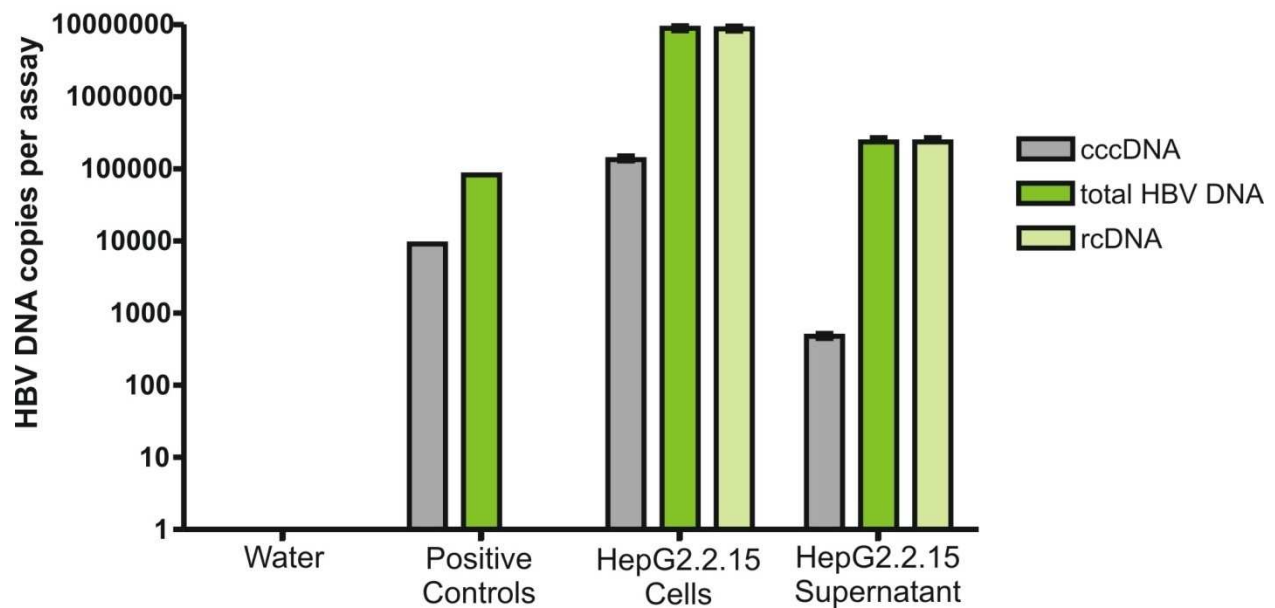
Figure 3.12: *In vitro* **quantification of HBV DNA.** HepG2.2.15 cell cultures were analysed by qPCR to establish the HBV DNA profiles of cell extracts and supernatants. pTZ-HBVcccR and pCH-9/3091 were included as positive controls (data not shown). Both HepG2.2.15 cell extracts and supernatants retain significantly large amounts of total HBV DNA with 8.83×10^6 and 2.36×10^5 copies respectively. Of this, rcDNA remains the major form of viral DNA, accounting for 98% of total HBV DNA isolated form cells and 100% isolated from supernatant. HepG2.2.15 samples were analysed in triplicate and error bars indicate the SEM.

3.2 Non-radioactive Southern blot hybridisation is proficient at detecting HBV DNA

Southern blot hybridisation has been extensively used to quantify total HBV DNA as well as isolate rcDNA and cccDNA from replicative intermediates (Farza et al., 1988; Gong et al., 1998; Gripon et al., 2002). Agarose gel electrophoresis permits fractionation of the different forms of HBV DNA, based on size and conformation, resulting in a distinct band migration pattern. Southern blot hybridisations are most often performed using radio-labelled probes, however DIG probes have been shown to be efficient for the sensitive detection of HBV DNA (Ren and Nassal, 2001). Therefore to eliminate radioactivity exposure, an HBV DIG probe was designed that was complementary to the *pre-S1* region to detect total HBV DNA. HepG2.2.15 cell cultures and HBV transgenic mice were used to develop this DIG-based Southern blot hybridisation technique, subsequently allowing HBV DNA quantification.

3.2.1 *Hin*dIII enzymatic digestions enhance HBV fractionation and reduce genomic DNA interference

HBV band migration patterns were improved by *Hin*dIII digestion, as shown by the HepG2.2.15 cell extracts in Figure 3.13. Three distinct bands were observed corresponding to the rcDNA, dsDNA replicative intermediates, and cccDNA. As the HBV genome does not contain a *Hin*dIII restriction site, all forms of HBV DNA remain intact, whereas the co-precipitated genomic DNA is digested at multiple restriction points. As cccDNA is covalently closed, it migrates more rapidly than the partially double stranded rcDNA, during agarose gel electrophoresis. The rcDNA, dsDNA and cccDNA migrate with equivalent sizes of approximately 4 kilobases (kb), 3.2

kb and 1.9 kb respectively (Brechot et al., 1981; Gong et al., 1998; Gripon et al., 2002; Ren and Nassal, 2001; Sells et al., 1987; Sells et al., 1988; Summers et al., 1990).   As dsDNA represents all linear double stranded HBV replicative intermediates present during infection, a migration pattern between 3.2 kb and 2 kb is often detected.   HBV single stranded DNA (ssDNA) migrates to approximately 1.6 kb, and therefore may co-migrate with cccDNA. Although the HBV genome does not contain a *Hin*dIII restriction site, it does contain a single *Eco*RI site.   A double digestion, with *Hin*dIII and *Eco*RI, should cleave the HBV genome at the single *Eco*RI restriction site resulting in a distinct 3.2 kb band.   Double digestion of HepG2.2.15 cells produced three migratory bands, one of which corresponded to the 3.2 kb band expected. As a result of this only *Hin*dIII enzymatic enhancement of HBV DNA was performed.

Following *Hin*dIII digestion, HepG2.2.15 cell samples and HBV transgenic mouse liver samples were concentrated to increase the amount of total HBV DNA.   Ethanol precipitation enabled significant improvement in HBV DNA detection during Southern blot hybridisation, as shown in Figure 3.14.   As a result of the small size of the HBV genome, it is difficult to detect viral DNA by Southern blotting, even though there may be numerous copies of the virus within the sample. This is evident in the detection limit determined during Southern blot hybridisation, using the DIG-labelled HBV DNA probe.   The HBV genome is approximately 3.2 kb in length, therefore a single copy of viral DNA is equivalent to approximately 0.4×10^{-9} ng of DNA.   The minimum Southern blot hybridisation limit of detection was found to be 0.019 ng of plasmid DNA.   This plasmid concentration corresponds to 5.67×10^6 copies of HBV DNA.   By concentrating the *in vitro* and *in vivo* HBV samples, the detection of the different viral DNA species is enhanced, which in turn facilitates accurate quantification.   By isolating the cccDNA from other HBV DNA species, unambiguous quantification of this replication intermediate should be attainable.
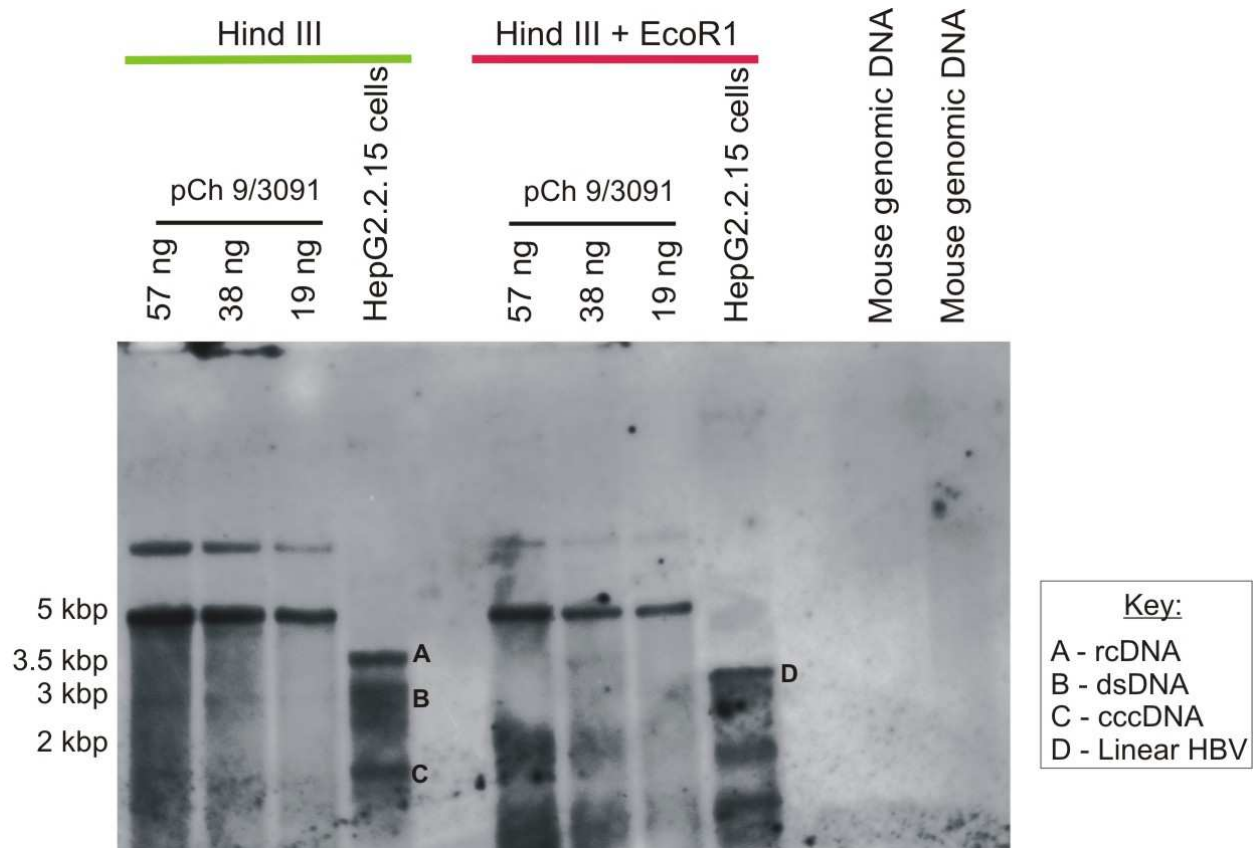
Figure 3.13: Southern blot analysis of enzymatic digestions performed on HepG2.2.15 cell extracts. Total nucleic acid isolated from HepG2.2.15 cells digested with *Hin*dIII migrate to form three distinct bands namely rcDNA (A); dsDNA (B); and cccDNA (C), detected by chemilumenescence using a DIG-labelled HBV probe. HBV ssDNA may migrate with cccDNA. Double digests of HepG2.2.15 cells, with *Hin*dIII and *Eco*RI, migrate to form three bands where (D) represents the 3.2 kb band representing total HBV DNA. Additional HBV DNA artifacts are detected below the 3 kb marker in *Hin*dIII/*Eco*RI digestions. Controls include standard dilutions of pCH9/3091 (positive control) and mouse genomic DNA (negative control).
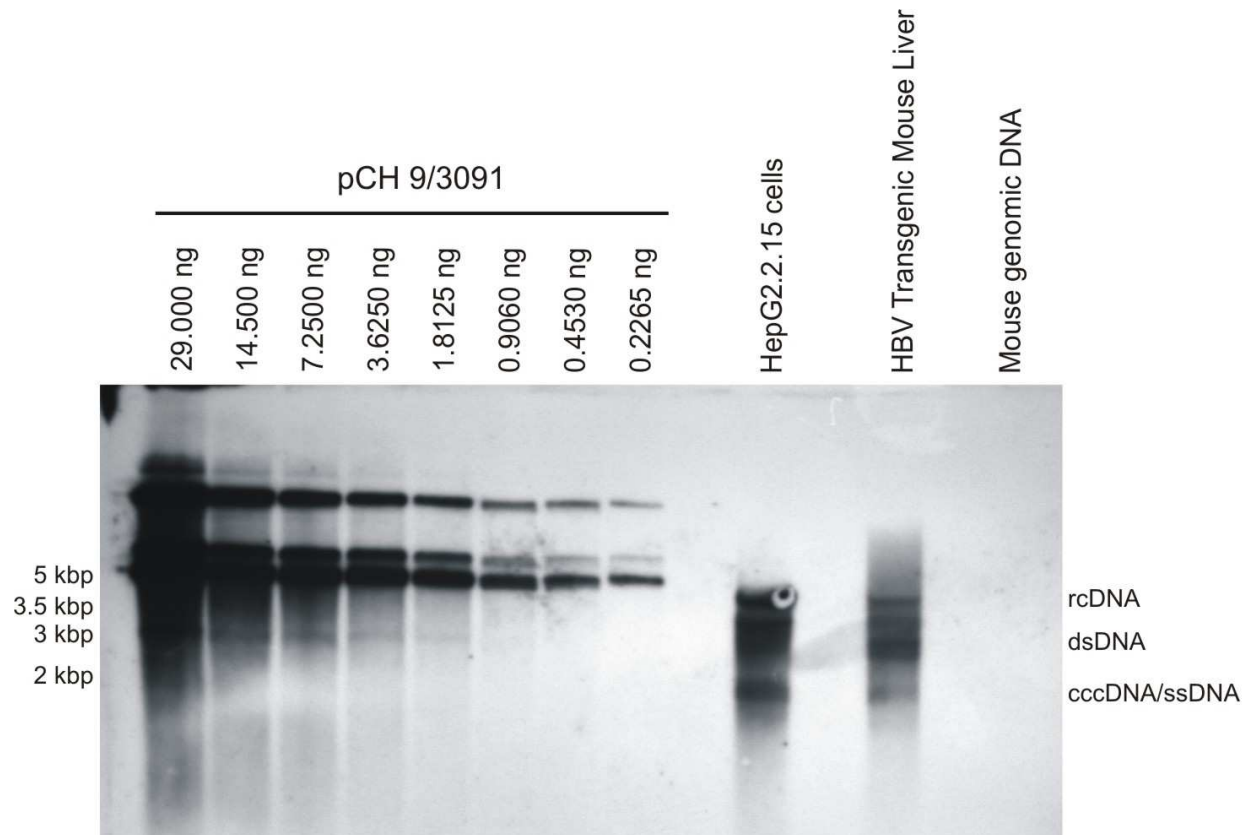
Figure 3.14: Evaluation of HBV samples, prepared from cultured cells and HBV transgenic mouse liver, using Southern blot hybridisation. The HepG2.2.15 cells form three distinct bands representing the rcDNA, dsDNA and cccDNA/ssDNA. The HBV transgenic mouse liver produces four migratory bands, namely an rcDNA fraction, two dsDNA fractions and a cccDNA fraction. In the HepG2.2.15 cells and the HBV transgenic mouse liver, the largest HBV DNA fraction is the dsDNA, indicating HBV replication intermediates. A pCH-9/3091 standard dilution was included as a positive control and mouse genomic DNA was included as a negative control.

3.2.2   Isolation of cccDNA from total HBV using Plasmid-Safe™ ATP-dependent DNase results in elimination of all DNA species

Both the HBV transgenic mouse liver and HepG2.2.15 cell extractions contain an association of rcDNA, dsDNA, ssDNA and cccDNA, as well as co-precipitated genomic DNA. Since the rcDNA is only partially double stranded, the dsDNA is linear, and the genomic DNA nicked, they are all susceptible to degradation by specific DNases. For example, the S1 nuclease cleaves single stranded DNA (Wong et al., 2004), mung bean nuclease degrades single and triple stranded DNA (Mazet-Wagner et al., 2006), and Plasmid-Safe™ ATP-dependent DNase (Epicentre Biotechnologies) digests linear double stranded DNA and to a lesser extent single stranded DNA, however has no effect on circular or supercoiled DNA (Mazet-Wagner et al., 2006; Singh et al., 2004). These nucleases have all been used to remove single stranded, linear, and rcDNA forms of HBV, as well as degrade sheared genomic DNA, whilst leaving the cccDNA intact. In theory the S1 and mung bean nucleases should cleave the rcDNA and sheared genomic DNA, leaving only the cccDNA. But neither nuclease is able to cleave the dsDNA replicative intermediates. This limits their use to serum samples only, where the rcDNA form of HBV predominates and there are no replicative intermediates present. The Plasmid-Safe™ ATP-dependent DNase on the other hand, should degrade the dsDNA, genomic DNA and the rcDNA leaving only the cccDNA. This is based on the cccDNA's covalently closed circular DNA form, would be resistant to degradation by this nuclease. This would be practical for cccDNA isolation from both the serum and liver samples. Therefore to isolate cccDNA, HBV transgenic mouse liver samples and HepG2.2.15 cells were incubated with Plasmid-Safe™ ATP-dependent DNase.

Samples were subsequently analysed by Southern blot, as shown in Figure 3.15. Three bands were detected in the lane containing HepG2.2.15 cells treated with *Hin*dIII whilst nothing was detected in the lane containing the cccDNA isolation. A similar result was obtained for the HBV transgenic mouse liver samples. qPCR was additionally used to determine the effects of various Plasmid-Safe™ ATP-dependent DNase concentrations on cccDNA found within transgenic mouse liver samples. Figure 3.16 illustrates that cccDNA is not completely protected from DNase degradation as expected. Instead, as enzyme concentrations are increased, cccDNA levels decrease. Untreated HBV transgenic mouse liver samples contain up to 1.05×10^6 copies of cccDNA whereas samples treated with 5 U and 10 U of DNase contain an average of 2.43×10^5 and 4.56×10^4 copies respectively. This poses a problem as the DNase seems to be degrading all forms of the virus DNA, including the cccDNA, making it difficult to quantify cccDNA alone. Therefore the Plasmid-Safe™ ATP-dependent DNase treatments were not investigated further.
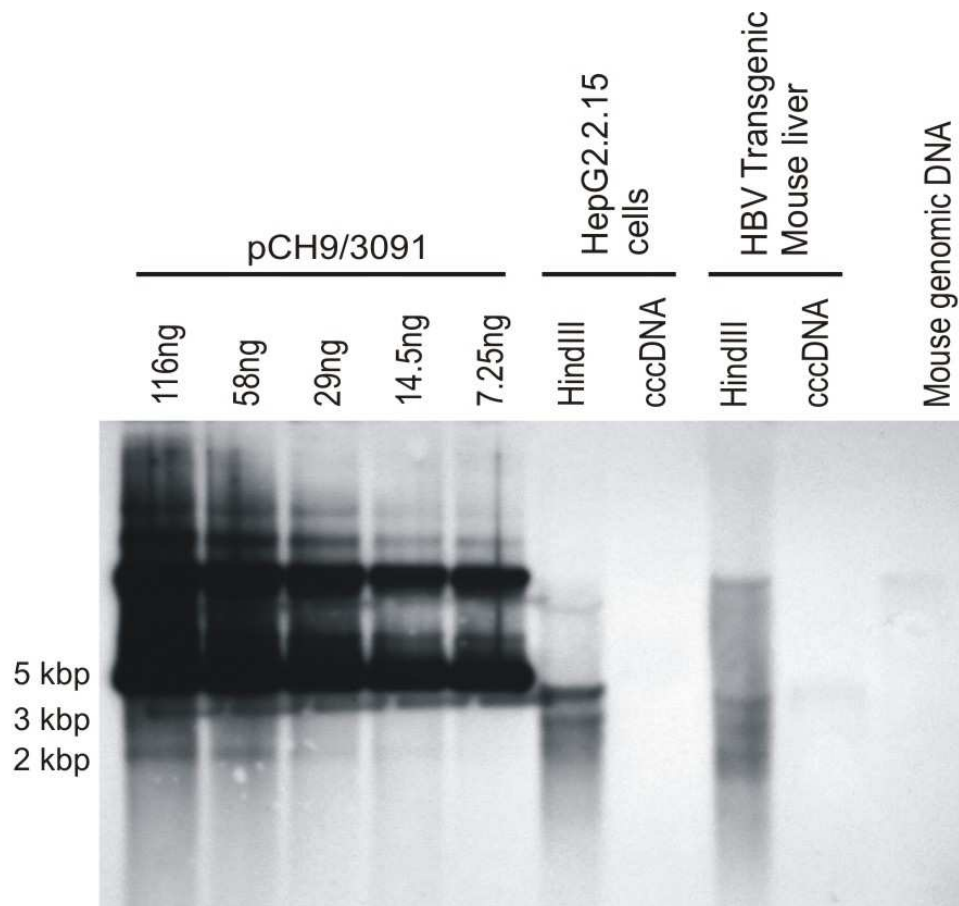
Figure 3.15: Isolation of cccDNA from total HBV DNA fractions analysed by Southern blot hybridisation. *Hin*dIII digestions of HepG2.2.15 cell extracts and HBV transgenic mouse liver results in a typical HBV banding pattern. No bands were detected in the HepG2.2.15 cell and HBV transgenic mouse liver cccDNA extracts. Plasmid-Safe™ ATP-dependent DNase treatments (shown in the cccDNA lanes) digested all HBV fractions, including the cccDNA. Positive controls include a 5-fold dilution series of pCH 9/3091, and negative controls include mouse genomic DNA.

ATP-dependent DNase qPCR
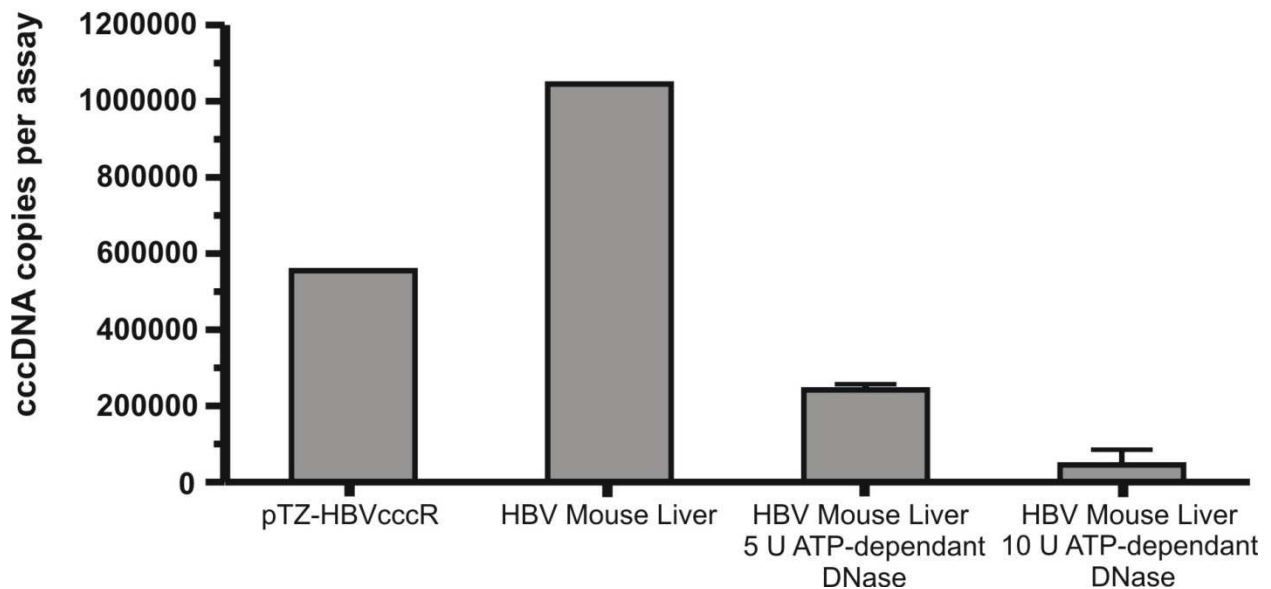
Figure 3.16: cccDNA elimination is proportional to DNase concentration. HBV transgenic mouse DNA extracts were treated with either 5 U or 10 U of Plasmid-Safe™ ATP-dependent DNase for 30 minutes and analysed by qPCR. pTZ-HBVcccR was included as an internal control. The number of cccDNA copies is drastically reduced in the 5 U DNase treated versus untreated HBV mouse liver samples.   Increasing DNase concentrations to 10 U further diminishes the amount of cccDNA.   Errors bars indicate the SEM where n = 2.

3.2.3   HBV DNA migratory patterns correlate to predicted molecular weight configurations

The HBV migration patterns detected using Southern blot hybridisation correspond to previously described molecular weights for *Hin*dIII digested HBV cccDNA, ssDNA, rcDNA and dsDNA (Brechot et al., 1981; Ren and Nassal, 2001; Sells et al., 1988; Summers et al., 1990). Although the migratory patterns are determined using molecular weight markers, this is not an indication of their actual molecular weights, but instead of their relative DNA conformations. The rcDNA migrates within the 5 kb and 4 kb region, which is much slower than the migration of the cccDNA. This is because the rcDNA is an open, double stranded circular form of HBV, as it contains an incomplete plus stand and a gap region. The cccDNA, being covalently closed, becomes supercoiled and therefore migrates to approximately 1.9 kb.   The replicative intermediates migrate between 3.2 kb and 2 kb as a result of their linear double stranded conformation. The ssDNA migrates to approximately 1.6 kb, just below cccDNA. Therefore although HBV DNA is 3.2 kb in size, the migration of the genome is either hindered or enhanced by its conformation. From these distinct differences in agarose gel molecular weight migration, all four forms of HBV DNA can be identified from a single DNA extraction.

To resolve the migratory patterns of HBV DNA, the O'GeneRuler DNA ladder mix, was used as a molecular weight marker during gel electrophoresis. However the ladder had to be excised from the gel before alkaline transfer since it interferes with probe hybridisation during Southern blot analysis. This is a result of the high concentration of DNA within the ladder. As the ladder contains such high concentrations of DNA, when compared to the viral DNA concentrations, excess probe binds non-specifically to the ladder. This poses a major problem as the HBV DNA bands are not visible during agarose gel electrophoresis, and as a result cannot be assigned a

molecular weight. To circumvent this the distance that the molecular weight markers migrated during gel electrophoresis was measured, in millimetres, as shown in Figure 3.17a. Following Southern blot hybridisation, the DNA migratory patterns were compared to the gel electrophoresis molecular weight marker measurements (Figure 3.17b). The establishment of this molecular weight pattern aided the identification of the HBV rcDNA, cccDNA, ssDNA and dsDNA or replication intermediates.

The O'GeneRuler DNA ladder mix spans the 1 kb to 10 kb molecular weight range. Key molecular weight positions, namely 5; 4; 3; 2; 1.5 and 1 kb, were measured based on the migration of pCH-9/3091 and the predicted migration pattern of HBV DNA. The 5-, 4-, and 3 kb positions were found to sit at 131 mm, 134 mm, and 139 mm respectively, whilst the 2-, 1.5-, and 1 kb positions were located at the 148 mm, 156 mm and 167.5 mm marks respectively. Southern blot hybridisation of HBV transgenic mouse liver (Figure 3.17b) indicates that the rcDNA is positioned at approximately 4 kb, whilst two dsDNA species are situated between the 3 kb and 2 kb markers, and finally the cccDNA and ssDNA is located between 2 kb and 1.5 kb. It remains difficult to completely isolate cccDNA from ssDNA as a result of their migration patterns. This correlates with previous examples of HBV Southern blot migration (Brechot et al., 1981; Ren and Nassal, 2001) however it is unique in that both rcDNA and cccDNA are detected in a single reaction. This obviates the need for additional enzymatic restriction digestion to eliminate cccDNA, or to linearise the HBV DNA.
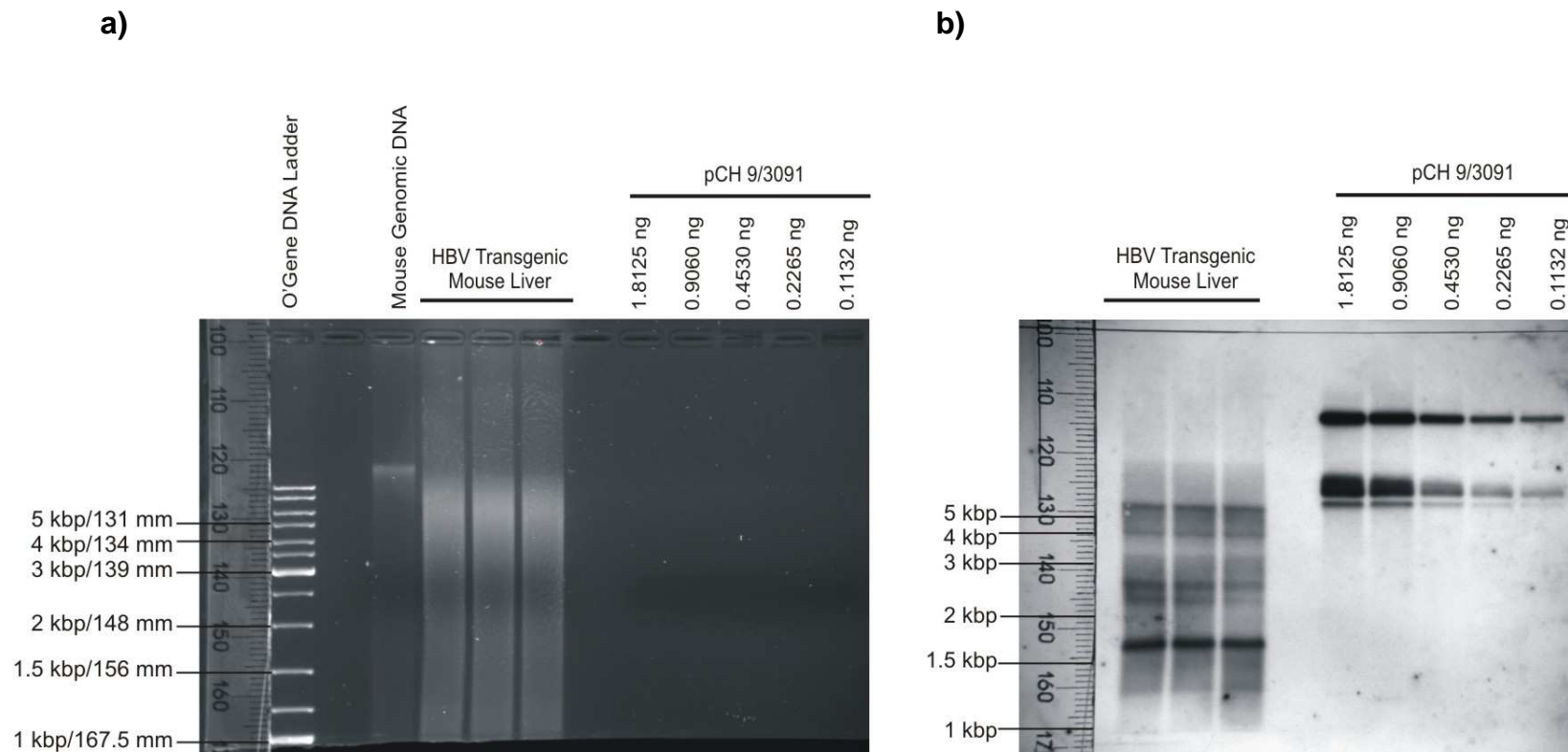
Figure 3.17: Calculating the relative molecular weight migration patterns of HBV DNA species during Southern blot hybridisation. a) Agarose gel electrophoresis of HBV transgenic mouse liver shows a single smeared pattern with no distinctive bands. The pCH-9/3091 dilution series is not visible. The ruler is lined up with the O'GeneRuler DNA ladder and shows the distance each molecular weight marker moved during electrophoresis. Measurements for the molecular weight markers are shown. b) Southern blot hybridisation film. Distinct HBV DNA bands are present in the transgenic mouse liver and pCH-9/3091 samples. The ruler determines the relative positions of the molecular weight markers, allowing the HBV DNA bands to be classified as rcDNA, dsDNA, cccDNA and ssDNA.

3.2.4   HBV DNA concentrations in HepG2.2.15 cell and HBV transgenic mouse liver extracts

Southern blot hybridisations with DIG-labelled HBV probes were used to determine the concentrations of rcDNA, dsDNA and cccDNA within HepG2.2.15 cell samples and media supernatants, as well as transgenic mouse liver extracts. A pCH-9/3091 dilution series was included in all hybridisations for precise quantification of HBV DNA. The HBV band migratory patterns for transgenic mouse liver and HepG2.2.15 cells are shown in Figure 3.18. Southern blot film images were analysed using ImageJ software (Abramoff, 2004; Girish and Vijayalakshmi, 2004), to determine HBV DNA concentrations. A pCH-9/3091 standard curve was created by determining the integrated density of each DIG-hybridised DNA band and plotting it against the plasmid concentration in nanograms (ng). Integrated density is defined as the area multiplied by the mean gray value, where the mean grey value is grey pixilation density dependent (Abramoff, 2004).   The area was kept constant throughout analysis, thereby measuring only the change in each samples' mean grey value.   Background noise was deducted and the image inverted to improve grey pixel measurements. Total HBV DNA concentrations as well as rcDNA to cccDNA ratios were established by determining the integrated intensity of rcDNA, dsDNA and cccDNA bands, which were subsequently evaluated against the pCH 9/3091 standard curve. Ethanol concentrations required to efficiently detect HBV DNA were taken into account and final quantifications were adjusted accordingly.
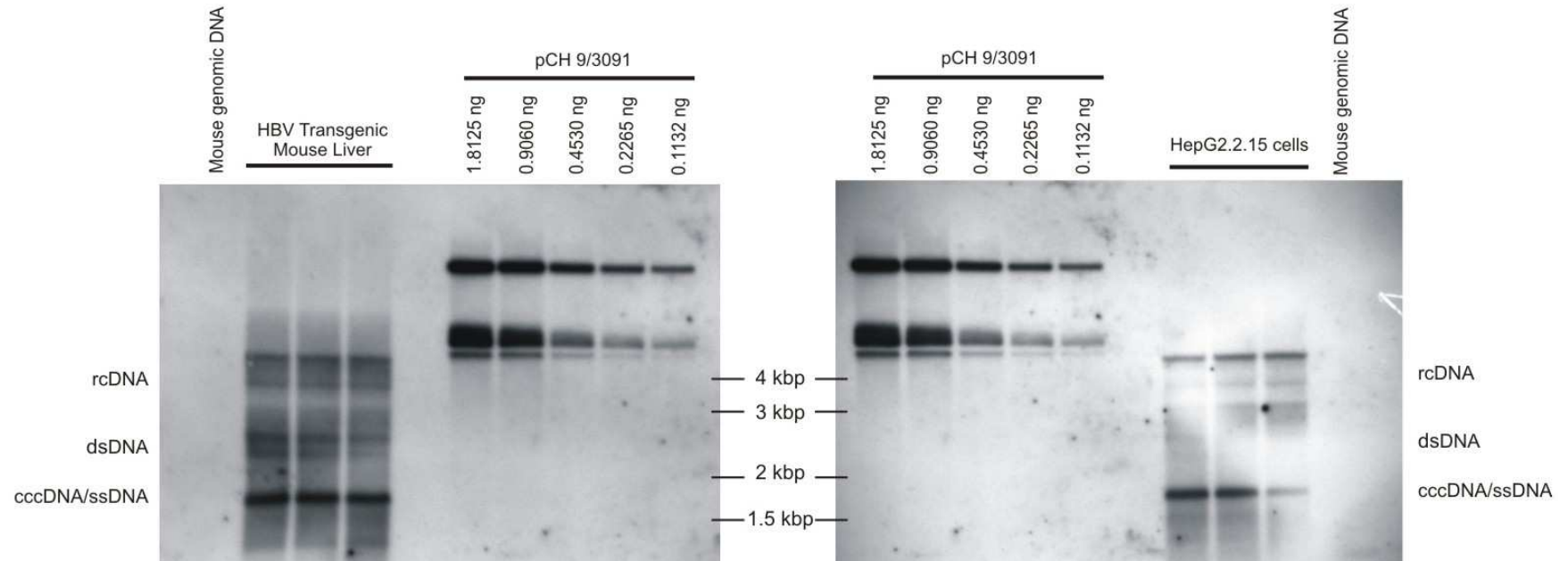
Figure 3.18: Southern blot hybridisation films of DIG-labelled HBV DNA species. The migratory patterns of HBV DNA in transgenic mouse livers and HepG2.2.15 cell extracts correspond to rcDNA, dsDNA replicative intermediates and cccDNA/ssDNA. Chemiluminescent detection is based on 25 µl aliquots of ethanol concentrated and *Hin*dIII digested samples. The pCH9/3091 standard curve was used to determine the concentration of each HBV DNA species, relative to the integrated density of each band. Mouse genomic DNA was included as a negative control.

The HBV DNA profiles of transgenic mouse liver and HepG2.2.15 cells were determined by chemiluminescent detection, from which the DNA copy number was calculated (Figure 3.19). HepG2.2.15 serum samples however, could not be quantified, most likely as a result of viral DNA levels below the predetermined detection limit. Mouse genomic DNA was included as a negative control to verify the specificity of the probe and exclude any chemiluminescence detection as a result of non-specific binding. Transgenic mouse liver was found to contain slightly more total HBV DNA, with 1.92×10^6 copies per assay (77 copies per cell), than HepG2.2.15 cells with a total of 1.81×10^6 copies per assay (36 copies per cell). rcDNA and cccDNA profiles were established based on the integrated intensity of individual bands, as a result of distinct migration patterns observed by HBV DNA species. Integrated viral DNA was not detected in transgenic mouse livers, most likely as a result of low copy numbers. The proportion of rcDNA to cccDNA was found to be higher in HepG2.2.15 cells, at 1.82 times more rcDNA, compared to transgenic mouse liver with only 1.50 times. Accordingly, transgenic mouse liver harbours more cccDNA than HepG2.2.15 cell lines, indicating inherent differences between viral replication in these two HBV model systems. Although HBV DNA was successfully quantified using Southern blot hybridisation and chemiluminescence detection, the process required sample concentration, restriction endonuclease digestion, and lengthy post-detection analysis. This, coupled with high detection limits, restricts the use of hybridisation as an efficient method of quantifying HBV viral loads.

Southern blot quantification of HBV
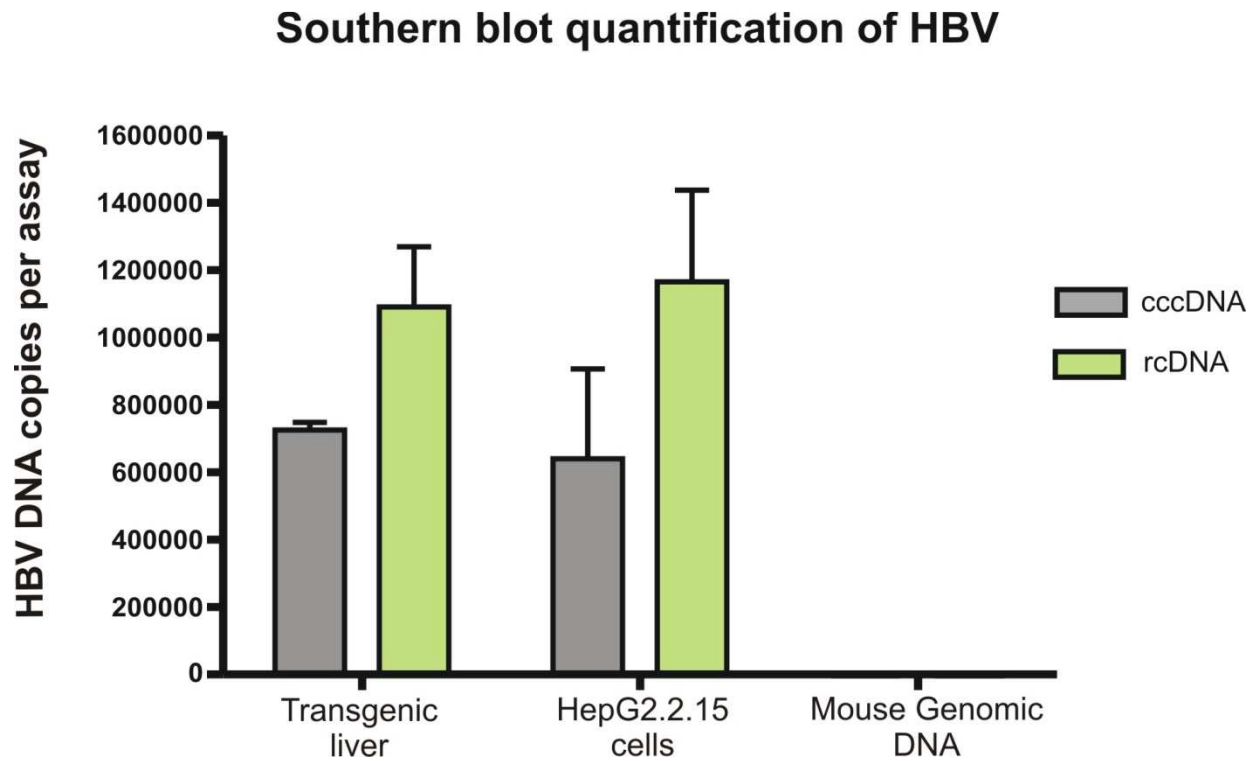
Figure 3.19: Resolution of HBV DNA profiles by Southern blot hybridisation. Transgenic mouse liver samples contain an average of 7.26×10^5 copies of cccDNA and 1.09×10^6 copies of rcDNA. HepG2.2.15 cells retain less cccDNA than transgenic liver with only 6.41×10^5 copies, but more rcDNA at 1.17×10^6 copies. No HBV was found in mouse genomic DNA. Error bars denote the SEM where n = 3.

3.3   Validation of the Real-time HBV qPCR assay against the 'gold   standard'

   of Southern blot hybridisation

There is a number of qPCR methods available for HBV DNA quantification (Brechtbuehl et al., 2001; He et al., 2002; Jun-Bin et al., 2003; Singh et al., 2004), yet they fail to substantiate HBV detection efficiencies with Southern blot hybridisation.   To determine whether HBV DIG-based Southern blot hybridisation and qPCR assay quantify HBV DNA in an equivalent manner, identical HepG2.2.15 cell culture extracts and HBV transgenic mouse liver samples analysed by Southern blot hybridisation, were evaluated by qPCR. Most importantly, minimum detection limits need to be taken into account as this determines whether occult or low levels of infection can be quantified.   By comparing the capacity of these two assays to accurately quantify HBV DNA, the most efficient method can be determined.

3.3.1   Detection limits are drastically reduced when using qPCR

HBV transgenic mouse liver, HepG2.2.15 cell extracts and HepG2.2.15 supernatants previously analysed and quantified by DIG-based Southern blot hybridisation were evaluated by HBV qPCR. Figure 3.20 shows absolute quantification and HBV DNA profiles of these three samples as well as qPCR and Southern blot detection limits. pTZ-HBVcccR and pCH-9/3091 were included as cccDNA and total HBV DNA positive controls (data not shown). Genomic DNA from wild-type mice and water blanks were included as negative controls. The HBV qPCR assay efficiently amplifies cccDNA and total HBV DNA in all three samples, unlike Southern blot hybridisation which was unable to detect any viral DNA in HepG2.2.15 supernatants. The qPCR quantification of both HBV DNA species in HepG2.2.15 supernatants is indicative of the

low minimum detection limits observed for this assay. Southern blot hybridisation was unable to detect such low copy numbers as a result of its elevated minimum detection limit.

qPCR analysis revealed that transgenic mouse liver retained an average of 2.54×10^6 copies of total HBV DNA (101.6 copies per cell) and 8.65×10^5 copies of cccDNA (34.6 copies per cell) (Figure 3.20), with an HBV DNA ratio of 66% rcDNA to 34% cccDNA. HepG2.2.15 cell extracts contained 1.69×10^6 copies of total HBV DNA (33.8 copies per cell) and only 8.29×10^4 copies of cccDNA (1.65 copies per cell) whilst HepG2.2.15 supernatants included 1.89×10^4 copies/µl of total HBV DNA and 157 copies/µl of cccDNA (Figure 3.20). rcDNA therefore remains the primary HBV DNA isolate in HepG2.2.15 cell cultures. These results coincide with the HBV DNA profiles observed during absolute quantification of *in vitro* and *in vivo* samples (section 3.1.5), indicating that rcDNA and cccDNA profiles remain relatively consistent in HBV model systems.

From this, HBV qPCR has emerged as a more sensitive method for viral DNA detection, allowing quantification of very low levels of viral replication. This may be important in clinical analysis of occult infections as well as monitoring drug responses during anti-viral treatments. But sensitivity alone does not validate this technique. HBV quantification results should correlate with those obtained during Southern blot hybridisation to justify the accuracy of the qPCR assay.

Absolute quantification of HBV DNA
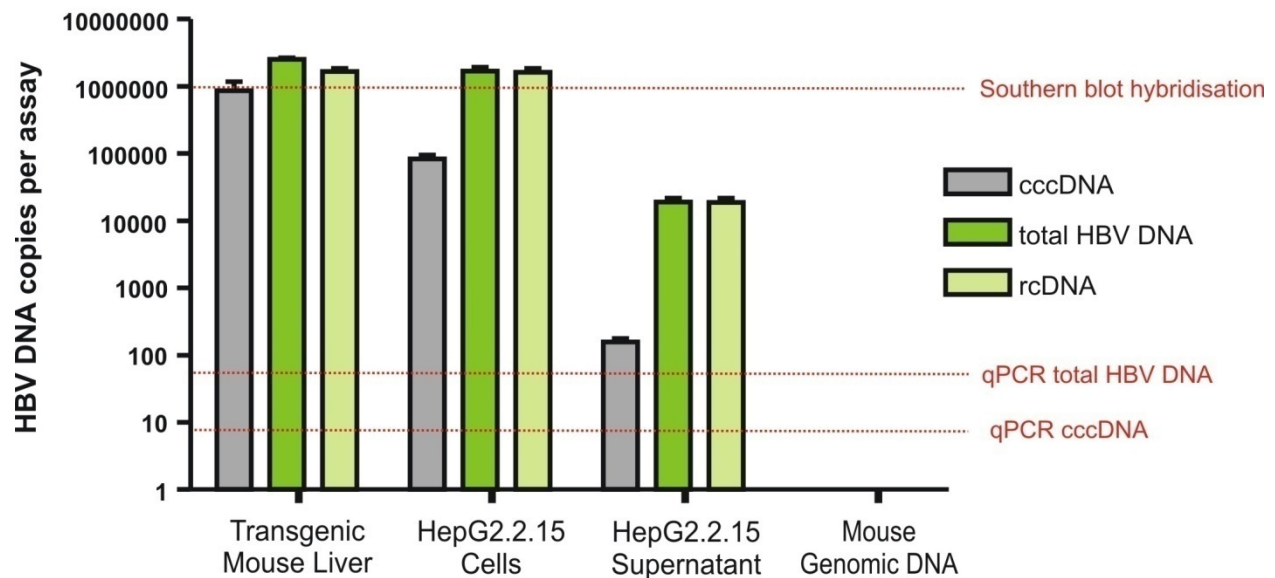
Figure 3.20: qPCR quantification of in vitro and in vivo HBV model systems. HBV transgenic mouse livers, HepG2.2.15 cells and HepG2.2.15 supernatants were analysed by qPCR. Transgenic mouse liver and HepG2.2.15 cells maintain high levels of total HBV DNA indicating active replication. Minimum detection limits are shown in red [1]. The low cccDNA and total HBV DNA minimum detection limits associated with qPCR allow quantification of HBV DNA from HepG2.2.15 supernatants, unlike Southern blot hybridisation. Mouse genomic DNA was included as a negative control. Error bars indicate the SEM where n = 3.

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[1] *Note: Southern blot detection limit amended from 5.67×10^6 to 1.42×10^6 copies of HBV DNA to compensate for adjustments made as a result of ethanol concentrations not performed during qPCR analysis.*

3.3.2   HBV qPCR results are corroborated by Southern blot hybridisation

To validate the HBV qPCR assay, viral DNA quantification was evaluated against Southern blot hybridisation from HBV transgenic mouse livers, HepG2.2.15 cell extracts and HepG2.2.15 supernatants. Table 3.3 displays paired cccDNA, total HBV DNA and rcDNA results, from both techniques. Values are described as the average number of HBV DNA copies per assay. Two-tailed paired Student's *t* tests were performed on each data set to establish if variation observed between Southern blot hybridisation and qPCR quantification were statistically significant. HBV transgenic mouse livers and HepG2.2.15 cells have corresponding results, showing no statistical significance. As HBV DNA present in HepG2.2.15 supernatants is not detected by Southern blotting, the qPCR results are statistically significant. This statistical significance is indicative of the increased sensitivity already observed by qPCR. Figures 3.21 and 3.22 graphically represent the results obtained for HBV transgenic mouse livers and HepG2.2.15 cell extracts. The HBV DNA profiles are uniformly distributed between Southern hybridisation and qPCR. cccDNA detection in HepG2.2.15 cell extracts shows the most marked difference between these two techniques. Southern blot hybridisation appears to overestimate the number of cccDNA copies. This may be a result of HBV ssDNA and cccDNA co-migration, as it is difficult to distinguish between these two intermediates by Southern blot. qPCR however, does not amplify these intermittent HBV replication intermediates efficiently. Therefore, as this HBV qPCR assay has shown equivalence with the 'gold standard' for HBV quantification and displays increased sensitivity, it remains a good candidate for monitoring HBV infection, patient's response to therapy and may be used to determine anti-viral efficacies of novel therapeutics. Most importantly, cccDNA can be monitored independently of rcDNA, allowing better investigation of HBV replication strategies and inhibition associated with RNAi based therapies.

Table 3.3: Quantification of HBV DNA using Southern blot hybridisation and qPCR

	HBV DNA copies per assay ($\pm$SEM)		*p*-value
	Southern Blot Hybridisation	*HBV qPCR*	
	cccDNA		
HBV Transgenic Mouse Liver	7.26×10^5 ($\pm 2.24 \times 10^4$)	8.65×10^5 ($\pm 3.13 \times 10^5$)	0.6825
HepG2.2.15 Cells	6.41×10^5 ($\pm 2.65 \times 10^5$)	8.29×10^4 ($\pm 1.19 \times 10^4$)	0.1804
HepG2.2.15 Supernatant	0	157 (± 22)	0.0183**
	Total HBV DNA		
HBV Transgenic Mouse Liver	1.92×10^6 ($\pm 2.30 \times 10^5$)	2.54×10^6 ($\pm 1.43 \times 10^5$)	0.0513
HepG2.2.15 Cells	1.81×10^6 ($\pm 2.89 \times 10^4$)	1.69×10^6 ($\pm 2.39 \times 10^5$)	0.6831
HepG2.2.15 Supernatant	0	1.89×10^4 ($\pm 2.89 \times 10^3$)	0.0224**
	rcDNA		
HBV Transgenic Mouse Liver	1.09×10^6 ($\pm 1.78 \times 10^5$)	1.67×10^6 ($\pm 1.93 \times 10^5$)	0.2190
HepG2.2.15 Cells	1.17×10^6 ($\pm 2.72 \times 10^{5)}$)	1.61×10^6 ($\pm 2.31 \times 10^5$)	0.0840
HepG2.2.15 Supernatant	0	1.88×10^4 ($\pm 2.87 \times 10^3$)	0.0226**

* mean values (n=3)
** Statistical significance < 0.05 (5%)

HBV transgenic mouse liver
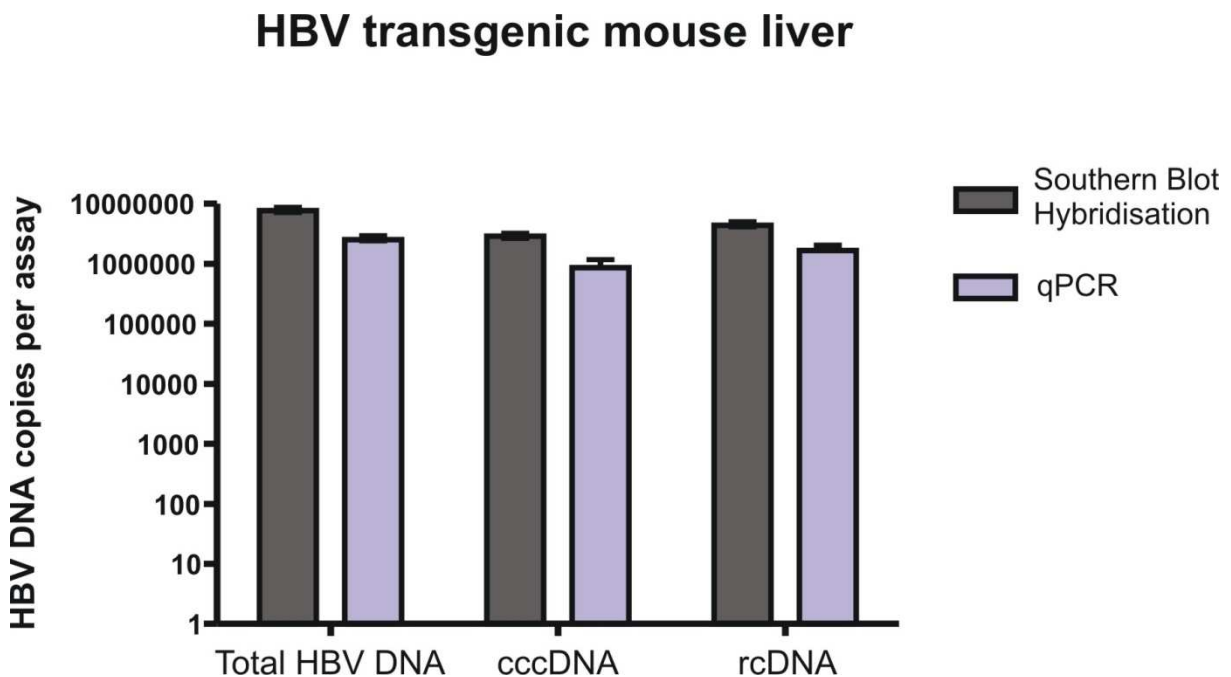
Figure 3.21: In vivo validation of HBV qPCR. Southern blot hybridisation and qPCR quantifications correlate across both forms of HBV DNA. No statistical significance was observed between total HBV DNA, cccDNA, or rcDNA quantification. Both techniques established rcDNA as the predominant DNA species in transgenic mouse livers. The difference was only marginal however, as cccDNA was also recovered at high concentrations. Samples were prepared in triplicate and error bars indicate the SEM.

HepG2.2.15 cells
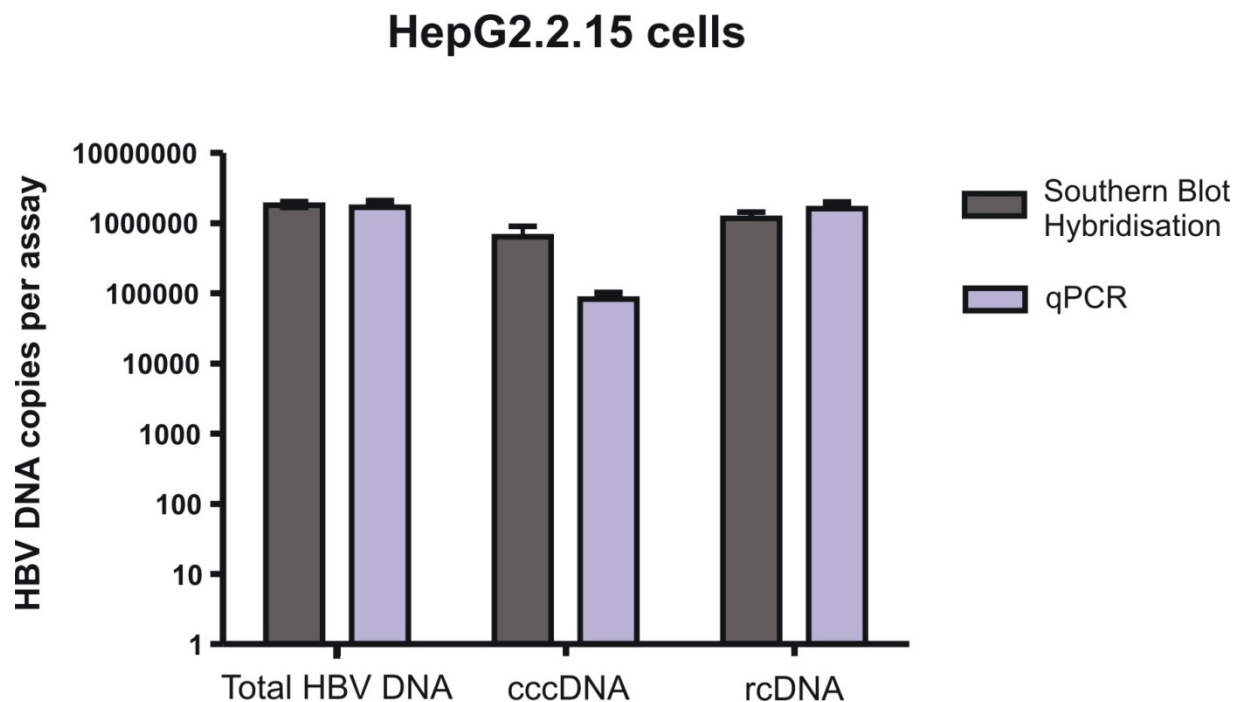
Figure 3.22: In vitro validation of HBV qPCR. Southern blot hybridisation and qPCR show equivalent results for total HBV DNA and rcDNA quantification. cccDNA copy numbers however do show variation between the two techniques, however this difference is not statistically significant. Samples were prepared in triplicate and error bars indicate the SEM.

3.4 The *in vivo* efficacy of anti-HBV miRNAs is illustrated by real-time qPCR

Several RNAi-based therapies have been successfully developed to inhibit HBV transcription and reduce viral loads (Arbuthnot et al., 2005; Hilleman, 2003). Anti-HBV knockdown efficiencies are predominantly determined by HBsAg ELISAs, DNA of circulating viral particle equivalents and relative intrahepatic HBV RNA concentrations. cccDNA levels however are not regularly monitored. Considering cccDNA drives viral replication and may remain dormant in the nucleus of infected hepatocytes, awaiting re-activation, it is important to quantify this form of HBV DNA during anti-viral therapy. Therefore HBV qPCR and RT-PCR was used to establish the *in vivo* RNAi silencing efficiencies of U6 shRNA5 (Carmona et al., 2006), CMV miRNA-122/5 (Ely et al., 2008), Lin-122/5 and ITR-122/5 (Chattopadhyay et al., 2009). Plasmid vectors expressing HBV replication-competent pgRNA, miRNA shuttles or linear expression cassettes, and *Renilla* luciferase reporter genes were administered by means of hydrodynamic tail vein injections. Mock HBV infections and negative controls were also included. Serological and intrahepatic markers of infection, including total HBV DNA, cccDNA and HBV RNA, were analysed relative to reporter gene delivery efficacy or mock HBV infections.

3.4.1 HBV replication is inhibited by RNAi-based therapeutics

Serological HBV DNA quantification was performed on murine serum samples collected at days 3 and 5 post injection. A significant decrease in circulating HBV DNA equivalents was observed at day 3 in all four RNAi treated isolates when normalised to mock injected mice, as illustrated in Figure 3.23. The U6 shRNA5, CMV miRNA-122/5 and Lin-122/5 cassettes all achieved 95% knockdown compared to ITR-122/5 with approximately 90% knockdown. An increase in viral

DNA equivalents was observed 5 days after injection, however U6 shRNA5 and Lin-122/5 maintained between 85 – 90% knockdown indicating prolonged HBV inhibition. Mice were sacrificed on day 5, allowing quantification of intrahepatic HBV RNA and DNA. RT-PCR was used to quantify total HBV RNA levels relative to the *GAPDH* housekeeping gene mRNA. Declining viral RNA levels were observed in all RNAi treated samples (Figure 3.24). CMV miRNA-122/5 displays the greatest knockdown efficiency at 90%, followed by U6 shRNA5 at 86%, and finally Lin-122/5 and ITR-122/5 at 81%. Attenuation of HBV RNA levels as well as the serological decline in circulating viral particle equivalents both correlate with effective RNAi-based silencing of HBV replication. Furthermore, this indicates that U6 shRNA5, CMV miRNA-122/5, Lin-122/5 and LTR-122/5 are all capable of inhibiting HBV replication and the subsequent release of new viral particles. However, to determine whether these RNAi-effectors influence cccDNA, an intrahepatic HBV DNA profile is required.
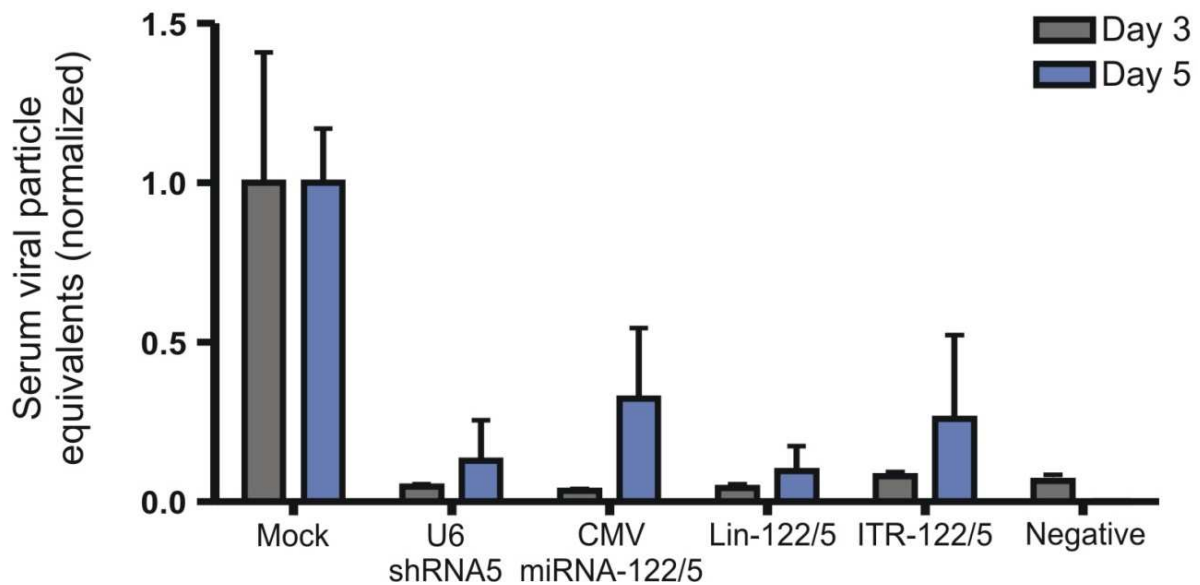
Figure 3.23: Decline in serum HBV DNA. Total HBV DNA was quantified at days 3 and 5 post injection, relative to the mock. U6 shRNA5, CMV miRNA-122/5, Lin-122/5 and ITR-122/5 all show exceptional inhibition of total HBV DNA after 3 days, however viral loads increase somewhat at day 5, most likely as a result of plasmid DNA clearance. U6 shRNA5 and Lin-122/5 exhibit the most efficient prolonged HBV inhibition, unlike CMV miRNA-122/5 and ITR-122/5. Experiments were performed in triplicate and error bars indicate the SEM.

Intrahepatic HBV RNA
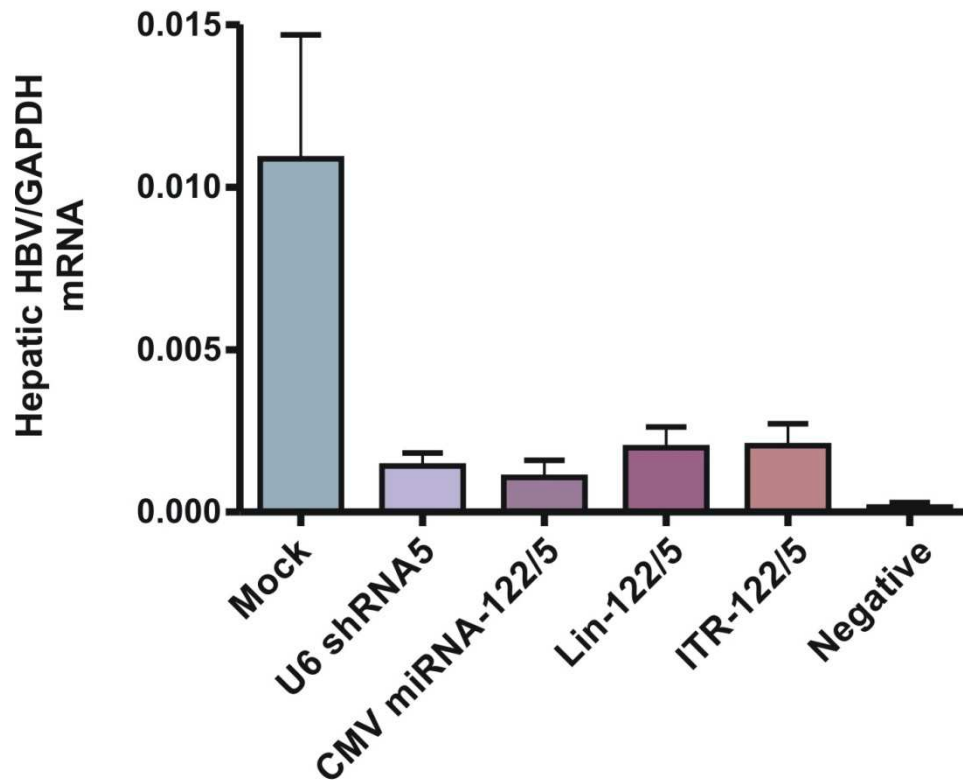
Figure 3.24: Relative quantification of intrahepatic HBV RNA. RT-PCR was used to quantify total HBV RNA relative to the *GAPDH* mRNA. Efficient HBV RNA silencing is observed by all RNAi effecters indicating HBV transcriptional inactivation. CMV miRNA-122/5 displayed 90% knockdown efficiency, whilst U6 shRNA5, Lin-122/5 and ITR-122/5 demonstrated over 80% knockdown. Error bars indicate the SEM where n = 3.

3.4.2   RNAi silencing regulates cccDNA levels

To examine the influence RNAi effecters have on cccDNA, qPCR was used to analyse the HBV DNA profiles of murine liver samples collected at day 5. Mice injected with HBV plasmid DNA were used as positive controls whilst mice injected with *Renilla* luciferase reporter plasmids only (psiCHECK2.2) were included as negative controls. pCH-9/3091 and pTZ-HBVcccR were included during qPCR analysis as internal standards. Intrahepatic total HBV DNA, cccDNA and *Renilla* luciferase were individually quantified by real-time PCR. Reporter plasmids were included during each hydrodynamic injection to control for transgene delivery efficiency. Therefore, each sample's total HBV DNA and cccDNA results were normalised to psiCHECK2.2 levels. Figure 3.25 shows the quantification of intrahepatic total HBV DNA and cccDNA, relative to *Renilla* luciferase plasmid delivery. CMV miRNA-122/5 is the most efficient inhibitor of total HBV DNA, with 54% knockdown, however no cccDNA knockdown was detected. Therefore rcDNA synthesis is inhibited, but CMV miRNA-122/5 has no direct influence on established cccDNA pools. Despite the increase in cccDNA, statistically there is no difference between Lin-122/5 treated samples and the mock. Lin-122/5 and ITR-122/5 showed poor total HBV DNA knockdown, and no inhibition of cccDNA indicating inefficient clearance of intrahepatic viral DNA. U6 shRNA5 demonstrated a total HBV DNA knockdown efficiency of 49%, and an unexpected cccDNA knockdown efficiency of 53%. Consequentially, U6 shRNA5 appears to have an inhibitory effect on cccDNA, possibly by preventing the redirection of newly synthesised rcDNA to the nucleus. This in turn would inhibit the development of a nuclear cccDNA pool.
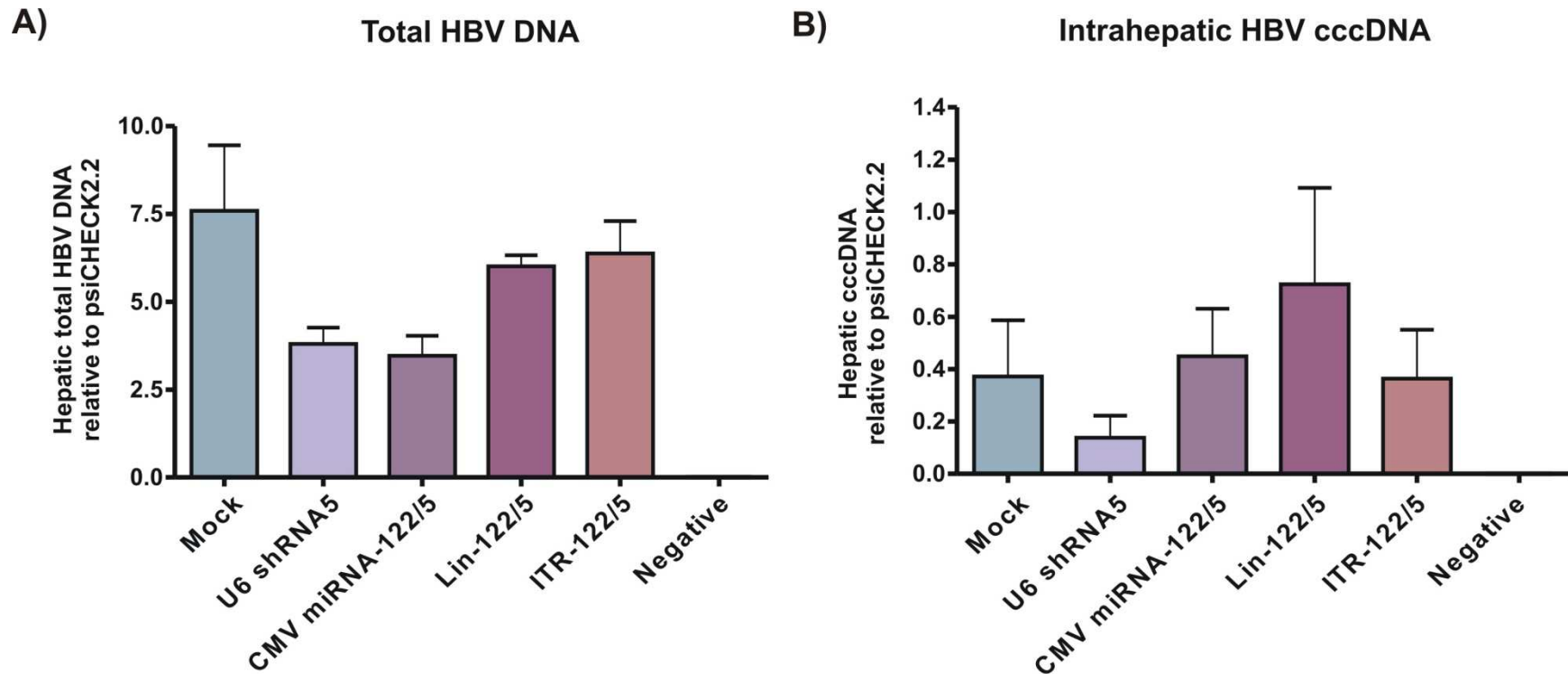
Figure 3.25: Quantification of hepatic HBV DNA relative to delivery efficiency. HBV DNA and the *Renilla* luciferase reporter plasmid were quantified using qPCR. Total HBV DNA (A) and cccDNA (B) profiles were resolved from murine liver samples collected 5 days post-infection. CMV miRNA-122/5 knocked down total HBV DNA the most efficiently, followed by U6 shRNA5 which additionally repressed cccDNA. Lin-122/5 and ITR-122/5 had no marked effect on total HBV DNA or cccDNA. Samples were analysed in triplicate and error bars indicate the SEM.

CHAPTER 4 – DISCUSSION

4.1. Efficient real-time HBV qPCR for viral DNA quantification

The ability to accurately detect and quantify serum and intrahepatic HBV DNA has become an essential component of chronic HBV management and innovative therapeutic design. Quick and cost-effective techniques, facilitating the rapid detection of viral DNA, are largely required in hyper-endemic regions such as Sub-Saharan Africa. Various commercially available HBsAg ELISA and conventional endpoint or competitive PCR-based assays are currently used for the clinical diagnosis of acute and chronic HBV infections. Additionally, these techniques are employed to determine anti-HBV drug efficacies, during therapeutic research or patient treatment regimens. However, as described in section 1.1.6, empty viral vesicles interfere with the accuracy of the HBsAg ELISA, and conventional PCR requires separate detection and quantification platforms. Real-time qPCR on the other hand, is not influenced by viral antigens, and is capable of detecting and quantifying HBV DNA in single reaction.

Absolute quantification of HBV has, more recently, become an important diagnostic tool, allowing the sensitive detection of viral DNA from sera and liver biopsies. Circulating viral particles are often used as markers of infection, indicative of either HBV clearance associated with acute infections or persistence leading to chronicity. Brechtbuehl and colleagues (Brechtbuehl et al., 2001) have described an efficient Lightcycler™ real-time PCR system with SYBR green fluorescence, to detect and quantify the HBV *surface* gene, allowing total HBV DNA quantification. Although this system permits the sensitive detection of HBV viral loads, it is unable to differentiate between rcDNA and cccDNA.

Viral mutagenesis, epigenetic regulation and minichromosome formation of cccDNA results in a persistent HBV genome (Vivekanandan et al., 2008), that is able to remain dormant during anti-viral therapy (reviewed by Zoulim, 2004). Monitoring intrahepatic cccDNA is crucial during drug development or chronic HBV management, as viral escape mutants and occult infections will readily be detected. Identifying occult infections is very important in HIV and HBV endemic areas, as co-infection increases the risk of developing liver disease (Salmon-Ceron et al., 2005; Thio et al., 2002). The HBV qPCR assay described here is able to accurately quantify total HBV DNA as well as differentiate between the two forms of the viral genome, thus providing a suitable alternative to current HBV diagnostic tools. Differential detection of viral DNA species is achieved by exploiting the gap region, found only in rcDNA. Additionally, the cccDNA primers were found to not amplify rcDNA non-specifically, as determined by three independent experiments, and genomic DNA is not amplified by either primer sets. Singh and colleagues (Singh et al., 2004) have developed a Lightcycler™ based assay that is able to quantify cccDNA using fluorescent probes, whilst He (He et al., 2002) and Jun-Bin (Jun-Bin et al., 2003) have developed chimaeric primers and FRET based cccDNA detection systems. A drawback of these systems is that they rely on the enzymatic degradation of rcDNA for accurate cccDNA quantification. Furthermore, Southern blot hybridisation was not used to validate the qPCR efficiency.

Plasmid-Safe™ ATP-dependent DNase was originally employed to isolate cccDNA from total viral DNA. According to previously described HBV PCR detection and quantification systems (Laras et al., 2006; Singh et al., 2004; Wong et al., 2004) enzymatic degradation of partially dsDNA and linear DNA, either with Plasmid-Safe™ ATP-dependent DNase or S1 nuclease, is a prerequisite for accurate cccDNA quantification. However during this analysis, DNase treatments were found to diminish both *in vitro* and *in vivo* cccDNA levels. Furthermore, *in vivo*

qPCR analysis of increasing enzyme units, indicate cccDNA copy numbers are inversely proportional to DNase concentrations. As a result of this, enzymatic isolation of cccDNA was eliminated from further HBV DNA qPCR analysis. Despite this, cccDNA and total HBV DNA primers were found to be sensitive and highly specific for their target sequences.

4.1.1. qPCR minimum detection limits enhance low level HBV DNA quantification

The accuracy and sensitivity of the HBV qPCR assay was successfully validated with Southern blot hybridisation. Although both radioactive and non-radioactive Southern blot analysis has been extensively used to quantify total HBV DNA and cccDNA levels (Farza et al., 1988; Gong et al., 1998; Gripon et al., 2002; Ren and Nassal, 2001), only limited sensitivity is achieved. Distinguishing cccDNA from ssDNA is also problematic, as both migrate to similar positions during agarose gel electrophoresis. Gripon and colleagues (Gripon et al., 2002) as well as Gong (Gong et al., 1998) and Summers (Summers et al., 1990) describe cccDNA as the single band or smear located just below the 2 kb marker, whilst others (Abdelhamed et al., 2003; Chen et al., 2007) describe the same band as only ssDNA. Here, a single band of approximately 1.9 kb was observed during Southern blot hybridisation (Figures 3.13 and 3.18) which correlates to cccDNA. However no distinct ssDNA band was observed around 1.5 kb (Figure 3.18), suggesting that cccDNA and ssDNA may co-migrate. This, in turn, explains the overestimation of cccDNA observed during Southern blot hybridisation when compared to qPCR analysis.

DIG hybridisation probes displayed poor minimum detection limits when compared to qPCR. This was particularly evident during HepG2.2.15 supernatant analysis, where no HBV DNA was detected using Southern blot hybridisation; however a distinct HBV DNA profile was obtained using qPCR. The HBV qPCR assay minimum detection limits (10 copies of cccDNA and 84 copies of total HBV DNA per assay) correspond to other real-time PCR systems (Table 4.1).

cccDNA detection sensitivity is in fact, superior to these HBV quantification systems. This will assist with successfully identifying occult infections and determining if novel anti-HBV therapeutics are capable of eliminating episomal cccDNA pools.

Table 4.1: Sensitivity of HBV real-time qPCR systems

	HBV DNA copies per assay		HBV DNA copies/ml	
	cccDNA	*Total HBV DNA*	*cccDNA*	*Total HBV DNA*
This study (Bloom 2010)	10	84	2.0×10^3	1.7×10^4
Brechtbuehl *et al.* (2001)	N/A	10^2	N/A	1.3×10^4
Singh *et al.* (2004)	25	25	-	-
Jun-Bin *et al.* (2003)	50	N/A	-	N/A
He *et al.* (2002)	100	N/A	-	N/A
Wong *et al.* (2004)	50	50	10^4	10^4
Laras *et al.* (2006)	100	10	-	-

4.2.   HBV DNA profiles differ between *in vitro* and *in vivo* model systems

HBV *in vitro* and *in vivo* model systems, like HepG2.2.15 cell cultures and transgenic mice, are often used to determine anti-HBV efficacy when developing new therapeutics.   If a novel therapeutic is shown to inhibit HBV replication *in vitro*, the drug is then assessed *in vivo*.   HBV qPCR was found to be an efficient method of quantifying both cccDNA and total HBV DNA levels in both these model systems. Furthermore, the ratio of rcDNA to cccDNA may be established.   Although these systems both support active HBV infection, major differences between the HBV DNA profiles of HepG2.2.15 cells and transgenic mouse livers was noted. One would expect HepG2.2.15 cells to have a similar cccDNA profile to that of transgenic mouse liver (40% cccDNA to 60% rcDNA) considering both are hepatocellular in origin.   But, 95% of HepG2.2.15 cell extracts is rcDNA, which corresponds more with transgenic mouse serum and HepG2.2.15 supernatants.   This suggests that the HepG2.2.15 cell line has a very high HBV replication rate, which may not mimic natural infection.   In addition, anti-HBV therapeutics are likely to have varying effects in these two systems.   Antivirals that inhibit reverse transcription of pgRNA to rcDNA may appear to be more efficient in HepG2.2.15 cell lines than in transgenic mice, merely as a result of the vast rcDNA pools found in these cell lines, however cccDNA may remain unaffected.   This emphasises the need to monitor both forms of viral DNA when screening antiviral efficacy.

4.2.1. cccDNA in HBV transgenic mouse model

There are conflicting opinions surrounding the ability of HBV transgenic mice to produce cccDNA.   It is generally believed that these mice do not support viral replication through a

cccDNA intermediate (Chisari, 1996; Dandri et al., 2005) as pgRNA is instead transcribed directly from the integrated HBV genome. However others have found that HBV transgenic mice are able to accommodate small amounts of cccDNA (Guidotti et al., 1995; Raney et al., 2001). However in most of these studies, HBV DNA was quantified using Southern blot hybridisation where bands corresponding to cccDNA and ssDNA co-migrate and are regarded as ssDNA (Chen et al., 2007; Farza et al., 1988). This is an inaccurate assumption considering that both viral DNAs have similar migration profiles. During this analysis, cccDNA was identified in both intrahepatic and serological samples isolated from transgenic mice. Furthermore, it was determined that up to 40% of intrahepatic total HBV DNA is cccDNA. Southern blot hybridisation was found to overestimate cccDNA levels when compared to qPCR as a result of HBV linear or ssDNA co-migration.

4.3. RNAi inhibits HBV replication

The *in vitro* and *in vivo* silencing of HBV using RNAi based therapies was confirmed with qPCR and RT-PCR. Decreases in circulating viral particles, pgRNA and intrahepatic total HBV DNA were established using all four RNAi-effecters. Silencing of pgRNA halted viral replication which resulted in reduced rcDNA levels. Intrahepatic cccDNA viral pools however remained unaffected by CMV miRNA-122/5, Lin-122/5 and ITR-122/5. U6 shRNA5 however, managed to inhibit cccDNA, presumably by inhibiting rcDNA nuclear translocation. Overall RNAi does not appear to destroy established episomal cccDNA, however efficiently inhibits RNA translation and therefore reverse transcription and replication of HBV. This corresponds with results reported by Starkey and colleagues (Starkey et al., 2009) who found that anti-HBV shRNAs were unable to reduce established cccDNA pools. Overall, the post-transcriptional silencing of

HBV will result in a decline in circulating virions and surface antigen levels, which will give the impression of viral clearance. However this may be misleading, as the cccDNA remains unaffected and once therapy ceases, re-infection may occur. It is therefore critical to monitor cccDNA levels when designing new therapeutics to determine their effect on established cccDNA pools.

4.4. Conclusion

In summary, the HBV qPCR assay described here is able to detect and quantify rcDNA and cccDNA from murine serum and liver samples as well as HepG2.2.15 cell cultures. Vast differences between the HBV DNA profiles of transgenic mouse liver and HepG2.2.15 cell extracts was noted. Specifically, HepG2.2.15 cell cultures produce substantial amounts of rcDNA when compared to cccDNA, whilst transgenic mouse liver samples contained a significant amount of cccDNA. As both these model systems are used during anti-HBV effecter design, the inherent differences between them may skew the results. This highlights the importance of monitoring the HBV DNA profiles of model systems during therapeutic research.

This qPCR allows the precise monitoring of HBV infection as a result of the capacity for both intrahepatic viral DNA and circulating viral particle quantification. This remains important during the clinical evaluation of chronic HBV or HIV/HBV co-infections. Serum total HBV DNA quantification may be used during routine HBV diagnostics. Hepatocellular cccDNA quantification may indicate disease progression or response to therapy, although liver biopsies remain invasive. Furthermore, occult infections associated with viral latency and cccDNA persistence may readily be quantified using this HBV qPCR assay, as low detection limits give rise to increased sensitivity when compared to Southern blot hybridisation. When compared to

other qPCR techniques, this assay provides the most sensitive cccDNA detection platform however total HBV DNA detection limits could be improved. To further decrease minimum detection limits and thus increase the sensitivity of the assay, fluorescent hybridisation probes could be designed to bind to the gap region or *pre-S1* sequence, allowing more accurate amplicon detection. Alternatively, various fluorophores may be linked directly to the primers. This would allow multiplexing of total HBV DNA and cccDNA quantification therefore improving qPCR efficiency.

CHAPTER 5 – APPENDIX

5.1 Vector NTI plasmid maps and sequences

5.1.1   pCH-9/3091 plasmid map

PreCore

Nco I (6032)

CMV Promoter

Pst I (5662)

Core Protein

Ava I (534)

Bam HI (1005)

PreS1

Eco RI (1281)

PreS2

Apa LI (4973)

pCH-9 3091

6338 bp

Ava I (1410)

Polymerase

Bam HI (1771)

Surface

Ampicillin

Apa LI (3727)

Nco I (2655)

Bam HI (2683)

T3 Promoter

Ava I (2746)

Sma I (3274)

Apa LI (2862)

Ava I (3272)

X Protein

Xma I (3272)

PreCore

5.1.2   pHBV 1.3x plasmid map

Apa LI (7105)

Apa LI (6608)

Zeo

Sma I (6353)

Ava I (6351)

Xma I (6351)

Ava I (6341)

Nco I (6261)

Nco I (5822)

Kan

Plac

Hin dIII (277)

Nco I (587)

Ava I (678)

Apa LI (794)

HBx ORF

PreC

Core ORF

Ava I (1653)

PreS1

Eco RI (2433)

PreS2

Ava I (2562)

Bam HI (2923)

Surface ORF

Pst I (2953)

Nco I (3807)

Ava I (3898)

Apa LI (4014)

HBx ORF

PreC

Apa LI (4720)

ccdB ORF

pCR-XL-HBV-A1 + BCP + pA

7543 bp

5.1.3   pTZ-HBVcccR M13 sequence

The pTZ-HBVcccR sequence results using M13 forward primers. The cccDNA forward and reverse primers amplify the highlighted target sequence.

ggsgggggtctmtacgactcactatagggaagcttgcatgcaggcctctgcagtcgacgggcccgggatccgattctgccgtaccgt

ccgaccacggggcgcacctctctttacgcggactccccgtctgtgcc<mark>ttctcatctgccggaccgtgtgcacttcgcttcacctccgcac</mark>

<mark>gtcgcatggagaccaccgtgaacgcccaccaaatattgcccaaggtcttacataagaggactcttggactctcagcaatgtcaacga</mark>

<mark>ccgacctcgaggcatacttcaaagactgtttgtttaaagactgggaggagttggggggaggagattaggttaaaggtctttgtaccagga</mark>

<mark>ggctgtaggcataaattggtctgcgcaccagcaccatgcaactttttcacctctgcctaatcatctcttgttcatgtcct</mark>actgttcaagcctc

caagctgtgccttgggtggctttggggcatggacattgaccgaattcgaatctagatgcattcgcgaggtaccgagctcgaattcactgg

ccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatcgccttgcagcacatcccccctttcgccagctggcgta

atagcgaagaggcccgcaccgatcgcccttcccaacagttgcgcagcctgaatggcgaatggaaattgtaagcgttaatattttgttaa

aattc

KRISTIE BLOOM

5.2 Laboratory protocols and reagents

5.2.1 Common Reagents

Tris-borate-EDTA (TBE) Buffer

For a $5 \times$ TBE stock solution: 54 g Tris base, 27.5 g boric acid, and 20 mL of 0.5 M EDTA (pH 8.0) was dissolved in a final volume of 1000 ml.

Tris-acetate-EDTA (TAE) Buffer

A $50 \times$ TAE stock solution was made by dissolving 242 g Tris base in 750 ml of best quality water. To this, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were added. The stock solution was made up to 1000 ml. For $1 \times$ TAE (40 mM Tris acetate, 1 mM EDTA), 20 ml of $50 \times$ TAE stock solution was diluted in 980 ml best quality water.

$6 \times$ Loading dye

0.125 g of bromophenol blue was mixed with 30 ml glycerol and 60 ml $10 \times$ TBE, to a final volume of 100 ml with best quality water (final concentrations: 0.125% bromophenol blue; 30% glycerol; 6× TBE)

$20 \times$ Sodium chloride sodium citrate (SSC)

A stock solution of $20 \times$ SSC was made by mixing 87.7 g Sodium chloride and 44.1 g of Sodium citrate with best quality water to a final volume of 500 ml (pH 7.0)

5.2.2   Chemically competent cells

A 50 µl aliquot of DH5α *E. Coli* stock was mixed with 10 ml LB medium (10 g tryptone, 5 g yeast extract, 7.5 g sodium chloride to a final volume of 1000ml in best quality water) and was incubated gently shaking at 37℃ overnight. Two mi llilitres if this culture was mixed with 40 ml fresh LB and incubated for 2 h in a shaking incubator at 37℃, and centrifuged at 600 × *g* for 15 min. The supernatant was removed and the bacterial cells re-suspended in 1 ml transformation buffer (100 mM calcium chloride, 10 mM PIPES, 15 % glycerol [pH 7.0]) after which an additional 19 ml of transformation buffer was added. The solution was incubated on ice for 30 min, followed by centrifugation at 200 × *g* for 10 min. The supernatant was decanted and the culture re-suspended in 1.5 ml transformation buffer. Chemically competent cells were stored at -70℃.

5.2.3   pTZ-HBV cccR and pTZ-HBVrcR restriction digest analysis

Roche Plasmid DNA extraction Kit

Bacterial cultures were centrifuged at 2000 × *g* for ten minutes. The supernatant was removed and the pellet was resuspended in 250 µl of suspension buffer with RNase following which 250 µl of lysis buffer was added to the resuspension and the sample was mixed by gentle inversion. The sample was incubated for 5 min at 25℃. Three hundred and fifty microlitres of chilled binding buffer was added to the sample which was mixed gently by inversion. The sample was then placed on ice for 5 minutes and centrifuged at 13 000 × *g*. The supernatant was transferred into a high pure filter tube with a collection tube. The sample was centrifuged at 13 000 × *g* for 60 s after which the flow through was discarded. Seven hundred mirolitres of wash

buffer II was added to the column and the sample was centrifuged at 13 000 × *g* for 60 s. The flowthrough was discarded and the column centrifuged for an additional 60 s at 13 000 × *g*. The flowthrough was discarded once again with the collection tube. The column was placed in a 1.5 ml microcentrifuge tube and 100 μl of best quality water was added to the column. The column was centrifuged for 60 s at 13 000 × *g*. The elution was stored at - 20℃.

*Eco*RI digestion protocol

Twenty units of *Eco*RI enzyme (New England BioLabs, MA, USA) were mixed with 1× NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM DTT (New England Biolabs)), and 100 ng of plasmid DNA (either pTZ-HBVcccR or pTZ-HBVrcR) to a final volume of 15 μl with sterile water.

*Xba*I/*Bam*HI double digestion protocol

Ten units of *Xba*I enzyme (New England Biolabs) and 10 U *Bam*HI enzyme (New England Biolabs) were mixed with the following: 1× NEBuffer 2 (New England Biolabs), 10× BSA, and 100 ng of plasmid (either pTZ-HBVcccR or pTZ-HBVrcR) to a final volume of 15 μl.

Gel Electrophoresis

Ten microlitre aliquots of each digest were mixed with of 6× Loading dye and subjected to electrophoresis in a 1.5% agarose gel (1.50 g molecular grade agarose, 100 ml 1× TAE buffer) containing 0.0025% Ethidium Bromide. Electrophoresis was performed for 1 hour at 150V.

5.2.4   QIAmp mini tissue protocol

Fifty milligrams of HBV transgenic mouse liver was placed in a 1.5ml microcentrifuge tube and 150 µl of ATL buffer was added to the sample. Following this, 20 µl of Proteinase K was added to the sample which was mixed by vortexing and incubated at 56℃ for 3 hours. The sample was microcentrifuged briefly and then 200 µl of buffer AL was added to the sample. The mixture was pulse vortexed and incubated at 70℃ for 10 min utes.   Two hundred and thirty microlitres of 100% ethanol was added to the sample and mixed by pulse vortexing. The sample was then transferred to a QIAamp spin column with a collection tube and centrifuged at $6000 \times g$ for 1 min. The collection tube was replaced and 500 µl of Buffer AW1 was added. The sample was centrifuged at $6000 \times g$ for 1 min. The collection tube was discarded and replaced. Five hundred microlitres of Buffer AW2 was added to the column which was centrifuged at $9\,000 \times g$ for 3 minutes. The column was placed in a 1.5ml microcentrifuge tube and the collection tube discarded. Two hundred microlitres of best quality water was added to the column and it was allowed to stand for 1 min, followed by centrifugation at $6000 \times g$ for 1 min. The sample was stored at -20 ℃.

5.2.5   DNA concentration and purity

DNA extracted from transgenic mice, non-transgenic mice, and HepG2.2.15 cells was subjected to spectrophotometry in order to determine concentration and purity. A 1 µl aliquot of each extraction was analysed on the NanoDrop® ND-1000 UV-Vis spectrophotometry (NanoDrop Technologies) with the NanoDrop ND-1000 3.3 software (Coleman Technologies Inc., Florida, USA).   Purity was assessed based on the A260/A280 reading, whilst concentration was expressed in the form of nanograms per microlitre.

5.2.6   Guanidine single-step RNA isolation

Reagents: **Denaturing Solution** (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol)

2 M Sodium acetate (16.42 g sodium acetate, 35 ml glacial acetic acid and 40 ml best quality water. Glacial acetic acid was used to adjust the pH to 4.0 following which the volume was made up to 100 ml with best quality water)

Water saturated phenol (100 g phenol crystals were dissolved in best quality water at 60°C, following which the upper phase was used for extractions)

DEPC-treated water (1 ml Diethyl pyrocarbonate(DEPC) per 1000 ml of best quality water. The solution was incubated at 37°C for 16 h and then autoclaved for 20 min at 121°C and 1 kg/cm^2. The DEPC-treated water was left to cool and stored at room temperature)

One hundred milligrams of liver tissue was homogenised in 1 ml denaturing solution following which 0.1 ml 2M sodium acetate (pH 4) was added and the sample mixed by inversion. Once the solution had thoroughly mixed, 1 ml of water-saturated phenol was then added, followed by 0.2 ml chloroform. The solution was mixed thoroughly before incubating for 20 min at 4°C. The sample was then centrifuged for 20 min at 10 000 $\times$ g for 20 min at 4°C. The upper aqueous phase was aliquoted into a new 2 ml microcentrifuge tube and 1 volume of cold, 100% isopropanol was added. The new solution was incubated for 20 min at -20°C followed by centrifugation at 10 000 $\times$ g for 10 min at 4°C. The supernatant was removed an d 0.3 ml of denaturing solution was used to re-suspend the RNA pellet followed by 0.3 ml cold, 100% isopropanol. The sample was incubated at -20°C for 20 min and then centrifuged at 10 000 $\times$ g for 10 min at 4°C. One millilitre of 75% ethanol w as used to re-suspend the RNA pellet once the

supernatant had been discarded. The sample was incubated at room temperature for 10 min and centrifuged for 5 min at 10 000 × g (4℃). The supernatant was removed and the pellet left to air dry following which it was re-suspended in 100 μl DEPC-treated water. Samples were stored at -70℃.

5.2.7 QuantiTect reverse transcription protocol

Additional reagents: DEPC-treated water (see appendix 5.2.6)

For reverse transcription of RNA to cDNA, all reagents and solutions were prepared and stored on ice. 1 μg aliquots of RNA were thawed and mixed with 1 x gDNA Wipeout buffer (Qiagen) to a final volume of 14 μl with DEPC-treated water. Samples were incubated at 42℃ for 2 min and then returned to ice. Six microlitres of reverse transcription master mix (1 μl Quantiscript Reverse Transcriptase, 4 μl 5 × Quantiscript RT Buffer and 1 μl RT Primer Mix) were added to the RNA samples which were then mixed gently and incubated at 42℃ for 20 minutes. Finally samples were incubated at 95℃ for 3 min and then s tored at -20℃ until qPCR analysis.

5.3 Multiple Alignments

Multiple alignments of three different HBV A1 subtypes from Germany, Uganda and Gambia. Regions that correspond completely to one another are shown in yellow whilst highly complementary regions are in blue.

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                                            1                                                  50
  HBV A1 Uganda (AY934772)     (1) TTCCACAGCTTTTCCACCAAGCTCTGCAAGATCCCAGAGTCAGGGGCCTGT
  HBV A1 Gambia (AY934764)     (1) TTCCACAGCTTTTCCACCAAGCTCTGCAAGATCCCAGAGTCAGGGGCCTGT
HBV A1 Germany (AY738139)      (1) TTCCACTGCCTTTCCACCAAGCTCTGCAGGATCCCAGAGTCAGGGGTCTGT
               Consensus       (1) TTCCACAGCTTTTCCACCAAGCTCTGCAAGATCCCAGAGTCAGGGGCCTGT
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                                        51                                               100
HBV A1 Uganda (AY934772)    (51)  ATCTTCCTGCTGGTGGCTCCAGTTCAGGAACACTCAACCCTGTTCCAAAT
HBV A1 Gambia (AY934764)    (51)  ATTTTCCTGCTGGTGGCTCCAGTTCAGGAATAGTAAAACCCTGTTCCGAAT
HBV A1 Germany (AY738139)   (51)  ATTTTCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAAACCCTGCTCCGAAT
              Consensus     (51)  ATTTTCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAACCCTGTTCCGAAT
                                        101                                              150
HBV A1 Uganda (AY934772)   (101)  ATTGCCTCTCACATCTCGTCAATCTCCTCGAGGACTGGGGACCCTGCGTC
HBV A1 Gambia (AY934764)   (101)  ATTGCCTCTCACATCTCGTCAATCTCCTCGAGGACTGGGGACCCTGCACC
HBV A1 Germany (AY738139)  (101)  ATTGCCTCTCACATCTCGTCAATCTCCGCGAGGACTGGGGACCCTGTGAC
              Consensus    (101)  ATTGCCTCTCACATCTCGTCAATCTCCTCGAGGACTGGGGACCCTGCG C
                                        151                                              200
HBV A1 Uganda (AY934772)   (151)  GAACATGGAGAACATCACATCAGGATTCCTAGGACCCCTGCTCGTGTTAC
HBV A1 Gambia (AY934764)   (151)  GACCATGGAGAACATCACATCAGGATTCCTAGGACCCCTGCTCGTGTTAC
HBV A1 Germany (AY738139)  (151)  GAACATGGAGAACATCACATTAGGATTCCTAGGACCCCTGCTCGTGTTAC
              Consensus    (151)  GAACATGGAGAACATCACATCAGGATTCCTAGGACCCCTGCTCGTGTTAC
                                        201                                              250
HBV A1 Uganda (AY934772)   (201)  AGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTA
HBV A1 Gambia (AY934764)   (201)  AGGCGGGGTTTTTCTTGTTGACAAAAATCCTCACAATACCGCAGAGTCTA
HBV A1 Germany (AY738139)  (201)  AGGCGGTGTTTTTCTTGTTGACAAGAATCCTCACAATACCACAGAGTCTA
              Consensus    (201)  AGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTA
                                        251                                              300
HBV A1 Uganda (AY934772)   (251)  GACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGCCCACCCGTGTGTCT
HBV A1 Gambia (AY934764)   (251)  GACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCT
HBV A1 Germany (AY738139)  (251)  GACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCT
              Consensus    (251)  GACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCT
                                        301                                              350
HBV A1 Uganda (AY934772)   (301)  TGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTC
HBV A1 Gambia (AY934764)   (301)  TGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTC
HBV A1 Germany (AY738139)  (301)  TGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTC
              Consensus    (301)  TGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTC
                                        351                                              400
HBV A1 Uganda (AY934772)   (351)  CTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATA
HBV A1 Gambia (AY934764)   (351)  CTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATA
HBV A1 Germany (AY738139)  (351)  CTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATA
              Consensus    (351)  CTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATA
                                        401                                              450
HBV A1 Uganda (AY934772)   (401)  TTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTATTGGTTCTTCTGGA
HBV A1 Gambia (AY934764)   (401)  TTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTATTGGTTCTTCTGGA
HBV A1 Germany (AY738139)  (401)  TTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTATTGGTTCTTCTGGA
              Consensus    (401)  TTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTATTGGTTCTTCTGGA
                                        451                                              500
HBV A1 Uganda (AY934772)   (451)  TTATCAAGGTATGTTGCCCGTTTGTCCTCTGATTCCAGGATCCCACAACAA
HBV A1 Gambia (AY934764)   (451)  TTGTCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCCACAACAA
HBV A1 Germany (AY738139)  (451)  TTATCAAGGTATGTTGGCCGTTTGTCCTCTAATTCCAGGATCAACAACAA
              Consensus    (451)  TTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAA
                                        501                                              550
HBV A1 Uganda (AY934772)   (501)  CCAGTACGGGACCCTGCAGAACCTGCACGACTCCTGCTCAAGGCAACTCT
HBV A1 Gambia (AY934764)   (501)  CCAGCACGGGACCCTGCAGAACCTGCACGACTCCTGCTCAAGGCAACTCT
HBV A1 Germany (AY738139)  (501)  CCAGTACGGGACCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCT
              Consensus    (501)  CCAGTACGGGACCCTGCAGAACCTGCACGACTCCTGCTCAAGGCAACTCT
                                        551                                              600
HBV A1 Uganda (AY934772)   (551)  ATGTTTCCCTCATGTTGCCTGTACAAAACCTACGGATGGAAATTGCACCTG
HBV A1 Gambia (AY934764)   (551)  ATGTTTCCCTCATGTTGTTTGTACAAAACCTACGGACGGAAATTGCACCTG
HBV A1 Germany (AY738139)  (551)  ATGTTTCCCTCATGTTGCCTGTACAAAACCTACGGATGGAAATTGCACCTG
              Consensus    (551)  ATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCACCTG
                                        601                                              650
HBV A1 Uganda (AY934772)   (601)  TATTCCCATCCCATCATCTTGGGCTTTCGTAAAATACCTATGGGAGTGGGG
HBV A1 Gambia (AY934764)   (601)  TATTCCCATCCCATCATCATGGGCTTTCGCAAAATACCTATGGGAGTGGGG
HBV A1 Germany (AY738139)  (601)  TATTCCCATCCCATCGTCCTGGGCTTTCGCAAAATACCTATGGGAGTTGG
              Consensus    (601)  TATTCCCATCCCATCATC TGGGCTTTCGCAAAATACCTATGGGAGTGGG
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                                            651                                              700
HBV A1 Uganda  (AY934772)  (651) CCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGG
HBV A1 Gambia  (AY934764)  (651) CCTCAGTCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGG
HBV A1 Germany (AY738139)  (651) CCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGG
           Consensus       (651) CCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGG
                                            701                                              750
HBV A1 Uganda  (AY934772)  (701) TTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTG
HBV A1 Gambia  (AY934764)  (701) TTCGTATGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTG
HBV A1 Germany (AY738139)  (701) TTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTATATGGATGATGTG
           Consensus       (701) TTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTG
                                            751                                              800
HBV A1 Uganda  (AY934772)  (751) GTTTTTGGGGGCCAAGTCTGTACAACATCTTGAGTCCCTTTATACCGCTGT
HBV A1 Gambia  (AY934764)  (751) GTATTGGGGGCCAAGTCTGTACAACATCTTGAGTCCCTTTATACCGCTGT
HBV A1 Germany (AY738139)  (751) GTATTGGGGGCCAAGTCTGTACAGCATCGTGAGTCCCTTTATACCGCTGT
           Consensus       (751) GTATTGGGGGCCAAGTCTGTACAACATCTTGAGTCCCTTTATACCGCTGT
                                            801                                              850
HBV A1 Uganda  (AY934772)  (801) TACCAATTTTCTTTTGTCTTTGGGTATACATTAAACCCTAACAAAACAA
HBV A1 Gambia  (AY934764)  (801) TACCAATTTTCTTTTGTCTTTGGGTATACATCTAAACCCTAACAAAACAA
HBV A1 Germany (AY738139)  (801) TACTAATTTTCTTTTGTCTCTGGGTATACATTTAAACCCTAACAAAACAA
           Consensus       (801) TACCAATTTTCTTTTGTCTTTGGGTATACATTTAAACCCTAACAAAACAA
                                            851                                              900
HBV A1 Uganda  (AY934772)  (851) AGAGATGGGGTTATTCCCTAAACTTCATGGGATATGTAATTGGAAGTTGG
HBV A1 Gambia  (AY934764)  (851) AAAGATGGGGTTATTCCCTAAACTTCATGGGTTACATAATTGGAAGTTGG
HBV A1 Germany (AY738139)  (851) GAAGATGGGGTTATTCCCTAAACTTCATGGGTTACATAATTGGAAGTTGG
           Consensus       (851) AAAGATGGGGTTATTCCCTAAACTTCATGGGTTACATAATTGGAAGTTGG
                                            901                                              950
HBV A1 Uganda  (AY934772)  (901) GGTACATTGCCACAGGATCATATTATACAAAAAATCAAACACTGTTTTAG
HBV A1 Gambia  (AY934764)  (901) GGATCATTGCCACAGGATCATATTGTACAAAAAATCAAAAATTGTTTTAG
HBV A1 Germany (AY738139)  (901) GGAACTTTGCCACAGGATCATATTGTGCAAAAGATCAAACACTGTTTTAG
           Consensus       (901) GGAACATTGCCACAGGATCATATTGTACAAAAAAATCAAACACTGTTTTAG
                                            951                                              1000
HBV A1 Uganda  (AY934772)  (951) AAAACTTCCTCTCAATCGACCTATTGATTGGAAAGTATGTCAACGAATTG
HBV A1 Gambia  (AY934764)  (951) AAAACTTCCTATTAATAGGCCTATTGATTGGAAAGTATGTCAAAGAATTG
HBV A1 Germany (AY738139)  (951) AAAACTTCCTGTTAACAGGCCTATTGATTGGAAAGTATGTCAAAGAATTG
           Consensus       (951) AAAACTTCCT TTAATAGGCCTATTGATTGGAAAGTATGTCAAAGAATTG
                                            1001                                             1050
HBV A1 Uganda  (AY934772)  (1001) TGGGTCTTTTTGGGCTTTGCCGCGCTCCATTTACACAATGTGGTTACCCGGCC
HBV A1 Gambia  (AY934764)  (1001) TGGGTCTGTTGGGCTTTGCAGCTCCCTTTACACAATGTGGATACCCTGCC
HBV A1 Germany (AY738139)  (1001) TGGGTCTTTTTGGGCTTTGCTGCTCCATTTACACAATGTGGATATCCTGCC
           Consensus       (1001) TGGGTCTTTTTGGGCTTTGC GCTCCATTTACACAATGTGGATACCCTGCC
                                            1051                                             1100
HBV A1 Uganda  (AY934772)  (1051) TTAATGCCCTTGTATGCATGTATACAGGCGAAACAGGCTTTTACTTTCTC
HBV A1 Gambia  (AY934764)  (1051) TTAATGCCTTTGTATGCGTGTATACAAGCTAAACAGGCTTTTACTTTCTC
HBV A1 Germany (AY738139)  (1051) TTAATGCCTTTGTATGCATGTATACAAGCTAAACAGGCTTTCACTTTCTC
           Consensus       (1051) TTAATGCCTTTGTATGCATGTATACAAGCTAAACAGGCTTTTACTTTCTC
                                            1101                                             1150
HBV A1 Uganda  (AY934772)  (1101) GCCAACTTACAAGGCCTTTCTAAGTAAACAGTATATGAACCTTTACCCCG
HBV A1 Gambia  (AY934764)  (1101) GCCAACTTACAAGGCCTTTCTAAGTAAACAGTATGTGAACCTTTACCCCG
HBV A1 Germany (AY738139)  (1101) GCCAACTTACAAGGCCTTTCTAAGTAAACAGTACATGAACCTTTACCCCG
           Consensus       (1101) GCCAACTTACAAGGCCTTTCTAAGTAAACAGTATATGAACCTTTACCCCG
                                            1151                                             1200
HBV A1 Uganda  (AY934772)  (1151) TTGCGCGGCAACGGCCTGGTCTATGCCAAGTGTTTGCTGACGCAACCCCC
HBV A1 Gambia  (AY934764)  (1151) TTGCCCGGCAACGGCCTGGTCTGTGCCAAGTGTTTGCTGACGCAACCCCC
HBV A1 Germany (AY738139)  (1151) TTGCTCGGCAACGGCCTGGTCTGTGCCAAGTGTTTGCTGACGCAACCCCC
           Consensus       (1151) TTGC CGGCAACGGCCTGGTCTGTGCCAAGTGTTTGCTGACGCAACCCCC
                                            1201                                             1250
HBV A1 Uganda  (AY934772)  (1201) ACTGGCTGGGGCTTGGCTATTGGCCATCAGCGCATGCGTGGAACCTTTGT
HBV A1 Gambia  (AY934764)  (1201) ACTGGGTGGGGCTTGGCCATTGGCCATCAGCGCATGCGCGGAACCTTTGT
HBV A1 Germany (AY738139)  (1201) ACTGGCTGGGGCTTGGCCATAGGCCATCAGCGCATGCGTGGAACCTTTGT
           Consensus       (1201) ACTGGCTGGGGCTTGGCCATTGGCCATCAGCGCATGCGTGGAACCTTTGT
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                                          1251                                          1300
HBV A1 Uganda  (AY934772)  (1251) GGCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCYGCCTGTTTTGCTC
HBV A1 Gambia  (AY934764)  (1251) GGCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCAGCTTGTTTTGCTC
HBV A1 Germany (AY738139)  (1251) GGCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCCGCTTGTTTTGCTC
             Consensus     (1251) GGCTCCTCTGCCGATCCATACTGCGGAACTCCTAGC GCTTGTTTTGCTC
                                          1301                                          1350
HBV A1 Uganda  (AY934772)  (1301) GCAGCCGGTCTGGAGCAAAMCTCATCGGGACTGATAATTCTGTCGTCCTC
HBV A1 Gambia  (AY934764)  (1301) GCAGCCGGTCTGGAGCAAAACTCATTGGGACTGATAATTCTGTCGTCCTC
HBV A1 Germany (AY738139)  (1301) GCAGCCGGTCTGGAGCAAAGCTCATCGGAACTGACAATTCTGTCGTCCTC
             Consensus     (1301) GCAGCCGGTCTGGAGCAAA CTCATCGGGACTGATAATTCTGTCGTCCTC
                                          1351                                          1400
HBV A1 Uganda  (AY934772)  (1351) TCGCGGAAATATACATCGTTTCCATGGCTGCTAGGGTGTACTGCCAACTG
HBV A1 Gambia  (AY934764)  (1351) TCTCGGAAATATACCTCCTTTCCATGGCTGCTAGGCTGTACTGCCAACTG
HBV A1 Germany (AY738139)  (1351) TCGCGGAAATATACATCGTTTCCATGGCTGCTAGGCTGTGCTGCCAACTG
             Consensus     (1351) TCGCGGAAATATACATCGTTTCCATGGCTGCTAGGCTGTACTGCCAACTG
                                          1401                                          1450
HBV A1 Uganda  (AY934772)  (1401) GATTCTTCGCGGGACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCCG
HBV A1 Gambia  (AY934764)  (1401) GATTCTTCGCGGGACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCCG
HBV A1 Germany (AY738139)  (1401) GATCCTTCGCGGGACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCCG
             Consensus     (1401) GATTCTTCGCGGGACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCCG
                                          1451                                          1500
HBV A1 Uganda  (AY934772)  (1451) CGGACGACCCCTCACGGGGCCGCTTGGGTCTCTATCGTCCCCTTCTCCGT
HBV A1 Gambia  (AY934764)  (1451) CGGACGACCCCTCTCGGGGCCGCTTGGGACTCTATCGTCCCCTTCTCCGT
HBV A1 Germany (AY738139)  (1451) CGGACGACCCCTCTCGGGGCCGCTTGGGACTCTCTCGTCCCCTTCTCCGT
             Consensus     (1451) CGGACGACCCCTCTCGGGGCCGCTTGGGACTCTATCGTCCCCTTCTCCGT
                                          1501                                          1550
HBV A1 Uganda  (AY934772)  (1501) CTGCCGTACCGTCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC
HBV A1 Gambia  (AY934764)  (1501) CTGCCGTACCGACCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC
HBV A1 Germany (AY738139)  (1501) CTGCCGTTCCAGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC
             Consensus     (1501) CTGCCGTACCG CCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC
                                          1551                                          1600
HBV A1 Uganda  (AY934772)  (1551) GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGC
HBV A1 Gambia  (AY934764)  (1551) GTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC
HBV A1 Germany (AY738139)  (1551) GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGC
             Consensus     (1551) GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGC
                                          1601                                          1650
HBV A1 Uganda  (AY934772)  (1601) ACGTTGCATGGAGACCACCGTGAACGCCCATCAGATCCTGCCCAAGGTCT
HBV A1 Gambia  (AY934764)  (1601) ACGTTGCATGGAGACCACCGTGAACGCCCATCAGATCCTGCCCAAGGTCT
HBV A1 Germany (AY738139)  (1601) ACGTTGCGTGGAGACCACCGTGAACGCCCATCAGATCCTGCCCAAGGTCT
             Consensus     (1601) ACGTTGCATGGAGACCACCGTGAACGCCCATCAGATCCTGCCCAAGGTCT
                                          1651                                          1700
HBV A1 Uganda  (AY934772)  (1651) TACATAAGAGGACTCTTGGACTCCCAGCAATGTCAACGACCGACCTTGAG
HBV A1 Gambia  (AY934764)  (1651) TACATAAGCGGACTCTTGGACTCCCAGCAATGTCAACGACCGACCTTGAG
HBV A1 Germany (AY738139)  (1651) TACATAAGAGGACTCTTGGACTCCCAGCAATGTCAACGACCGACCTTGAG
             Consensus     (1651) TACATAAGAGGACTCTTGGACTCCCAGCAATGTCAACGACCGACCTTGAG
                                          1701                                          1750
HBV A1 Uganda  (AY934772)  (1701) GCCTACTTCAAAGACTGTGTGTATAAAGACTGGGAGGTGTTGGGGGAGGA
HBV A1 Gambia  (AY934764)  (1701) GCCTACTTCAAAGACTGTGTGTTTAAAGACTGGGAGGAGTTGGGGGAGGA
HBV A1 Germany (AY738139)  (1701) GCCTACTTCAAAGACTGTGTGTTTAAGGACTGGGAGGAGCTGGGGGAGGA
             Consensus     (1701) GCCTACTTCAAAGACTGTGTGTTTAAAGACTGGGAGGAGTTGGGGGAGGA
                                          1751                                          1800
HBV A1 Uganda  (AY934772)  (1751) GATTAGGTTAATGATCTGTGTATTAGGAGGCTGTAGGCATAAATTGGTCT
HBV A1 Gambia  (AY934764)  (1751) GATTAGGTTAAAGGTCTTTGTATTAGGAGGCTGTAGGCATAAATTGGTCT
HBV A1 Germany (AY738139)  (1751) GATTAGGTTAAAGGTCTTTGTATTAGGAGGCTGTAGGCATAAATTGGTCT
             Consensus     (1751) GATTAGGTTAAAGGTCTTTGTATTAGGAGGCTGTAGGCATAAATTGGTCT
                                          1801                                          1850
HBV A1 Uganda  (AY934772)  (1801) GCGCACCAGCACCATGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTA
HBV A1 Gambia  (AY934764)  (1801) GCGCACCATCATCATGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTA
HBV A1 Germany (AY738139)  (1801) GCGCACCAGCACCAYGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTA
             Consensus     (1801) GCGCACCAGCACCATGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTA
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                                        1851                                    1900
HBV A1 Uganda (AY934772)  (1851) CATGTCCCACTGTTCAAGCCTCCAAGCTGTGCCTTGGATGGCTTTGGGGC
HBV A1 Gambia (AY934764)  (1851) CATGTCCCACTCTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
HBV A1 Germany (AY738139) (1851) CATGTCCCACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
             Consensus    (1851) CATGTCCCACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
                                        1901                                    1950
HBV A1 Uganda (AY934772)  (1901) ATGGACATTGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTC
HBV A1 Gambia (AY934764)  (1901) ATGGACATTGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTC
HBV A1 Germany (AY738139) (1901) ATGGACATTGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTC
             Consensus    (1901) ATGGACATTGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTC
                                        1951                                    2000
HBV A1 Uganda (AY934772)  (1951) TTTTTTGCCTTCTGACTTCTTTCCTTCAGTCCGGGATCTACTTGATACAG
HBV A1 Gambia (AY934764)  (1951) GTTTTTGCCTTCTGACTTCTTTCCTTCCGTCCGAGATCTCCTAGATACCG
HBV A1 Germany (AY738139) (1951) GTTTTTGCCTTCTGACTTCTTTCCTTCCGTCAGAGATCTCCTAGACACCG
             Consensus    (1951) GTTTTTGCCTTCTGACTTCTTTCCTTCCGTCCGAGATCTCCTAGATACCG
                                        2001                                    2050
HBV A1 Uganda (AY934772)  (2001) CCTCAGCTCTGTATCGGGAAGCCTTAGAGTCTCCTGAGCATTGCTCACCT
HBV A1 Gambia (AY934764)  (2001) CCTCAGCTCTGTACCGGGAAGCATTAGAGTCTCCTGAGCATTGCTCACCT
HBV A1 Germany (AY738139) (2001) CCTCAGCTCTGTATCGAGAAGCCTTAGAGTCTCCTGAGCATTGCTCACCT
             Consensus    (2001) CCTCAGCTCTGTATCGGGAAGCCTTAGAGTCTCCTGAGCATTGCTCACCT
                                        2051                                    2100
HBV A1 Uganda (AY934772)  (2051) CACCATACAGCACTCAGGCAAGCCATTCTCTGCTGGGTGGAATTAATGAC
HBV A1 Gambia (AY934764)  (2051) CACCACACAGCACTAAGGCAAGCCATTCTTTGTTGGGGGGAATTAATGAC
HBV A1 Germany (AY738139) (2051) CACCATACTGCACTCAGGCAAGCCATTCTCTGCTGGGGGGAATTGATGAC
             Consensus    (2051) CACCATACAGCACTCAGGCAAGCCATTCTCTGCTGGGGGGAATTAATGAC
                                        2101                                    2150
HBV A1 Uganda (AY934772)  (2101) TCTAGCTACCTGGGTGGGTAATAATTTGGAAGATCCAGCATCCAGGGATC
HBV A1 Gambia (AY934764)  (2101) TCTAGCTACCTGGGTGGGCAATAATTTGGAAGATCCAGCATCCAGGGATC
HBV A1 Germany (AY738139) (2101) TCTAGCTACCTGGGTGGGTAATAATTTGCAAGATCCAGCATCCAGGGATC
             Consensus    (2101) TCTAGCTACCTGGGTGGGTAATAATTTGGAAGATCCAGCATCCAGGGATC
                                        2151                                    2200
HBV A1 Uganda (AY934772)  (2151) TAGTAGTCAATTATGTTAATACTAACATGGGCCTAAAGATCAGACAACTA
HBV A1 Gambia (AY934764)  (2151) TAGTAGTCAATTATGTTAATACTAACATGGGCCTAAAGATCAGGCAACTA
HBV A1 Germany (AY738139) (2151) TAGTAGTCAATTATGTTAATACTAACATGGGTTTAAAAATCAGGCAACTA
             Consensus    (2151) TAGTAGTCAATTATGTTAATACTAACATGGGCCTAAAGATCAGGCAACTA
                                        2201                                    2250
HBV A1 Uganda (AY934772)  (2201) TTGTGGTTTCATATTTCTTGCCTTACTTTTGGAAGAGAAACTGTCCTTGA
HBV A1 Gambia (AY934764)  (2201) TTGTGGTTTCATATTTCCTGCCTTACTTTTGGAAGAGAAACTGTACTTGA
HBV A1 Germany (AY738139) (2201) TTGTGGTTTCATATATCTTGCCTTACTTTTGGAAGAGAGACGGTACTTGA
             Consensus    (2201) TTGTGGTTTCATATTTCTTGCCTTACTTTTGGAAGAGAAACTGTACTTGA
                                        2251                                    2300
HBV A1 Uganda (AY934772)  (2251) GTATTTGGTCTCCTTCGGAGTGTGGATTCGCACTCCTCCAGCCTATAGAC
HBV A1 Gambia (AY934764)  (2251) GTATTTGGTCTCTTTCGGAGTGTGGATTCGCACTCCTCCAGCTTATCGAC
HBV A1 Germany (AY738139) (2251) ATATTTGGTCTCTTTCGGAGTGTGGATTCGCACTCCTCCAGCCTATAGAC
             Consensus    (2251) GTATTTGGTCTCTTTCGGAGTGTGGATTCGCACTCCTCCAGCCTATAGAC
                                        2301                                    2350
HBV A1 Uganda (AY934772)  (2301) CACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGA
HBV A1 Gambia (AY934764)  (2301) CACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGA
HBV A1 Germany (AY738139) (2301) CACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGA
             Consensus    (2301) CACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGA
                                        2351                                    2400
HBV A1 Uganda (AY934772)  (2351) CGACGAGACCGAGGTAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAG
HBV A1 Gambia (AY934764)  (2351) CGACGGGACCGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAG
HBV A1 Germany (AY738139) (2351) CGACGGGACCGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAG
             Consensus    (2351) CGACGGGACCGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAG
                                        2401                                    2450
HBV A1 Uganda (AY934772)  (2401) ACGAAGATCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTC
HBV A1 Gambia (AY934764)  (2401) ACGAAGATCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTC
HBV A1 Germany (AY738139) (2401) ACGCAGATCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTC
             Consensus    (2401) ACGAAGATCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTC
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                                        2451                                              2500
HBV A1 Uganda (AY934772)  (2451)  AATGTTAGTATTCCTTGGACTCATAAGGTGGGAAATTTTACTGGGCTTTA
HBV A1 Gambia (AY934764)  (2451)  AATGTTAGTATTCCTTGGACACATAAGGTGGGAAATTTTACTGGGCTTTA
HBV A1 Germany (AY738139) (2451)  AATGTTAGTATTCCTTGGACTCATAAGGTGGGAAACTTTACGGGGCTTTA
               Consensus  (2451)  AATGTTAGTATTCCTTGGACTCATAAGGTGGGAAATTTTACTGGGCTTTA
                                        2501                                              2550
HBV A1 Uganda (AY934772)  (2501)  TTCCTCTACTGTCCCTATCTTTAATCCTGAATGGCAAACTCCTTCCTTTC
HBV A1 Gambia (AY934764)  (2501)  TTCCTCTACTGTACCCATCTTTAATCCTGAATGGCAAACTCCTACTTTTC
HBV A1 Germany (AY738139) (2501)  TTCCTCTACAGTACCTATCTTCAATCCTGAATGGCAAACTCCTTCCTTCC
               Consensus  (2501)  TTCCTCTACTGTACCTATCTTTAATCCTGAATGGCAAACTCCTTCCTTTC
                                        2551                                              2600
HBV A1 Uganda (AY934772)  (2551)  CTAAAATCCATTTACATGAGGACATTATTAATAGGTGTCAGCAATTTGTG
HBV A1 Gambia (AY934764)  (2551)  CCAAGATTCATTTACATGAGGACATTGTTAATAGGTGTCAACAATTTGTG
HBV A1 Germany (AY738139) (2551)  CTAAGATTCATTTACAAGAGGACATTATTAATAGGTGTCAACAATTTGTG
               Consensus  (2551)  CTAAGATTCATTTACATGAGGACATTATTAATAGGTGTCAACAATTTGTG
                                        2601                                              2650
HBV A1 Uganda (AY934772)  (2601)  GGTCCTCTCACTGTAAATGAAAAGAGAAGATTGAAATTAATTATGCCTGC
HBV A1 Gambia (AY934764)  (2601)  GGTCCTCTCACAGTAAATGAAAAAAGAAGATTAAAATTGATTATGCCTGC
HBV A1 Germany (AY738139) (2601)  GGCTCTCTCACTGTAAATGAAAAAAGAAGATTGAAATTAATTATGCCTGC
               Consensus  (2601)  GGTCCTCTCACTGTAAATGAAAAAAGAAGATTGAAATTAATTATGCCTGC
                                        2651                                              2700
HBV A1 Uganda (AY934772)  (2651)  TAGATTTTTTCCTAACAGCACCAAATATTTGCCTCTAGACAAAGGGATTA
HBV A1 Gambia (AY934764)  (2651)  TAGATTCTATCCTAACAGTACGAAATATTTGCCCCTAGACAAAGGAATTA
HBV A1 Germany (AY738139) (2651)  TAGATTCTATCCTACTCACACTAAATATTTGCCCTTAGACAAAGGAATTA
               Consensus  (2651)  TAGATTCTATCCTAACAGCAC AAATATTTGCCCCTAGACAAAGGAATTA
                                        2701                                              2750
HBV A1 Uganda (AY934772)  (2701)  AACCTTATTATCCTGATCAAGTAGTTAATCATTACTTCCAGACCCGACAT
HBV A1 Gambia (AY934764)  (2701)  AACCTTATTATCCTGATCATGTAGTTAATCATTACTTCCAGACCCGACAT
HBV A1 Germany (AY738139) (2701)  AACCTTATTATCCAGATCAGGTAGTTAATCATTACTTCCAAACCAGACAT
               Consensus  (2701)  AACCTTATTATCCTGATCA GTAGTTAATCATTACTTCCAGACCCGACAT
                                        2751                                              2800
HBV A1 Uganda (AY934772)  (2751)  TATTTACATACTCTTTGGAAGGCTGGGATTCTATATAAGAGGGAAACTAC
HBV A1 Gambia (AY934764)  (2751)  TATTTACATACTCTTTGGAAGGCTGGTATTCTATATAAGAGGGAAACTAC
HBV A1 Germany (AY738139) (2751)  TATTTACATACTCTTTGGAAGGCTGGTATTCTATATAAGAGGGAAACCAC
               Consensus  (2751)  TATTTACATACTCTTTGGAAGGCTGGTATTCTATATAAGAGGGAAACTAC
                                        2801                                              2850
HBV A1 Uganda (AY934772)  (2801)  ACGTAGCGCCTCATTTTGCGGGTCACCATACTCTTGGGAACAAGAGCTAC
HBV A1 Gambia (AY934764)  (2801)  ACGTAGCGCCTCATTTTGCGGGTCACCATATTCTTGGGAACAAGAGCTAC
HBV A1 Germany (AY738139) (2801)  ACGTAGCGCATCATTTTGCGGGTCACCATATTCTTGGGAACAAGAGCTAC
               Consensus  (2801)  ACGTAGCGCCTCATTTTGCGGGTCACCATATTCTTGGGAACAAGAGCTAC
                                        2851                                              2900
HBV A1 Uganda (AY934772)  (2851)  ATCATGGGAGGTTGGTTACCCAAAACCTCGCAAAGGCATGGGGACAAATCT
HBV A1 Gambia (AY934764)  (2851)  ATCATGGGAGGTTGGTTACCAAAACCTCGCAAAGGCATGGGGACGAATCT
HBV A1 Germany (AY738139) (2851)  AGCATGGGAGGTTGGTCATCAAAACCTCGCAAAGGCATGGGGACGAATCT
               Consensus  (2851)  ATCATGGGAGGTTGGTTACCAAAACCTCGCAAAGGCATGGGGACGAATCT
                                        2901                                              2950
HBV A1 Uganda (AY934772)  (2901)  TTCTGTTCCCAATCCTCTGGGATTCTTTCCCGATCATCAGTTGGACCCTG
HBV A1 Gambia (AY934764)  (2901)  ATCTGTTCCCAACCCTCTGGGATTCTTTCCCGATCATCAGTTGGACCCTG
HBV A1 Germany (AY738139) (2901)  TTCTGTTCCCAACCCTCTGGGATTCTTTCCCGATCATCAGTTGGACCCTG
               Consensus  (2901)  TTCTGTTCCCAACCCTCTGGGATTCTTTCCCGATCATCAGTTGGACCCTG
                                        2951                                              3000
HBV A1 Uganda (AY934772)  (2951)  CATTCGGAGCCAATTTCAAACAATCCAGACTGGGACTTCAACCCCATCAAG
HBV A1 Gambia (AY934764)  (2951)  CATTCGGAGCCAACACAAACAATCCAGATTGGGACTTCAACCCCATCAAG
HBV A1 Germany (AY738139) (2951)  CATTCGGAGCCAACTCAAACAATCCAGATTGGGACTTCAACCCCATCAAG
               Consensus  (2951)  CATTCGGAGCCAACTCAAACAATCCAGATTGGGACTTCAACCCCATCAAG
                                        3001                                              3050
HBV A1 Uganda (AY934772)  (3001)  GACCACTGGCCACAAGCCAACCAGGTAGGAGTGGGAGCATTCGGCCCAGG
HBV A1 Gambia (AY934764)  (3001)  GACCACTGGCCACAAGCCAATCAGGTAGGAGTGGGAGCATTTGGGCCAGG
HBV A1 Germany (AY738139) (3001)  GACCACTGGCCAGCAGCCAACCAGGTAGGAGTGGGAGCATTCGGGCCAGG
               Consensus  (3001)  GACCACTGGCCACAAGCCAACCAGGTAGGAGTGGGAGCATTCGGGCCAGG
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                                           3051                                                3100
 HBV A1 Uganda (AY934772)  (3051) GTTCACTCCCCCACACGGAGGTGTTTTGGGGTGGAGCCCTCAGGCTCAGG
 HBV A1 Gambia (AY934764)  (3051) GTTCACCCCTCCTCACGGAGGTGTTCTGGGGTGGAGCCCTCAGGCTCAGG
HBV A1 Germany (AY738139)  (3051) GCTCACCCCTCCACACGGGGGTATTTTGGGGTGGAGCCCTCAGGCTCAGG
             Consensus     (3051) GTTCACCCCTCCACACGGAGGTGTTTTGGGGTGGAGCCCTCAGGCTCAGG
                                           3101                                                3150
 HBV A1 Uganda (AY934772)  (3101) GCATATTGGCTACAGTGCCAGCAGTGCCTCCTCCTGCCTCCACCAATCGG
 HBV A1 Gambia (AY934764)  (3101) GCATATCGACTACTGTGCCAGCAGTTCCTCCTCCTGCCTGCACCAATCGG
HBV A1 Germany (AY738139)  (3101) GCATATTGACCACAGTGTCAACAATTCCTCCTCCTGCCTCCACCAATCGG
             Consensus     (3101) GCATATTGACTACAGTGCCAGCAGTTCCTCCTCCTGCCTCCACCAATCGG
                                           3151                                                3200
 HBV A1 Uganda (AY934772)  (3151) CAGTCAGGAAGGCAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCA
 HBV A1 Gambia (AY934764)  (3151) CAGTCAGGAAGGCAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCA
HBV A1 Germany (AY738139)  (3151) CAGTCAGGAAGGCAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCA
             Consensus     (3151) CAGTCAGGAAGGCAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCA
                                           3201          3221
 HBV A1 Uganda (AY934772)  (3201) TCCTCAGGCCATGCAGTGGAA
 HBV A1 Gambia (AY934764)  (3201) TCCTCAGGCCATGCAGTGGAA
HBV A1 Germany (AY738139)  (3201) TCCTCAGGCCATGCAGTGGAA
             Consensus     (3201) TCCTCAGGCCATGCAGTGGAA
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