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Real-time PCR quantitation of hepatitis B virus DNA using automated sample preparation and murine cytomegalovirus internal control

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Summary

Quantitation of circulating hepatitis B virus (HBV) DNA is important for monitoring disease progression and for assessing the response to antiviral therapy. Several commercial and 'in house' assays for HBV DNA quantitation have been described but many of these have limitations of relatively low sensitivity and limited dynamic range. This study describes the development and evaluation of a FRET-based real-time PCR assay designed to overcome these limitations and to provide accurate quantitation of DNA from all eight genotypes of HBV (A – H). The assay employs a fully automated nucleic acid extraction system permitting high sample throughput with minimal 'hands-on' time and incorporates a murine cytomegalovirus (mCMV) internal control to prevent false negative results and under-reporting due to unrecognised problems with viral lysis, DNA purification or PCR amplification. Sensitivity, assessed by Probit analysis at the 95% detection level, was 24.4 IU/ml, associated with an extremely wide dynamic range (~ 9 $\log_{10}$). Coefficients of variation were low for both intra-assay and inter-assay variability (CV%, 7% – 11%) and quantitative data correlated well ($R^2 = 0.97$) with the Digene hybrid capture assay. This assay provides an ideal system for therapeutic monitoring and for studying the relationship between HBV viral load and stage of disease.

Key words: hepatitis B virus, HBV DNA, chronic hepatitis, real-time PCR, automation, therapeutic monitoring

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Introduction

Hepatitis B virus (HBV) has infected two billion people across the globe and given rise to at least 350 million persistently infected carriers. Carriers are at increased risk of developing cirrhosis and hepatocellular carcinoma, and as a consequence HBV is now estimated to be the tenth leading cause of death worldwide (Lavanchy, 2004). In recent years, measurement of the circulating HBV DNA level has come to be regarded as the most direct and reliable means of monitoring HBV infection and has been widely used in pre-treatment evaluation and clinical staging (Mommeja-Marin et al., 2003; Chu et al., 2002). Quantitative HBV DNA assays have also been employed for assessing infectivity, for monitoring antiviral therapy and for detecting the emergence of drug resistance (Berger et al., 2001; Corden et al., 2003).

Numerous commercial and 'in-house' molecular assays for HBV DNA using a variety of hybridisation technologies including dot blot, hybrid capture, branched DNA signal amplification and polymerase chain reaction (PCR) have been described over the past decade (Zaaijer et al., 1994; Hendricks et al., 1995; Poljak et al., 2001) but many of these have relatively low sensitivity and limited dynamic range. The recent introduction of fluorescence resonance energy transfer (FRET)-based real-time PCR (Ho et al., 2003; Weiss et al., 2004; Stelzl et al., 2004; Sum et al., 2004) has been particularly advantageous for HBV DNA quantitation because it provides high sensitivity with a much broader dynamic range (typically $\geq 7 \log_{10}$) than alternative assay types. In addition, real-time PCR assays offer greater accuracy, are more rapid and pose less risk of amplicon contamination than other PCR formats because they permit simultaneous amplification and detection in a closed vessel system.

We describe here a novel real-time quantitative assay for HBV DNA which utilises high throughput robotic sample preparation and a murine cytomegalovirus (mCMV) internal control. The assay has been designed to enable accurate quantitation of all eight genotypes of HBV (A–H) and to monitor the efficiency of viral lysis, DNA extraction, PCR amplification and signal detection for each sample.

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Materials and Methods

Clinical samples and controls

Residual EDTA plasma and serum from clinical samples referred for diagnostic HBV marker analysis were stored at -20°C. These included samples from 32 patients with chronic HBV infection who had previously had their serum HBV DNA levels quantified by Digene Hybrid Capture® II assay and samples from 38 patients with HBeAg positive chronic HBV infection who were being treated with Lamivudine. A consecutive series of 256 HBV DNA positive plasma samples from patients of known e antigen status was also analysed (181 HBeAg positive and 75 anti-HBe positive). Controls included HCV-RNA positive plasma samples from 32 patients with chronic hepatitis C infection and 48 samples from healthy blood donors. Samples from blood donors were provided by the National Blood Service and the HBV DNA proficiency panel was obtained from Quality Control for Molecular Diagnostics (www.qcmd.org). Genotyped HBV samples (genotypes confirmed by dideoxy sequencing, Norder et al., 1993) were kindly donated by S. Ijaz (Health Protection Agency, London, UK). For assay calibration, the WHO HBV International Standard 97/746 (one million IU/ml) was supplied by the UK National Institute for Biological Standards and Control (Saldanha et al., 2001). For use as an internal control, the Smith strain of murine cytomegalovirus (American Type Cell Collection, ATCC no. VR-1399) was cultured in the murine fibroblast cell line C1217 (Selgrade et al., 1981). Culture supernatant was harvested, clarified and stored in aliquots at -70°C. The titre of the clarified mCMV supernatant was estimated by the limit dilution/Poisson distribution method (Simmonds et al., 1990) using a nested PCR primer set described previously (Tedder et al., 2002a).

Automated nucleic acid extraction

Viral DNA for quantitation by real-time PCR was extracted automatically on a Qiagen BioRobot 9604 utilising QIAamp96 Virus Kit reagents (Qiagen, Hilden, Germany) according to the manufacturer's

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protocol. Ten μl of mCMV supernatant (at 500 copies/μl) were added to each ml of lysis buffer (AL buffer), equivalent to approximately 1000 copies of mCMV per 200 μl plasma/serum sample or ~ 250 copies of mCMV DNA per PCR reaction. Briefly, 200 μl of plasma/serum were added to 200 μl of the mCMV-containing viral lysis buffer and incubated at 56°C for 10 min after which 230 μl of 100% ethanol were added. The mixture was passed through a 96-well QIAamp vacuum plate and washed twice. DNA was eluted from the matrix in 86 μl of nuclease-free water.

Real-time PCR primers and TaqMan probes

One hundred and twenty six sequences representing all eight HBV genotypes (A-H) were downloaded from the GenBank nucleotide database and aligned using the program ClustalX v1.81 (Thompson et al., 1997). A highly conserved region of the S gene was selected for the design of real-time PCR primers and a fluorogenic 5' nuclease assay probe (TaqMan probe). A single base mismatch in genotype B was accommodated by inclusion of a mixed nucleotide (G/T) in the probe sequence. Sequences of the HBV primers and dual labelled TaqMan probe are as follows: HBVTAQ1 (sense, 5'-GTG TCT GCG GCG TTT TAT CA); HBVTAQ2 (antisense, 5'-GAC AAA CGG GCA ACA TAC CTT); HBVTAQPR (sense, 5' *FAM*-CCT CT(T/G) CAT CCT GCT GCT ATG CCT CAT C-*TAMRA*).

Murine cytomegalovirus primers and TaqMan probe were based on GenBank accession number U68299: mCMVTAQ1 (sense, 5'-AAC CCG GCA AGA TTT CTA ACG, nucleotides 95916-95936); mCMVTAQ2 (antisense, 5'-ATT CTG TGG GTC TGC GAC TCA, nucleotides 96004-95984); mCMVTAQPR (sense, 5' *VIC*-CTA GTC ATC GAC GGT GCA CAT CGG C-*TAMRA*, nucleotides 95952-95976). Primers and probes were synthesised by MWG Biotech (UK) Ltd. BLAST searches (Altschul et al., 1990) predicted that the HBV and mCMV primer/probe sets would be specific for their respective targets and would not be expected to cross react with any other microbial or human sequences.

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Real-time PCR amplification and detection

Viral DNA extracted from plasma/serum was amplified and quantified in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The reaction was prepared using 25 µl TaqMan® Universal PCR Master Mix (containing AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTPs with dUTP, passive reference dye and optimized buffer components; Applied Biosystems, product number 4304437) with 0.4 µM primers, 0.2 µM probes and 20 µl extracted DNA (equivalent to approximately 50 µl of plasma/serum) in a 50 µl total reaction volume. An initial 2 min incubation at 50°C to activate UNG (Uracil-N-glycosylase) and destroy potential carry-over amplicons was followed by 10 min at 95°C to activate the thermostable DNA polymerase. Subsequently, 45 cycles of 95°C for 15 secs and 60°C for 1 min were performed in 9600 emulation mode.

Digene hybrid capture assay

The Digene Hybrid Capture® II HBV DNA Test was used in accordance with the manufacturer's instructions (Digene Corp. Gaithersburg, MD, USA). The standard test format was employed giving a dynamic range of 1.4×10^5 to 1.7×10^9 HBV copies/ml. Briefly, denatured HBV DNA in the test sample was hybridised with an HBV-specific RNA probe mix and the resulting RNA:DNA hybrids captured onto wells of a microtitre plate coated with hybrid-specific antibodies. The captured hybrids were detected and quantified by means of an alkaline phosphatase labelled anti-hybrid antibody in conjunction with a chemiluminescent substrate. Calibration of the Digene assay is based on a series of manufacturer's HBV DNA plasmid standards quoted as 1.42×10^5, 2.83×10^7, 5.66×10^8 and 1.7×10^9 copies/ml.

HBsAg and HBeAg assays

Hepatitis B surface antigen (HBsAg) was detected by enzyme immunoassay (Abbott Murex HBsAg Version 3, reference GE34/36) used according to the manufacturer's instructions. HBsAg was quantitated using a reverse passive haemagglutination test (Serodia® HBs antigen test kit, Fujirebio Inc.,

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Japan). HBV e-antigen (HBeAg) was measured with an 'in house' assay (Ferns and Tedder, 1985) using commercially available reagents (Murex Biotech Ltd., UK, HBeAg/anti-HBe GE19 reagents).

Results

Calibration of 'in house' standard against the WHO HBV International Standard 97/746

Two hundred and fifty ml of an HBeAg positive serum from an HBV-infected blood donor were aliquoted and frozen at -70°C for use as an 'in house' standard. The titre of this material was found to be 2 x 10^8 IU/ml when calibrated against the WHO HBV International Standard 97/746. The amplification efficiency of the 'in house' standard was the same as that of the WHO standard as judged by the identical slopes of their superimposed calibration curves (Figure 1). A typical amplification plot generated by the ABI Prism 7000 software from calibration standards used to construct the standard curve is shown in Figure 2. HBV amplification efficiency was not affected by the presence of ~ 250 copies of the internal control mCMV DNA in each reaction (data not presented). NB. All of the data presented in this paper were derived from assays in which the mCMV internal control was utilised.

Lower limit of detection and dynamic range

The lower limit of detection of the assay was assessed by testing multiple replicates of dilutions of the WHO HBV International Standard 97/746. Dilutions equal to 100, 50, 25, 10, and 5 IU/ml were tested in batches of eight replicates on three separate assay runs, giving a total of 24 replicates at each dilution (Table 1). Using Probit analysis (Arcus QuickStat Biomedical v1.0 software), the number of positive results at each dilution was used to calculate the HBV DNA concentration giving a 95% probability of detection. The 95% detection limit was determined to be 24.4 IU/ml. Samples of HBV genotypes A-G exhibited equivalent amplification/detection efficiency, as predicted by the GenBank database analysis used for primer and probe design. HBV genotype H was not available for testing but would be expected on sequence grounds to be amplified and detected with the same efficiency as the other seven genotypes.

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The dynamic range of the assay was found to be approximately 9 $\log_{10}$ (10 – 10^{10} IU/ml). Diluting samples of known titre in either serum or plasma produced identical results (data not presented).

Specificity

Forty eight normal human sera were tested in the assay and all gave negative results. Thirty one of 32 plasma samples from patients with chronic hepatitis C infection also gave negative results and the remaining sample was subsequently shown to be doubly infected with HBV and HCV. Samples of normal human genomic DNA and DNA extracted from HIV infected human lymphocytes were also shown to be non reactive in the assay as was human cytomegalovirus DNA. In a series of one hundred assays carried out over a two year period no evidence of cross contamination was observed between adjacent wells containing the top calibration HBV standard (10^8 IU/ml) and normal human serum.

Reproducibility

Inter-assay coefficient of variation (CV% based on HBV DNA concentration in IU/ml), calculated from seven consecutive assays run over a two month period, was 11.1% for a low titre sample (200 IU/ml) and 8.5% for a high titre sample (10^7 IU/ml). The intra-assay coefficient of variation was 7.0% on seven repeats of 10^5 IU/ml and 7.1% on seven repeats of 10^3 IU/ml. The threshold cycle for the mCMV internal control signal was typically between 31 and 33 cycles for all samples. Rarely (less than 0.3% of specimens) test samples exhibited a delayed mCMV threshold cycle indicating either inhibition of the PCR reaction or inefficiency of nucleic acid extraction, for example due to a blocked well in the QIAamp vacuum plate. In such cases repeat quantitation of the sample at a 1:10 dilution was generally successful.

Correlation with Digene hybrid capture assay

Thirty two samples from patients with chronic HBV infection which had had HBV DNA concentrations quantified by Digene hybrid capture assay were reanalysed using the real-time PCR assay. Correlation between the two assays was excellent (R^2 = 0.97) over the shared 4 $\log_{10}$ dynamic range (Figure 3). For

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the purposes of this comparison IU/ml determined by the real-time PCR assay were converted into copies/ml using a multiplication factor of five. This conversion was required because quantitative data generated by the Digene assay are expressed in copies/ml. The conversion factor was based on the international collaborative study of Saldhana et al (2001) in which the WHO International Unit was shown to be equivalent to approximately five HBV genome copies.

Performance on clinical samples

In a consecutive series of 256 HBV DNA positive plasmas, the geometric mean HBV DNA titre of the 181 HBeAg containing samples was 5×10^6 IU/ml. The geometric mean titre of the 75 anti-HBe positive samples was significantly lower ($P < 0.001$) at 2×10^4 IU/ml.

The real-time PCR assay was successfully used to monitor the response to antiviral therapy in 38 patients with HBeAg positive chronic HBV infection. A typical case is illustrated in Figure 4. This patient exhibited a dramatic ($> 4 \log_{10}$) decline in circulating HBV DNA concentration soon after commencing Lamivudine followed by a more gradual decline over the subsequent 15 months. The decline in HBV DNA concentration was accompanied by normalisation of serum alanine amino transferase (ALT) levels. Following cessation of Lamivudine therapy HBV DNA concentration was shown to increase by approximately $3 \log_{10}$. The changing levels of circulating HBV DNA in this patient were reflected by concordant changes in HBsAg titre and accompanied by seroconversion from HBeAg to anti-HBe positivity.

Performance on quality assurance panel

Results obtained by testing a coded panel of HBV proficiency samples supplied by Quality Control for Molecular Diagnostics (QCMD) are shown in Figure 5. Once again HBV DNA concentrations measured in IU/ml by the real-time PCR assay were converted into copies/ml using a multiplication factor of five

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(Saldanha et al., 2001). There was a good correlation between the data generated by the real-time PCR assay and the titres provided by QCMD on decoding the panel (R^2 = 0.9985).

Discussion

A variety of commercial assays for HBV DNA quantitation are now available and routinely used in diagnostic laboratories worldwide. Although generally reliable and convenient, many of these assays offer only a relatively limited dynamic range so that clinical samples frequently have to be diluted and retested in order to avoid exceeding the upper limit of quantitation (Hendricks et al., 1995; Poljak et al., 2001; Kessler et al., 2000). The novel real-time PCR assay described here exhibits an extraordinarily wide dynamic range (~ 9 $\log_{10}$) and efficiently overcomes this limitation. The advantage of this assay characteristic is well illustrated by Figure 4 in which HBV DNA concentrations vary over a range of ~ 8 $\log_{10}$ within a single patient. Figure 4 also illustrates the importance of extreme assay sensitivity especially when monitoring patients on antiviral therapy; 18 of the 21 plasma samples analysed in this case would have been below the level of detection by Digene hybrid capture assay. The lower limit of detection of the real-time PCR assay described in this study (Table 1) matches that of the most sensitive tests yet described (Weiss et al., 2004; Sum et al., 2004). Pre-extraction centrifugation and/or use of a larger plasma/serum volume could further increase the sensitivity of the assay if required (Gobbers et al., 2001; Burgener et al., 2003). The use of 'safety' primers (Heermann et al., 1996) based on the primer set described here could also be considered. Such primers would reduce the small theoretical risk of inefficient amplification in case of the emergence of HBV strains with sequence variation corresponding to the 3' end of the current primers.

Good reproducibility is an essential requirement of nucleic acid quantitation tests and the very low inter-assay and intra-assay variability (CV%, 7% – 11%) of this real-time PCR compares favourably with other protocols (Stelzl et al., 2004). Most previously described real-time PCR assays have not included an

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internal control in each sample from the lysis step onwards. This is important because such a strategy controls each sample for the efficiency of nucleic acid purification as well as revealing the presence of PCR inhibitors. The present assay, by employing whole virions (mCMV) rather than plasmid DNA (Weiss et al., 2004) as internal control, goes even further by controlling for the efficiency of the viral lysis step itself. mCMV is readily available, non-hazardous to humans and easy to culture and harvest. It has proven useful for this purpose in other assays (Tedder et al., 2002a) and is now universally employed as an internal control for all DNA virus 'in house' PCR protocols in our laboratory.

Statistically significant differences in HBV DNA titres between HBeAg positive and anti-HBe positive samples were observed in this study and similar differences have been reported by others using real-time PCR and other assays (Ho et al., 2003; Jardi et al., 2001; Tedder et al., 2002b). To be able accurately to compare HBV DNA results generated by different laboratories using different tests it is essential to have a well validated, internationally accepted HBV standard which can be used for assay calibration (Saldanha et al., 2001). We therefore used the World Health Organisation International HBV Standard 97/746 as the primary calibration standard and test results are therefore expressed in International Units per ml (IU/ml). By using a multiplication factor of five to convert IU/ml into copies/ml (Saldanha et al., 2001) an excellent correlation was observed between the results of the real-time PCR and the results of the Digene hybrid capture assay. Similarly, using the same correction factor, the results generated by the real-time PCR assay were in good agreement with the values defined by the QCMD proficiency panel.

By using robotic technology for automated nucleic acid extraction and by exploiting real-time fluorescence for detection of amplicons, the length of time required to generate results has been greatly reduced in comparison with conventional PCR-based assays. High throughput is possible as all stages are conducted in a standard 96 well microtitre plate format. The total assay duration for 86 patient samples is approximately 4.5 hours of which only 1 hour involves 'hands-on' activity. The assay is

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suitable for both plasma and serum samples, and reagent, royalty and consumables costs are significantly lower than for commercial assays.

In conclusion, the novel real-time PCR assay described in this paper offers several important advantages for HBV DNA quantitation. These include, i) an extremely wide dynamic range and a 95% detection limit of 24.4 IU/ml, ii) very high intra-assay and inter-assay reproducibility, iii) primers and probes designed to accurately quantify all 8 genotypes of HBV, iv) calibration based on the WHO International Standard 97/746, v) an excellent performance with the QCMD proficiency panel and a high correlation with Digene hybrid capture assay over 4 $\log_{10}$, vi) the inclusion of mCMV to control for the efficiency of all stages of the assay including viral lysis, vii) a rapid high throughput system with minimal 'hands-on' time due to robotic nucleic acid extraction and real-time amplicon detection, and viii) significant financial savings in comparison with commercial assays. We consider that this assay represents an ideal system in a busy diagnostic laboratory for monitoring the virological response in chronically infected patients undergoing antiviral therapy and for studies designed to explore the relationship between HBV viral load and stage of disease.

Acknowledgments

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Figure Legends

Figure 1

Calibration of the 'in house' standard against the WHO HBV International Standard 97/746. The 'in house' standard dilutions (diamond symbols) shown here are 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 fold, and the WHO standard dilutions (cross symbols) are 10^1, 2 x 10^1, 10^2, 2 x 10^2, 2 x 10^3 and 2 x 10^4 fold.

Figure 2

Typical amplification plot generated by ABI Prism 7000 software. Reading from left to right the curves represent the following calibration standards: 10^8 IU/ml, 10^7 IU/ml, 10^6 IU/ml, 10^5 IU/ml, 10^4 IU/ml, 10^3 IU/ml, 10^2 IU/ml and 10 IU/ml.

Figure 3

Correlation between HBV DNA titres in 32 HBsAg positive samples measured by Digene hybrid capture assay and by the novel real-time PCR assay described in this study.

Figure 4

Monitoring of HBV DNA titre (solid line) and HBsAg titre (dashed line) in a patient with chronic hepatitis B infection over a three year period. The duration of antiviral therapy with Lamivudine is indicated by the stippled box. Seroconversion from HBeAg to anti-HBe is shown at the top of the Figure together with serum ALT data for each time point (normal range for ALT, 10 – 35 U/ml).

Figure 5

Results obtained by blind testing the QCMD hepatitis B proficiency panel. NB. A negative result (i.e. below the lower limit of detection) is represented by 1.00E+00 in this Figure.

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Figure 1

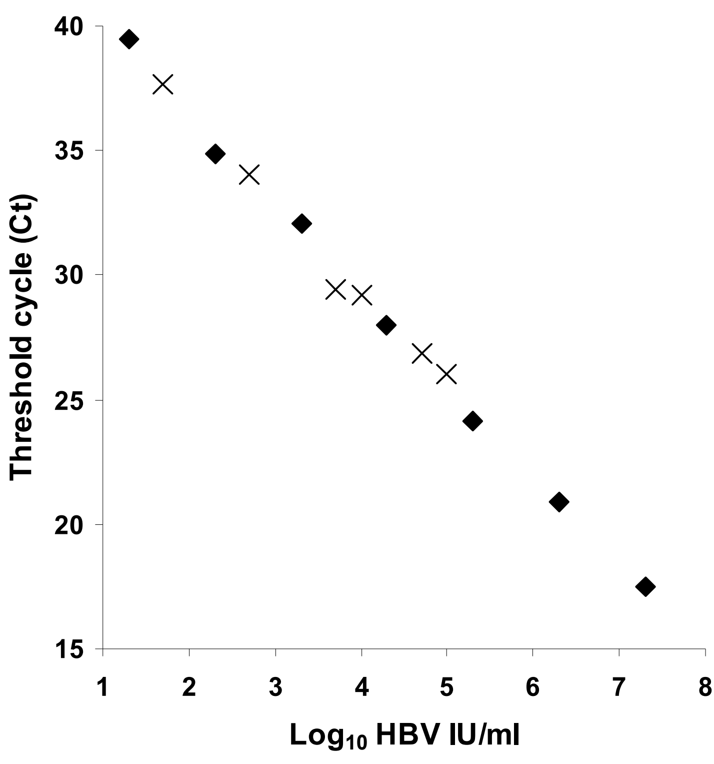

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Figure 2

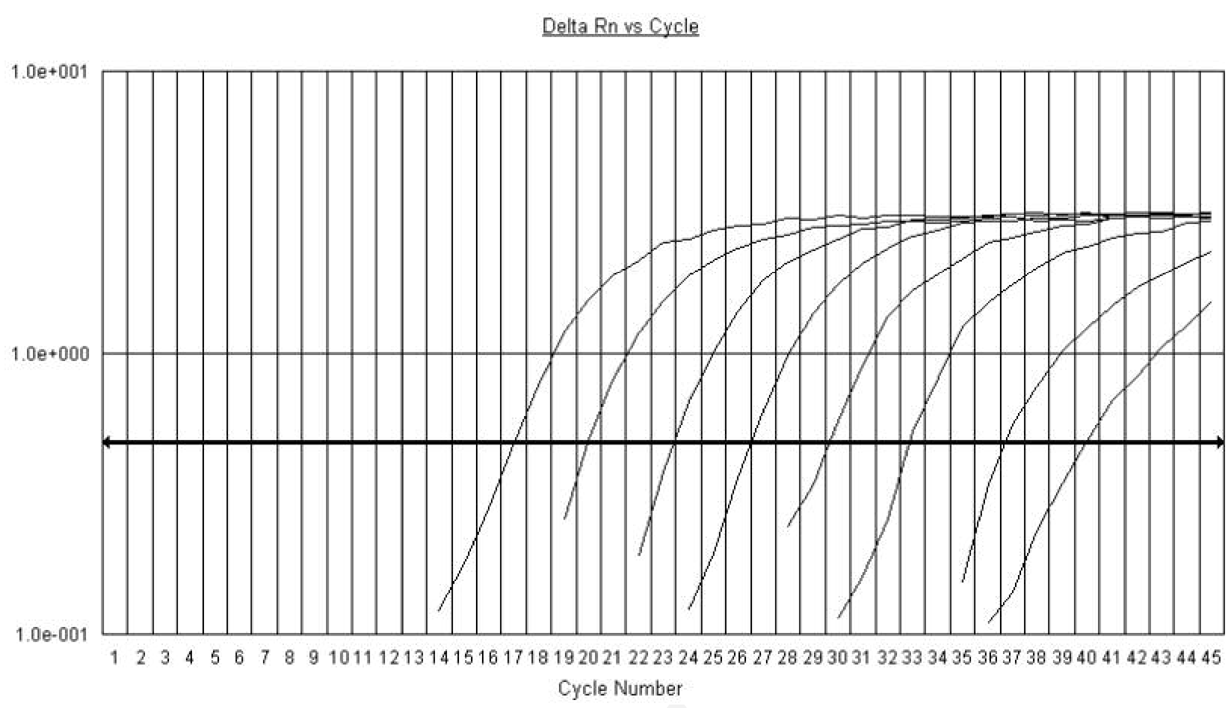

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Figure 3

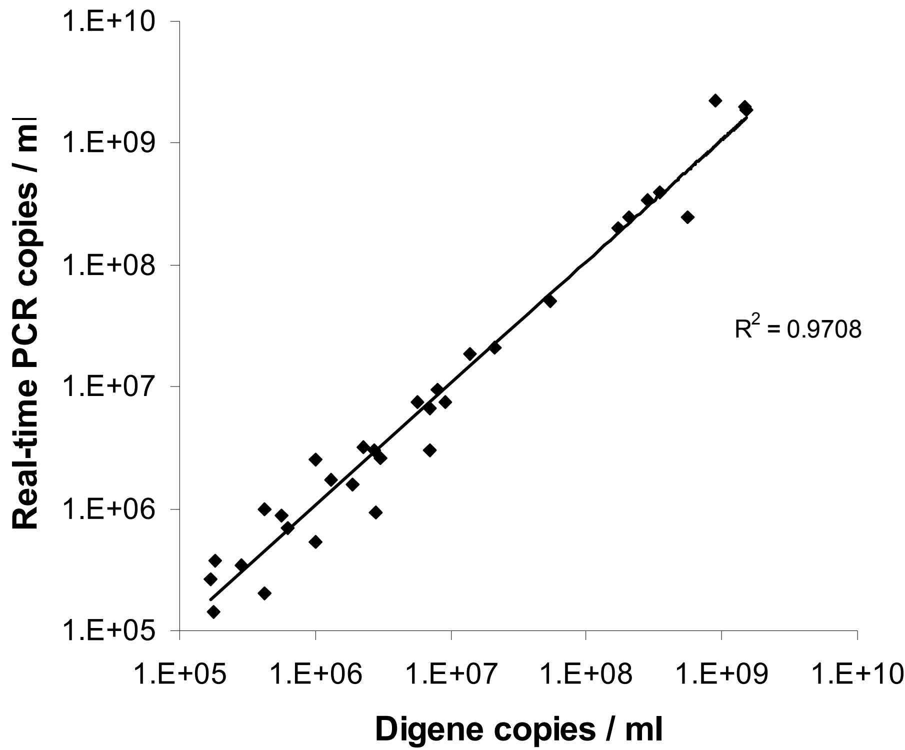

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Figure 4

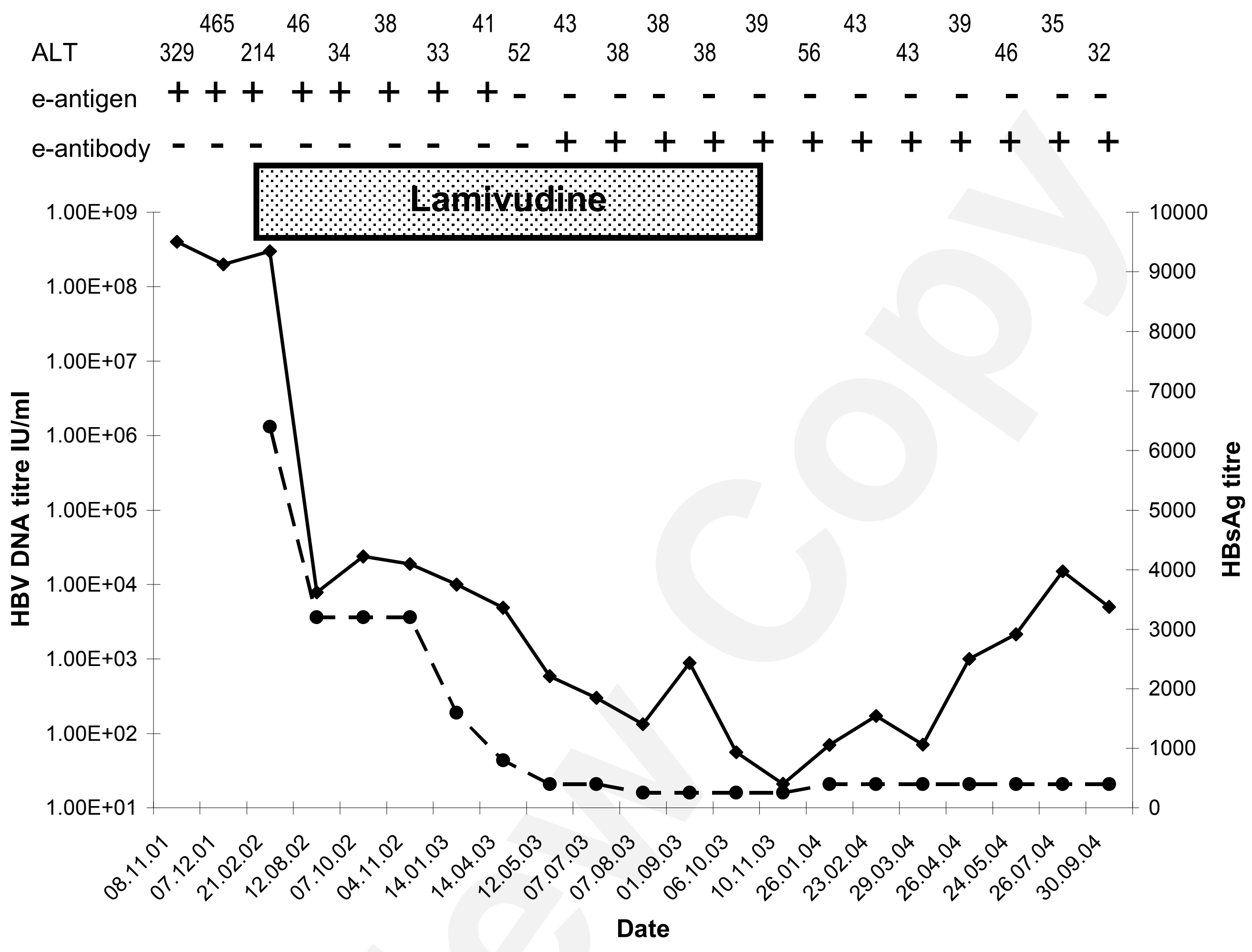

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Figure 5

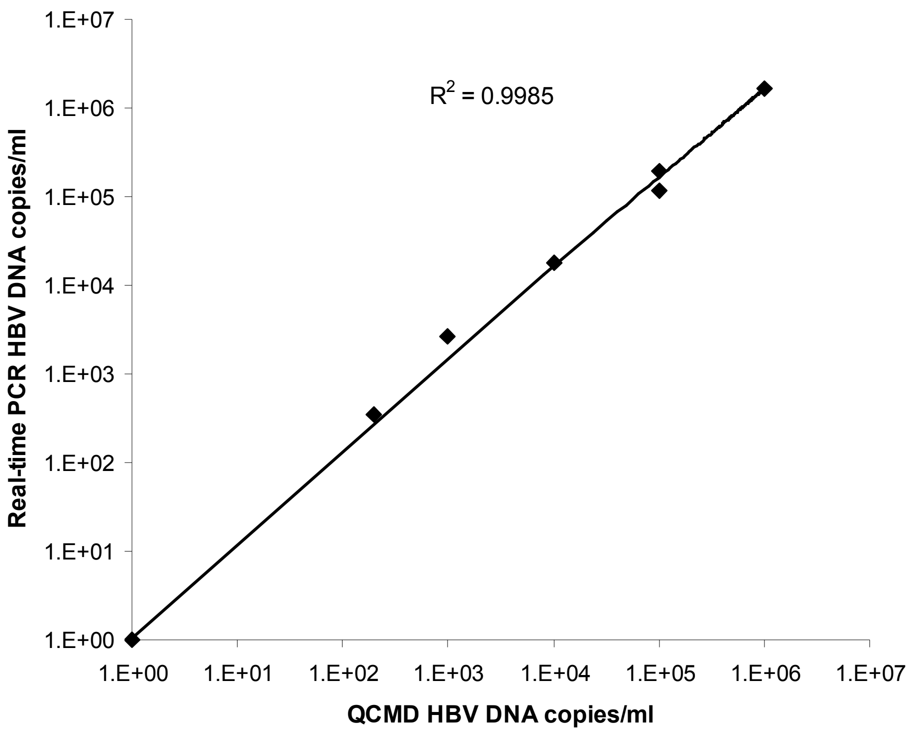

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Table 1 Real-time PCR assay data used for Probit analysis

HBV Concentration [a] IU/ml	Number Positive/Tested	Percentage Positive
100	24 / 24	100.0%
50	24 / 24	100.0%
25	23 / 24	95.8%
10	17 / 24	70.8%
5	15 / 24	62.5%
0	0 / 24	0.0%

[a] Based on dilutions of the WHO HBV International Standard 97/746 in normal human plasma

Review Copy