

## Real Time PCR Usage in the Quantification of Hepatitis B Virus DNA-Clinical Applications in Disease Management

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### ABSTRACT

Simultaneous quantification and detection of Hepatitis B virus (HBV) DNA plays significant role in diagnosing and monitoring infection related to HBV as well as assessing therapeutic response. Variability among HBV genotypes and the huge range of clinical HBV DNA levels presents challenges for PCR-based amplification techniques. High sensitivity, wide linear range, good reproducibility, and genotype inclusivity, combined with a small sample volume requirement and low cost, make this novel quantitative HBV Real-Time PCR assay particularly well suited for application to large clinical and epidemiological studies. Serum DNA levels are a prognostic factor, and contribute to define the phase of chronic hepatitis B (CHB) infection, the treatment indication, and allow an assessment of the efficacy of antiviral therapy. High levels of HBV DNA are an independent risk factor for cirrhosis and hepatocellular carcinoma HCC in Asia. Recent advances in antiviral therapy, based on the development of new and more powerful nucleotide analogues, have dramatically improved chronic hepatitis B management, including the prevention of allograft reinfection in those patients undergoing liver transplantation for HBV related disease. Advances in the molecular diagnosis of drug resistance using highly sensitive methodologies such as DNA Amplification by PCR can further detect upcoming viral resistance at an early stage when the variant represents only a minor fraction of the total viral population. Such new tools are especially relevant for patients at high risk for disease progression or acute exacerbation.

**Keywords:** Liver cirrhosis, DNA amplification, HBV genotypes, vaccination, DNA quantification, molecular probes.

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### INTRODUCTION

Hepadnaviruses utilize an unusual replication strategy. On infection, the partially double-stranded open circular genomic DNA is transported to the hepatocyte nucleus, where host-cell enzymes convert it to a relaxed circular fully double-stranded molecule. From this replicative form is generated a covalently closed circular (ccc) DNA, which associates with cellular histones to form a viral minichromosome (1,2). The HBV (hepatitis B virus) ccc DNA remains in the cell nucleus

and serves as the transcriptional template for HBV-RNA production.

Hepatitis B virus (HBV) is a partially double stranded DNA containing virus of Hepadnaviridae family [1]. Hepatitis B virus (HBV), a 3.2 kb *Orthohepadnavirus*, is a well-known agent of acute and chronic hepatitis, with an estimated 350 million chronic carriers around the world [2, 3]. HBV cause the Hepatitis B which may be acute or chronic in nature. As an estimate more than 2 billion people are infected with

HBV and 350 millions are chronically infected. Hepatitis is associated with high rate of morbidity and mortality. It is a fatal disorder due to complications of cirrhosis, hepatocellular carcinoma (HCC) and portal hypertension. It enters into the liver by blood circulation. The new DNA strand are

generated by reverse transcription of mRNA intermediate which is longer than that of its genome and is about 3.5 kb in length, the mRNA is called as pre-genomic RNA or PgrRNA. The HBV genome encodes four partially overlapped open reading frames (ORF).

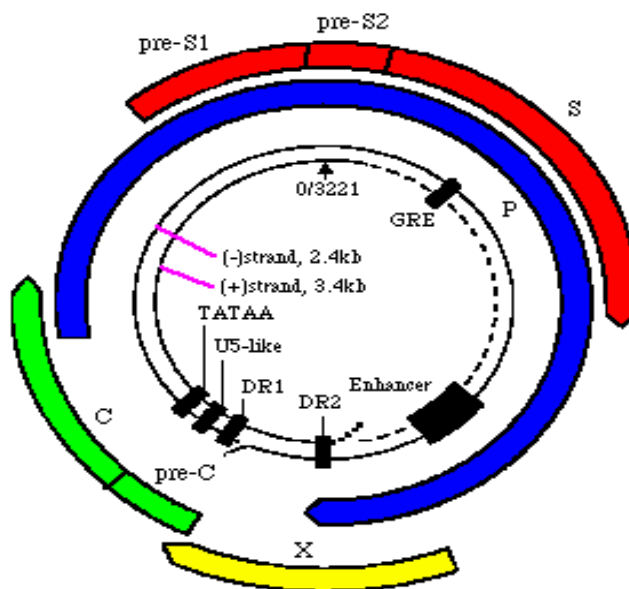


Figure 1: Structure of HBV Genome

### Transmission

Hepatitis B is largely transmitted through exposure to bodily fluids containing the virus. This includes unprotected sexual contact, blood transfusions, re-use of contaminated needles and syringes, vertical transmission from mother to child during childbirth, and so on. Without intervention, a mother who is positive for the hepatitis B surface antigen confers a 20% risk of passing the infection to her offspring at the time of birth. This risk is as high as 90% if the mother is also positive for the hepatitis B e antigen. Transmission from mother to child during childbirth without intervention, a mother who is positive for HBsAg confers a 20% risk of passing the infection to her offspring at the time of birth. Roughly 16-40% of unimmunized sexual partners of individuals with hepatitis B will be infected through sexual contact. The risk of transmission is closely related to the rate of viral replication in the infected individual at the time of exposure. Other risk factors for developing HBV infection include working in a health care setting,

transfusion, dialysis, acupuncture, tattooing, extended over ease travel and residence in an institution. However hepatitis B viruses cannot spread by casual contact, such as holding hands, sharing eating utensils or drinking glasses, breast feeding, kissing, hugging, coughing or sneezing [5].

### Molecular tools for HBV detection

Virology techniques developed over the past 20 years are being used to diagnose and monitor chronic viral infections, such as those caused by hepatitis B virus (HBV) and hepatitis C virus (HCV). Molecular biology tools can be used to detect and quantify viral genomes, sequence them, assign them to a phylogenetic clade or subclade (genotype or subtype), and identify clinically relevant nucleotide or amino acid substitutions, such as those associated with resistance to antiviral drugs. Recent advances include real-time target amplification methods for detecting and quantifying viral genomes and next-generation sequencing (NGS) techniques. Other new assays detect and quantify viral antigens, whereas point-of-care tests and alternatives to biologic tests

that require whole-blood samples have been developed.

### Target Amplification Methods

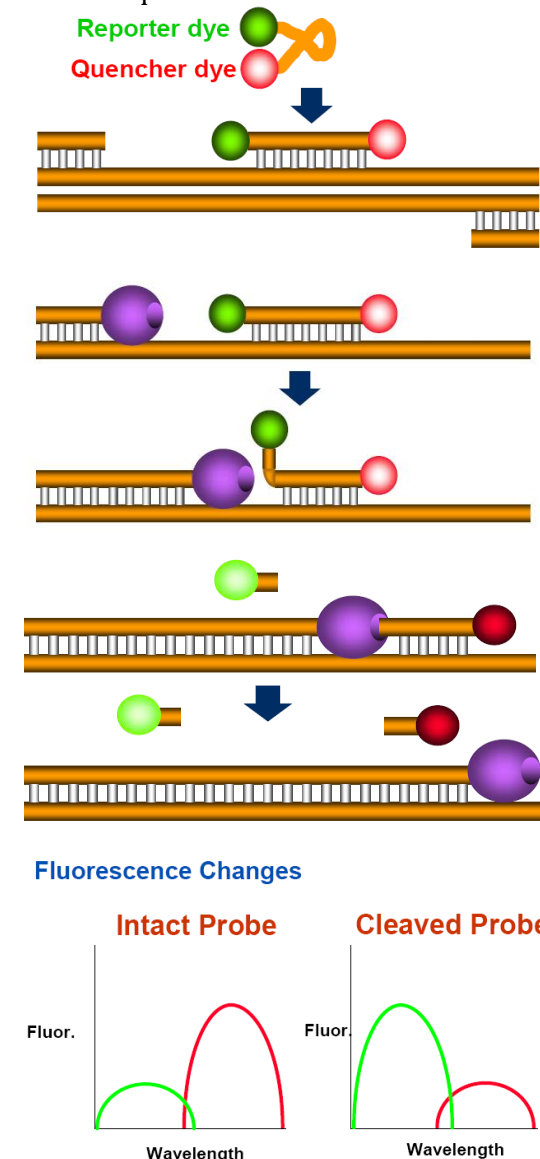
First-generation target amplification techniques have been widely used to diagnose HBV and HCV infections and to monitor responses to antiviral therapies. In PCR or Transcription Mediated Amplification (TMA) assays, amplicons are detected at the end of the amplification process by their specific hybridization to immobilized oligonucleotide probes; the amplicon-probe hybrids are detected by an enzymatic reaction. They are quantified based on competitive amplification of the viral template with a known amount of synthetic standard added to each reaction tube; the relative amounts of viral template and standard amplicons are measured and the results are interpreted with a standard curve established in parallel.

Because amplification reactions are saturable, these methods have a narrow dynamic range of quantification. As a result, high levels of virus are not always accurately quantified and require reanalysis after samples are diluted, whereas low levels of virus (such as in patients receiving antiviral therapy) are often not detectable. This problem was solved by development of real-time target amplification techniques in which quantification takes place during the exponential phase of the amplification reaction. In addition, the reaction is run in a closed system, which prevents carryover contamination and improves specificity.

### Real-Time PCR

PCR uses a number of temperatures and a thermostable DNA polymerase to create double-stranded DNA amplicons. In assays for HBV, nucleic acids are isolated from a sample and the HBV DNA is directly amplified by PCR. Each complete PCR cycle doubles the number of DNA copies. The principle of Real-Time PCR is to detect amplicon synthesis during the PCR reaction and there by deduce the starting amount of viral genome in a clinical sample. A fluorescent probe is linked to a quencher and annealed to the target sequence, between the sense and antisense PCR primers. During each PCR reaction, as the DNA polymerase extends the primer, its intrinsic nuclease activity degrades the

probe, releasing the reporter fluorochrome (Figure 2, 3). The amount of fluorescence released during the amplification cycle and detected by the system is proportional to the amount of amplicons generated in each PCR cycle. Software is used to calculate the threshold cycle in each reaction (Ct), which has a linear relationship with the initial amount of nucleic acid. In each PCR run, parallel processing of a panel of quantified standards is used to establish a standard curve for quantification.



**Figure 2: An overview of working mechanism of TaqMan Probe in Real Time PCR for HBV DNA quantification.**

Hepatitis-B viral antigen and antibodies detectable in the blood follows acute infection. Numerous tests are used for the

detection of Hepatitis B viral infection which involves serum or blood tests that detect either viral antigen (protein produce by the virus) or antibodies produce by the host against the Hepatitis B viral protein. HBV testing begins with serological blood tests used to detect antibodies to HBV [6]. Hepatitis B surface antigen (HBsAg) is a most frequently used marker to screen for the presence of this infection. It is the first detectable viral antigen, appearing during the infection. However in early infection, this antigen may not be present and it may be undetectable later in the infection stage as it is being cleared by the host, which is disadvantageous for HBV detection. Molecular tests help to determine the activity of HBV infection, the selection of patients for the treatment, and the efficacy of antiviral therapy, identifying the development of HBV drug resistant strains [7]. Sample preparation is the major weakness in molecular tests, and improvement is constantly introduced to decrease the variability of techniques and the risk of contamination, such as ready-to-use reagents and automation of the extraction procedure. Overall HBV antibody test have a strong positive predictive value for exposure to HBV, but may miss patient who have not yet develop antibody, called sero conversion or have an insufficient levels antibodies to detect [8]. Immunocompromised individuals infected with HBV may never develop antibodies to virus and never test positive using HBV antibody screening. Anti-HBV antibody indicate exposure to the virus, but cannot be determine if on going infection is present but initial stage of infection is very low amount of antibodies which are not detectable by serological methods. During HBV infection body immune system take a span of time for generation of antibodies against HBV infection (protein produced by Hepatitis B virions), this period of time is called 'Window Period' [9]. During this 'window' in which the host remains infected but is successfully clearing the virus, IgM antibodies to Hepatitis-B core antigen (Hbc IgM) may be the only serological evidence of disease. After the appearance of HBsAg another Ag named as the HBeAg will appear. Traditionally, the presence of

HBeAg in the host serum is associated with much higher rates of viral replication and enhanced infectivity. During the natural course of an infection, the HBeAg may be cleared an Ab to the 'e' Ag (Anti-HBe) will arise immediately afterwards [10]. This conversion is usually associated with a dramatic decline in viral replication. If the host is able to clear the infection, eventually the HBsAg will become undetectable and will be followed by IgG antibody to the Hepatitis-B surface antigen and core antigen (Anti-HBs and Anti-HBc IgG). Individual who remains HBsAg positive for at least 6 months are considered to be Hepatitis-B carrier. Carriers of virus may have chronic Hepatitis-B, which would be reflected by elevated serum alanine aminotransferase (ALT) level and inflammation of liver as revealed by biopsy [11]. Particularly those who acquired the infection as adults have very little viral multiplication and hence may be at little risk of long term complications or transmitting infection to others. Various advanced PCR based detection techniques have been developed for qualitative and quantitative detection of HBV infection [12].

**HBV Quantification by Real Time Utilizing TaqMan Probe Assay**

The HBV viral load can be monitored utilizing COBAS TaqMan 48 Real Time PCR from Roche. This technique uses thermostable recombinant enzyme DNA polymerase (ZO5) for reverse transcription and as well as PCR amplification [13]. Under the appropriate, conditions utilizing the  $Mn^{2+}$ , ZO5 DNA polymerase involve the reverse transcription and PCR amplification together with real time detection of amplicon from processed specimen. In this technique dual labeled fluorescent probe called TaqMan is being utilized for real time detection of amplicon accumulation, which will be monitored by the emission intensity of fluorescent reporter dyes released during amplification process [14].

#### **TaqMan Probe Molecular Chemistry**

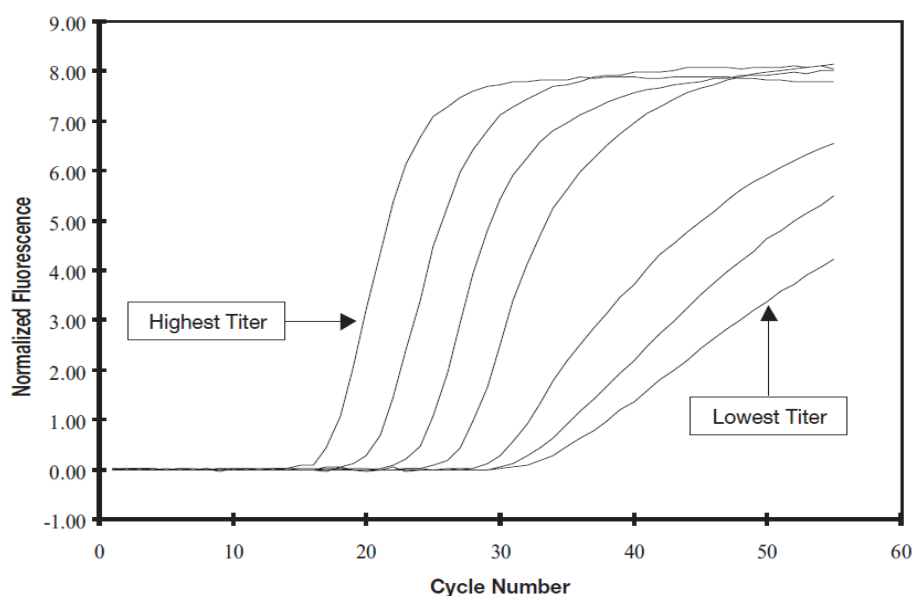
TaqMan probe consists of oligonucleotide sequence labeled with reporter and quencher dye. The TaqMan probes are designed in such a way, which contains complimentary sequence for target. The reporter region of the TaqMan probe binds

to the 5' end of the target. When these dual labeled fluorescent probes are present in bound state the quencher region emits the fluorescence which is absorbed by the reporter region. This type of emission of fluorescence of quencher region and absorbance based on the 'forster type energy transfer effect' (FRET) (as shown in figures 2 and 3). During the amplification reaction the probes hybridized to a target sequences at 5' and is cleaved by the 5'-3' exonuclease activity of the thermostable Z05 DNA polymerase. When the thermostable Z05 DNA polymerase come in contact with hybridized probe during polymerization, reporter & quencher dyes are released and separated. Quenching no longer occurs and the fluorescent activity of quencher dye increases [15].

#### Hepatitis B Virus DNA Quantification

Amplification of HBV DNA & HBV quantitation standard DNA is measured independently at different wavelengths. Emission intensity of individual reporter dye effectively increases in each cycle that allows independent identification of HBV DNA & HBV quantitation standard DNA. This intensity of signal is related to the amount of starting material at the beginning of PCR. Real Time PCR accumulates continuous collection of fluorescent signals from one or more polymerase chain

reaction over a range of cycles. Quantitative Real Time PCR is the conversion of fluorescent signal from each reaction into numerical value for each sample by means of graph (shown in figure 4). As the concentration of the virus increases, the growth curves shift to earlier cycles. Therefore the leftmost growth curve corresponds to the highest viral titer level whereas the rightmost growth curve corresponds to the lowest viral titer level. Graphical representation of fluorescent signal from an ideal v/s actual reaction over 40 cycles of real time PCR in an ideal PCR, there are two phases, a baseline where the signal is below the level of instrument detection followed by the persistent geometric increases in fluorescence that continue over the remaining cycle of the experiment. However, in an actual PCR, there are four phases as in ideal reaction, there is a baseline followed by a geometric phase [16]. However, the amplification becomes less than ideal leading to a linear phase & finally a plateau where no further increase in signal occur over the remaining cycle. The COBAS TaqMan 48 Analyzer automatically determines the HBV DNA titer for the specimen or control. The HBV DNA titer is expressed in International Units (IU)/mL.



**Figure 3. Graphical presentation for conversion of fluorescent signal from each reaction into numerical value for each sample.**

**Significance of HBV DNA Quantification**

HBV has eight genotypes (A-H) due to variation in nucleotide sequences greater than 8%. Various publications are found that the clinical course and outcome of antiviral therapy dependent on the genotype of the infecting HBV strains. After that real time come in field of molecular diagnostic of epidemic diseases. The Roche Real Time PCR accurately identifies the genotype in one step reaction by means of primer specificity. It provides a useful tool for rapid detection and would make large scale longitudinal HBV related studies simple & feasible. TaqMan hepatitis B virus (HBV) analyte specific reagent is designed for the quantification of HBV DNA in serum or plasma. Analytical sensitivity and precision were assessed with commercially available HBV standards, while clinical serum specimens from HBsAg seropositive patient and healthy blood donor were used to determine clinical sensitivity, specificity and correlation with other commercially available assays [17]. Analytical studies yielded a limit of detection of 2.4 IU/ml, with good linearity and correlation with expanded HBV DNA titre over a range. Clinical sensitivity and specificity of the assay combined with automated sample processing are both 100%. TaqMan HBV providing sensitive and accurate quantification of HBV DNA levels over a range of 8 logs 10 IU/ml. Hepatitis B virus (HBV) infection continues to be a leading cause of chronic liver disease, with more than 350 million people chronically infected worldwide. Chronic HBV carriers are at increased risk for the development of cirrhosis and hepatocellular carcinoma. The direct detection of HBV DNA in serum or plasma has become an important tool in diagnosis of chronic HBV infection. Furthermore, serum HBV DNA level may be an important prognostic indicator as well as an important marker for measuring therapeutic response and to the development of resistance to antiviral agents, a variety of commercially available HBV DNA assays reporting in standardized units with improved sensitivity and wide dynamic ranges are currently available [18]. The COBAS TaqMan HBV test is one of the commercially available Real Time PCR assay

designed for the quantitative detection of HBV DNA in human serum and plasma. The benefits of nucleic acid amplification and detection using Real Time PCR include substantial reduction in labor, decreased test turnaround time, and reduced potential for contamination with exogenous DNA. The disadvantages of technically demanding manual sample preparation methods are also numerous. Use of automated sample processing in clinical diagnostic laboratories provides a labor-saving approach to reducing the number of failed sample preparations, while potentially limiting the occurrence of specimen to specimen contamination during processing. This approach may also reduce laboratory space requirements and decreased dependence on laboratory technologists. Nucleic acid extraction from biologic specimens is technically demanding, and reliable PCR result depends on it. The amount of intra hepatic HBV DNA was significantly lower in occult HBV infection than in overt disease. It is known that maintained high levels of HBV DNA are associated with progressive liver disease. Serum DNA levels are a prognostic factor, and contribute to define the phase of chronic hepatitis B (CHB) infection, the treatment indication, and allow an assessment of the efficacy of antiviral therapy. High levels of HBV DNA are an independent risk factor for cirrhosis and hepatocellular carcinoma HCC in Asia. Recent advances in antiviral therapy, based on the development of new and more powerful nucleotide analogues, have dramatically improved chronic hepatitis B management, including the prevention of allograft reinfection in those patients undergoing liver transplantation for HBV related disease.

**CONCLUSION**

Serological markers are key elements in diagnosing acute hepatitis B virus (HBV) infection and determining its possible evolution towards chronicity. Once treatment of chronic HBV is initiated with approved anti-hepadnaviral agents, such as lamivudine, interferon-alpha, or adefovir dipivoxil, the measurement of HBV DNA in serum can not only help monitor treatment efficacy but also indicates breakthrough

infection should drug resistance emerge. Should chronic carriage ensue, those persons who are unable to resolve HBV infection enter into a low replication phase of infection marked by the seroconversion of HBeAg to anti-HBe. This change occurs in about 10% (5%-20%) of chronic adult carriers per year. The inactive carrier state is marked by continued HBsAg positivity, in contrast to a drop in HBV DNA levels to less than  $10^5$  copies/mL. In addition, ALT levels and anti-HBc IgM decline and normalize; liver histology shows a significant reduction in necro-inflammation. Advances in the molecular diagnosis of drug resistance using highly sensitive methodologies such as DNA Amplification by PCR can further detect upcoming viral resistance at an early stage when the variant represents only a minor fraction of the total viral population. Such new tools are especially relevant for patients at high risk for disease progression or acute exacerbation.

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**Conflict of Interest: None**

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