

# Clinical Performance Characteristics of Hepatitis B Virus Quantification by Combining Artus-DSP Assay with Rotor Gene Q-Real-Time PCR

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**Abstract:** *Background:* A simple and reliable DNA extraction & quantification method of hepatitis B virus (HBV) is critical in developing a viral load measurement for HBV infection. Current commercially available plasma Hepatitis B Virus (HBV), DNA extraction & quantification methods are less sensitive and require optimization, which restrict wide adoption in clinical laboratories. This study offers a report on the quantitative viral load- artus® HBV RG PCR assay by combining QIAamp® DSP Plasma DNA extraction with Rotor gene Q-Real-time PCR (Rotor gene Q-PCR). *Methods:* Plasma was separated by centrifugation after collection of whole peripheral blood, which was then stored at -20° C until process. DNA was extracted as per instruction & guidelines of manufacturer. The recovered eluted DNA was mixed into PCR master-mix. The Q-PCR assay performance, including linearity, diagnostic accuracy and reliability were determined. Viral load compared of the no viral load, low viral titer & high viral titer from the clinical samples for evaluation. *Results:* The average % yield for HBV DNA standards used was within in the range (SD 0.04 to 0.13, CV% 0.97 to 2.22). The coefficient of variation (CV) of HBV quantification for low and high titer samples was 2.23% and 2.37%, respectively. The real-time assay linearity was demonstrated with a slope of -3.03 to -3.50 and R<sup>2</sup> values of 0.98 to 0.99. Accuracy and reliability of the Rotor gene Q-PCR assay was confirmed with the reference panel, and there was a strong correlation between the two results (R<sup>2</sup> = 0.99, < 0.01) & found no major difference in observed viral load. *Conclusions:* The Rotorgene Q-PCR performance with artus-DSP assay is reliable and wide linear range quantification method and can be used for sensitive detection of plasma HBV with low nucleic acids content.

**Keywords:** Hepatitis B Virus, Quantitative Standards, DNA amplification, Taqman Technology

## 1. Introduction

Hepatitis B Virus infection has been known as a common cause of liver diseases in humans ranging from self-limiting acute hepatitis to active chronic hepatitis, liver cirrhosis, liver failure and hepatocellular carcinoma [6]. Approximately 95% of adults, exposure to HBV develops to an acute infection without clinical symptoms or with an inflammatory disease of the liver (acute hepatitis), which usually gets resolved in about 6 months, whereas the remaining 5 % fails to control the viral infection, developing to chronic disease or cancer [17]. Hepatitis B virus is prevalent worldwide, with an estimated 400 million people infected globally [28]. About 350 million people have chronic HBV infections [5], [11], with annually more than 750,000 deaths worldwide [12]. India has the major etiology of chronic liver diseases with more than 40 million carriers of HBV and also has ethnically diverse population [2]. With 50 million carriers, India has the second largest population of individuals with chronic Hepatitis B infection worldwide [27].

HBV is diagnosed with serological markers; include HBsAg, anti-HBsAg, HBeAg, anti-HBeAg, anti-HBc, serum alanine transaminase (ALT), aspartate transaminase (AST) tests and quantification of HBV DNA, whose measurement is applied in the management of HBV infections [3]. HBV DNA viral load play a critical role in determining the phase of infection and to decide the treatment [11]. Lower level of HBV DNA is associated with a lower risk of a hepatocellular carcinoma development than a higher level [5]. HBV DNA levels

change during different phases of infection in chronically infected individuals [8]. As per guidelines of world health organization for the prevention and treatment with person of chronic HBV infection, HBV DNA quantification is recommended for the treatment [29]. HBV DNA quantification studies have helped researchers to consider guidelines for the assessment of diagnosis and treatment responses. HBV DNA monitoring is therefore a best predictor and has been recommended for efficient management of HBV [21], [23]. Various HBV DNA quantitative assays are now available and it is important to use an accurate HBV monitoring tool. Real-time PCR has become the standard diagnostic technology and is widely used for HBV detection with high sensitivity, wider linear range, and reproducibility [24], [19]. For making same level results, HBV DNA load is universally reported in IU/ml that have been calibrated with the World Health Organization international standard for HBV DNA [20]. There are different viral detection limit assay available of wider range [7], but for treatment prospect of HBV, a more sensitive assay with a lower limit of detection of lower than 15 IU/ml is recommended for early detection of viral load [13], [10].

Plasma HBV DNA levels are unstable with time progress and depend upon the infection phase. HBV DNA detection in whole peripheral blood is a reliable marker of active HBV replication and is more useful in the diagnosis of infection comparison to immunoassay [15]. It had also been verified that fast processing from plasma DNA after plasma collection was necessary to produce realistic results [22].

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Thus more specific method is required which can handle large sample input volume to increase sensitivity for HBV DNA detection assay so that it could reach recommended limit of detection [9]. There are a few commercial assays available which fulfill to identify the recommended lower limit viral load. Therefore, a simple, reliable DNA extraction & amplification method is important for sensitive HBV DNA detection. The HBV DNA extraction with DSP viral kit following amplification through Rotor gene Q-PCR is a validated & CE-IVD approved method. The extracted HBV DNA by DSP method is applied into artus HBV RG PCR pre-mixture and the performance characteristics of the artus-DSP assay was evaluated and promised to high DNA recoveries with good reproducibility. The method illustrated that artus-DSP Q-PCR assay is the reliable, sensitive detection assay of HBV DNA and the method also give new insight on the application of plasma samples with low DNA content.

## 2. Methods

Ethics statement; This study was performed & approved at Core Diagnostics, Guru-gram & the institutional review board, Kumaun University Nainital. All subjects were recommended by clinician to identify HBV DNA viral load. Test requisition form was received from all subjects before this study & their identity has not been disclosed.

### 2.1 Samples

For this prospective study, a total of 56 clinical samples were taken for the evaluation of HBV DNA Viral Load in the laboratory. The whole peripheral blood samples from patients suspected with HBV were collected between December 2016 to July 2017 and plasma separated immediately. All samples were stored at -20°C until use. The relevant history and other details of the patients were noted from reports and test requisition form.

### 2.2 Extraction of HBV DNA

An assay (artus-DSP) using QIAamp® DSP virus extraction procedure was performed as per guidelines. The QIAamp® DSP virus extraction procedure allows the selective binding of viral nucleic acid in silica-based membrane. The viral DNA is purified from 500 µl of plasma and finally eluted in 50 µl of elution buffer. Reactions for amplification were performed similar to artus® HBV RG PCR. The Lower detection limit of the artus® HBV RG PCR kit using the DSP virus kit was claimed to be 10.22 IU/ml. Performance characteristics, including linear range, accuracy and analytical reliability, were studied using HBV DNA standards and clinical samples.

### 2.3 Real-time PCR

The Real-time HBV PCR assay with artus® HBV RG PCR kit was performed according to the manufacturer's instructions. The Q-PCR reactions were performed on a Rotor gene Q Real-Time PCR System. The cycling

parameters consisted of incubation at 95°C for 15 sec, 55°C for 30 sec & 72°C for 15 sec with 45 times repeated cycles.

### 2.4 Establishment of Standard Curves

For standardization of the quantitative HBV DNA detection assays, the HBV QS standards with known copy numbers ( $1 \times 10^1$ ,  $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$  &  $1 \times 10^5$  IU/µl) were used in the experiment. The quantitative values of standards & samples were plotted against the concentration of log<sub>10</sub> IU/ml, which resulted in typical amplification curves.

### 2.5 Percent yield of HBV DNA

The HBV DNA was extracted from plasma samples with low (4.0 log<sub>10</sub> IU/ml) and high (6.0 log<sub>10</sub> IU/ml)–viral load using respected protocol, recommended reagents & DNA-extraction column. The amplified HBV DNA was quantified using the artus Q-PCR assay. The % yield of HBV DNA was calculated as: actual yield/ theoretical yield \*100%.

### 2.6 Diagnostic correlation

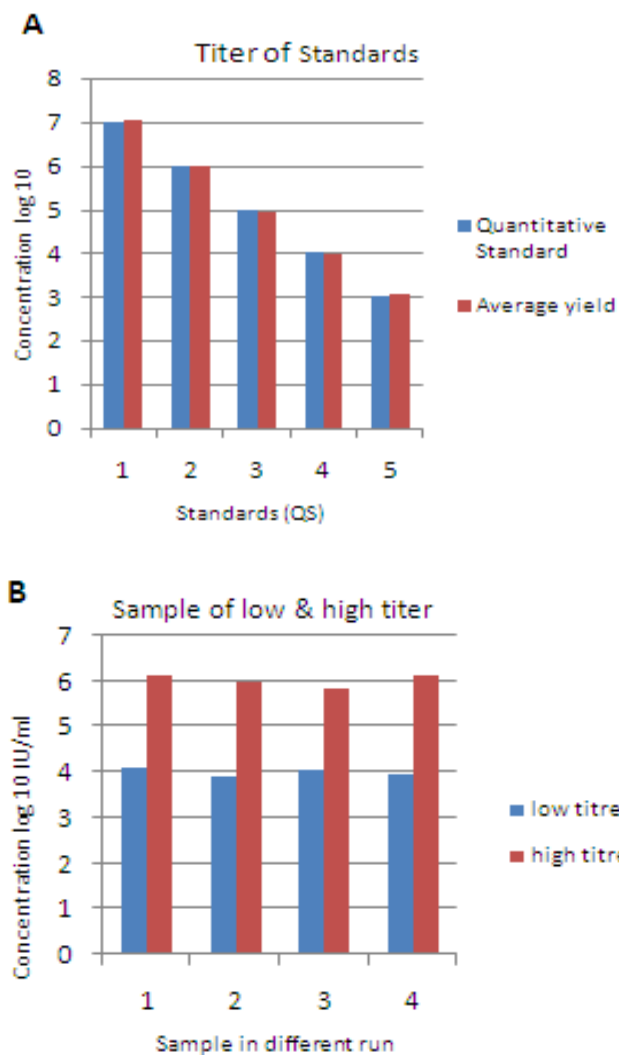
The accuracy & reliability of the artus-DSP Q-PCR assay was determined by analyzing the known low-concentration WHO standard (3.93 log<sub>10</sub> IU/ml) & diagnostics correlation of no titer, low titer & high titer with reference samples.

## 3. Statistical Analysis

The quantitative results were log transformed for further analysis. The continuous variables were expressed as mean and standard deviation (SD). The correlation analysis between the artus-DSP Q-PCR and reference method (CAP-CTM Real-time PCR-Taqman Technology) were analyzed by Pearson's product moment correlation coefficient and linear regression equation. The Bland-Altman plot was also used in order to represent the degree of agreement ( $\bar{d} \pm 1.96*SD$ ) between quantitative results obtained from both methods. All statistical analyses were performed using the Microsoft-excel.

## 4. Results

Clinical performance characteristics of among 56 clinical samples tested, 47 samples were detected by the artus-DSP assay with HBV DNA viral loads ranging 11.8 IU/ml to  $4 \times 10^8$  IU/ml (median, 2530 IU/ml). Likewise, 26 samples were analyzed by reference method (CAP-CTM Real-time PCR-Taqman technology) ranging 70 IU/ml to 20123310 IU/ml of HBV DNA viral load (median, 943 IU/ml). Out of 26 (46.4%) samples that were quantified by both method, the differences in quantification mean were 2.82 log<sub>10</sub> IU/ml (artus-DSP) and 2.81 log<sub>10</sub> IU/ml (reference method), respectively.

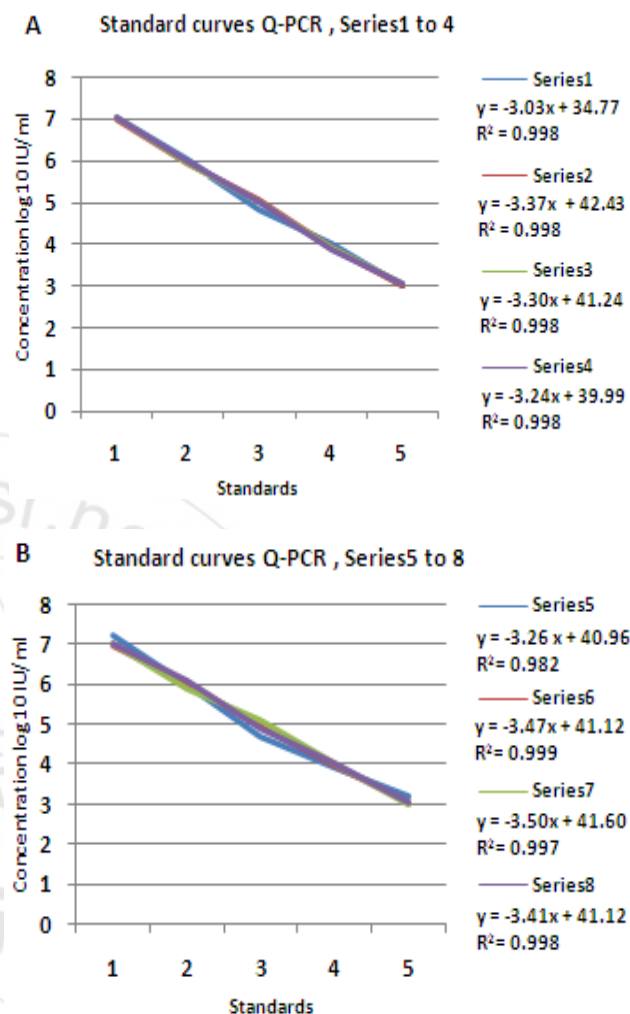


**Figure 1:** Yield of HBV DNA titer with standards and clinical samples; (A) The average % yield of HBV DNA concentration obtained using quantitative standards QS1 (100.57), QS2 (99.74), QS3 (99.17), QS4 (99.14), QS5 (101.67); (B) The average viral load for high-titer (6.018 log<sub>10</sub> IU/ml) and low-titer (4.001 log<sub>10</sub> IU/ml) for HBV DNA concentration obtained using the DNA-extraction column method.

The value of average % yield for high-titer QS1 & low-titer QS5 samples were slightly higher than rest of the QS titers, QS2, QS3 & QS4. However, the difference was not found to be statistically significant (Figure 1 A). At the same time, the average value of viral load observed for the low-titer sample and for the high-titer, obtained with the DSP DNA-extraction column were 4.001 (SD 0.089, CV% 2.239) and 6.018 (SD 0.143, CV% 2.378) respectively (Figure 1 B).

The slopes of the standard curves were generally at -3.03 to -3.50. The consistency of the standards was evaluated by a correlation coefficient ( $R^2$ ) of 0.98 to 0.99, which indicates the linearity of the values plotted in the standard curves (Figure 2). All the relevant data were detected according to the standard curves in order to judge the clinical performance & characteristics of the present method including, linearity and diagnostic accuracy. Linearity was promised between

31.6 and  $2 \times 10^7$  IU/ml in each assay, using quantitative standards. The assay linearity was assessed as the difference between the detected values and the nominal values assigned to the standards.



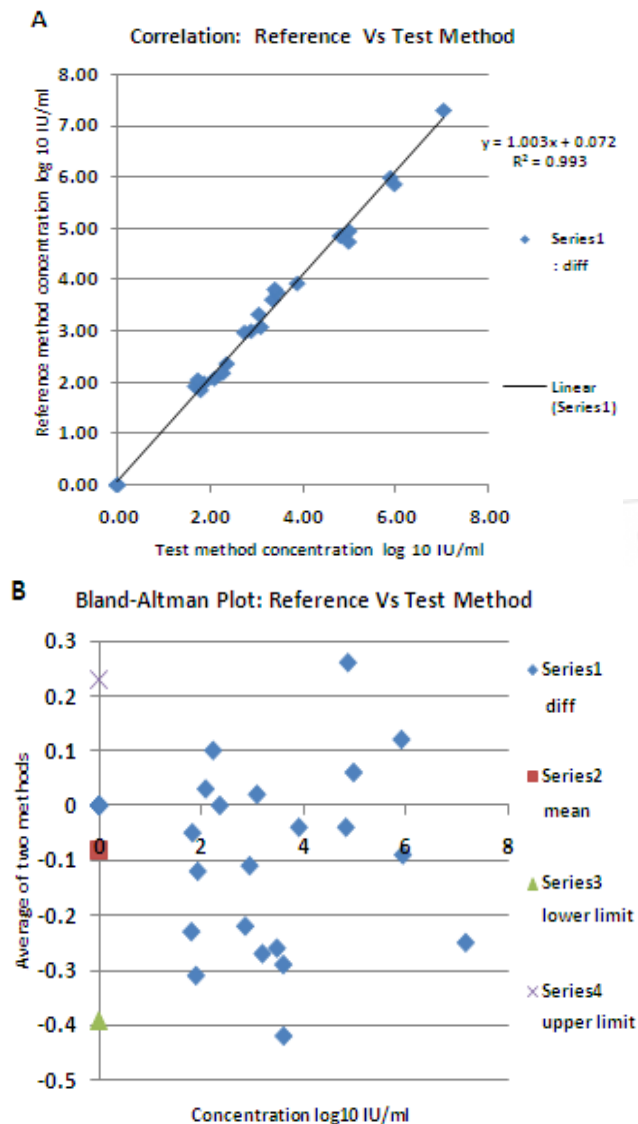
**Figure 2:** Performance of standards curves in Q-PCR assay; Standard curves (concentration log<sub>10</sub> IU/ml) applied in the Q-PCR assay for the determination of results by using QS1, QS2, QS3, QS4, and QS5. (A) Series1 to 4 applied in correlation study of two methods used. (B) Series5 to 8 applied to determine viral load for low titer & high titer samples.

Diagnostic accuracy of artus-DSP Q-PCR assay was determined by analyzing the dilution of the 8500 IU/ml WHO standard. The positive value was analyzed with respect of referring concentration levels.

Correlation with 26 reference clinical samples was accessed for viral load comparison. Samples of no viral load, low viral load and high viral load were analyzed using the artus-DSP Q-PCR and the reference method using CAP-CTM Real-time PCR-Taqman Technology. As shown in Figure 3 A, correlation analysis for the paired quantitative results demonstrated a significantly positive correlation between the two assays ( $R^2 = 0.993$ ,  $P < 0.01$ ). A Bland-Altman plot was also used to determine the agreement between the two assays (Figure 3 B). Using this method, the differences between the

HBV DNA viral load log<sub>10</sub> IU/ml concentrations of two assays were plotted against the averages of the two methods.

method can be utilized for the sensitive & wide linear detection of HBV quantification.



**Figure 3:** (A) Correlation; Comparison of paired quantitative result ( $R = 0.996$ ;  $P < 0.01$ ) between the two assays observed by linear regression equation & Pearson product moment correlation coefficient. (B) Level of agreement; In Bland-Altman plot, the mean difference between artus-DSP-QPCR and the reference method calculated  $-0.081$  log<sub>10</sub> IU/ml (SD  $0.158$  log<sub>10</sub> IU/ml) with limits of agreement of  $-0.391$  to  $0.229$  log<sub>10</sub> IU/ml.

As shown in Figure 3 B nearly all the differences of the paired viral loads were within the range of the mean difference, only two out of 26 values lies just outside the limit. The mean value of difference between these two groups was  $-0.081$  (log<sub>10</sub> IU/ml), with a SD of  $0.158$  log<sub>10</sub> IU/ml. The Bland-Altman analysis expects the values to cluster around the mean of the differences, and certainly within 2 standard deviations of the mean. Assuming the result differences was normally distributed in a 95% prediction interval, to accept the test method depends on the level of precision needed to the particular technology. Since the range of differences between the reference and test method is very low (i.e.  $< 2$  standard deviations of difference), hence this

## 5. Discussion

Hepatitis B virus infection counts with a wide range of clinical symptoms and as this infection have a heavy economic and social burden for both patients and society. Therefore, there is a need for wide range reliable markers to improve the management of this infection. Lower HBV DNA level is associated with a lower risk of a hepatocellular carcinoma (HCC) development than a higher level [5]. Since the viral load of HBV patients varies greatly, Q-PCR assays with a wide detection range are highly desirable. Available real-time PCR assay for HBV DNA quantization have diverse and not the low level of limit of detection as recommended. [4]

In this study, we verified the performance specifications of Real-time HBV detection of European Conformity (CE)-marked IVD-licensed artus-DSP assay for quantification of HBV DNA. The Clinical Laboratory Improvement Amendments (CLIA) specified that clinical laboratories should verify the manufacturer's performance specifications in the respective laboratory [26]. Testing of the performance characteristics, including analytical linear range, accuracy and reliability was performed. The research has demonstrated that the detection of low level of HBV DNA was essential in order to determine the necessity of antiviral treatment [19]. The improved detection sensitivity of HBV DNA detection assays is essentially dependent upon an increased sample input volume [25]. Therefore, a large volume of sample is advantageous for the preparation of increased amounts of DNA template. The amplification efficiency of Q-PCR with DNA prepared by DSP was satisfactory, demonstrating the DSP-based DNA extraction is compatible with Q-PCR. The retention of DNA by membrane is majorly affected by DNA size and pore size of filtration membrane [1]. As reported, the plasmid DNA (3.0 to 17 kbp) transmission significantly decreased with increasing pore size of the membrane, which indicated that the pore size played a key role in affecting DNA yield [16]. In this work, HBV DNA yield obtained from artus-DSP method for low viral titer & high viral titer were satisfactory, indicating HBV DNA can be effectively retained by column devices. The gain & loss of DNA amplification signal can be described by the adsorption of DNA to the consumable items & while handling process of repeated freeze-thaw cycles [14], however it was not found any significant difference between % yields obtained. From the results of performance evaluations, it appeared that the artus-DSP Q-PCR assay was valuable method. The detection range was demonstrated of  $11.8$  IU/ml to  $4 \times 10^8$  IU/ml, with a slope of  $-3.03$  to  $-3.50$  and  $R^2$  values of  $0.98$  to  $0.99$ , indicating the reliability of the artus-DSP method. [18]. The Bland-Altman analysis indicated no major difference between the two methods. The difference of the values obtained outside the limit could be due to repeated freeze-thaw of the samples [14]. Hence the results provided evidence for the performance of this assay that was compatible in analyzing plasma samples of different viral loads.

In conclusion, the artus-DSP Q-PCR assay is reliable and wide linear range quantification method. The method can be used for the sensitive detection of plasma HBV. This study finds it to be a powerful tool to achieve optimal monitoring of infection level and timely treatment advice. In addition, with its advantage in handling large volumes of sample, artus-DSP method holds the potential for dealing with low nucleic acids content.

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