

Several concerns about the primer design in the universal molecular beacon real-time PCR assay and its application in HBV DNA detection

Xiaomin Li · Yong Huang · Chen Song · Meiping Zhao · Yuanzong Li

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Abstract A universal hepatitis B virus (HBV) DNA detection kit is appealing for the worldwide diagnosis and monitoring of the treatment of different mutant types of hepatitis B virus. A sensitive and reproducible real-time PCR assay based on the universal molecular beacon (U-MB) technique was developed for the detection of HBV DNA in serum. The U-MB probe used in the assay has no interaction with the HBV DNA sequence. The U-MB technique not only reduced the cost of HBV detection but also had the potential for the development of a universal detection kit for different mutant HBV types and other DNA systems. To demonstrate its clinical utility, 90 serum samples were analyzed using the U-MB real-time PCR method. In the experiments we found that several crucial factors needed to be considered in the primer design, such as the avoidance of formation of severe primer–dimer and primer self-hairpin structure. With the optimized primer sets, satisfactory results were obtained for all the tested samples. We concluded that this assay would be an excellent candidate for a universal HBV DNA detection method.

Keywords Universal molecular beacon (U-MB) · Real-time PCR · HBV DNA · Primer design · Clinical analysis

Introduction

Hepatitis B virus (HBV) infects approximately 400 million people worldwide. HBV can cause pathology ranging from self-limited illness to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1]. The presence of hepatitis B surface antigen (HBsAg) in serum indicates hepatitis B virus infection, but does not provide information on the replicative state of the virus. Naturally occurring escape mutants of HBV with various mutations in the surface antigen gene could have no detectable HBsAg in serum [2]. Hepatitis B e antigen (HBeAg) has been considered a viral replication marker, but the precore point mutant HBVs cause HBeAg negative phenotype irrespective of their status of replication [3]. These HBV DNA positive individuals with aberrant HBV serology are infective and pose a risk, especially as blood donors or health care workers. Therefore, the measurement of HBV DNA in serum has become an important tool to identify individuals with viral replication, to monitor patients on therapy, and to predict whether antiviral therapy will be successful. During the past few years, real-time detection PCR (RTD-PCR) assays have been developed to overcome the problems of conventional PCR assays [4, 5]. Such real-time PCR assays are based on the fluorescent labeling of oligonucleotide probes that bind specially to the PCR amplicon and have afforded effective results [6–11]. Nevertheless some difficulties in the clinical use of these RTD-PCR assays are evident: because the fluorescent probes are specific for the target, they may not be very sensitive or accurate; furthermore, for different unknown mutant types the probe design would be troublesome; and the cost is really high. Therefore we introduced the universal molecular beacon (U-MB) RTD-PCR assay for HBV DNA. The U-MB [12] has no interaction with the target HBV DNA sequence, so it

X. Li · Y. Huang · C. Song · M. Zhao (✉) · Y. Li (✉)
The Key Laboratory of Bioorganic Chemistry & Molecular
Engineering and Institute of Analytical Chemistry,
College of Chemistry & Molecular Engineering,
Peking University,
Beijing 100871, China
e-mail: mpzhao@pku.edu.cn
e-mail: yzli@pku.edu.cn

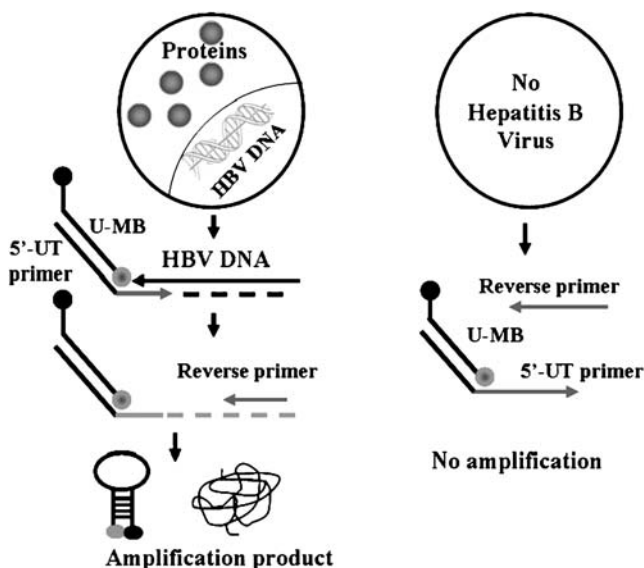


Fig. 1 Principle of the U-MB real-time PCR assay for the HBV DNA assay. In the HBV DNA system, the fluorescence quenching signal indicates the target amplification: first the 5'-UT primer extends, then the reverse primer extends and replaces the U-MB; finally the fluorescence is quenched. In the HBV DNA absent system, no target amplification takes place and fluorescence remains constant

can theoretically be applied in different mutant types and the cost would be decreased. Also, this U-MB assay has the potential for the development of a commercial kit for any clinical gene detection and only the primers will be designed in the target system by customers themselves. The principle of the U-MB real-time PCR assay has been described previously in detail [12]. Figure 1 illustrates the principle of detecting HBV DNA by the U-MB assay. Briefly, this technique is based on the concept that the 5'-universal-tailed (5'-UT) primer consists of two parts, one is the 5'-universal tail which specifically hybridizes to the U-MB and the other part hybridizes to target HBV DNA (target-specific part). During the amplification process, the U-MB is displaced by the extension of the reverse primer and the fluorescence is quenched, which accounts for the existence of target HBV DNA. To get reliable results, some concerns about the primer design in the system are addressed.

Experimental

Reagents and serum samples

The U-MB has a universal sequence of (AG)₁₀ in its loop part. The loop and one arm of the stem of the U-MB are complementary to the 5'-end of the 5'-UT primer. The U-MB is labeled with 6-carboxyfluorescein (FAM) at the 5'-end and 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) at the 3'-end. The sequence of U-MB is 5'-FAM-CCCGG(AG)₁₀CCGGG-DABCYL-3'. All the primers and their target gene sites are listed in Table 1. The underlined portions highlight the hybridization parts between or within the primers. Primers and U-MB were synthesized and purified by Sangon Company (Shanghai, China).

Ninety serum samples were collected from the Peking University Hospital and serum samples were divided into four groups based on serologic markers of HBV infection: group 1 consists of 11 serum samples which were HBeAg positive; group 2 consists of 34 serum samples which were HBeAb positive; group 3 consists of 10 serum samples which were HBsAg and HBcAb positive; group 4 consists of 35 serum samples which were HBsAg negative regardless of antibody status.

Serological assay

HBsAg, HBeAg, HBeAb, HBsAb, and HBcAb were assayed using a commercial enzyme-linked immunoassay-serum gold-labeled rapid cassette test (Aikang, Beijing, China) and the data were provided by Peking University Hospital.

DNA extraction

The serum samples were stored at -20 °C and used without any pretreatment. DNA was extracted from serum using a TIANamp Virus DNA/RNA Kit purchased from Tiangen Biotechnology Co. (Beijing, China). According to the manufacturer's instructions, 60 μL of DNA extraction

Table 1 Sequences of primers, target gene sites, and expected PCR product sizes

Primers	Sequence (5'-3')	Target sites
P1	(CT) ₁₀ CCGGGAGTTGGGGGAGGAGATTAG	Within the x and core gene
P2	GAAGTCAGAAGGCAAAAACG	232 bp
P3	(CT) ₁₀ CCGGGCTGGCCAAAATTCGCAGT	Within the surface antigen gene
P4	GTGCAGGTCTTGCATGGCCC	229 bp
P5	(CT) ₈ CTCTCCGGGCCTGCACCGAACATGGAGAG	Within the surface antigen gene
P6	ATATGATAAAAAGCCGCAGACAC	259 bp

Underlined portions show the hybridization parts between or within the primers

solution was obtained from 200 μL of each serum sample. The DNA solutions were stored at $-80\text{ }^\circ\text{C}$ until further use.

Real-time PCR

PCR amplification was performed with the U-MB and three different sets of PCR primers respectively. A 50- μL volume of reaction mixture for real-time PCR contained 1 \times reaction buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 0.1% Triton X-100), 0.4 mM dNTPs each (Tiangen Biotechnology Company, Beijing, China), 1.5 unit Vent_R (exo-) DNA polymerase (NEB, UK), 100 nM 5'-UT primer, 100 nM U-MB, and 160 nM reverse primer. The serum DNA extracted was thawed and 5 μL of it was directly used for each reaction. For the no template control (NTC), 5 μL of ddH₂O was used. The PCR amplification was carried out in a sealed tube in a Stratagene Mx3000p real-time PCR instrument (USA) with a program of initial denaturation 94 $^\circ\text{C}$ for 10 min, followed by 40 cycles of 94 $^\circ\text{C}$ denaturation for 50 s, 55 $^\circ\text{C}$ annealing for 30 s, 72 $^\circ\text{C}$ extension for 1 min, and finally 25 $^\circ\text{C}$ for 1 min to cool down for high fluorescence quenching efficiency. Fluorescence measurements were taken at the end of the cooling down segment. A parameter *C_{dt}* is used to indicate the cycle number at which the fluorescence quenching rate (*Q_f*) equals a threshold value of 5%. To demonstrate the accuracy of the method in quantification, DNA extracted solution of one HBeAg positive sample with moderate load of HBV DNA was created in a 5-log range by 1:10 serial dilution. *C_{dt}* values of each diluted sample were obtained. All the samples were measured in duplicate.

Results and discussion

Recent advances in real-time PCR have facilitated the development of sensitive detection of HBV DNA [13–15]; however, each of the cited papers reported different kinds of fluorescence probe in HBV DNA real-time PCR detection and there was little data about the repeated applications of the probes. For those reported probes, HBV DNA may become undetectable for different HBV mutants.

This paper reports an HBV DNA detection system using a U-MB real-time PCR assay. Theoretically, the U-MB has no interaction with the target sequences and the U-MB system proposed can be extended to any kind of real-time PCR system. This U-MB real-time PCR technique has been successfully applied in the point mutation detection [12]. However, in the HBV DNA detection system, three pairs of primer sets were designed for this assay and only one set employed was approved successfully. This will be discussed in detail below.

Concerns about primer design in the U-MB real-time PCR assay

The target-specific part of the three 5'-UT primers coupled with their corresponding reverse primers has been applied in HBV DNA detection with good detection results [7, 10, 16]. But in this method, when the target-specific part was incorporated into the 5'-UT primer, the three primer sets showed different amplification specificities (Fig. 2). With each set of primers, PCR was performed in the presence of HBV DNA template and in the absence of the template as no template control (NTC) for comparison. Upon analysis of the amplification curve, for the P1/P2 set, a fluorescence decrease was observed in the input of the HBV DNA template system with accumulating cycles, but not in the NTC system (Fig. 2a); for the P3/P4 set, the fluorescence decreased in both the input of the HBV DNA template system and the NTC system with accumulating cycles (Fig. 2b); for the P5/P6 set, the fluorescence decreased dramatically within the first several cycles in both systems (Fig. 2b). So only the P1/P2 set was suitable and therefore chosen for the statistical serum HBV DNA detection.

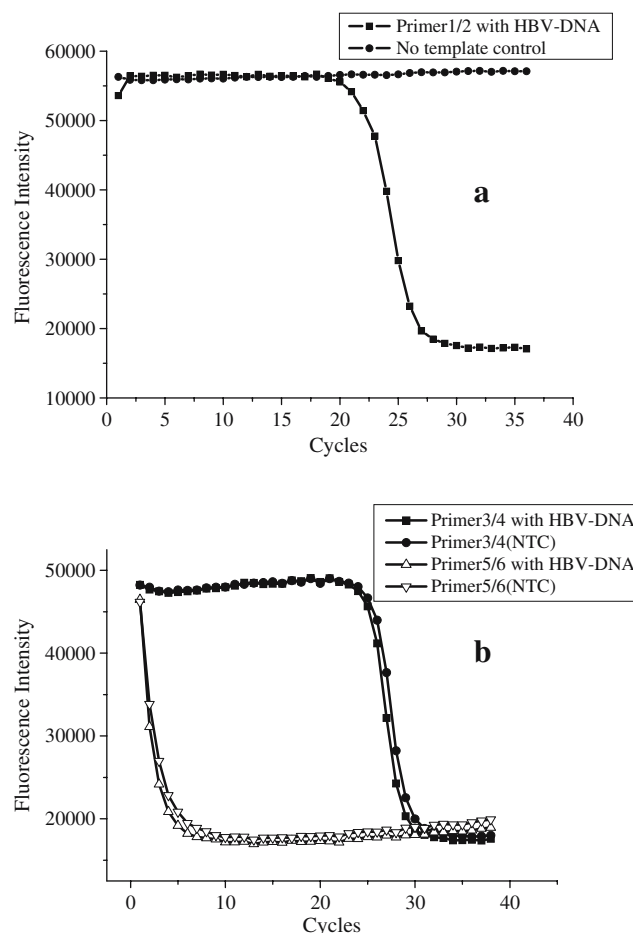


Fig. 2 Different primer sets using U-MB real-time PCR assay. Amplification plots of set P1/P2 (a), sets P3/P4 and P5/P6 (b)

In an attempt to clarify the reasons for the unexpected results of the two primer sets, further analysis was carried out. According to the agarose gel electrophoresis results of the PCR products in the no template system with the P3/P4 or P5/P6 set, the P3/P4 set approximated to a 60-bp oligonucleotide and the P5/P6 approximated to a 40- to 50-bp oligonucleotide (data not shown). This phenomenon proved that amplifications took place. To the best of our knowledge concerning the most common nonspecific amplification information, we concluded that there were primer–dimer or other nonspecific hybridizations appearing in the two primer sets. Based on careful inspection, for the P3/P4 set the 3'-end of P4 is complementary to P3 leading to the formation of primer–dimer; for the P5/P6 set, the P5 has a 3'-end with six bases complementary to the P5 itself forming hairpin hybridization structure. For the P3/P4 set, U-MB hybridizes with the 5'-UT primer (P3) which will further form the primer–dimer with the reverse primer (P4). When the extension occurs, the U-MB could be replaced and cause a significant drop of fluorescence in both the HBV DNA positive and negative systems, so a false positive result will be obtained. For the P5/P6 set, the U-MB hybridizes to the 5'-tail of the 5'-UT primer (P5) and the 3'-end of the P5 forms a six-bp hairpin structure that exists in large excess compared with hybridization to the target DNA. Once the reaction initiates, the DNA polymerase will extend the P5 from the 3'-end leading to the replacement of the U-MB in the first several cycles, and the fluorescence decreased dramatically to a plateau.

The different behaviors of the primer pairs suggest that the rational primer design is really crucial. Primer design has two essential and distinct phases: physical design and selectivity design. Selectivity refers to the ability of a primer to bind to a single location within the initial “pool” of DNA. Physical design of primers involves the consideration of factors such as GC content, annealing and melting temperatures, oligonucleotide structure, and primer length. In the U-MB assay, the primer length of the 5'-tailed primer does not comply with the rule. According to our results, there are two aspects of the primer design that are more crucial for the U-MB assay. One is the formation of primer–dimer, like the P3/P4 set, which is a product of duplex formation between two primers. Formation of this duplex not only reduces the concentration of free primers in the reaction solution, but also initiates the formation of nonspecific DNA product [17, 18]. Although the primer–dimer sometimes produces false positive results, the mechanism of its formation is unclear [19]. It is suggested that complementation of the 3'-end of the primers' segments initiates the annealing between the two primers at low temperature leading to the extension. To confirm this hypothesis, a contrastive thermal PCR program was set up as initial denaturation, followed by 30 cycles of denatur-

ation, annealing, and extension without cool down segment then followed by 20 cycles containing the cool down segment. The temperatures and the time were set as the same as described in the “Real-time PCR” section. In the first 30 cycles without cool down segment, the fluorescence quenching was delayed about ten cycles in the no template control (NTC) system, but no change in the HBV DNA positive system was observed (data not shown). This phenomenon illustrated that a low temperature (25 °C) in the cool down segment accelerated the formation of primer–dimer. Therefore, in the PCR program the low temperature step should be carefully used to prevent possible hybridization between primers. Since the concentrations of the primers are much higher than the other DNA existing in the system, it is suggested that hybridization between two primers with more than three continuous base pairs at the 3'-end of primer should be avoided.

Another aspect is the formation of the hairpin structure of the 5'-tailed primer at its 3'-end. Like the P5/P6 set, this 5'-UT primer (P5) probably does not hybridize to the template to amplify the target fragment and the 3'-end of P5 may extend to replace the U-MB in the first several cycles, so the fluorescence decreased dramatically to a plateau. It is also supposed that the low temperature stabilized the hairpin structure leading to the fluorescence decrease. However, when the cooling down segment in the original PCR program was removed the fluorescence of the system still decreased to a very low plateau (data not shown). This meant that the six-bp hairpin-stem was very stable, independent of the cool down segment. When the 3'-end hybridization part of the P5 was decreased to four bases, there was no fluorescence decrease in the 40 cycles with the NTC system, and it could be used in the HBV DNA detection (data not shown). This suggested that more than four-bp self-hairpin structures should be avoided and the no template control experiment is a critical part that could discern whether the primer could be used or not.

Selection of DNA polymerase

The DNA polymerase selection in the U-MB real-time PCR assay is also essential. Vent_r (exo-) DNA polymerase has been applied in this assay. It has strand displacement activity and lost the 5'-exonuclease activity [20]. The displacement activity ensures the quenching of the fluorescence when strand extension replaced the U-MB, while the lack of 5'-exonuclease activity guarantees the integrality of the U-MB since the 5'-end of the U-MB is complementary to 5'-UT. The Taq DNA polymerase [21, 22] is cheaper and is commonly used in PCR systems. However, it cannot be used in the present system due to the presence of the 5'-exonuclease activity. This activity will hydrolyze the 5'-end of U-MB, leading to the separation between the fluorophore

and the quencher from the beginning of the amplification. Thus fluorescence increase instead of fluorescence quenching could be observed (data not shown). The use of Vent_R (exo-) DNA polymerase in the U-MB real-time PCR assay increased the cost a bit compared with using the Taq DNA polymerase. The cost of Taq DNA polymerase per reaction was about US \$0.2, and the cost of Vent_R (exo-) DNA polymerase was about US \$0.4 per reaction. But in view of the overall cost of the assay, the cost increase due to the use of the Vent_R (exo-) DNA polymerase was much lower than using a new MB for different detections.

Correlations between HBV DNA levels and qualitative serological data in clinical samples

Ninety serum samples studied in this assay were divided into four groups based on serological markers of HBV infection: 11 HBeAg positive samples, 34 HBeAb positive samples, 10 HBsAg and HBeAb positive samples, and 35 HBsAg negative samples. We evaluated the correlation between the immunological HBV status and the U-MB real-time PCR results. The U-MB real-time PCR detected HBV DNA in 76% (42/55) of all the HBsAg positive samples. HBV DNA positive rate was 100% in HBeAg positive serum samples, indicating that expression of HBeAg strongly correlated with a positive result in the PCR assay. That suggests that HBeAg takes the vital role in the risk of transmission of HBV. HBV DNA positive rate was 76% in HBeAb positive serum samples, and negative

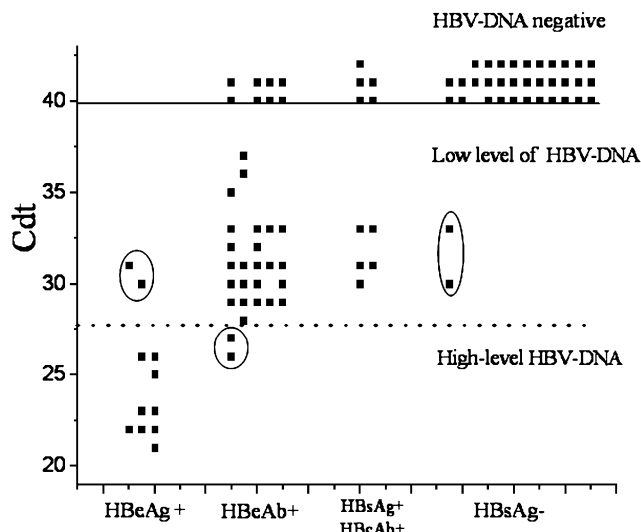


Fig. 3 *Cdt* level of the HBV DNA in HBeAg positive, HBeAb positive, HBsAg and HBeAb positive, and HBsAg negative serum samples. Results are divided into three parts: the dotted line indicates the cut off value to distinguish high level HBV DNA load from low level HBV DNA load, the solid line indicates the cut off value for the HBV DNA detected from that undetected by the U-MB real-time PCR assay

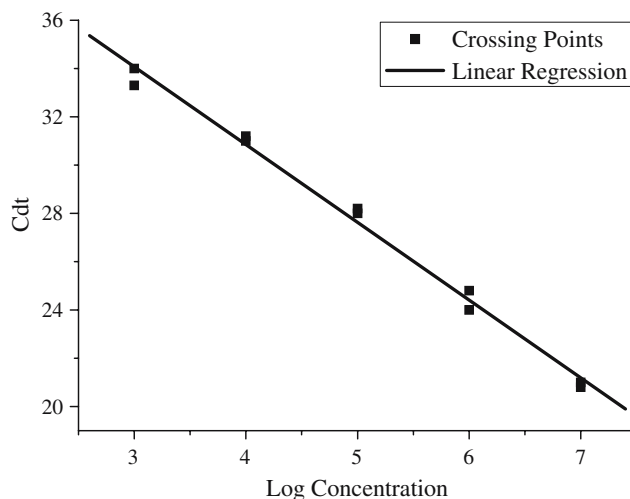


Fig. 4 Standard curve obtained from a 5-log range by 1:10 serial dilution of HBV DNA sample expressed as logarithm of concentration vs. the cycle in which the signal exceeded the threshold point (Q_f equals a threshold value of 5%). Each sample was run in duplicate and the results for each replicate are shown (■)

in the remaining 24% serum samples. The inability to detect HBV DNA in some HBsAg samples has been reported [23, 24]. It has been suggested that HBsAg in serum may arise from a low level of surface antigen expression stemming from defective virus or occurring from integrated HBV independent of viral replication [23, 25]. A negative U-MB real-time PCR result in an HBsAg positive sample also suggests a lack of HBeAg and viral replication. HBV DNA positive rate was 50% in HBsAg and HBeAb positive serum samples. Contrary to common opinion, the disappearance of HBsAg from the serum and the development of HBsAb may not reflect complete virological recovery from acute HBV infection. HBV DNA, and possible HBV virions, may be present in the serum. In our study, there were two such samples. This confirms that the real-time PCR for HBV DNA detection is very important and complementary to the immunological method.

Cdt indicates the quantitative level of HBV DNA. Lower *Cdt* values mean high numbers of copies of HBV DNA;

Table 2 Intraexperimental and interexperimental variability of the U-MB real-time PCR method for the HBV DNA detection

Sample no.	Reproducibility of Intra-assay			Reproducibility of Inter-assay		
	\bar{Cdt}	SD	CV (%)	\bar{Cdt}	SD	CV (%)
9	21.2	0.2	0.2	21.0	2.0	9.5
30	25.1	0.2	0.7	23.7	1.2	5.1
35	27.2	0.3	1.1	26.7	1.5	5.6
48	32.8	0.2	0.8	32.7	1.5	4.6
90	33.7	0.5	1.4	34.0	1.0	2.9

higher *Cdt* values mean low numbers of copies. Compared with the four groups of serum samples, they can be divided into three parts (Fig. 3): a high level of HBV DNA, a low level of HBV DNA, and below the detection limit. Among those HBV DNA positive results, the *Cdt* values of 82% (9/11) in HBeAg positive serum samples were lower than 27 cycles (2 samples excepted) and in HBeAb positive, HBsAg and HBcAb positive serum samples, the *Cdt* values of 95% were larger than 27 (2 of the HBeAb positive samples excepted). There were marked differences in HBV DNA levels between HBeAg positive serum samples and HBeAg negative serum samples (for those HBeAb positive or HBsAg positive). As expected, the HBeAg positive serum samples had markedly higher viral load (lower *Cdt*) than HBeAg negative serum samples. The two exceptions of the HBeAg positive serum samples have *Cdt* values higher than 27 and may get well from the HBV infection, the HBV DNA replication is slower but the HBeAg has not vanished. The two HBeAg negative but HBV DNA high load samples may represent precore variants which cannot be detected by the HBeAg immunology test. So, in fact the real-time PCR may prove superior to the immunological assay when HBeAg is chosen as a marker of potential infectivity because of its ability to identify patients with HBeAg precore mutant. Furthermore, the *Cdt* results of the quantitative level of the 90 serum samples suggested the potential capability of the developed U-MB real-time PCR assay in identification of the different sites of the HBV infection. The good specificity and sensitivity also showed this assay would be an excellent candidate for the quantification of hepatitis B virus in clinical application.

Accuracy and reproducibility study of the HBV DNA U-MB assay

The accuracy of the U-MB assay was determined by testing HBeAg positive sample. A linear relationship between the *Cdt* values and the logarithm of serial dilution numbers of the DNA extraction solution of the HBeAg positive sample is presented in Fig. 4 ($r > 0.99$).

Intraexperimental and interexperimental variability were studied by using five HBV DNA positive serum samples. These five samples were assayed in triplicate in one experiment for intraexperimental analysis and in three independent experiments for interexperimental analysis. The results of the average *Cdt* along with the standard deviation (SD) and percentage coefficients of variation (CV %) are shown in Table 2. The data proved good reproducibility of the U-MB real-time PCR assay compared with those previously reported results [26, 27]. The good linear relationship between the *Cdt* values and the logarithm of serial dilution numbers of the DNA extraction solution of the HBeAg positive sample proved that the

present method will be reliable for relatively quantitative measurement of the copies of HBV DNA.

Conclusion

By considerate design of the primer sets, we have developed a highly sensitive and reproducible real-time PCR assay for the detection of HBV DNA based on the U-MB technique. A major advantage is the U-MB, which theoretically could be used in various HBV mutant types, may alleviate the troublesome work of fluorescent probe design, and reduce the detection cost. The method furthermore has the potential to allow the development of a detection kit for HBV or other DNA based on the U-MB real-time PCR assay in the future.

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