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# A novel colorimetric PCR-based biosensor for detection and quantification of hepatitis B virus

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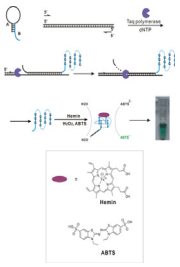
## HIGHLIGHTS

- We have established an operation-convenient and cost-effective way for detection HBV.
- This method has shown good performance with a broad range of linearity and high sensitivity.
- The results of detection is reported with macroscopic colorimetric signals.
- The strategy takes the key advantage of the TaqMan technology and use inexpensive DNA sensor.
- This approach can detect HBV DNA qualitatively or quantificationally.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Hepatitis B virus (HBV) can cause viral infection that attacks the liver and it is a major global health problem that put people at a high risk of death from cirrhosis of the liver and liver cancer. HBV has infected one third of the worldwide population, and 350 million people suffer from chronic HBV infection. For these reasons, development of an accurate, sensitive and expedient detection method for diagnosing, monitoring and assessing therapeutic response of HBV is very necessary and urgent for public health and disease control. Here we report a new strategy for detection of viral load quantitation of HBV based on colorimetric polymerase chain reaction (PCR) with DNAzyme-containing probe. The special DNAzyme adopting a G-quadruplex structure exhibited peroxidase-like activity in the presence of hemin to report colorimetric signal. This method has shown a broad range of linearity and high sensitivity. This study builds important foundation to achieve the specific and accurate detection level of HBV DNA with a low-cost and effective method in helping diagnosing, preventing and protecting human health form HBV generally all over the world and especially in developing countries.



## 1. Introduction

Hepatitis B virus (HBV) can cause a potential life-threatening liver infection and pathology ranging from self-limited illness to chronic hepatitis, cirrhosis and hepatocellular carcinoma [1]. Approximately 400 million people are infected globally by HBV and about one third of the world population has been infected once in their lives [2]. It is an important health hazard for the worldwide population and the most serious type of viral hepatitis.

Presently, serologic immunity and nucleic acid testing are the most commonly used methods for HBV diagnosis, prevention and treatment in clinical medicine [3]. The HBV serological tests such as

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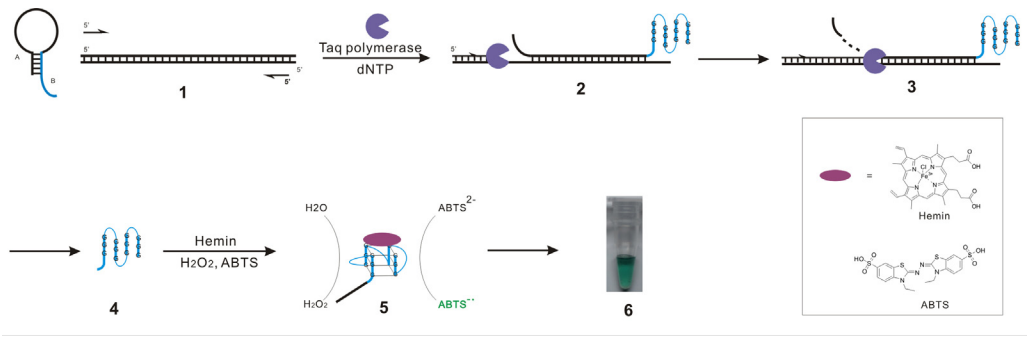


**Fig. 1.** Strategy of the colorimetric PCR-based detection of HBV. (1) Melting of double-stranded DNA and hairpin loop during PCR denaturation. (2) Hybridization of probe and primers to targeted ssDNA respectively during PCR annealing step. (3) Cleaving of the hybridized probe by DNA polymerase during primers extension. (4) Release of G-quadruplex sequence after cleavage by DNA polymerase. (5) Horseradish peroxidase-mimicking DNAzymes formation with the addition of hemin, reacting with ABTS and $H_2O_2$. (6) Colorimetric test result. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ELISA assays are used to distinguish acute, self-limited infections from chronic HBV infections and monitor vaccine-induced immunity by testing for a series of serological markers of HBV. But this method can only be used to qualitative diagnosis with low sensitivity and cause false-positive frequently. Besides these, nucleic acid detection of HBV DNA based on the PCR techniques is also used to quantify HBV viral load and track the effectiveness of therapeutic drugs [4]. With PCR amplification, specific segment of viral DNA can be magnified and a large number of copies of the target DNA sequence are produced across several orders of magnitude [5]. Compared to the traditional immunological assays, the nucleic acid assays show higher sensitivity, accuracy and specificity, which get rid of the evident limit for the accurate detection of HBV DNA in the progression of disease as well as the therapeutic effect. Because the nucleic acid assays can solve these problems which were brought about by immunological test and offer several obvious advantages, it is the most reliable diagnosis technique for detecting and monitoring HBV infection as well as assessing therapeutic response.

TaqMan technique in nucleic acid assays is one of the most popular methods used for detection of HBV DNA. The said technique uses a TaqMan probe containing a fluorophor and a quencher at both the ends respectively. In the single-stranded form, florescence is not detected because of Fluorescence Resonance Energy Transfer (FRET). When the PCR extension progresses, a DNA polymerase with the activity of 5′-3′ exonuclease leads to degrading the fluorophor-modified DNA probe that will anneal to the target strand [6]. But the main limitation of the TaqMan method is the high-cost modified flurogenic oligonucleotide probes and sophisticated equipments, which are restricted to well-equipped laboratories and less accessible to many ordinary users.

Herein, we report a novel approach for both qualitative and quantitation of HBV DNA based on our previous work [7]. This method takes the key advantage of the TaqMan technology (i.e., the elegant use of the 5′-exonuclease activity of Taq polymerase) [8], and applies a low-cost catalytic DNA molecular beacon as probe [9]. In the process of PCR amplification, Taq DNA polymerases cleave the probe and release a DNAzyme sequence [10–12], which has been embedded in the probe. After PCR amplification, the DNAzyme can form G-quadruplex and bind with hemin, possessing a peroxidase-like activity which catalyzes the oxidation of different substrates by $H_2O_2$ to generate either colorimetric or fluorometric signals [13–15].

## 2. Experimental

### 2.1. Materials

Hemin was purchased from Alfa Aesar; T4 polynucleotide kinase, Taq DNA polymerase, Taq buffer and dNTPs were purchased from TransGen Biotech (Beijing, China). UNG was purchased form Takara. FastStart Taq DNA polymerase was purchased from Roche. [γ-$^{32}$P] ATP was purchased from Furui Biological Engineering (Beijing, China). ABTS was purchased from Wolsen (Xi'an, China). $H_2O_2$ was purchased from Bodi Chemical Holding Co., Ltd. (Tianjin, China) and the oligonucleotides were synthesized from Sangon Biotech (Shanghai, China).

### 2.2. Apparatus

The detection of the absorbance produced by oxidized ABTS was performed with a Varioskan Flash (Thermo Scientific). The absorbance wavelength was 414 nm. About 40 readings with a 30 s interval were recorded. The PCR were performed on a C1000 thermal cycler (Bio-Rad).

### 2.3. Labeling reaction

Probe-HBV-2 probes and mark were 5′ end-labeled with [γ-$^{32}$P] ATP and T4 polynucleotide kinase to a specific activity of 10 units $\mu L^{-1}$.

### 2.4. PCR for colorimetric detection and $^{32}$P labeled probe

The PCR was performed in 50 μL volume containing 2 μL HBV DNA template, 1.5 μM forward primer, 1 μM reverse primer, 0.6 μM probe, 1.2 mM dNTPs (600 μM dUTP, 200 μM dATP, 200 μM dCTP, 200 μM dGTP), 5 units of FastStart Taq DNA polymerase, 1 unit of UNG, 0.5 mM $MgCl_2$, 1× Taq polymerase buffer (15 mM tris-HCl(pH 8.2), 30 mM KCl, 5 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.002% BSA). The PCR procedure was as follows: 20 °C for 10 min; 95 °C for 2 min followed by 40 cycles of 94 °C for 30 s and 60 °C for 90 s.

### 2.5. Clinical serum sample extraction and the serum calibration curve preparation

The DNA was extracted form the HBV infection and normal clinical serum samples from HBV patients or normal population with QIAamp DNA Blood Mini Kit (Qiagen). $10^8$ copies $mL^{-1}$ clinical serum samples were chosen to dilute with negative serum by decuple to 10 copies successively. These samples were extracted with QIAamp DNA Blood Mini Kit (Qiagen) to build the calibration curve preparation.

### 2.6. Colorimetric assay

NaCl at the concentration of 400 mM was added to the PCR products and heated at 94 °C for 5 min and incubated at room temperature for 30 min afterwards. Finally, hemin (2 μM), ABTS

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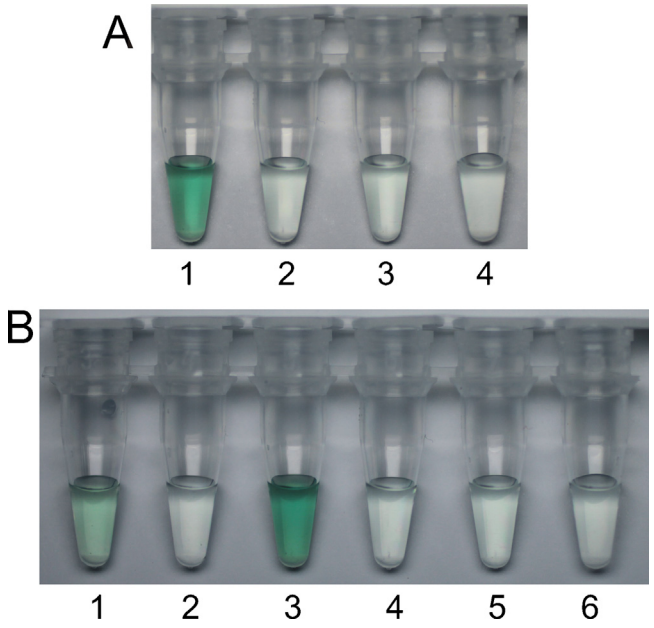


**Fig. 2.** Colorimetric detection results with different probes. (A) Colorimetric detection of HBV genome with probe-HBV-1. (1) PCR reaction containing target DNA by using probe-HBV-1 with amino modifier at 3′-end. 2) Negative control: PCR reaction containing no target DNA by using probe-HBV-1 with amino modifier at 3′-end. 3) PCR reaction containing target DNA by using probe-HBV-1 without modification. 4) Negative control: PCR reaction containing no target DNA by using probe-HBV-1 without modification. (B) Colorimetric PCR reaction with three different probes. Tube 1: probe-HBV-1 with HBV DNA targets; Tube 2: probe-HBV-1 without targets; Tube 3: probe-HBV-2 with targets; Tube 4: probe-HBV-2 without targets; Tube 5: probe-HBV-3 with targets; Tube 6: probe-HBV-3 without targets.

(2.4 mM) and $H_2O_2$ (2 mM) were added, followed by absorbance detection of $ABTS^-$ at 414 nm immediately.

## 3. Results and discussion

### 3.1. The principle of colorimetric detection

The principle of our detection strategy is depicted in Fig. 1. The DNA probe was designed to form a hairpin structure at room temperature. After denaturation step of PCR, the concentration of probes is much higher than that of HBV dsDNA templates, so the chance for probe to hybridize with the targeted HBV single strand DNA is much higher than that of its complementary single strand DNA. Then, in the annealing step of PCR, the loop domain of the probe hybridizes to the conserved region of HBV genome, which will be amplified by two primers. The stem part of the probe contains blocking sequence A (black) and a DNAzyme sequence B (blue) that could report the detection result through oxidation reaction [16–20]. The blocking sequence A is used to prohibit DNAzyme sequence B fold into catalytic G-quadruplex structure at room temperature to decrease the background of colorimetric assay [7]. As the PCR proceeding, the probe formed a stable duplex with target sequence of HBV genome and Taq DNA polymerase with 5′-3′ exonuclease activity can cleave the obstruction of part A, consequently released the part-B. After PCR amplification, the cleaved fragment of the probe could form a stable G-quadruplex structure and bind with hemin to exhibits the peroxidase-like activity to oxidize ABTS in the presence of $H_2O_2$, leading to produce a colorimetric green product.

### 3.2. One-step PCR

According to our previous procedure, this method required two consecutive PCR steps to achieve the successful detection of a target DNA, which included the amplification of target sequence and subsequently the cleavage of probe [7]. In our pilot experiment, hairpin HBV probe (probe-HBV-1) constructed according to our previous work was added into the PCR reaction mixture from the beginning. However, no colorimetric change was observed finally (Fig. 2A and Table S1). We speculated that the catalytic beacons probe could be extended from the 3′ end by DNA polymerase unexpectedly, which could interfere the forming of right structure of DNAzyme and the following colorimetric reaction as well. Nearly the same probe, but with amino modifier at the 3′ end (probe-HBV-1, Table S2), has been introduced into our PCR amplification because the modification on the probe could prohibit the undesired extension caused by the DNA polymerase. A PCR containing the modified probe was performed with or without template as positive and negative control respectively, which was followed by the addition of hemin, $H_2O_2$, and ABTS in the end. As illustrated in Fig. 2A, the positive control reaction turned into green product that can easily be discriminated by naked eyes, while the negative control remained colorless. Therefore, the 3′ amino modifier on probe could obviously improve the detection result and was generally applied in our following experiments.

### 3.3. Evaluation and selection of different HBV probes

Three different hairpin probes (probe-HBV-1, probe-HBV-2and probe-HBV-3) and corresponding primers were designed against the different conserved regions of the HBV genome including overlapping genes encoding X-protein, S gene and X gene region [2,21,22] (Table S2, S3 & S4). Under the optimized PCR amplification conditions of corresponding probes, the different colorimetric results by using those three pairs of probes were turned out (Fig. 2B). It was demonstrated that probe-HBV-2 for S gene region of HBV genome exhibited the best colorimetric result comparative to that of other probes. Furthermore, this region of HBV genome is rather conserved in different genotypes of HBV strains and can be extensively and accurately used to detect all HBV genotypes. Therefore, the probe-HBV-2 was proved to be the best fitted probe for HBV detection based on our method. The selected sequences are:

Primer-HBV-Forward-2:CCTGGTTATCGCTGGATGTGT, Primer-HBV-Reverse-2:GGACAAACGGGCAACATACCTT, Probe-HBV-2: CCCTACCCATTCATCCTGCTGCTATGCCTCATCTTCTTTGGG-TAGGGCGGGTTGGGAAA-$NH_2$

### 3.4. $^{32}P$ labeled selected probe PCR reaction

To verify the probe had been cleaved by DNA polymerase successfully, a 5′ $^{32}P$ labeled probe -HBV-2 was added into the PCR reaction. The PAGE analysis of the PCR reaction was illustrated in Fig. 3. As we expected, in the positive control containing HBV genome, a 13-nt fragment was released from probe-HBV-2 during the PCR reaction (lane 2, Fig. 3). Comparatively, in the negative control, without HBV target, the probe displayed no cleavage (lane 3, Fig. 3). When Taq DNA polymerase encounters the hybridized complementary duplex between the target site of HBV genome and the probe-HBV-2, it cleaves the probe at the second nucleotide of stable double-stranded domain. Then, a 13-nt $^{32}P$-labeled fragment from probe-HBV-2 was released. The result is consistent with the previous research about 5′-3′ exonuclease activity of DNA polymerase [8]. So these results prove that the final colorimetric result of positive control was caused by the cleavage of probe to release enzymes as reporter in the process of PCR amplification.

### 3.5. Optimization for conditions of PCR reaction and detection

To apply this method conveniently in clinical detection of HBV DNA, we combine our method with uracil *N*-glycosylase (UNG)

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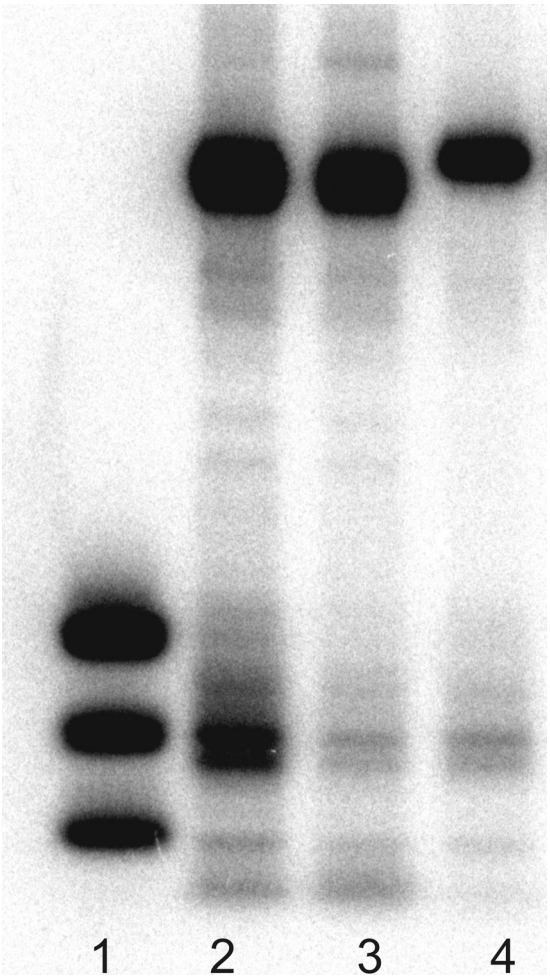


**Fig. 3.** PAGE analysis of the cleavage of isotope-labeled probe-HBV-2 in PCR amplification. Lane 1, from top to bottom 17-nt, 13-nt, 9-nt DNA marker; lane 2, PCR

treatment system, which is most commonly used method to prevent the contamination caused by PCR amplicon [23]. To overcome the shortage of low-efficiency PCR amplification caused by dUTP, we optimized the PCR conditions, such as the PCR temperature, cycles, times, buffer constitution, the quantity of enzyme, concentration of sensor and primer and concentration of ion. After a large number of experiments, we finally confirmed the optimal PCR conditions as follows: 50 μL volume reaction containing 2 μL HBV DNA template, 1.5 μM forward primer, 1 μM reverse primer, 0.6 μM probe, 1.2 mM dNTPs (600 μM dUTP, 200 μM dATP, 200 μM dCTP, 200 μM dGTP), 2.5 units of FastStart Taq DNA Polymerase, 1 unit of UNG, 0.5 mM $MgCl_2$, 1× Taq polymerase buffer (15 mM tris-HCl (pH 8.2), 30 mM KCl, 5 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.002% BSA) with 20 °C for 10 min; 95 °C for 2 min followed by 40 cycles of 94 °C for 30 s and 60 °C for 90 s. We have verified the high efficient PCR amplification under this reaction condition with different concentration of samples (Fig. S1).

### 3.6. Colorimetric detection and quantification of HBV DNA

In this strategy, after PCR reaction, hemin, ABTS and $H_2O_2$ were added and the colorimetric signals of different concentrations of HBV DNA were recorded (Fig. 4A). We optimized the colorimetric detection condition, such as the concentration of NaCl, hemin, ABTS and $H_2O_2$, reaction and detection time and so on. Finally, the optimal detection condition was picked out as: by adding 400 mM NaCl into buffer after PCR amplification, heating at 94 °C for 5 min and incubation at room temperature for 30 min afterwards. Then, hemin (2 μM), ABTS (2.4 mM) and $H_2O_2$ (2 mM) were added into reaction mixture. Under the optimized condition, samples containing different concentration of HBV DNA (from 10 to $10^7$ copies) has been detected, the colorimetric results revealed a gradual increase of color intensity from light to dark green, while the negative control containing no HBV target remained colorless (Fig. 4A). As few as 10 copies of HBV DNA could be qualitatively detected with naked eyes in 50 $\mu L^{-1}$ reaction mixture. The method represents a simply visual way for HBV DNA detection, which refrains from the advanced equipments and harsh detection conditions. With the aid of simple UV-spectrometer of microplate-reader, more accurate quantitative detection could be realized. A time-dependent optical absorption changes (414 nM) were recorded (experiments were conducted in triplicate), and the relationship between different concentrations of HBV DNA and absorbance was studied (Fig. 4B). The optical density was proportional to the concentration of HBV DNA over the range of 10–$10^7$ copies. The inset is a calibration curve with colorimetric intensity data at 414 nm obtained from the spectrum, corresponding to different concentrations of HBV DNA (the average values of triplicate data were used for plotting at 3 min). The calibration curve revealed that there was a good linear relationship between absorbance and the concentration of HBV DNA with a correlation coefficient of 0.9788, which showed linear dynamic ranges from 10 to $10^7$ copies. In our work, we tested the detection limit from $10^7$ to 10 copies by serial dilutions, and the 10 copies is the lowest concentration in samples that we could dilute and reproducible detected. So a minimum detection for 10 copies of HBV DNA was achieved based on our method. Moreover, this method showed good reproducibility (Fig. S2) and could be modified into fluorometric version by using tyramine as the substrate of DNAzyme reporter [24,25] (Fig. S3).

### 3.7. Comparation with TaqMan assay

TaqMan assay is the most advanced technique for HBV detection, which is widely used in clinical diagnose, so we compared our colorimetric assay with the quantitative TaqMan PCR assay. Seven different concentrations of clinical HBV serum samples and seven negative clinical serum samples were measured with the TaqMan method. Then we applied our colorimetric assay to detect the same clinical sample in triplicate, meanwhile consecutive serum samples ranging from $10^1$ to $10^8$ copies $mL^{-1}$ were determined in triplicate to record the time-dependent optical absorption changes (Fig. 5A) and the calibration curve which used the average values of triplicate data was obtained plotting at 3 min (Fig 5B). It showed a good linear relationship between absorbance and the concentration of HBV DNA. The correlation coefficient reached 0.9822 and their formulation was $FI = 0.0731\ \lg(C_{HBVDNA}) + 0.0319$. By the formulation, the $\lg(C_{HBVDNA})$ of the clinical serum sample was calculated comparing with the value of TaqMan assay (Table S7). The results revealed the excellent proximity of estimated values in curve showed the correlation between the two assays was very high, whose correlation coefficient attained 0.913 (Fig. 6). Besides, the other negative clinical samples determined by TaqMan assay were also negative measured in triplicate by our colorimetric method, which failed to produce a detectable PCR product (Table S8).

This detection method described here appears to have several obvious features and advances as follows: (1) Comparing to TaqMan assay based on sophisticated real-time PCR

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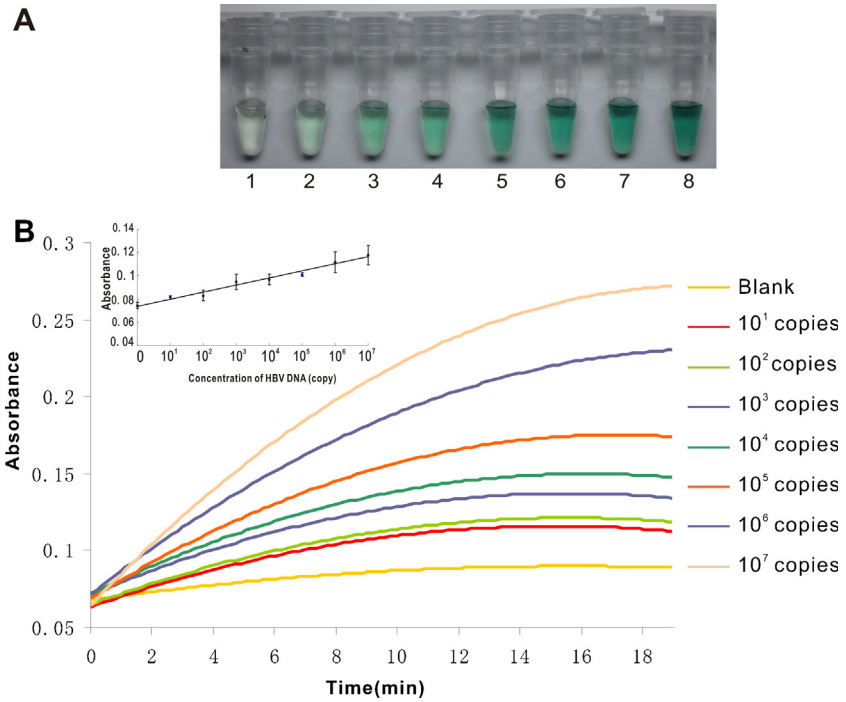


**Fig. 4.** Colorimetric detection of HBV DNA. (A) Photograph of the colorimetric detection of different concentration of HBV DNA: Tube 1, negative control (without HBV DNA); Tubes 2–8 containing 10, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ copies of HBV DNA, successively. (B) Time-dependent colorimetric detection of different concentrations of HBV DNA. The inset was a calibrated curve of the average absorbance (414 nm) at 3 min plotted against the number of HBV DNA. The solid line indicates linear least squares fitting between 10 and $10^7$ copies of HBV DNA, and their formulation is FI = 0.0061 lg($C_{HBVDNA}$) + 0.0678 ($R^2$ = 0.9788). The error bars were determined by standard deviation (SD) of the triplicate data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

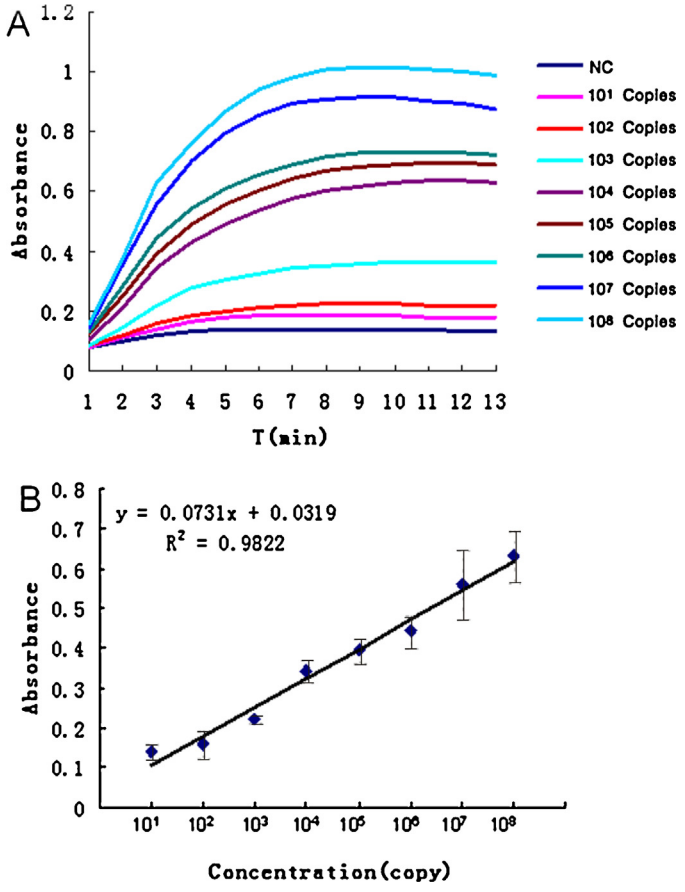


**Fig. 5.** Calibration curve establishment of HBV serum samples. (A) Time-dependent colorimetric detection of different concentrations of HBV DNA. (B) Calibrated curve of the average absorbance (414 nm) at 3 min plotted against the number of HBV DNA. The solid line indicates linear least squares fitting between 10 and $10^8$ copies of HBV DNA, and their formulation is FI = 0.0731 lg($C_{HBVDNA}$) + 0.0319 ($R^2$ = 0.913). The error bars were determined by standard deviation (SD) of the triplicate data.

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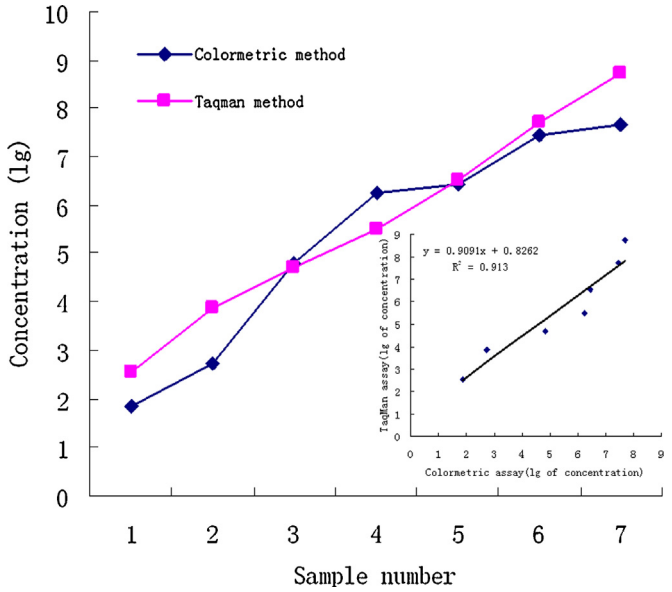


**Fig. 6.** Comparison of colorimetric and TaqMan assay. (A) Serum samples of consecutive concentration were measured by colorimetric and TaqMan assay. Red points represent the values of TaqMan assay and blue points represent the values of colorimetric assay. The inset was the comparison of the values by colorimetric and TaqMan assay. *X*-axis represents the lg value of concentration by colorimetric assay and *Y*-axis represents the lg value of concentration by TaqMan assay. The correlation coefficient is 0.913. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

machine and expensive fluorescence-modified probe, our method require only ordinary PCR machine and no fluorescence-modified probe to achieve accurate genetic detection. Therefore, the cost of this method would be much cheaper than that of TaqMan assay. (2) The colorimetric qualitative detection can be discriminated by naked eyes directly without any help of other sophisticated equipments. Any laboratory equipped with a normal PCR machine is enough to carry out the colorimetric detection without the limit of advanced laboratory-setting. Besides, flexibility of reported ways could be chosen which can be detected by colorimetric or fluorescent results for different detection purposes. (3) In contrast to TaqMan assay, our method revealed the comparable sensitivity, specificity with similar detection time. Moreover, the colorimetric based assay is more robust in HBV DNA diagnosis, which implies the future application of our method in clinical detection.

## 4. Conclusion

At present, the HBV genome detections mainly depend on real-time PCR by using non-specific fluorescent dyes that intercalate with any double-stranded DNA (SYBR Green and YOYO-114) or sequence-specific DNA probes like TaqMan, but both of them have their own disadvantages and limits. Though fluorescent dyes are popular and affordable in developing country, this non-sequence-specific method will cause unspecific and false positive detection results. The sequence-specific approach represented by the TaqMan method shows high accuracy and specificity. But this method requires fluorophore-labeled oligonucleotide probes and sophisticated real-time PCR machine, which is less accessible to ordinary users, particularly in developing countries. We have developed the novel colorimetric approach which can circumvent restrictions of those two methods and keep the advantages of them, namely accuracy and cost-effective. This method can not only achieve the favorable detection level for HBV DNA specifically, sensitively and accurately, but also break through the limitation of expensive equipments and reagent of the existing method. Furthermore, the UNG system was applied to prevent the contamination caused by the PCR amplicon that the setting-limited laboratory still can use the method. Therefore, with the high infection of HBV all over the world, we wish our method could make a contribution to popularizing the HBV DNA diagnosis especially in developing countries for the better preventing, monitoring and protecting human health form HBV as well as instructing the treatment of HBV infection.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.05.032.

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