# Chapter 22

# A Novel Colorimetric PCR-Based Biosensor for Detection and Quantification of Hepatitis B Virus

**Li Yang, Mei Li, Feng Du, Gangyi Chen, Afshan Yasmeen, and Zhuo Tang**

## Abstract

Hepatitis B virus (HBV) can cause viral infection that attacks the liver and it is a major global health problem that puts 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 an 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 all over the world, and especially in developing countries.

**Key words** Hepatitis B virus, Molecular diagnosis, Biosensor, G-quadruplex, DNAzyme, Peroxidase-like activity, Colorimetric PCR

## 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 ELISA assays are used to distinguish acute, self-limited infections from chronic HBV infections and monitor vaccine-induced

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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 are 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 fluorophore 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 fluorophore-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 equipment, which are restricted to well-equipped laboratories and less accessible to many ordinary users.

G-quadruplexes are higher-order DNA and RNA structures formed from G-rich sequences that are built around tetrads of hydrogen-bonded guanine bases. The criteria for a potential quadruplex sequence is restricted to: $G_{3-5}N_{L1}G_{3-5}N_{L2}G_{3-5}N_{L3}G_{3-5}$, where $N_{L1-3}$ are loops of unknown length, within the limits $1 < N_{L1-3} < 7$ nt. DNAzymes are catalytic single-stranded deoxyribonucleic acids that are obtained through in vitro selection. The DNAzyme we use has been found to exhibit peroxidase-like activity by forming G-quadruplex structure and binding with hemin. In recent years, this DNAzyme has been used to design various colorimetric or chemiluminescent assays. 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 Materials

### 2.1 Preparation of $^{32}$P Labeled Probe

1. T4 polynucleotide kinase (10 U/μL; TransGen Biotech, Beijing, China).
2. [γ-$^{32}$P] ATP (Furui Biological Engineering, Beijing, China).
3. Probe-HBV-2 (Sangon Biotech, Shanghai, China) is dissolved in sterile water.

### 2.2 Clinical Serum Sample Extraction

1. QIAamp DNA Blood Mini Kit (Qiagen).
2. HBV infection clinical serum samples (from HBV patients).
3. Normal clinical serum samples (from normal population).

### 2.3 Amplification

1. HBV DNA template (the DNA is extracted from the HBV infection clinical serum samples) is dissolved in sterile water, stored at −20 °C.
2. Primers (forward primer: 1.5 μM, reverse primer: 1 μM; Sangon Biotech, Shanghai, China) are dissolved in sterile water, stored at −20 °C.
3. Probe ($^{32}$P labeled, 0.6 μM; Sangon Biotech, Shanghai, China) is dissolved in sterile water, stored at −20 °C.
4. 1.2 mM dNTPs (600 μM dUTP, 200 μM dATP, 200 μM dCTP, 200 μM dGTP; TransGen Biotech, Beijing, China).
5. FastStart Taq DNA polymerase (5 U; Roche).
6. Taq DNA polymerase (5 U; TransGen Biotech, Beijing, China).
7. UNG (1 U; Takara).
8. $MgCl_2$ (0.5 mM).
9. 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; TransGen Biotech, Beijing, China).
10. C1000 thermal cycler (Bio-Rad).

### 2.4 Colorimetric Detection

1. 400 mM NaCl solution is prepared by dissolving 0.2340 g NaCl in 10 ml of ultrapure water.
2. Hemin (Alfa Aesar) is dissolved in DMSO.
3. ABTS (2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)) (Wolsen, Xi'an, China) is dissolved in ultrapure water.

4. $H_2O_2$ (Bodi Chemical Holding Co., Ltd., Tianjin, China) is diluted in ultrapure water.
5. Varioskan Flash ($\lambda = 414$ nm, 40 readings with a 30 s interval are recorded; Thermo Scientific).

### 2.5 TaqMan Assay

1. Hepatitis B Viral DNA Quantitative Fluorescence Diagnostic Kit (48 Tests; Sansure Biotech, Changsha, China).
2. Real-Time PCR System (Thermo Scientific).

## 3 Methods

### 3.1 The Principle of the Colorimetric Detection

1. The principle of our detection strategy is depicted in Fig. 1. The DNA probe containing three parts of sequences (A, B, C) is 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 (part C) 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

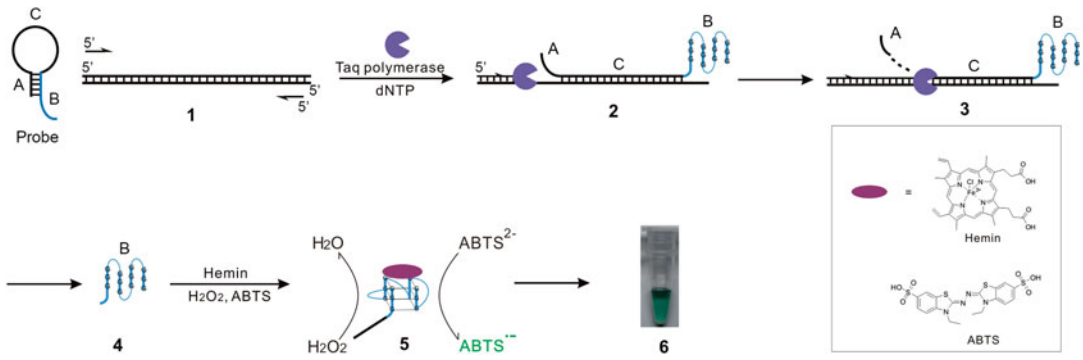


**Fig. 1** Strategy of the colorimetric PCR-based detection of HBV. A part of the probe is the blocking sequence, B represents the sequence for DNAzyme forming G-quadruplex, loop C is the complementary sequence of the conserved region of HBV genome. (*1*) Melting of double-stranded DNA and hairpin loop 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

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. With the exception of the HBV sequence, the blocking sequence and the sequence for DNAzyme forming G-quadruplex can be used for detection of other targets.

### 3.2 DNA Extraction from Clinical Serum Sample

1. Extraction of DNA from the HBV infection and normal clinical serum samples from HBV patients or normal population is carried out using QIAamp DNA Blood Mini Kit as described by the manufacturer (Qiagen).

### 3.3 The Serum Calibration Curve Preparation

1. $10^8$ copies/mL clinical serum samples are chosen to dilute with negative serum by decuple to ten copies successively. These samples are extracted with QIAamp DNA Blood Mini Kit (Qiagen) to build the calibration curve.

### 3.4 Manual Design of Probe and Primers

1. According to our previous procedure, this method requires two consecutive PCR steps to achieve the successful detection of a target DNA, which include the amplification of target sequence and subsequently the cleavage of probe [7]. In our experiment, three different hairpin probes (probe-HBV-1, probe-HBV-2, and probe-HBV-3) and corresponding primers are designed against the different conserved regions of the HBV genome including overlapping genes encoding X-protein, S gene, and X gene region.
2. Under the optimized conditions, Fig. 2 shows that probe-HBV-2 for S gene region of HBV genome exhibits 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 has been 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: CCCTACCCA TTCATCCTGCTGCTATG CCTCATCTTCTT **TGGGTAGGG** **CGGGTTGGGAAA** -$NH_2$ (*see* **Note 1**).

   The sequence marked with box represents the stem part of Probe-HBV-2. Sequence in the second box would be closed

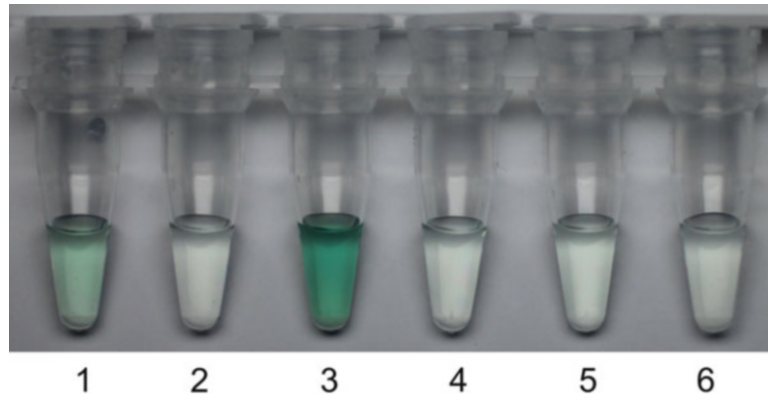


**Fig. 2** Colorimetric PCR reaction with three different probes. *Tube 1*: probe-HBV-1 with HBV DNA targets; *tube 2*: probe-HBV-1 without targets; *tube 3*: probe-HBV-2 with targets; *tube 4*: probe-HBV-2 without targets; *tube 5*: probe-HBV-3 with targets; *tube 6*: probe-HBV-3 without targets

at room temperature. The underlined sequence could anneal to S gene region of HBV genome and is the loop domain of the probe. Sequence for DNAzyme forming G-quadruplex is shown in boldface.

### 3.5 One-Step PCR

1. Thaw 10× Taq polymerase buffer, dNTPs, and primers.
2. The PCR is performed in 50 μL volume containing 2 μL HBV DNA template, 1.5 μM Primer-HBV-Forward-2, 1 μM Primer-HBV-Reverse-2, 0.6 μM Probe-HBV-2, 1.2 mM dNTPs (600 μM dUTP, 200 μM dATP, 200 μM dCTP, 200 μM dGTP), 5 U of FastStart Taq DNA polymerase, 1 U of UNG (*see* **Note 2**), 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).
3. The negative control is prepared without HBV DNA.
4. The amplification conditions are 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.

### 3.6 $^{32}$P Labeled Selected Probe PCR Reaction

1. To verify the probe had been cleaved by DNA polymerase successfully, Probe-HBV-2 and mark are 5′ end-labeled with [γ-$^{32}$P] ATP. The reaction mixture containing oligonucleotides with 10 μCi [γ-$^{32}$P] and 10 U of T4 polynucleotide kinase is incubated for 1 h at 37 °C for DNA phosphorylation.
2. The labeled product is purified by 10% denaturing PAGE.
3. The 5′ $^{32}$P labeled probe-HBV-2 is added into the PCR reaction.
4. The PCR product is used for PAGE analysis. As shown in Fig. 3, in the positive control containing HBV genome, a

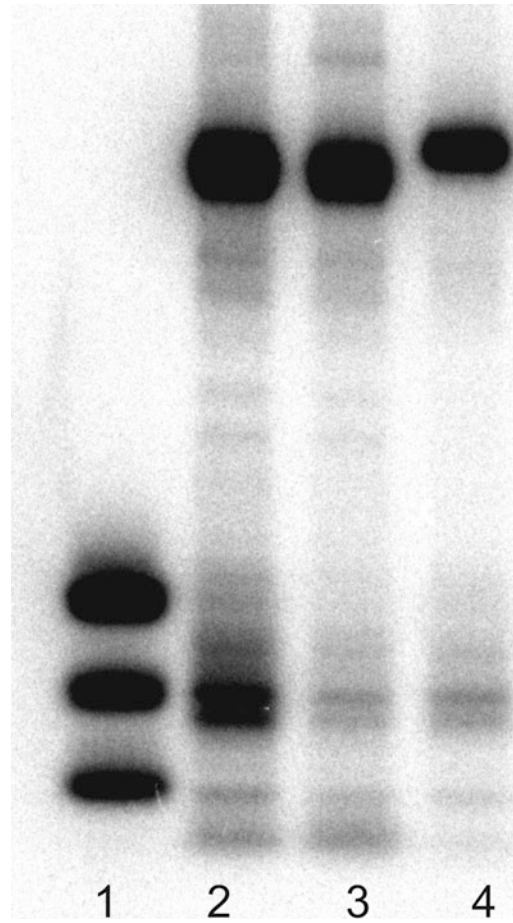


**Fig. 3** PAGE analysis of the cleavage of isotope-labeled probe-HBV-2 in PCR amplification. *Lane 1*, from top to bottom 17-nt, 13-nt, 9-nt DNA marker; *lane 2*, PCR reaction containing isotope-labeled probe-HBV-2 and HBV DNA targets; *lane 3*, negative control: PCR reaction without HBV DNA targets; *lane 4*, $^{32}$P-labeled probe-HBV-2 as marker

13-nt fragment is 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 is released. The result is consistent with our previous research about 5′–3′ exonuclease activity of DNA polymerase. So these results prove that the final colorimetric result of positive control is caused by the cleavage of probe to release enzymes as reporter in the process of PCR amplification.

### 3.7 Colorimetric Detection

1. NaCl at the concentration of 400 mM (*see* **Note 3**) is added to the PCR products.
2. The reaction mixture is heated at 94 °C for 5 min.
3. The reaction mixture is incubated at room temperature for 30 min.
4. Hemin (2 μM), ABTS (2.4 mM), and $H_2O_2$ (2 mM) (*see* **Note 4**) are added successively.
5. Colorimetric signals of different concentrations of HBV DNA are recorded (Fig. 4a). Samples containing different concentrations of HBV DNA (from 10 to $10^7$ copies) has been detected, the colorimetric results reveal a gradual increase of color intensity from light to dark green, while the negative control containing no HBV target remained colorless. As few as ten 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 equipment and harsh detection conditions.

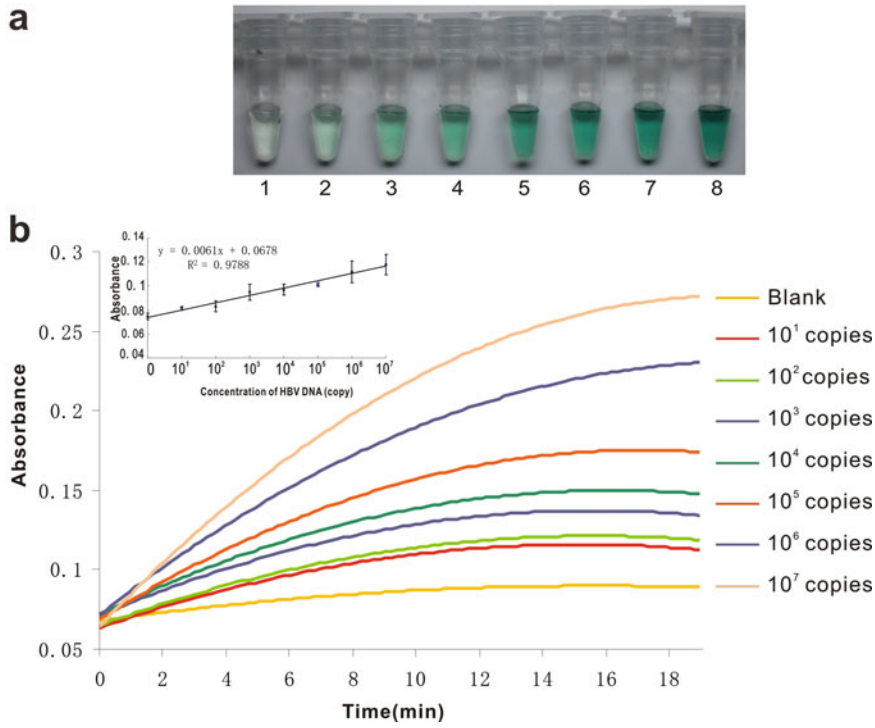


**Fig. 4** Colorimetric detection of HBV DNA. (**a**) Photograph of the colorimetric detection of different concentration of HBV DNA: *tube 1*, negative control (without HBV DNA); *tubes 2–8* containing 10, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ copies of HBV DNA, successively. (**b**) Time-dependent colorimetric detection of different concentrations of HBV DNA. The inset is 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 correlation coefficient is 0.9788. The error bars were determined by standard deviation (SD) of the triplicate data

6. Absorbance detection of $ABTS^{-}$ is performed at 414 nm. About 40 readings with a 30 s interval are recorded. With the aid of simple UV-spectrometer of microplate-reader, more accurate quantitative detection can be realized. A time-dependent optical absorption change (414 nm) is recorded (experiments are conducted in triplicate), and the relationship between different concentrations of HBV DNA and absorbance is studied (Fig. 4b). The optical density is 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 are used for plotting at 3 min). The calibration curve reveals that there is a good linear relationship between absorbance and the concentration of HBV DNA with a correlation coefficient of 0.9788, which shows linear dynamic ranges from 10 to $10^7$ copies. We test the detection limit from $10^7$ to 10 copies by serial dilutions, and the ten copies is the lowest concentration in samples that we can dilute and detect reproducibly. So a minimum detection for ten copies of HBV DNA is achieved based on our method.

### 3.8 Comparation with TaqMan Assay

1. TaqMan assay is the most advanced technique for HBV detection, which is widely used in clinical diagnosis, so we compare our colorimetric assay with the quantitative TaqMan PCR assay. Seven different concentrations of clinical HBV serum samples and seven negative clinical serum samples are measured with the TaqMan method.
2. Prepared reagents according to the instruction of Hepatitis B Viral DNA Quantitative Fluorescence Diagnostic Kit.
3. 200 μL of negative control, positive control, quantitative reference A–D, and the sample under test is respectively added to seven clean 1.5 ml microcentrifuge tubes, each containing 300 μL of DNA extraction solution. Mix completely by vibrating for about 10 min.
4. Add 100 μL of DNA extraction solution 2-mix to each tube. Mix for 10 s, then quiescence at room temperature for 10 min.
5. Place the microcentrifuge tubes into the magnetic bead separator for 3 min after a short centrifugation, then sucked out the solution slowly.
6. 600 μL DNA extraction solution 3 and 200 μL DNA extraction solution 4 are added to each tube. Mix for 5 s.
7. Place the microcentrifuge tubes into the magnetic bead separator again after a short centrifugation. 3 min later, there are two layers of the solution. Discard the lower layer and transfer all of the microcentrifuge tubes to tube rack.

8. Add 50 μL of PCR-mix to each tube. Mix completely. The mixed solution is transferred to clean 0.2 ml PCR tube respectively.
9. Apply the mixed solution for Real-Time PCR under the instruction of Hepatitis B Viral DNA Quantitative Fluorescence Diagnostic Kit.
10. Apply colorimetric assay to detect the same clinical sample in triplicate; meanwhile consecutive serum samples ranging from $10^1$ to $10^8$ copies/mL are determined in triplicate to record the time-dependent optical absorption changes (Fig. 5a) and

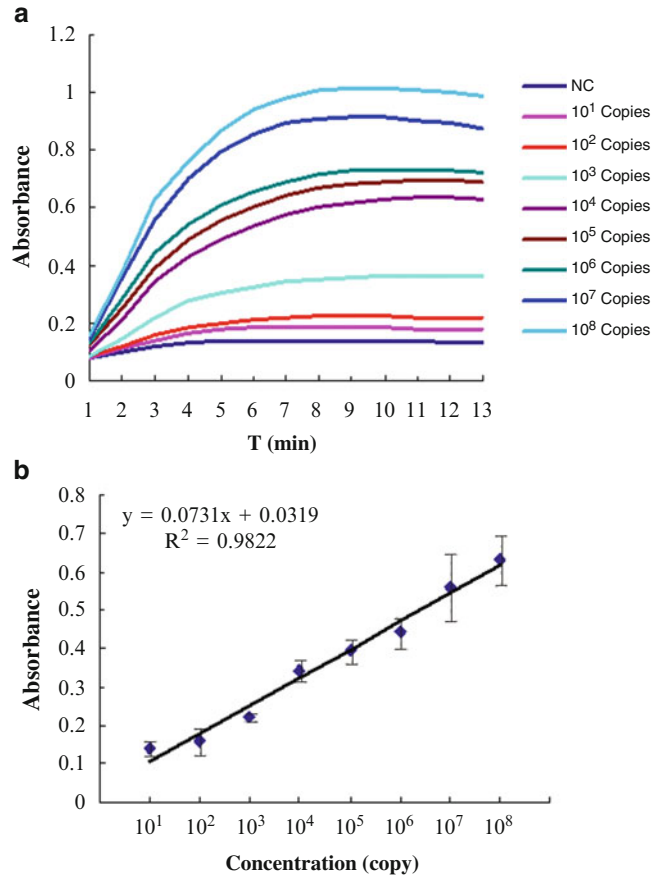


**Fig. 5** Calibration curve establishment of HBV serum samples. (**a**) Time-dependent colorimetric detection of different concentrations of HBV DNA (414 nm). (**b**) Calibrated curve of the average absorbance (414 nm) at 3 min plotted against the number of HBV DNA. The *solid line* indicates linear least squares fitting between 10 and $10^8$ copies of HBV DNA, and their formulation is $FI = 0.0731\ lg(C_{HBVDNA}) + 0.0319$ ($R^2 = 0.9822$). The correlation coefficient is 0.9822. The error bars were determined by standard deviation (SD) of the triplicate data

the calibration curve which used the average values of triplicate data is obtained plotting at 3 min (Fig. 5b). It shows a good linear relationship between absorbance and the concentration of HBV DNA. The correlation coefficient reached 0.9822 and their formulation is FI = 0.0731 lg($C_{HBVDNA}$) + 0.0319. The results reveal the excellent proximity of estimated values in curve showed the correlation between the two assays is very high, whose correlation coefficient attained 0.913 (Fig. 6). Besides, the other negative clinical samples determined by TaqMan assay are also negative measured in triplicate by our colorimetric method, which failed to produce a detectable PCR product.

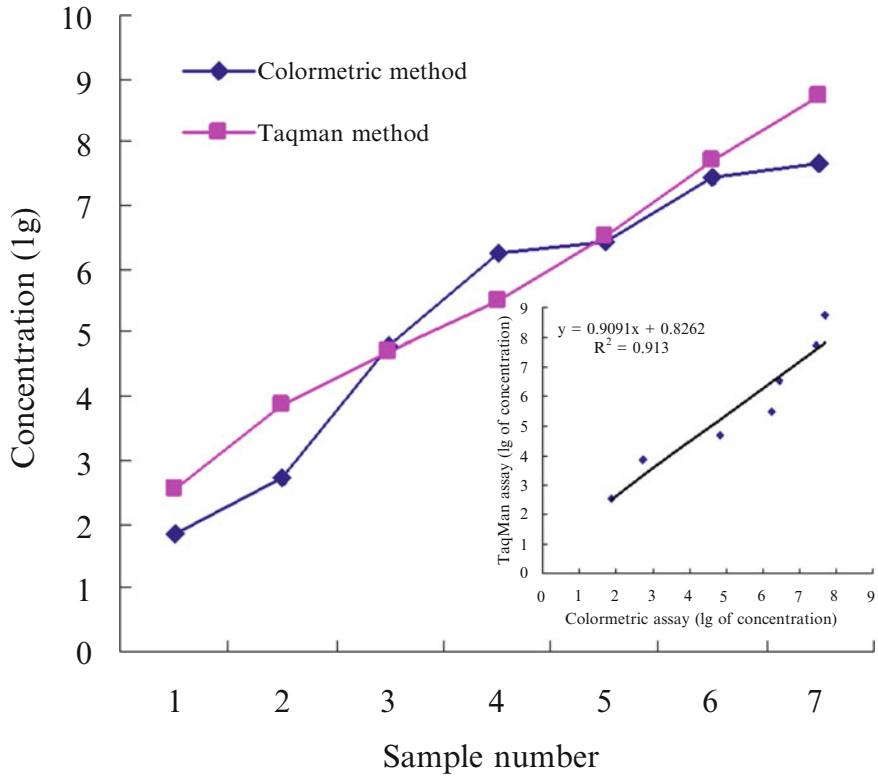


**Fig. 6** Comparison of colorimetric and TaqMan assay. Serum samples of consecutive concentration were measured by colorimetric and TaqMan assay. *Red points* represent the values of TaqMan assay and *blue points* represent the values of colorimetric assay. The inset is the comparison of the values by colorimetric and TaqMan assay. The concentration of target used for experiment was copies/ml. "lg" represents the denary logarithm value of the concentration of target. *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

## 4 Notes

1. 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. The probe with amino modifier at the 3′ end has been introduced into our PCR amplification because the modification on the probe could prohibit the undesired extension caused by the DNA polymerase.
2. Uracil N-glycosylase (UNG) treatment system is most commonly used method to prevent the contamination caused by PCR amplicon. To overcome the shortage of low-efficiency PCR amplification caused by dUTP, we optimized the PCR conditions, containing the PCR temperature, cycles, times, buffer constitution, the quantity of enzyme, concentration of sensor and primer, and concentration of ion.
3. NaCl solution can help the original probe form hairpin structure, which keep solution colorless. So the concentration of the NaCl solution should be tested to hold the background of original probe solution.
4. To ensure the result of colorimetric reaction, Hemin, ABTS, and $H_2O_2$ are freshly prepared.

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