Supporting Information (SI)

Fluorogenic bidirectional displacement probe-based real-time isothermal DNA amplification and specific visual detection of products

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1. Materials and Methods

1.1 Materials and Clinical Specimens.

Bst 2.0 WarmStart™ DNA polymerase (8 U/μL), dNTPs (10 mM), Mg_2SO_4 (100 mM), and 10× Isothermal Amplification Buffer containing 200 mM Tris-HCl, 500 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, and 1% Tween-20, are all provided by the New England BioLabs Ltd. (Ipswich, MA, USA). Urea and hydroxyl naphthol blue (HNB) are from Lemongreen Ltd. (Shanghai, China). 10000× SYBR Green I is purchased from the Life Technologies Ltd. (New York, USA). Betaine is from the Sigma-Aldrich Ltd. (UK). Nuclease-free sterile water and the human genomic DNA (female) standard are from the Life Technologies. Polyacrylamide and the pUC57 vector are from Sangon Biotech Ltd. (Shanghai, China).
The HBV TaqMan qPCR diagnosis kit with HBV positive serum standards (2×10^6, 2×10^5, 2×10^4, and 2×10^3 IU mL^{-1}) and the HBV negative serum standard are purchased from the DAAN Gene Co., Ltd. Of Sun Yat-sen University (Guangzhou, China). The human lung cancer cell, the human blood cell, the 1× phosphate buffered saline (1× PBS), the human serum, and the human plasma are kept in our labs. A total of 12 clinical specimens' nucleic acids from suspicious patients with HBV and the reference viruses' genomic nucleic acids for specificity assays including HBV, hepatitis C virus (HCV) and human papillomavirus (HPV) are all kindly provided by the People's Hospital of Jilin Province in China. Before collecting real samples, the hospital staff has obtained the informed consent for any experimentation with human subjects. All experiments have been performed in compliance with the relevant laws and institutional guidelines, and the institutional committee has approved the experiments.

1.2 Artificial Plasmid DNAs and Primer Design.

The part sequences of HBV polymerase gene and S protein (S) gene are cloned into a pUC57 vector as the artificial plasmid target DNAs by Sangon Biotech (Shanghai) to develop FBD-IDA reactions, respectively. Particularly, the sequence of polymerase gene contains four SNPs in the reverse transcriptase (rt) region, which are the mutations rtL80M (C-A, wild-mutant), rtA181T (G-A), rtM204V (A-G), and rtM204I (G-T) that mediate HBV resistant to an antiviral drug of Lamivudine.[1, 2] Detailed sequences of the fragments are available in the Table S1. The used primers of OF, OR, FP, QP, AF, and AR are all designed by using the PrimerExplorer V4 software available on the web site (http://primerexplorer.jp/e). The FP and QP design is based on the design instruction of inner primers (FIP and BIP) in LAMP. Similarly, the OF (OR) and AF (AR) design should be according to the design instruction of F3 (LF) and B3c (LB) in LAMP. In FBD probe-based HBV IDA, the FAM (6-carboxyfluorescein) is added into the FP by using the aminohexyl (C6) linker at the 5'-end nucleotide of oligonucleotides and the Dabcyl (4'-Carboxy-N,N-dimethyl-4-aminoazobenzene) is added into the QP by using the internal modified base, amino-modifier C6 dT. All the primers and modified oligonucleotides are synthetized and high-pressure liquid chromatography purified by the Sangon Biotech Ltd, the sequence information of which are shown in Table S1.

1.3 Development of One-pot Real-time and Visual FBD-IDA Assays

The one-pot real-time FBD-IDA assays are conducted in the 96 microplates from the Life Technologies Ltd. The optimized mixture (10 μL) is composed of the following components: 0.8 μM FBD probes (the ratio of FP and QP is 0.8 μM to 0.8 μM), 0.2 μM each of OF and OR, 1.6 μM each of AF and AR, 1.0 μL of 10× Isothermal Amplification Buffer, 1.4 mM of each dNTP, 8 U Bst 2.0 WarmStart™ DNA polymerase, 0.8 M Betaine, 6 mM $MgSO_4$, and 1.0 μL templates. In establishing the visual products detection assays, the optimized mixture is similar to real-time FBD-IDA mixture above only supplemented by 120 μM HNB, but the FP in the FBD probe should be labeled with the FAM fluorophore. The reaction mixture is firstly incubated at 37°C for 10 min to fully prepare the FBD probes, then incubated at 63°C for 90 min to execute the reaction, and finally heated at 80°C for 20 min to terminate the reaction. As required, 5.0 μL of amplification products are used to do the 15% denatured polyacrylamide gel electrophoresis containing urea (DPAGE) to screen whether their products have a series of elongated FAM-labeled single-stranded amplicons to confirm the amplification.

In addition, to investigate the melting temperature of the probe, the probe is pre-annealed with equal amount (1.6 μM) of FAM-labeled FP and Dabcyl-labeled QP primers of HBV S gene incubated in 25-μL mixture containing 20 mM Tris-HCl (pH 8.8 at 25 °C), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween-20, and 0.8 M Betaine at 37 °C for 10 min. The dissociation curve from 50 °C to 94 °C is determined by measuring the raw and derivative fluorescence relative intensity change. All the reactions and real-time measuring are accomplished by using an ABI 7500 fast device (Applied Biosystems, ThermoFisher scientific in USA).

Fluorescent images of FBD-IDA visual product detection and the TaqMan qPCR, including measuring the produced green fluorescence intensity at the 530 nm wavelength, are all conducted by using a Maestro Ex IN-VIVO Imaging System (CRI Maestro, USA) which can analyze the fluorescent emission spectra and intensity. In the system, a blue light at 455 nm wavelength with auto-exposure time is taken as the excitation light and the emitted light is accepted by a large area CCD camera through a 495 nm long-

pass filter. The images are captured in a range of 500 nm to 720 nm through the liquid-crystal tunable filter automatically tuned with 10 nm increment.

1.4 Evaluation of the FBD-IDA assays

The human genomic DNA, the extracted DNAs from the human lung cancer cell and blood cell, the extracted HBV negative serum standard, and the reference viruses' genomic nucleic acids including HBV genomic DNA, HPV genomic DNA, HCV genomic RNA are used as the templates to test the specificities of FBD-IDA with 3 independent runs for each template. To detect the mutant gene, SNPs are introduced into the FP, QP, and AF (Table S1). The isovolumetric nuclease-free sterile water taken as the template serves as a no-target control (NTC).

Linearity of quantification of FBD-IDA is evaluated by using ten-fold dilutions of the artificial plasmid target DNAs with the part sequence of HBV S gene (from 4×10^6 to 4×10^0 copies number per microliter, cpm). The linearity of quantification is also estimated by using the extracted HBV positive serum standards with the quantified concentrations (ten-fold dilutions of standards from 2×10^6 to 2×10^3 IU mL^{-1}). Detection limit is determined by using the extracted DNAs from the serial dilutions of HBV positive serum standards (from 2×10^3, 200, 100, 50, 20, 10, 5, and 2 IU mL^{-1}) in batches of 5 replicates on two separate assay runs, giving a total of 10 replicates for each dilution. The HBV TaqMan qPCR is conducted as the parallel tests, using the same amount and type of templates. Linearity of quantification analysis and the probit analysis of detection limit are all carried out by the SPSS 17.0 (IBM, USA) software.

To estimate the tolerance of FBD-IDA to some biological substances that are residues in the extracted nucleic acids, the 1× phosphate buffered saline (1× PBS), human serum, and human plasma at various concentrations (1%, 2%, 5%, and 10%, v/v) are added deliberately into the reaction mixture. Three independent runs are conducted.

1.5 Detection Performance with Clinical Specimens

Clinical performance of the FBD-IDA assays for the detection of HBV is evaluated with a total of 12 clinical specimens' nucleic acids from suspicious patients with HBV. The real samples have been screened by the hospital staff with a frequently-used commercial HBV qPCR diagnosis kits. Of the 12 samples, 7 are confirmed to be HBV negative and 5 are HBV positive including a weak positive with the virus load of 522 IU mL^{-1} which is very close to the negative-defined value (below 500 IU mL^{-1}) according to the manufacturer's instructions.

2. Supplemented Tables and Figures

Table S1. List of used FBD-IDA primers and the cloned gene sequences.

Description	Sequence (5'-3') [a]
The cloned part sequence of HBV S gene	GGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTAT CAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCATCAACAACCAGCACCGGACCATGCAAAACCTGCACAACT CCTGCTCAAGGAACCTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGACGGAAACTGCACCTGTATTCCCATC CCATCATCTTGGGCTTTCGCAAGTAACCTATGGGAGTGGGCCTC
FP (S gene)	FAM-AGGTTTTGCATGGTCCGGTGGGTATGTTGCCCGTTTGT
QP (S gene)	CACCGGACCATGCAAAACC(T-Dabcyl)CCGTAGGTTTTGTACAGCA
OF (S gene)	GCCTCATCTTCTTGTTGGT
OR (S gene)	GATGGGATGGGAATACAGG
AF (S gene)	GCACAACTCCTGCTCAAGG
AR (S gene)	TTGTTGATGATCCTGGAATTAGAGG
The cloned part sequence of HBV polymerase gene (wild type)	TCTATGTTTCCCTCTTGTTGCTGTACAAAACCTTCGGACGGAAACTGCACTTGTATTCCCATCCCATCATCCTGGGCTT TCGCAAGATTCCTATGGGAGTGGGCCTCAGTCCGTTTCTC**C**TG**G**CTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCG TAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTAT**A**T**G**GATGATGTGGTATTGGGGGCCAAGACTGTACAACATCTTGA GTCCCTTTTTACCTCTATTACCAATTTTCTTTTGTCTTTGGGTATACATTTGAACCCTAATAAAA
The cloned part sequence of HBV polymerase gene (mutant type)	TCTATGTTTCCCTCTTGTTGCTGTACAAAACCTTCGGACGGAAACTGCACTTGTATTCCCATCCCATCATCCTGGGCTT TCGCAAGATTCCTATGGGAGTGGGCCTCAGTCCGTTTCTC***A***TG***A***CTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCG TAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTAT***G***T***T***GATGATGTGGTATTGGGGGCCAAGACTGTACAACATCTTGA GTCCCTTTTTACCTCTATTACCAATTTTCTTTTGTCTTTGGGTATACATTTGAACCCTAATAAAA
FP (mutant gene)	FAM-GCCCTACGAACCACTGAACACCTCAGTCCGTTTCTC***A***T
QP (mutant gene)	TGTTCAGTGGTTCGTAGGG(C-Dabcyl)CCAATACCACATCATC***A***A***C***AT
OF (mutant gene)	GCAAGATTCCTATGGGAGT
OR (mutant gene)	GACAAAAGAAAATTGGTAATAGAGG
AF (mutant gene)	AATGGCACTAGTAAACTGAG***T***C
AR (mutant gene)	TTCCCCCACTGTTTGGCTTT

[a] FAM, 6-carboxyfluorescein fluorophore; Dabcyl, 4'-Carboxy-N,N-dimethyl-4-aminoazobenzene. The underlined sequence sites are the sequences of double-stranded fragment in the FBD probe. The bold letters indicate the SNPs that are the mutations rtL80M (**C-*A***, **wild-*mutant***), rtA181T (**G-*A***), rtM204V (**A-*G***), and rtM204I (**G-*T***) in order of 5′-3′.

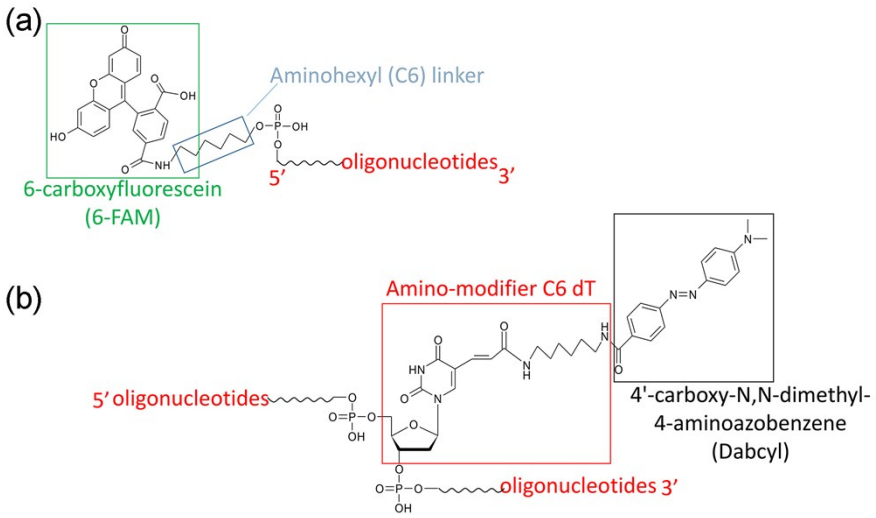

Fig. S1. The chemical structures of the FAM and Dabcyl groups added to the oligonucleotide primers. (a) The FAM is added by using the aminohexyl (C6) linker at the 5'-end nucleotide of oligonucleotides. (b) The Dabcyl is added by using the internal modified base of amino-modifier C6 dT. Apart from dT, there are also the amino-modifier C6 dA, dG, and dC (all not indicated) for the addition of Dabcyl. The modified oligonucleotides are synthetized by the Sangon Biotech Ltd, China.

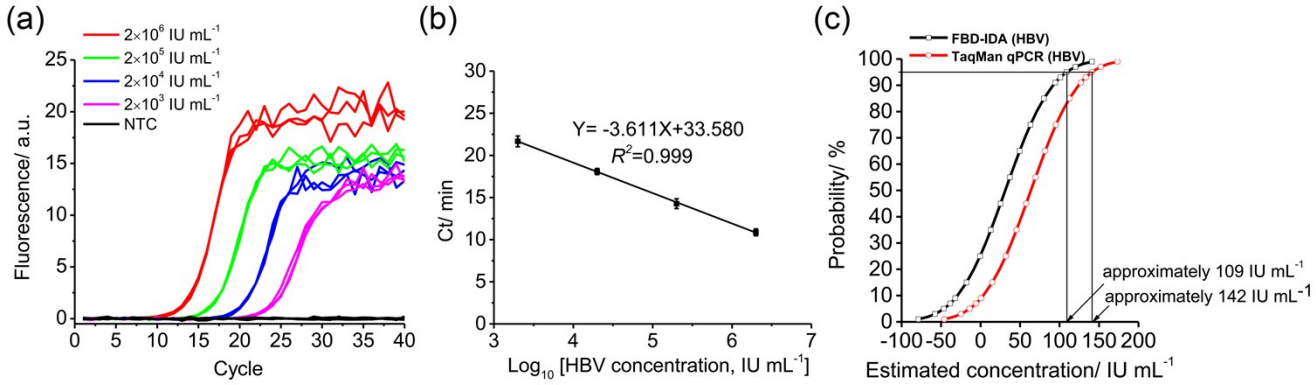

Fig. S2. (a) The detection of HBV positive serum standards (ten-fold dilutions from 2×10^6 to 2×10^3 IU mL^{-1}) by a commercial HBV TaqMan qPCR kit approved by the SFDA of China. Each test has three replicates. NTC, no-target control. (b) Linear relationship between Ct value and the logarithmic ($\log_{10}$) values of the HBV concentrations. Error bars are the SD (n=3). (c) Probit analysis of the limit of detection for FBD-IDA and TaqMan qPCR to test the dilutions of HBV positive serum standards (below 2×10^3 IU mL^{-1}). The 95% probability detection limit for FBD-IDA is about 109 IU mL^{-1} and for TaqMan qPCR is about 142 IU mL^{-1}.

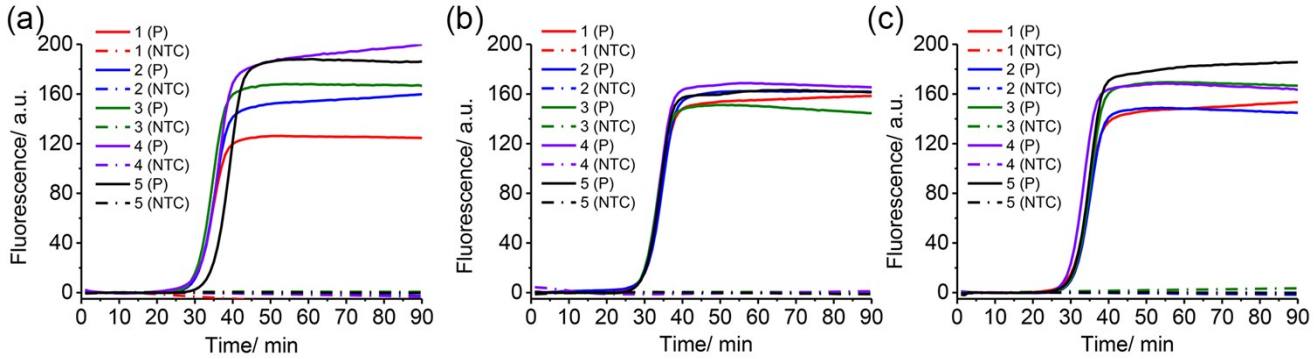

Fig. S3. Evaluation of the tolerance of real-time FBD-IDA to some biological substances, including the (a) 1× phosphate buffered saline (1× PBS), (b) human plasma, and (c) human serum at various concentrations. 1-5, 0%, 1%, 2%, 5%, and 10% (v/v) of the

biological substances added into the reaction mixture. P, the positive test with the artificial plasmid DNAs of HBV S gene (4×10^6 cpm). NTC, the no-target control.

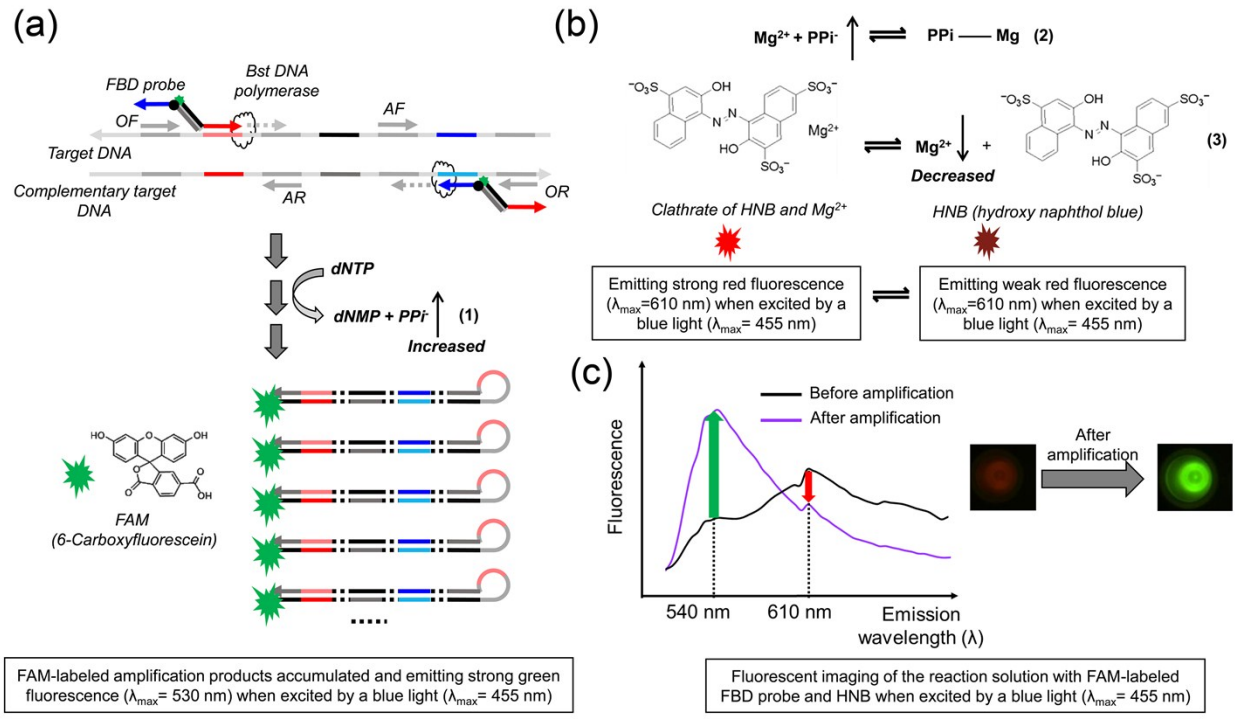

Fig. S4. Scheme of specific visual endpoint detection of FAM-labeled FBD probe-based IDA with the dye of HNB coupled. The FBD-IDA reaction system consists of FAM-labeled FBD probes, primers, target DNAs, *Bst* DNA ploymerase, dNTPs, soluble Mg^{2+}, HNB, and other ionic compositions of the buffer. The FP in the probe should be labeled with the FAM fluorophore, because FAM can share similar excitation light (e.g. a blue light with the excitation wavelength at 455 nm) with the HNB, just like SYBR Green I.[3] The change of fluorescence is mainly attributed to the amount change of dNTPs, soluble Mg^{2+}, and the FAM-labeled amplification products.

(a) During the amplification, dNTPs in the mixture are consumed in the DNA synthesis causing many pyrophosphate ions (PPi^-) accumulated (**Reaction 1**)[4, 5] and lots of FAM-labeled amplification proudcts are also generated. (b) Accumulated PPi^- chelates the soluble Mg^{2+} in the mixture to form the PPi–Mg complex (**Reaction 2** in forward direction)[5], which leads to the **Reaction 3** in forward direction due to the reduction of decrease of soluble Mg^{2+}. (c) After amplification, when the reaction solution is excited by a blue light, fluorescence intensity at 530 nm is greatly enhanced but it at 610 nm is reduced, and the color is changed from dark red to green in fluorescent imaging.

References

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