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An internal amplification control for quantitative nucleic acid analysis using nanoparticle-based dipstick biosensors

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ABSTRACT

Quantitative analysis of virus nucleic acids is essential for monitoring the efficacy of medical treatment based on the copy numbers of virus's RNA or DNA in blood. To quantitatively detect virus nucleic acids in blood, here an internal amplification control (IAC) coupled with a nanoparticle-based DNA biosensor was proposed. The IACs with a specific sequence were designed and spiked into serum before nucleic acids extraction. Sequences of the IACs and the targets only differ in the base order of one PCR priming site; thus, the IACs and the targets are identical in Tm, giving the same amplification efficiency during PCR. To visually detect amplicons, a dipstick biosensor based on streptavidin-functionalized nanoparticles is employed. By comparing color densities of a test zone with an IAC zone on the biosensor, the content of the target in serum can be semi-quantitatively analyzed. This approach has achieved the detection of HBV DNA at approximately 100 copies of the pathogen load. The feasibility of this method is demonstrated by successful semi-quantification of pathogen load in 30 clinical samples from HBV-infected patients. These data indicate that the introduction of an IAC and nanoparticle-based dipstick-type biosensor could be a powerful tool in point of care testing (POCT).

1. Introduction

The dipstick-type biosensor technique (Jaroenram et al., 2009; Shyu et al., 2002), which has been used in gold-immunochromotography assay (Chiao et al., 2004; Ju et al., 2010), can facilitate the rapid reaction and a visual inspection. The biosensor can be conveniently applied for point of care testing (POCT), especially for virus screening before an emergent blood transfusion. Current application of the biosensor method is mainly for the detection of protein antigens of pathogenic micro-organisms. Although the development of the third-generation of monoclonal antibodies has greatly enhanced the sensitivity of immunologic diagnosis, false negativity caused by the following reasons has limited its application: (i) antibody titers in biological samples may be lower than the immunological detection limit in an early stage of infection when there is only a low level of antibodies. Individual variation of immune function could also affect the antibodies production, and (ii) antigenic variation and rare serological subtypes can cause the false negative results. These situations have limited the feasibility of immunological methods for the diagnosis of infection in a timely manner.

In recent years, nucleic acid test (NAT) has been widely used for clinical diagnosis (Brojer et al., 2004; Dettori et al., 2009; Grabarczyk et al., 2009; Hoffmann et al., 2006). Compared to the traditional antigen–antibody based assays, NAT is advantageous for early diagnosis of the disease because viral infection can be detected in a few days of infection (Kok et al., 2010); hence the "window period" of virus detection can be greatly shortened. For example, the window periods of human immunodeficiency virus (HIV), Hepatitis B Virus (HBV), and Hepatitis C Virus (HCV) were shortened to 11 days, 25 days and 59 days by employing NAT for safety screening of blood (Chiao et al., 2004).

NAT using a dipstick-type biosensor has been proposed for the rapid and visual detection of pathogen-specific nucleic acids (Jaroenram et al., 2009; Kiatpathomchai et al., 2008). In comparison with NAT assays based on fluorescence or chemiluminescence (Jaroenram et al., 2009; Kiatpathomchai et al., 2008; Kok et al., 2010; Mugasa et al., 2009; Puthawibool et al., 2009; Soliman and El-Matbouli, 2009), a dipstick biosensor does not require special detection equipments (Jaroenram et al., 2009;

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Kalogianni et al., 2009; Konstantou et al., 2009; Litos et al., 2009; Toubanaki et al., 2009). However, most of reported dipstick-based DNA assays only give qualitative results (Chua et al., 2011; Puthawibool et al., 2009), and this is not sufficient for a sensitive and accurate determination on the status of pathogen's infection or replication in a body. It is thus necessary to develop a quantitative or semi-quantitative dipstick-based DNA assay.

Unlike real-time PCR, dipstick-based DNA assay is of an end-point detection type. It is difficult to achieve a quantitative detection only by measuring the intensity of a test zone. Although semi-quantitative detection of genetically modified organisms (GMO) in food samples was possible by analyzing the test zone densitometrically (Shyu et al., 2002), variations of PCR amplification efficiencies among samples greatly affect the accuracy of the results. One way to improve the accuracy of quantification would be the use of a competitive internal amplification control (IAC) in PCR. Although IAC has been applied for dipstick-based DNA biosensor (Chua et al., 2011), the IAC was only used for monitoring the success of PCR. Since the sequence of the IAC is independent of the target of interest, there could be amplification bias between the IAC and the target; thus, it is impossible to employ this IAC for quantifying the target in a sample. To allow the IAC to be the internal quantification standard of a target, a particularly designed IAC was proposed here for quantifying virus loads in a blood sample. Because the sequence composition of the proposed IAC is same as the corresponding sequences in the target, amplification bias between the target and the target-specific IAC is largely eliminated. Although the IAC and its corresponding target DNA have an equal length and an equal base composition, the IAC and the target DNA will be colored at different positions of a strip because the dipstick-based assay is different from gel electrophoresis-based assay. The virus load in a serum sample is obtained by comparing the color density of a test zone with that of an IAC zone on the biosensor. If an IAC was added into a sample without any virus target, the IAC zone would still be color-developed. This makes it possible to use the IAC to monitor the reliability of the whole process including nucleic acid extraction, amplification, and dipstick-based detection. Based on this principle, various pathogens may be quantified conveniently by designing pathogen-specific IACs.

2. Materials and methods

2.1. Materials and instruments

Sheep anti-digoxigenin antibody (anti-Dig) was purchased from the Roche Molecular Biochemicals (Mannheim, Germany). Rabbit anti-fluorescein antibody (anti-FITC) was from the Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). *Taq* DNA polymerase was obtained from the TaKaRa Biotechnology Co., Ltd. (Dalian, China). DNA/RNA Extraction Kit was purchased from Qiagen Co., Ltd. (Shenzhen, China). All the oligonucleotides were synthesized and purified by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). Dipsticks were prepared by the Liming Biological Products Co., Ltd. (Nanjing, China) according to the authors' requirements. PCR was performed on a PTC-225 Peltier Thermal Cycler (MJ RESEARCH, Inc., USA). Digital camera, Sony P100, was purchased from Sony Co., Ltd. (Japan).

2.2. Recombinant plasmid construction

According to the authors' requirements, the recombinant HBV plasmid was prepared by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). Targets were amplified by PCR using single-stranded oligos, and the product was subcloned into the PCR2.1-TOPO plasmid. Then the recombinant plasmid was transformed into DH5α competent cells and identified by sequencing. Finally, the plasmid was purified with the use of SDS-alkaline lysis method, and the purified plasmid was quantified by UV spectrophotometry ($\lambda = 260$ nm).

2.3. Preparation of IAC

The preparation of HBV-specific IAC was taken as an example. The plasmid containing a genome sequence of HBV was used as a starting material of PCR. PCR primers are HBV–IAC–F (5′–CCA CCT CAA CCA TCC ACC AGT GTC ACC AAC CTC TTG TCC TC-3′) and HBV–IAC–R (5′-GAA GTA GAG GAC AAA CGG GCA ACA TA-3′). Underlined bases represent an IAC-specific sequence. As primer HBV–IAC–F has to tag the sequence in the 5′ end to supply the priming site for amplifying IAC in the next PCR, the lengths of the two primers for preparing IAC are different. However, this did not affect the preparation of qualified IAC, because the annealing temperature of PCR is below the Tm of both primers. The 173 bp PCR products were resolved by electrophoresis in a 2% (wt/vol) agarose gel, and purified by gel extraction using the TaKaRa agarose gel DNA purification kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Purified product was quantified by OD260 and used as the IAC for HBV analysis. The length and base-composition of IAC products are identical to those of HBV products except for a permutation of 22 bases at the 5′ end of the forward primer (the IAC-specific sequence described above).

2.4. Extraction of virus nucleic acid from blood

Blood specimens were supplied by Jiangsu Entry–Exit Inspection and Quarantine Bureau. One microliter of IAC (5000 copies/μl) was added to the blood specimens before extraction. Both viral and IAC nucleic acids were extracted from blood by a Viral DNA/RNA Isolation kit (Shenzhen PG Biotech, China). According to the manufacturer's instruction, 2 ml of whole blood was collected in a sterile centrifuge tube, and cellular components were removed by centrifugation ($1600 \times g$, 20 min). Serum containing the IAC and virus DNA was transferred to another sterile centrifuge tube and stored at -20 °C until DNA extraction. The above steps should be completed within 4 h after blood collection. For DNA extraction, 100 μl of samples were lysed in 100 μl of the DNA Extracting Solution 1 provided by the kit, and the mixture was centrifuged at $11700 \times g$ for 10 min. After the removal of supernatant, the precipitates were dissolved completely in 25 μl of the DNA Extracting Solution 2. Finally, the solution was heated at 100 °C for 10 min and centrifuged at $11{,}700 \times g$ for 10 min. The supernatant containing the target and IAC DNA was saved for subsequent steps.

2.5. Preparation of nanoparticle-based dipstick biosensor

The dry-reagent dipstick (6 mm × 60 mm) consists of an immersion pad, a conjugate pad, a nitrocellulose membrane, and an absorbent pad assembled on a plastic adhesive backing. Biotinylated bovine serum albumin (biotin–BSA) (4 mg/ml), anti-Dig (1 mg/ml) and anti-FITC (0.1 mg/ml) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) were dispensed onto the zones. On the nitrocellulose membrane, there are three zones: the quality control zone (conjugated with biotin–BSA), the IAC zone (conjugated with anti-Dig) and the test zone (conjugated with anti-FITC). Streptavidin-immobilized nanoparticles which were loaded on the conjugate pads were prepared by bioconjugating 0.1 mg/ml streptavidin and 1% nanoparticles (200–500 nm in size) in 0.01 M PBS (pH 7.4) at 4 °C over night. After the addition of 1% BSA for sealing, centrifugation was performed and the supernatant was removed. The streptavidin-immobilized nanoparticles were resuspended in 0.01 M PBS (pH 7.4) containing 0.1% BSA to a final concentration of