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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

0.6%. A 7.5 μl aliquot of nanoparticles was deposited on the conjugate pad of the biosensor. After assembled and cut at 6 mm widths with an automatic strip cutter, the dipsticks were stored dryly at room temperature until use.

2.6. PCR and dipstick-type detection

The 25 μl PCR reaction contains 0.2 mM of each dNTP, 0.4 μM HBV–Biotin–R primer (5′-Biotin–GAA GTA GAG GAC AAA CGG GCA ACA TA-3′) and HBV–FITC–F primer (5′-FITC–GCA GTC CCC AAC CTC CAA TCA C-3′), 0.1 μM HBV–Dig–F primer (5′-Dig–CCA CCT CAA CCA TCC ACC AGT G-3′), 1.25 U of TaKaRa *Taq* DNA polymerase, 1 × TaKaRa PCR Buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.3), 1.5 mM $MgCl_2$, and the DNA templates-containing supernatant described above. For PCR conditions, initial denature was carried out at 94 °C for 2 min, which was followed by 35–50 cycles of denaturing at 94 °C for 10 s, and annealing and extending at 70 °C for 30 s. The optimal number of PCR cycles was found to be 45. After amplification cycles, an final extension was carried out at 72 °C for 2 min. Negative controls, using water instead of DNA samples as the PCR templates, were included in each series of PCR to ensure the free of contamination. Three microliters of a PCR product mixed with 97 μl of the developing buffer were applied onto the conjugate pad of the dipstick near the nanoparticles. The PBS-based developing buffer contains 0.14 M NaCl, 2.7 mM KCl, 10 mM sodium phosphate, and 1.7 mM potassium phosphate (pH 7.4). Each dipstick was allowed to absorb at least 100 μl of a developing buffer. Images of the dipsticks were taken with the use of Syngene imaging system. Color densities of the zones were analyzed with the GeneTools Analysis Software (SynGene, Frederick, MD). To test the performance of the dipstick, oligonucleotides labeled at both ends (5′-Biotin–TGG TTG CTC TAG GAA AGC AGT ATT CTG AAG AGG CTT CTA A–FITC-3′) were used. The visual detection was completed within 15 min.

3. Results and discussion

3.1. Principle

The procedure of the proposed assay is illustrated in Fig. 1. A pathogen-specific IAC, which is prepared by PCR using a 5′-sequence tagged primer and a regular gene-specific primer in advance, is added to the serum samples. The IAC is co-extracted with the pathogen DNA. To accomplish an unbiased amplification during PCR, both the lengths and base-compositions of the IAC should be identical to those of the target of interest. The tagged sequence (marked with "the different sequences" in Fig. 1) of IAC is designed based on the corresponding sequence region in the target of interest. To increase the specificity and to save the PCR running-time, the combination of primer annealing and extension in the PCR is proposed. By using a two-step instead of three-step PCR, the PCR running-time is greatly reduced (45 cycles within 45 min). Following PCR, amplicons of Dig–IAC and FITC-target are obtained. During dipstick-type detection, the PCR products together with the developing buffer are loaded on the dipstick and migrate along the strip. After binding to the nanoparticles, IAC and the pathogen-target are captured by the immobilized

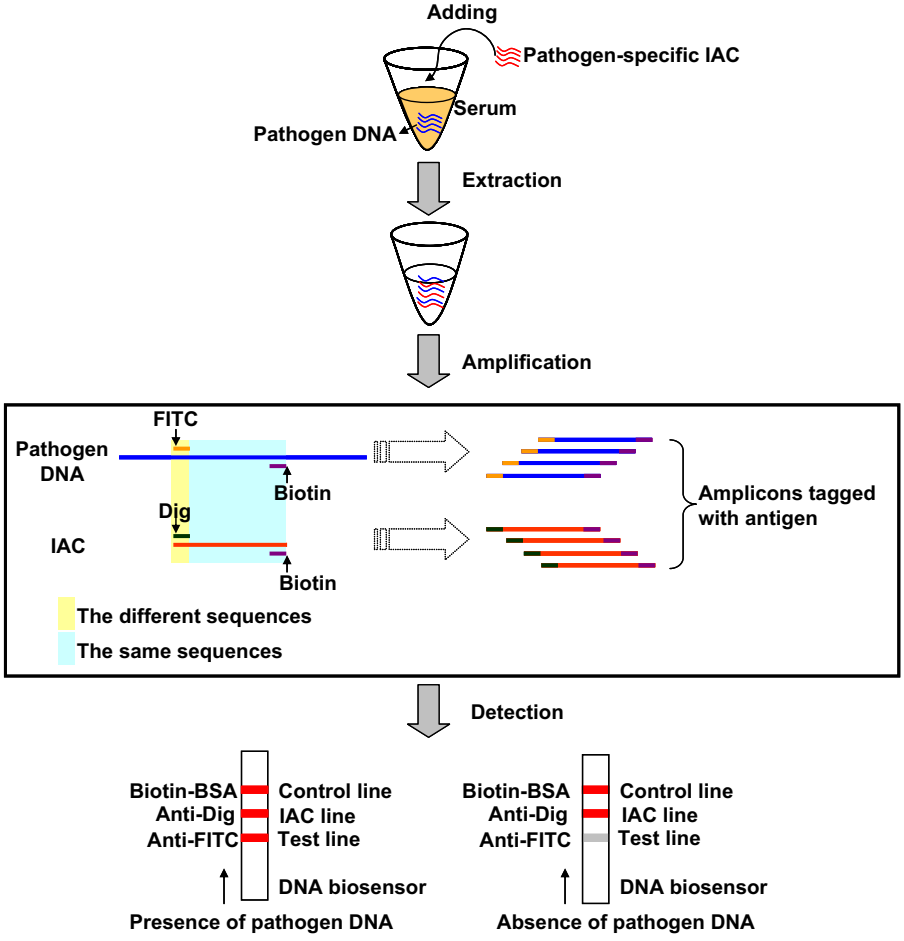

Fig. 1. Schematic illumination of the method for semi-quantitative NAT based on IAC and a visible nanoparticle-based dipstick biosensor.

anti-Dig at the IAC zone and the immobilized anti-FITC at the test zone, respectively. Color development is realized by the accumulation of a large number of nanoparticles at the two zones. The excess of nanoparticles bound to the immobilized biotin–BSA at a quality control zone confirms a satisfactory performance of the dipstick biosensor.

If no pathogen exists in the serum, color would not appear at the test zone. Thus, negative result is obtained when only two red zones are observed at the quality control and IAC zones. If the serum contains a pathogen, a positive signal should appear at the test zone. As the depth of color is proportional to the amount of PCR products, pathogen loads in the serums can be readily estimated by comparing the color intensity at the IAC zone with that at the test zone. In addition to quantification, the IAC zone can also be employed as a marker for evaluating whether or not the steps of DNA extraction and amplification have been successful. For example, if no color is observed at the IAC zones, it can be concluded that DNA extraction or amplification has failed.

3.2. Evaluation of the biosensor

As the double-labeled products of a pathogen-target and the corresponding IAC are the expected detecting targets of the nanoparticle-based biosensor, the specific response of the biosensor to the products should be investigated. As shown in Fig. 2A, single-labeled primers (Biotin or FITC or Dig at only one end of the primers) gave no signal at either the test zone or the IAC zone (strips 1–3), indicating that it is not necessary to remove the residual single-labeled primers in PCR products. However, false-positive signal could be caused by the double-labeled primer-dimer produced in PCR. Thus, it is essential to avoid the production of primer-dimer in PCR. As shown in the strip 4 of Fig. 2A, no signal was observed from the PCR negative control (using water instead of DNA as the PCR target), but positive signals at the test zone (specific to FITC) or the IAC zone (specific to Dig) were clearly observed when a target DNA (synthesized oligos or PCR amplicons) labeled at both ends with biotin and FITC (strips 5–7) or with biotin and Dig (strips 8–10), respectively, was applied to the biosensor. When PCR products originated from the IAC and the pathogen-target of interest, signals appeared at both the test zone and the IAC zone (strip 11). These results suggest that the biosensor is specific, and amplicons from a sample and the IAC can be discriminated by their different labels at one end.

To investigate whether or not a buffer with certain ionic strength and pH value is required for developing dipsticks, PBS buffer and water were used as developing reagents respectively. As shown in Fig. 2B, the PBS buffer gave a much higher sensitivity than water. When the sampling amount of PCR products was decreased to 10 fmol, no signal was obtained by using water as developing medium. However, an obvious signal at the anti-FITC zone was observed when PBS buffer was used as the developing solution. Accordingly, a proper developing buffer may be essential for achieving a high sensitivity of the biosensor. This is presumably because that the developing buffer could stabilize the streptavidin-immobilized nanoparticles and provide a suitable environment for an antigen–antibody reaction.

As raw PCR products contain a large amount of residual modified primers, the nanoparticles and antibodies on the dipstick would capture these primers. The competition between the modified primers and the detection targets could compromise the detection sensitivity. To improve the sensitivity, it is preferable to purify raw PCR products before sampling on the biosensor. However the purification process may bring the risk of cross-contamination due to the formation of amplicon–aerogel. To escape the purification step, sufficient amount of nanoparticles and antibodies on the biosensor becomes necessary to compensate for the reduction in the effective binding capacity caused by the residual primers in raw PCR products. Various biosensors prepared with different amounts of nanoparticles (from 0.06% to 0.6%) and antibodies (from 0.2 mg/ml to 1 mg/ml of anti-Dig, and from 0.06 mg/ml to 0.1 mg/ml of anti-FITC) were applied for the detection of raw PCR products and purified PCR products, respectively. As shown in Fig. 2C, the density at the detection band from raw PCR products was not significantly decreased in comparison with those from purified PCR products if the dipstick biosensor was prepared with 0.6% nanoparticles, 1 mg/ml anti-Dig and 0.1 mg/ml anti-FITC. These results indicated that the residual primers in the raw PCR products did not cause a significant loss of the sensitivity of the dipstick biosensor. Therefore raw PCR products can be directly dropped on the biosensor for the detection, which makes the operation faster and simpler.

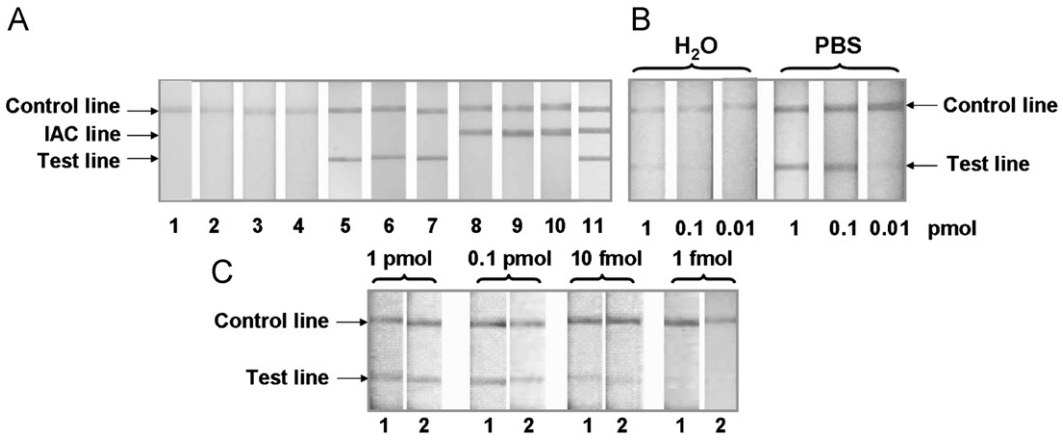

Fig. 2. Evaluation of the DNA biosensor: (A) response of the DNA biosensor to blank control (strip 1), biotin-labeled primer (strip 2), FITC-labeled primer (strip 3), Dig-labeled primer (strip 4), and seven HBV-positive samples including a synthetic oligonucleotide double-labeled with biotin and FITC (strip 5), raw PCR products double-labeled with biotin and FITC (strip 6), purified PCR products double-labeled with biotin and FITC (strip7), a synthetic oligonucleotide double-labeled with biotin and Dig (strip 8), raw PCR products double-labeled with biotin and Dig (strip 9), purified PCR products double-labeled with biotin and Dig (strip 10), 2-plex PCR products, one of which is labeled with biotin and FITC, the other of which is labeled with biotin and Dig (strip 11); (B) effect of different developing solutions (H_2O and PBS) on the intensity of the test zone. The various amounts of targets spotting on DNA biosensors are listed below the strips; and (C) comparison of biosensor results between the raw double-labeled PCR products (marked "1" below the strips) and the purified ones (marked "2" below the strips).

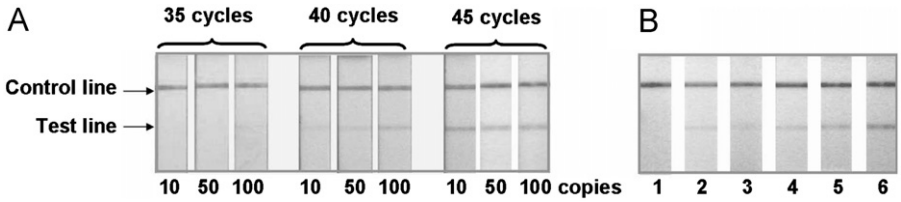

Fig. 3. Strip images from the dipstick assay of different amounts of plasmid targets using various PCR cycles (A) and serum samples with various virus loads (B). The virus loads of the serum samples are "N/A (not applicable, when the fluorescence of a sample does not rise significantly above the background noise, and hence does not cross the threshold)" (strip 1), 50 (strip 2), 4.72×10^2 (strip 3), 5.67×10^2 (strip 4), 1.81×10^4 (strip 5), and 1.33×10^6 (strip 6) copies/ml by the detection with real-time PCR. The sample of 50 copies/ml (strip 2) was prepared by diluting the sample of 4.72×10^2 copies/ml (strip 3).

To evaluate a suitable sampling range of the biosensor, different amounts of PCR products (1 pmol, 0.1 pmol, 10 fmol and 1 fmol) were applied on the biosensors. Positive results were obtained for all the samples (Fig. 2C). Thus, the detection sensitivity is at least 1 fmol of PCR products. Moreover, the sampling range could be at lease from 1 fmol to 1 pmol of PCR products. It is also observed that the band density was linearly enhanced with the increasing amount of the spotting sample, supporting the possibility of semi-quantification on the biosensors.

3.3. Sensitivity of the nucleic acid analysis on the biosensors

This assay is composed of two steps, PCR and biosensor detection. As mentioned above, the sensitivity for the biosensor detection step can reach the femtomol level (around 6×10^8 copies of PCR amplicons). Therefore, to detect a small amount of nucleic acid, for example, several copies of viral targets, PCR amplification of the target DNAs to femtomol levels is essential for successful detection. It is well known that the amount of amplicons depends on the cycling number of PCR. According to the results in Fig. 3A, 40 cycles and 35 cycles of PCR amplification were needed for detecting 10 copies and 100 copies of DNA templates by the biosensors, respectively. To achieve a higher sensitivity, 45 cycles within 50 min was routinely used for PCR, and this gave a strong signal for amplifying samples containing several tens of copies of DNA targets. Considering that increasing PCR cycles may lead to non-specific amplification, agarose gel electrophoresis of final PCR products was performed. Non-specific band did not appear in the electrophoregram of PCR products, indicating that specific amplification can be achieved even with 45 cycles of amplification.

To further evaluate the sensitivity of the method, 4 serum samples containing HBV pathogens with the concentrations of 4.72×10^2, 5.67×10^2, 1.81×10^4 and 1.33×10^6 copies/ml, were analyzed by the method. The concentrations of the target pathogens were determined by real-time PCR. As shown in Fig. 3B (strips 3, 4, 5 and 6), all of the samples were successfully diagnosed by the dipstick-type detection. Most importantly, a serum sample with 50 copies/ml of HBV load was also detected as a positive sample by the method (Fig. 3B, strip 2). It is concluded that this assay is sensitive enough for blood safety screening.

3.4. Optimization studies on semi-quantitative nucleic acid testing

The use of IAC as a quantification reference is the key to allow the semi-quantitative nucleic acid testing. Because of the amplification competition between the IAC and the DNA target, the spiking amounts of the IAC template and the concentration ratio of primers used for amplifying the target and its corresponding IAC should be optimized. As shown in Fig. 4, the primer-concentration ratio of 4:1 (HBV–FITC–F:HBV–Dig–F) gave the strongest bands at both the test zone and the IAC zone when

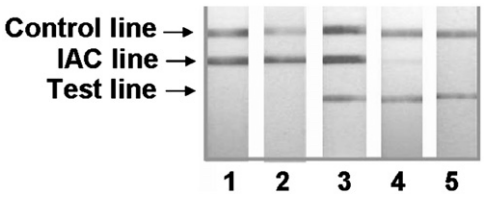

Fig. 4. Strip images from the dipstick assay of PCR amplicons amplified from the primers with the concentration-ratios (primer HBV–FITC–F: primer HBV–Dig–F) of 1:1 (strip 1), 2:1 (strip 2), 4:1 (strip 3), 8:1 (strip 4), and 16:1 (strip 5). The serum sample with a virus load of 100 copies was used as a detection target.

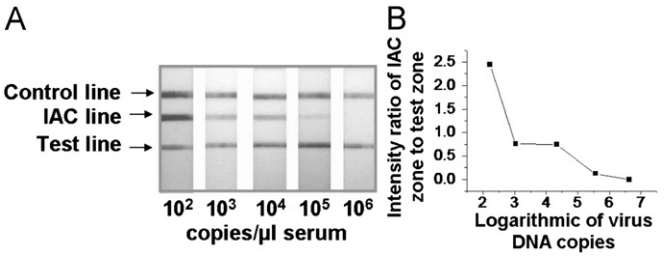

Fig. 5. Strip images (A) and semi-quantitative analysis (B) from the dipstick assay of serum samples with virus loads of 10^2 copies, 10^3 copies, 10^4 copies, 10^5 copies, and 10^6 copies, respectively. A semi-quantitative curve was obtained by comparing the virus loads from real-time PCR with intensity-ratios of the IAC zone vs. the test zone at the strips. The intensity was analyzed by Syngene imaging system.

5000 copies of the IAC was spiked into a serum sample with a virus load of 100 copies; thus, this primer-concentration ratio and this IAC spiking amount were used for the following detection. Serum samples with different virus loads ranging from 10^2 copies to 10^6 copies were successfully detected, and semi-quantitative results were obtained (see Fig. 5A for details). As shown in Fig. 5B, the band-intensity ratios of the IAC zone to the test zone gradually decreased with the increase of the virus loads; so it is easy to get virus loads in serums at a semi-quantitative level by measuring the band-intensity ratio of the IAC zone to the test zone. Although the test zone of 10^6 copies has lesser intensity than those of 10^4 and 10^5 copies of DNA in Fig. 5A, the intensity ratio (the test zone vs. the IAC zone in a dipstick) of the sample with 10^6 copies of DNA is much larger than those of the samples with 10^4 and 10^5 copies of DNA, suggesting that the number of the targets in the sample with 10^6 copies of DNA is much larger than that in the samples with 10^4 or 10^5 copies of DNA. Therefore the proposed IAC-based method is largely independent of the intensities of test zones between dipsticks, but mostly related to the ratio of the test zone vs. the IAC zone in a single dipstick. Usually it is very difficult to eliminate bias among assays due to variations in DNA extraction efficiency, amplification efficiency, and reaction efficiency on the biosensor, but it is easy to reduce

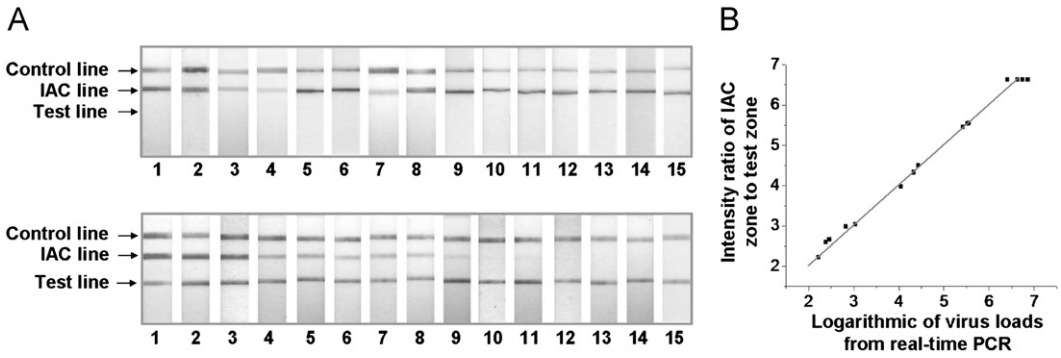

Fig. 6. Strip images of the dipstick assay for serum samples from 15 normal subjects (A), 15 HBV carriers (B), and comparison of the results from the HBV-positive samples between real-time PCR and semi-quantitative dipstick assay (C). Virus loads of the samples in the dipstick assay were obtained by analyzing the intensity ratio using the standard curve documented in Fig. 5B.

the bias within an assay by employing an IAC with a sequence similar to the target.

Conventionally, quantification relying on the intensity comparison among test zones at different dipsticks is very subjective in the dipstick-based method. But in the proposed method, this drawback was largely overcome by using an IAC with a sequence very similar to the target of interest to quantify the target in a sample. Relatively accurate quantification of a sample could be achieved by comparing the intensities at an IAC zone and a test zone in a single dipstick.

3.5. Application of the biosensor to clinical sample detection

To validate the semi-quantitative performance of the visible biosensor system, 30 serum samples, 15 from normal people and 15 from HBV carriers, were analyzed. The results showed that all the normal serums were detection-negative (Fig. 6A) and all the virus-positive serums were detection-positive (Fig. 6B). By naked-eye observation, the virus loads can be roughly determined as more than 10^6 copies if there is no visible band at the IAC zone (Fig. 6B, strips 12–15). The virus loads from 10^2 copies to 10^6 copies can be roughly estimated by visibly comparing the intensities at IAC zones and test zones with those from a series of standard samples in Fig. 5A, and a more accurate quantification can be achieved by analyzing the intensity ratios using the standard curve in Fig. 5B and the Syngene imaging system. The semi-quantitative results of the 15 HBV-positive samples calculated by using the standard curve as a reference are similar to those determined by real-time PCR (Fig. 6C), indicating that the biosensor is a practical tool for clinical application. The read-out process of the biosensor is relatively simple and inexpensive. The whole detection step can be accomplished within 15 min. Therefore, the system appears to be a convenient procedure for POCT.

4. Conclusion

Dipstick-type biosensor, often used as a visual and sensitive tool for protein detection, is recently improved for protein-labeled NAT. This improvement allows nucleic acid testing simple, convenient and inexpensive. In this study, it is firstly demonstrated that a semi-quantitative dipstick-type biosensor assay can be used to determine the virus loads through the introduction of an IAC strategy. Due to the specially designed IAC sequence, the amplification efficiency is almost identical between the target DNA and the corresponding IAC. Consequently, semi-quantification of the target is achieved by simply comparing the band-intensities between the IAC zone and the test zone at a dipstick. Moreover, a two-step PCR with a high annealing temperature was successfully used to increase the PCR specificity and to reduce the PCR time. The use of IAC could avoid false-negative results in the assay. The absence of signal band at both the IAC zone and test zone would indicate the failure of the assay itself. If only the control signal appears at the IAC zone in the strip, the assay is proposed to be successful, but no virus target is present in the sample. Therefore, in addition to the internal quantification reference, the IAC zone also serves as an internal positive-control in the assay. Since no special instrumentation is required by the assay, it is believed that pathogen diagnosis based on this biosensor should be a useful tool for NAT out of a conventional lab.

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