Clinica Chimica Acta 488 (2019) 202–208

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Development of up-converting phosphor technology-based lateral flow assay for quantitative detection of serum PIVKA-II: Inception of a near-patient PIVKA-II detection tool

Yi Huang[a,b,*,1], Songgao Zhang[c,1], Qingzhu Zheng[a,b], Yadong Li[a,b], Lili Yu[a,b], Qingwei Wu[a,b], Jiaying Zheng[a,b], Yanan Wu[a,b], Funan Qiu[a,d], Qi Gao[e,**], Jiancheng Zhang[a,f,*]

[a] Provincial Clinical College, Fujian Medical University, Fuzhou 350001, Fujian, China
[b] Department of Clinical Laboratory, Fujian Provincial Hospital, Fuzhou 350001, Fujian, China
[c] Fujian University of Traditional Chinese Medicine, Fuzhou 350122, Fujian, China
[d] Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Fuzhou 350001, Fujian, China
[e] Department of Applied Science, Beijing Hotgen Biotech Co. Ltd, Beijing 102600, Beijing, China
[f] Fujian provincial Key Laboratory, Fujian Provincial Hospital, Fuzhou 350001, Fujian, China

ARTICLE INFO

Keywords:
Up-conversion phosphor technology
Lateral flow assay
Hepatocellular carcinoma
PIVKA-II
Point of care testing

ABSTRACT

Background: Development of a test card based on up-conversion phosphor technology-based immune lateral flow (UPT-LF) assay as a near-patient detection tool for serum Prothrombin induced by vitamin K absence or antagonist II (PIVKA-II).

Methods: Up-converted phosphor nanoparticles (UCPs) were used to bind to PIVKA-II monoclonal antibodies as labeled probes to develop the test card for detecting serum PIVKA-II. The UPT-LF test card was evaluated by the limit of detection, linearity, stability, recovery rate, precision and interference. Preliminary clinical validation was conducted by detection of 498 serum samples from 228 patients with hepatocellular carcinoma (HCC), 170 patients with liver benign lesion (LBL) and 100 healthy controls (HC). Additionally, the correlation of serum PIVKA-II detection between UPT-LF assay and Chemiluminescence enzyme immunoassay (CLEIA) assay were performed.

Results: Modified and activated UCPs bound to monoclonal antibodies powerfully to form the luminescent labeled probes. Limit of detection and linear range of UPT-LF test card for serum PIVKA-II were 2.66 and 4.8–20,000 ng/ml, respectively. Test card had good 25 °C thermal and 4 °C validity period stability, 93.1%–99.2% of recovery rate, 2.6–5.8% and 5.4–8.9% of intra-assay and inter-assay CVs, and strong anti-interference ability for 8 common serum analytes. The sensitivity and specificity (vs LBL + HC group) of test card for HCC were 71.5% and 88.9%, respectively. The R2 between UPT-LF assay and CLEIA assay was 0.901.

Conclusions: UPT-LF assay provides a reliable, rapid and convenient test for quantitative detection of serum PIVKA-II as well as diagnosis of HCC by a point of care testing way.

1. Introduction

Prothrombin induced by vitamin K absence or antagonist II (PIVKA-II) is an abnormal prothrombin with loss of normal coagulation function. PIVKA-II was first reported to be overexpressed in the serum of hepatocellular carcinoma (HCC) patients by Libman et al. [1]. About 95% of HCC patients with tumor nodules > 5 cm in diameter have extremely increased PIVKA-II concentrations with a median of 6000 mAU/ml in sera [2]. Yoon et al. [3] found that the overall sensitivity and specificity of PIVKA-II for HCC are 48%–62% and 81%–98%, respectively, which are substantially higher than 39%–64% and 76%–91%, respectively, of α-fetoprotein (AFP). Given that serum PIVKA-II exceeds 90 mAU/ml, the sensitivity and specificity to predict the microvascular invasion of HCC cells can reach 70% and 63%, respectively [4]. Additionally, in reference to increased serum AFP, aberrantly increased serum PIVKA-II concentrations are reportedly

* Corresponding authors at: Provincial Clinical College, Fujian Medical University, Fuzhou 350001, Fujian, China
** Corresponding author at: Department of Applied Science, Beijing Hotgen Biotech Co. Ltd, Beijing 102600, Beijing, China
E-mail addresses: hyi8070@126.com (Y. Huang), qi.gao@hotgen.com.cn (Q. Gao), fjzhangjiancheng@126.com (J. Zhang).
[1] Yi Huang and Songgao Zhang contributed equally to this work.

https://doi.org/10.1016/j.cca.2018.11.020
Received 27 July 2018; Received in revised form 6 November 2018; Accepted 12 November 2018
Available online 13 November 2018
0009-8981/

responsible for the larger tumor volume, and proportion of poor differentiation and portal vein thrombosis in HCC patients [5,6]. As a result, PIVKA-II has been shown great potential in the management of diagnosis, illness assessment and prognosis judgment of HCC. Currently, serum PIVKA-II testing is conducted on the chemiluminescence enzyme immunoassay (CLEIA) assay, chemiluminescence immunoassay (CLIA), and liquid binding assay (LBA) [7–10]. However, these assays require relatively complex analyzer, which are limited in large biochemical department or specialist centralized laboratory and not suitable for point-of-care testing (POCT). Given the clinical need for near-patient assessment, a POCT assay may be a good alternative to serum PIVKA-II detection.

Up-conversion phosphor technology (UPT)-based immune lateral flow (UPT-LF) assay may be an excellent representative of numerous POCT methods [11,12]. Compared with the traditional bioluminescence probe, inorganic up-converted phosphor nanoparticles (UCPs) doped with the rare earth Ln^{3+}, a new generation of fluorescent bio-labeling material, have many advantages such as high luminescence stability, low background fluorescence interference, narrow absorption and emission spectrum, and long half-life, and have been widely applied in many prospects [13,14]. Meanwhile, the immune lateral flow test (LF), an assay widely used in semi quantitative analysis of analyte with the advantages of relatively short detection time and low cost, is not only suitable for laboratory testing, but also for POCT assay [15–17]. In the present study, we describe the development of a test card based on UPT-LF assay by using $NaYF_4$:Yb^{3+}, Er^{3+} nanoparticles co-doped with two types of rare earth Ln^{3+} element, yttrium (Yb^{3+}) and erbium(Er^{3+}), as the luminescent matrix material of the bio-labelling probe [18–20],and a strip as a solid-phase reaction medium. This system enabled the POCT detection of serum PIVKA-II concentrations in HCC patients.

2. Materials and methods

2.1. Materials and instruments

UCPs ($NaYF_4$:Yb^{3+}, Er^{3+}), UPT exclusive shell, lamination card, absorbent pad, nitrocellulose membrane (NC membrane, 503C), glass fiber SB08, PIVKA-II monoclonal antibody 1 (mIgG1), PIVKA-II monoclonal antibody 2 (mIgG2), goat anti-mouse IgG, PIVKA-II recombinant antigen and Heterophilic Blocking Reagent mouse IgG (HBR-IgG) were provided by the Beijing Hotgen Biotech. According to the manufacturer Beijing Hotgen Biotech, the specific epitopes of anti-PIVKA-II mIgG1 and mIgG2 are proprietary. 3-Aminopropyltriethoxysilane (APES), nonionic emulsifier CO-520 and tetraethoxysilane (TEOS) were purchased from Hangzhou Dadi Chemical. Lumipulse® G PIVKA-II detection kit (CLEIA) was from Fujirebio Inc.

Continuous Dispenser and Sprayer and High Speed Sensor Cutter were from Hangzhou Autokun Technology. UPT-3A-1800 biosensor was from Beijing Hotgen Biotech (Beijing, China). Lumipulse® G1200 (Fujirebio Inc.).

2.2. Reagents

Solutions were prepared with the following components and conditions: sample dilution (0.02 mol/l PBS, 0.15% BSA, and 0.1% NaN_3; pH 7.4), PIVKA-II recombinant antigen and antibody buffer (0.02 mol/l PB; pH 7.4), reaction system buffer (0.02 mol/l Tris-HCl, 0.1% Tween-20, and 0.15% BSA; pH 7.4), conjugate storage buffer (0.02 mol/l PBS, 0.1% BSA, 0.3% TritonX-100, 0.02% NaN_3; pH 7.4), and conjugate lyophilization buffer (5% trehalose, 1% BSA, 2% mannitol, 1% glycine, 0.01% TB, 0.5% casein, and 0.1% NaN_3; pH 7.4). All the reagents mentioned above were provided by Beijing Hotgen Biotech.

2.3. Patients and samples

All sera involved in this study were collected from Fujian Provincial Hospital from June 2015 to December 2017. The use of sera was approved by the Ethics Committee of Fujian Provincial Hospital. Sera were obtained from 100 healthy controls (HC) (median age 44 y; 47 males and 53 females), 170 patients with liver benign lesion (LBL), including 90 patients with chronic hepatitis B (CHB) (median age 48 y; 57 males and 33 females), 80 patients with liver cirrhosis (LC) (median age 63 y; 54 males and 26 females), 228 patients with HCC (median age 56 ys; 199 males and 29 females). All the HC subjects were serologically negative with hepatitis B surface antigen, normal in liver function, and had no masses under ultrasound or CT/MRI imaging. The patients with LC were confirmed by abdominal ultrasonography or liver tissue biopsy. All patients with CHB were serologically positive with hepatitis B surface antigen for more than six months. The patients with HCC were confirmed by pathological examination after liver biopsy under ultrasound guidance or liver surgery. All serum samples were preserved strictly in accordance with the following requirements: 5 ml of peripheral venous blood was collected under early morning fasting, left at room temperature for 2 h, centrifuged at 3000 rpm for 5 min to separate the serum, dispensed in two tubes, and frozen at −80 °C from 1.5 months to 2.0 y until use.

2.4. Development of UPT-LF test card

2.4.1. Silica coating and surface modification activation of UCPs

The UCPs (Supplementary Fig. 1(A)) were developed in accordance with the nanoparticle modification method [21]. The procedure was as follows: 40 mg UCPs and 95 ml cyclohexane were dissolved in water and sonicated to complete suspension, and 2.5 ml nonionic emulsifier CO-520 was added. After sonication in water for 20 min, 0.35 ml ammonia (30 wt%) and 0.09 ml TEOS were added for 24 h under the magnetic stirring. Then, 16 ml ethanol was added, and the supernatant was removed after centrifugation. Finally, the precipitate was dissolved with 90 ml isopropanol, followed by 0.9 ml APES addition, magnetic stirring for 2.5 h, centrifugation and washing for 3 times.

2.4.2. Assembly of the UPT-LF test card

The assembly of the UPT-LF strip was carried out as previously described [22]. The procedure of optimization was as follows: 5 mg modified and activated UCPs with protein binding activity bound to the labeled antibody mIgG1 (0.075 mg/ml) in 1 ml reaction system buffer to form a luminescent label probe. The UCPs- mIgG1 conjugates were diluted by conjugate storage buffer and conjugate lyophilization buffer, and then attached to the blank glass fiber SB08 at 0.1 ml/cm to form a conjugated pad. The pad was freeze-dried at −60 °C for 8 h. The blank glass fiber SB08 was treated with HBR-IgG and casein as a sample pad. The test antibody (mlgG2) and the control antibody (goat anti-mouse IgG) were diluted by antibody buffer to 2.0 and 0.6 mg/ml, respectively, and then the detection line (T line) and the quality control line (C line) were drawn apart from 3 mm on the NC membrane by using a continuous dispenser and sprayer, The NC membrane was dried at 37 °C for 2 h. The conjugated pad and sample pad were cut into 0.5 and 2.0 cm widths, respectively. The absorbent pad, NC membrane, the conjugated pad, and the sample pad were attached to the lamination card, successively, and then cut into 4 mm wide strips by using a high speed sensor cutter (Supplementary Fig. 2(B)). The strip was packed in a UPT exclusive shell (Supplementary Fig. 2(A)) to complete the entire assembly process. The relationship between the structure of strip and the window position of the shell is shown in Supplementary Fig. 2(C).

2.5. The procedure of UPT-LF assay

The peripheral venous blood of a resident patient was collected by medical staff and centrifuged at bedside by a compact microcentrifuge

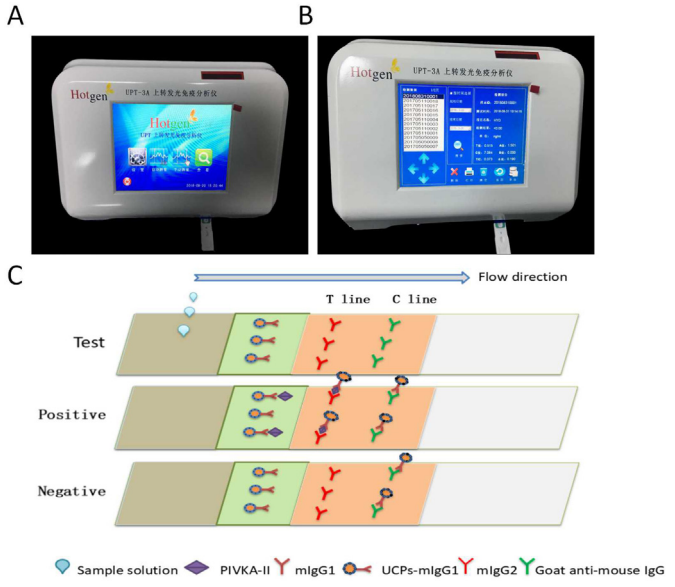

Fig. 1. Schematic diagram of the UPT-LF test card for the detection of serum sample. (A) The test card was scanned by UPT-3A-1800 biosensor. (B) The detection results were displayed on the screen of UPT-3A-1800 biosensor. (C) a schematic diagram of the immunoreaction on the strip.

at 3000 rpm for 5 min to separate the serum. 40 µl of serum was removed and mixed with 60 µl of sample diluent and then added to the sample adding window of the test card, and successively passed through the sample pad, conjugated pad, NC membrane and absorbent pad under the siphon effect of the absorbent pad (Fig. 1(C)). After 15 min, the test card was scanned in the detection hole of the UPT-3A-1800 biosensor (Fig. 1(A)), and the labeled probe launched the visible light of the 541.5 nm wavelength under the 980 nm NIR excitation light (Supplementary Fig. 1(B)). The biosensor converted the received light signals from T line and C line into electrical signals with "T value" and "C value", and the results were described as the ratio of T to C values (T/C values) (Fig.1(B)). The T/C value was positively correlated with the concentration of detected antigen.

2.6. Evaluation of the UPT-LF assay

2.6.1. Limit of detection (LoD) and linearity

The limit of blank (LoB) was calculated using 60 replications of blank (sample dilution with no analyte), LoB = mean $_{blank}$ + 2 (SD $_{blank}$). The LoD was calculated using 60 replications of low concentration sample (0.5 ng/ml) and LoD = LoB +2 (SD $_{low\ concentration\ sample}$) [23].

After two-fold serial dilutions to 40,000, 20,000, 10,000, 5000, 2500, 1250, 625, 312, 156, 78, 39, 19.5, 9.7, 4.8 and 2.4 ng/ml, each concentration of the PIVKA-II calibrator (PIVKA-II recombinant antigen) was detected twice, and the average value (C_o) was calculated. The linear regression curve was drawn by using log (C_o) as the ordinate X and log (T/C- mean $_{blank}$) as the abscissa Y. The regression equation was "Y = a × X + b". The a and b in the equation were the parameters of the test card, which determined the conversion relationship between the T/C value and the concentration of the detected antigen.

2.6.2. Stability

The PIVKA-II recombinant antigen and the sera of HCC, LC, CHB and HC were detected by UPT-LF test cards, which were stored at 25 °C for 0, 7, 15, 30 and 60 days (d), at 37 °C for 0, 1, 3, 5 and 7 d, and at 4 °C for 0 d and 18 months (m) (Reference to the validity period of other test cards based on the UPT-LF assay in Beijing Hotgen Biotech), respectively. Each sample was detected twice, and the relative deviation (δ) was calculated by comparing the results of test cards at different time points with that at 0 d. δ < 15% was considered acceptable.

2.6.3. Recovery rate

The PIVKA-II recombinant antigen was diluted to 55, 1000 and 15,000 ng/ml, respectively, and added to normal serum sample at a volume ratio of 1:9. The mixed samples and serum sample were repeatedly detected for 3 times, and the mean was calculated. The recovery rate was calculated in accordance with the following equation:

$$R = \frac{(V_0 + V_1) \times C - V_1 C_1}{V_0 C_0} \times 100\%$$

In the equation, the volume and concentration of PIVKA-II recombinant antigen was V_0 and C_0, respectively. The volume and concentration of serum sample was V_1 and C_1, respectively. The concentration of mixed sample was C. The recovery rate was R, and a recovery rate of 85%–115% was considered acceptable.

2.6.4. Precision

Serum samples with low concentration (26.3 ng/ml), medium concentration (1052.9 ng/ml) and high concentration (15,925.9 ng/ml) of PIVKA-II were selected for intra-assay and inter-assay precision evaluation. Three different concentrations of serum samples with 20 duplications were then tested to obtain intra-assay variation. These serum samples were tested on 20 different days, with each concentration having two replicates to obtain inter-assay variation. Precision was evaluated by intra-assay/inter-assay CV (%). The intra-assay CV < 10% and inter-assay CV < 15% were considered acceptable.

2.6.5. Interference

Distilled water and eight common interferents including 5 mg/dl AFP, 0.20 µg/ml carcinoembryonic antigen (CEA), 750 IU/ml rheumatoid factor (RF), 65 mg/dl bilirubin, 7 g/dl albumin, 12.30 mg/dl vitamin C, 0.35 g/dl hemoglobin (Hb) and 500 mg/dl triglyceride (TG) were added to serum samples from HC and HCC patient, with 7.7 ng/ml and 527.8 ng/ml PIVKA-II at a volume ratio of 1:9, respectively. The 8 mixtures and serum sample were detected for 3 times. The anti-interference performance of UPT-LF assay was evaluated by bias (δ %) = (concentration of mixture - concentration of serum sample) / concentration of serum sample ×100%. δ % < 15% was considered acceptable.

2.6.6. Cutoff, sensitivity and specificity for the diagnosis of HCC

The concentrations of serum PIVKA-II of 228 cases in HCC group, 170 cases in LBL group (80 cases with LC, 90 cases with CHB) and 100 cases in HC group were detected by the UPT-LF assay. The differences in serum PIVKA-II concentration between the HCC group and control group (LBL + HC), and among the control groups (LC, CHB, and HC) were assessed by Mann-Whitney *U* test and Kruskal-Wallis test, respectively. The diagnostic performance for HCC was analyzed by receiver operating characteristic (ROC) curve, and the best Youden index (YI) was used as the basis for determining the cutoff in diagnosis of HCC.

2.6.7. Comparison between UPT-LF assay and CLEIA assay

Serum PIVKA-II concentrations of 178 cases in HCC group, 60 cases in LBL group (30 cases with LC and 30 cases with CHB) and 35 cases in HC group were detected by UPT-LF test card and Lumipulse® G PIVKA-II detection kit (CLEIA), respectively. The sensitivity and specificity (vs LBL + HC) of PIVKA-II for HCC were compared between two assays, and the correlation of PIVKA-II concentrations of 178 HCC cases between two assays was evaluated. The LoD, linear range, and cutoff for HCC of Lumipulse® G PIVKA-II detection kit (CLEIA) were 1.37 mAU/ml, 5–75,000 mAU/ml, and 40 mAU/ml, respectively. In accordance with the instruction of the Lumipulse® G PIVKA-II detection kit from FUJIREBIO INC., 1 mAU/ml = 1 ng/ml.

3. Statistical analysis

Mann-Whitney *U* test was used to compare continuous measurement data with a non-normal distribution between two groups. Kruskal-Wallis test was employed while comparing three groups. The correlation between two assays was evaluated using Pearson's correlation coefficient (R). Statistical analysis was performed using the software SPSS 20.0 (SPSS), GraphPad Prism 5.0, and Excel. $P < 0.05$ was considered as statistically significant.

4. Results

4.1. Surface silicification and functionalization of UCPs

Given the synthesis methods, UCPs generally had a layer of hydrophobic oleic acid ligands on the surfaces [24], whereas nonionic emulsifier CO-520 may cause the particles to monodisperse as far as possible by reducing the surface-tension of the particles and enhancing the water solubility. The surface silicification and activation process of UCPs were shown in Supplementary Fig. 1(C). After TEOS and ammonia water were added, the nano-SiO_2 was formed and adhered to the surface of the particles, making the surface of the particles coated with a layer of nano-SiO_2 [25]. When a silane coupling agent APES was added in the reaction, one end of the nano-SiO_2 on the surface of UCPs can then be connected to silicon, whereas the other end can carry an active carboxyl group. As a result, the functionalized UCPs can bind to various bioactive molecules, such as antibodies.

4.2. Detection system of UPT-LF assay

The detection system consisted of PIVKA-II sample dilution, test card, and UPT-3A-1800 biosensor (Fig. 1). The test card consisted of the shell and internal strip. The shell included an upper shell and a bottom shell. The upper shell was successively equipped with a sample adding window, a sample scanning window and a chromatography end indicating window. The strip was put in the groove of the bottom shell, with the sample pad with HBR-IgG, conjugate pad with bio-probes, NC membrane with T (2.0 mg/ml) and C line (0.6 mg/ml), and absorbent pad by the direction of chromatography. The portable UPT-3A-1800 biosensor was an ideal candidate available for the serum PIVKA-II POCT test, with a relatively cheap infrared light source and rechargeable batteries.

4.3. LoD and linearity of UPT-LF assay

The Mean $_{blank}$, SD $_{blank}$ and SD $_{low\ concentration\ sample}$ were 0.112, 0.0064 and 0.0041, respectively, and the T/C values of LoB and LoD were 0.125 and 0.129, respectively, which corresponded to the concentrations of 2.25 and 2.66 ng/ml, respectively, under the linear regression equation (shown below).

The linearity of UPT-LF assay for the quantitative detection of PIVKA-II was presented in Fig. 2, where a significant linear relationship with PIVKA-II concentration ranging from 4.8 to 20,000 ng/ml ($R^2 = 0.984$, $P < 0.01$) was achieved. However, no linear relationship was observed for the PIVKA-II concentrations < 4.8 or > 20,000 ng/ml (data not shown). Accordingly, log (C_o) and log (T/C- mean $_{blank}$) of 13 concentrations from two-fold serial dilutions were calculated, and the linear regression equation was deduced as follows: $Y = 0.827X - 1.871$ ($N = 13$, $P < 0.01$).

4.4. Stability

The results of UPT-LF test cards stored at 25 °C for 0, 7, 15, 30, and 60 d were shown in Fig. 3(A) and (B), which bias (δ) calculated by comparing the results at different time points with that at 0 d was < 15%. Meanwhile, the results of test cards stored at 37 °C for 0, 1, 3, 5, and 7 d were presented in Fig. 3(C) and (D), with a calculated δ > 15% when the results at 7 d were compared with that at 0 d. Finally, the results of test cards stored at 4 °C for 0 d, and 18 m (recommended validity period) were displayed in Supplementary Fig. 3 and corresponded with a calculated δ < 15% when the results of test cards at 18 m were compared with that at 0 d.

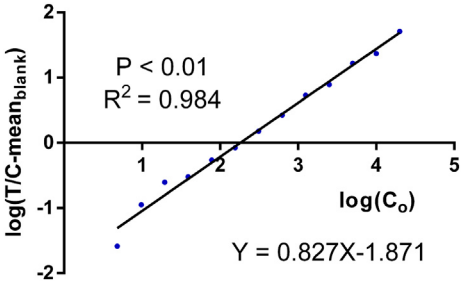

Fig. 2. Standard curve for UPT-LF assay. The T/C value is the mean of each diluted level detected twice; the mean $_{blank}$ and C_o are the background value and the diluted level (ng/ml) of PIVKA-II calibrator, respectively. The same base logarithms of T/C– mean $_{blank}$ and C_o are taken as Y and X axis, respectively, to draw the curve.

4.5. Recovery rate

The accuracy of UPT-LF assay was evaluated by recovery rate. Three different concentrations of serum were analyzed by adding the low, medium, and high concentrations of PIVKA-II recombinant antigen to normal serum sample. The recovery rates of low, medium and high concentrations were 93.1%, 97.7%, and 99.2%, respectively, which were between 85% and 115%.

4.6. Precision

The results of intra-assay and inter-assay precision determined by three different concentration PIVKA-II serum sample were shown in Table 1. The intra-assay CV was < 5%, and the inter-assay CV was < 10%.

4.7. Interference

The specificity of UPT-LF assay was evaluated by interference. Eight common interferents were added to serum samples from HC and HCC patient, respectively, and anti-interference ability of the UPT-LF assay was presented in Table 2, where all δ (%) of eight common interferents were < 10%.

4.8. Cutoff, sensitivity and specificity for HCC diagnosis

The quantitative detection results of the serum samples by UPT-LF assay were presented in Fig. 4(A). The concentration (C_o, ng/ml) of serum PIVKA-II in HCC group (median, 53.2 ng/ml, and inter-quartile range [IQR], 20.6–322.3 ng/ml) was significantly higher than that of the control group (LBL + HC group) (median, 9.2 ng/ml, and IQR, 4.8–15.4 ng/ml) ($Z = -13.7$, $P < 0.01$). The differences among the LC group (median, 10.8 ng/ml, and IQR, 7.0–20.1 ng/ml), CHB group (median, 10.7 ng/ml, and IQR, 5.4–16.3 ng/ml) and HC group (median, 6.7 ng/ml, and IQR, 3.1–14.2 ng/ml) were observed not to reach statistical significance ($X^2 = 5.67$, $P > 0.05$). ROC curve analysis (HCC vs LBI + HC group) indicated that the area under the ROC (AUC) of serum PIVKA-II was 0.85 ($P < 0.001$) (Fig. 4(B)). Given the optimal cutoff for the diagnosis of HCC was set at 25.3 ng/ml (T/C value of 0.29) by the maximum YI of 0.6, sensitivity and specificity of serum PIVKA-II for HCC were 71.5% and 88.9%, respectively.

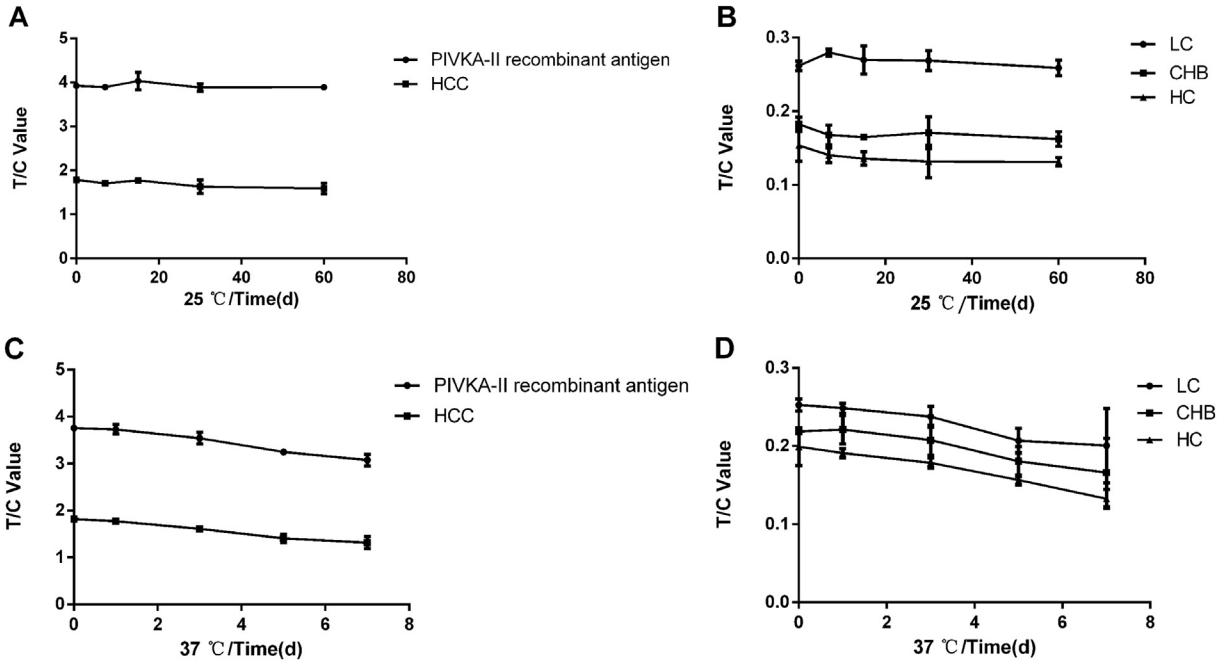

Fig. 3. Line chart of quantitative detection results for PIVKA-II recombinant antigen and PIVKA-II in sera of HCC, LC, CHB and HC by the test card stored at 25 ℃ and 37 ℃ for five time points. The quantitative detection results of the test card stored at 25 ℃ for 0, 7, 15, 30 and 60 d were presented in (A) and (B). The quantitative detection results of the test card stored at 37 ℃ for 0, 1, 3, 5 and 7 d were presented in (C) and (D).

Table 1
Intra-assay and Inter-assay precision of PIVKA-II in serum samples.

PIVKA-II	Intra-assay (N = 20)	Inter-assay (N = 20)
(ng/ml)	Mean (ng/ml) SD CV (%)	Mean (ng/ml) SD CV (%)
26.3	27.4 1.6 5.8	26.8 2.4 8.9
1052.9	1025.7 35.7 3.4	1064.1 69.2 6.5
15,925.9	15,884.5413.0 2.6	15,923.7859.9 5.4

N: number of serum samples.

4.9. Comparison between UPT-LF assay and CLEIA assay

Considering the concentration of 25.3 ng/ml set as the cutoff for the diagnosis of HCC in UPT-LF assay, the sensitivity and specificity were 70.2% and 90.5%, respectively, which were comparable to that of CLEIA assay with the sensitivity of 67.4% and specificity of 91.6%. In addition, a significant linear relationship of serum PIVKA-II concentrations was noted between UPT-LF assay and CLEIA assay; the linear regression equation was $Y = 0.08X - 4.50$ ($R^2 = 0.901$, $P < 0.01$) (Fig. 5).

5. Discussion

Ten carboxylated glutamic residues exist in the molecular structure of prothrombin precursor, and are modified to γ-carboxyglutamic acid (Gla) residues by the glutamyl carboxylase in hepatocytes [26]. Because vitamin K is deficient or glutamyl carboxylase activity is impaired, abnormal prothrombin devoid of one to ten Gla residues and no procoagulant activity is produced and released to the bloodstream in HCC patients [27]. Accordingly, serum PIVKA-II detection has comprehensively demonstrated its potential in the diagnosis of HCC. For example, a multicenter study comprised of 1034 serum samples from different regions of China indicated the higher accuracy of PIVKA-II than that of AFP in distinguishing HCC from various other liver lesions, including LC, liver metastasis, liver hemangiomas, and CHB. Additionally, the superiority of PIVKA-II to AFP was presented to be profound in the surveillance of early HCC with a diameter < 3 cm and AFP-negative HCC [28]. Similar observations were obtained by Pote et al. [29] and Zakhary et al. [30]. Furthermore, PIVKA-II combined with AFP or AFP-L3 was reported to be conductive to improving the diagnostic sensitivity for HCC, especially at early stage [31–34]. Besides the excellent performance for HCC diagnosis, serum PIVKA-II has been shown to be a valuable serological indicator responsible for the illness assessment and prognosis judgment of HCC [2,4–6].

With in-depth understanding of the relationship between PIVKA-II and HCC, several traditional assays for serum PIVKA-II have been introduced in succession; these assays include the radioimmunoassay [1], enzyme-linked immunosorbent assay (ELISA) [35], and conventional enzyme immunoassay (EIA) [36]. However, considering the disadvantages such as the long and labor-intensive process, insufficient sensitivity and precision, to name a few, existed in the above traditional

Table 2
Interference evaluation of UPT-LF assay.

	HC serum	Common interferents							
	HCC serum	AFP	CEA	RF	Bilirubin	Albumin	Vitamin C	Hb	TG
PIVKA-II (ng/ml)	7.7	8.1	7.9	8	8.2	7.9	7.9	8.3	8.1
	523.4	531.5	519.2	539.7	542.9	528.9	533.6	546.4	536.3
δ (%)		5.2	2.6	3.9	6.5	2.6	2.6	7.8	5.2
		1.5	−0.8	3.1	3.7	1.1	1.9	4.4	2.5

AFP: 5 mg/dl, CEA: 0.20 μg/ml, RF: 750 IU/ml, bilirubin: 65 mg/dl, albumin: 7 g/dl, vitamin C: 12.30 mg/dl, Hb: 0.35 g/dl, TG: 500 mg/dl.

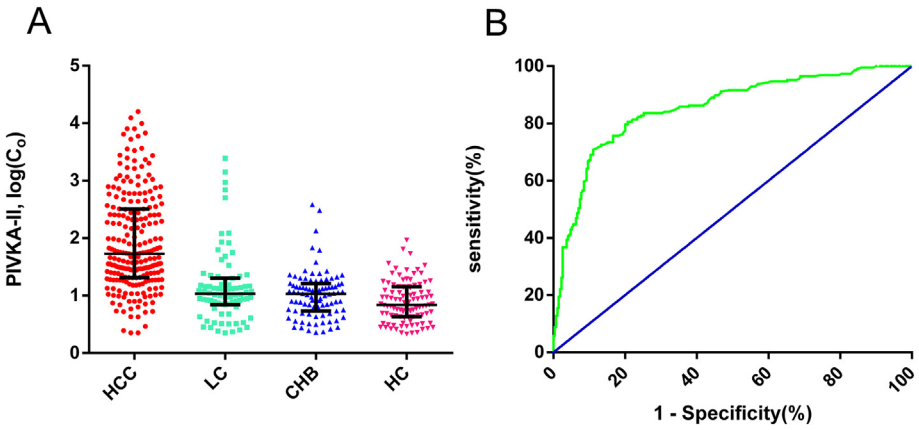

Fig. 4. Evaluation of the performance of UPT-LF assay for serum PIVKA-II detection in four groups by using scatter plot and the diagnostic performance for HCC by using receiver operating characteristic (ROC) curve. (A) The detection results ($\log(C_o)$) of UPT-LF assay for serum PIVKA-II in HCC, LC, CHB and HC group. The horizontal line represented median level of serum PIVKA-II, and the vertical line represented inter-quartile range. (B) The ROC for HCC (vs LBL + HC). The area under ROC (AUC) is 0.85 ($P < 0.001$).

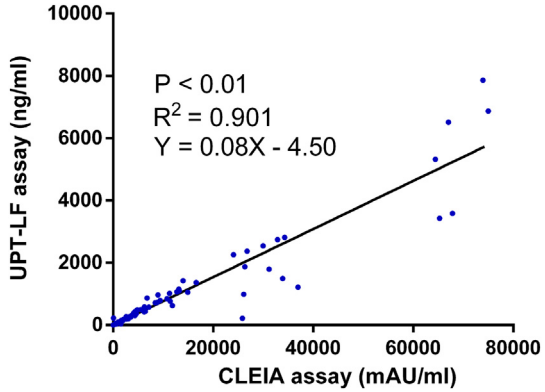

Fig. 5. Linear relationship between the UPT-LF assay and the CLEIA assay for the quantitative detection of serum PIVKA-II in 178 HCC patients. The linear regression equation is $Y = 0.08X - 4.50$, and correlation coefficient R^2 between two assays is 0.901 ($P < 0.01$).

assays, new assays with improved analytical performances for serum PIVKA-II detection have been developed in recent ys, including CLEIA [7,8], CLIA [9], LBA [10], a micro-total analysis system (μTAS) based on the LBA of electrokinetic analyte transport assay (EATA) as well as capillary zone electrophoresis (CZE) [7], and immunomagnetic reduction (IMR) assay [37]. For instance, as a representative of various PIVKA-II assays, Lumipulse® G system provided an automated CLEIA assay for the quantitative detection of serum PIVKA-II, with a total imprecision < 5% CV for the concentrations between 41 and 62,825 mAU/ml, a limit of qualification of 1.37 mAU/ml, and a wide linear range (5–75,000 mAU/ml) [8].

The luminescence principle of UCPs is anti-Stokes shift [38]; thus, a near infrared semiconductor laser can be used as the excitation source under such technology. Given the infrared light with the advantages of no effect on biological tissue or blood and low background noise [39], UCPs have a higher sensitivity and signal-to-noise ratio than that of conventional luminescent probes [14]. According to several previously reported studies, UPT-LF assay has been successfully applied for the quantitative detection of some proteins, nucleic acids, and bacteria in human specimens (such as whole blood or serum) [18,22,40–41].

In this study, we report a test card for the quantitative detection of serum PIVKA-II based on UPT-LF assay for the first time. In this test card, UCPs labeled anti-PIVKA-II monoclonal antibody is used as a probe, and the strip is used as a solid-phase carrier of immunoreaction. The developed UPT-LF assay was shown to be applicable to not only a wide linear range (4.8–20,000 ng/ml) for serum PIVKA-II, but also a high sensitivity with LoD of 2.66 ng/ml. The stability evaluation revealed the test card can be kept stable at 4 °C during validity period and has good thermal duration at room temperature. However, the thermal stability of test card at 37 °C is not as good as that at 25 °C, with a calculated $\delta > 15\%$ when the detection results at 7 d were compared with that at 0 d. This effect may be related to the degradation of the antibodies on the conjugate pad or the antibodies coated on the NC membrane stored at a relatively high temperature for a long time. Additionally, the recovery rates of UPT-LF assay for low, medium, and high concentrations of PIVKA-II recombinant antigen vary from 93.1% to 99.2%, the CVs of intra-batch and inter-batch are < 5% and < 10%, respectively, and all δ (%) of eight interferents are < 10%. All above evidences reveal the fact that the developed UPT-LF assay can reliably detect serum PIVKA-II concentration with excellent detection performance of good sensitivity, linearity, stability, accuracy, precision, and specificity. In particular, the UPT-3A-1800 biosensor equipped with the near infrared semiconductor laser as the excitation light source in detection system of UPT-LF assay, is an ideal immunoassay analyzer for POCT, with the advantages such as small size, easy to carry, and short detection time no > 15 min [12]. As such, the UPT-LF assay has become available for a near-patient detection of serum PIVKA-II concentration and hence enables “on-the-spot” decision-making and overcomes the dependence on sample shipment to special sites such as large biochemical department and specialist centralized laboratory, where serum PIVKA-II is measured on complex CLEIA analyzer in batches, and sometimes not on a daily basis. However, as a POCT, we have to acknowledge that there are challenges for this assay's use at the patient's bedside, since it requires centrifugation of whole blood followed by separation of the serum.

We further explore the performance of test card based on UPT-LF assay for HCC diagnosis. The serum PIVKA-II concentration in HCC group is significantly higher than that of the control group (LBL + HC group) ($P < 0.01$), whereas no significant differences are noted among the LC group, CHB group, and HC group ($P > 0.05$). Additionally, no significant differences in serum PIVKA-II concentrations were noted between females and males in our study, which is supported by the following statistical data from the Mann–Whitney *U* test: $Z = -0.628$, $P = 0.530$ in the HCC group; $Z = -0.255$, $P = 0.798$ in the LC group; $Z = -1.117$, $P = 0.264$ in the CHB group; and $Z = -0.237$, $P = 0.813$ in the HC group. As expected, UPT-LF assay results presented herein exhibit good diagnostic performance for HCC, given the significant differentiation of HCC from LBL and HC with an AUC reaching 0.85. As the concentration of 25.3 ng/ml is set as cutoff, the best performance of test card for HCC can be achieved, with the diagnostic sensitivity and specificity (vs LBL + HC) of 70.2% and 90.5%, respectively, which are comparable to that of commercially available CLEIA kit. In addition, on the basis of result of the linear regression analysis, a good linear relationship ($R^2 = 0.901$) is observed between UPT-LF assay and CLEIA assay for the quantitative detection of serum PIVKA-II in HCC patients.

Indeed, UPT-LF assay developed in this study provides a reliable, rapid and convenient test for the quantitative detection of serum PIVKA-II by a POCT way. This advancement may greatly contribute to

the further popularization of serum PIVKA-II detection, further improvement of the screening and diagnosis of HCC, and then reducing the mortality of patients.

Acknowledgement

This work was supported by the Medical Innovation Program of Fujian Province (No.2017-CX-4), and key project of Science and Technology Plan of Fujian Province (No.2014Y0009) to Yi Huang.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.11.020.

References

[1] H.A. Liebman, B.C. Furie, M.J. Tong, et al., Des-gamma-carboxy (abnormal) prothrombin as a serum marker of primary hepatocellular carcinoma, N. Engl. J. Med. Sci. 310 (1984) 1427–1431.
[2] S. Nakamura, K. Nouso, K. Sakaguchi, et al., Sensitivity and specificity of des-gamma-carboxy prothrombin for diagnosis of patients with hepatocellular carcinomas varies according to tumor size, AM. J. Gastroenterol. Sci. 101 (2006) 2038–2043.
[3] Y.J. Yoon, K.H. Han, D.Y. Kim, Role of serum prothrombin induced by vitamin K absence or antagonist-II in the early detection of hepatocellular carcinoma in patients with chronic hepatitis B virus infection, Scand. J. Gastroenterol. Sci. 44 (2009) 861–866.
[4] N. Pote, F. Cauchy, M. Albuquerque, et al., Performance of PIVKA-II for early hepatocellular carcinoma diagnosis and prediction of microvascular invasion, J. Hepatol. Sci. 62 (2015) 848–854.
[5] B.X. Truong, Y. Yano, V.T. VAN, et al., Clinical utility of protein induced by vitamin K absence in patients with chronic hepatitis B virus infection, Biomed. Rep. 1 (2013) 122–128.
[6] Y. Yano, F. Yamashita, K. Kuwaki, et al., Clinical features of hepatitis C virus-related hepatocellular carcinoma and their association with alpha-fetoprotein and protein induced by vitamin K absence or antagonist-II, Liver. Int. Sci. 26 (2006) 789–795.
[7] J. Choi, Y. Park, J.H. Kim, et al., Evaluation of automated serum des-gamma-carboxyprothrombin (DCP) assays for detecting hepatocellular carcinoma, Clin. Biochem. Sci. 44 (2011) 1464–1468.
[8] P. Sultanik, A. Ginguay, J. Vandame, et al., Diagnostic accuracy of des-gamma-carboxy prothrombin for hepatocellular carcinoma in a French cohort using the Lumipulse((R)) G600 analyzer, J. Viral. Hepat. Sci. 24 (2017) 80–85.
[9] H. Kinukawa, T. Shirakawa, T. Yoshimura, Epitope characterization of an anti-PIVKA-II antibody and evaluation of a fully automated chemiluminescent immunoassay for PIVKA-II, Clin. Biochem. Sci. 48 (2015) 1120–1125.
[10] I. Yamaguchi, K. Nakamura, H. Kitano, et al., Development of des-gamma-carboxy prothrombin (DCP) measuring reagent using the LiBASys clinical analyzer, Clin. Chem. Lab. Med. Sci. 46 (2008) 411–416.
[11] P.L. Corstjens, C.J. De Dood, D. Kornelis, et al., Tools for diagnosis, monitoring and screening of Schistosoma infections utilizing lateral-flow based assays and upconverting phosphor labels, Parasitol. Sci. 141 (2014) 1841–1855.
[12] X. Yang, L. Liu, Q. Hao, et al., Development and evaluation of up-converting phosphor technology-based lateral flow assay for quantitative detection of NT-proBNP in blood, PLoS One 12 (2017) e0171376.
[13] E.M. Chan, E.S. Levy, B.E. Cohen, Rationally designed energy transfer in upconverting nanoparticles, Adv. Mater. Sci. 27 (2015) 5753–5761.
[14] Y. Yang, D. Tu, W. Zheng, et al., Lanthanide-doped Sr_2YF_7 nanoparticles: controlled synthesis, optical spectroscopy and biodetection, Nanoscale. Sci. 6 (2014) 11098–11105.
[15] J. Hampl, M. Hall, N.A. Mufti, et al., Upconverting phosphor reporters in immunochromatographic assays, Anal. Biochem. Sci. 288 (2001) 176–187.
[16] H. Chen, Y. Guan, S. Wang, et al., Turn-on detection of a cancer marker based on near-infrared luminescence energy transfer from $NaYF_4$: Yb, Tm/$NaGdF_4$ core-shell upconverting nanoparticles to gold nanorods, Lang. Sci. 30 (2014) 13085–13091.
[17] M. He, Z. Liu, Paper-based microfluidic device with upconversion fluorescence assay, Anal. Chem. Sci. 85 (2013) 11691–11694.
[18] F. Hua, P. Zhang, F. Zhang, et al., Development and evaluation of an up-converting phosphor technology-based lateral flow assay for rapid detection of Francisella tularensis, Sci. Rep. Sci. 5 (2015) 17178.
[19] W. Hong, L. Huang, H. Wang, et al., Development of an up-converting phosphor technology-based 10-channel lateral flow assay for profiling antibodies against Yersinia pestis, J. Microbiol. Methods Sci. 83 (2010) 133–140.
[20] X. Liu, Y. Zhao, C. Sun, et al., Rapid detection of abrin in foods with an up-converting phosphor technology-based lateral flow assay, Sci. Rep. 6 (2016) 34926.
[21] J. Tang, L. Wang, Q. Li, et al., Synthesis and upconversion luminescence of high-quality water-soluble $NaYF_4$:Yb^{3+}, Er^{3+} nanoclusters, J. Nanosci. Nanotechnol. Sci. 16 (2016) 3802–3805.
[22] Y.M. Wu, Y. Cen, L.J. Huang, et al., Upconversion fluorescence resonance energy transfer biosensor for sensitive detection of human immunodeficiency virus antibodies in human serum, Chem. Commun. Sci. 50 (2014) 4759–4762.
[23] D.A. Armbruster, T. Pry, Limit of blank, limit of detection and limit of quantitation, Clin. Biochem. Rev. Sci. 29 (Suppl. 1) (2008) S49–S52.
[24] S. Wilhelm, T. Hirsch, W.M. Patterson, et al., Multicolor upconversion nanoparticles for protein conjugation, Theranostics Sci. 3 (2013) 239–248.
[25] J.R. Abdul, Y. Zhang, Biocompatibility of silica coated NaYF(4) upconversion fluorescent nanocrystals, Biomater. Sci. 29 (2008) 4122–4128.
[26] N. Pozzi, Z. Chen, D.W. Gohara, et al., Crystal structure of prothrombin reveals conformational flexibility and mechanism of activation, J. Biol. Chem. Sci. 288 (2013) 22734–22744.
[27] S. Takahashi, M. Kudo, H. Chung, et al., PIVKA-II is the best prognostic predictor in patients with hepatocellular carcinoma after radiofrequency ablation therapy, Oncol -Basel Sci. 75 (Suppl. 1) (2008) 91–98.
[28] J. Ji, H. Wang, Y. Li, et al., Diagnostic evaluation of des-gamma-carboxy prothrombin versus alpha-fetoprotein for hepatitis B virus-related hepatocellular carcinoma in China: a large-scale, multicentre study, PLOS ONE 11 (2016) e0153227.
[29] N. Pote, F. Cauchy, M. Albuquerque, et al., Performance of PIVKA-II for early hepatocellular carcinoma diagnosis and prediction of microvascular invasion, J. Hepatol. Sci. 62 (2015) 848–854.
[30] N.I. Zakhary, S.M. Khodeer, H.E. Shafik, et al., Impact of PIVKA-II in diagnosis of hepatocellular carcinoma, J. Adv. Res. Sci. 4 (2013) 539–546.
[31] T.S. Lim, D.Y. Kim, K.H. Han, et al., Combined use of AFP, PIVKA-II, and AFP-L3 as tumor markers enhances diagnostic accuracy for hepatocellular carcinoma in cirrhotic patients, Scand. J. Gastroenterol. Sci. 51 (2016) 344–353.
[32] J.A. Marrero, Z. Feng, Y. Wang, et al., Alpha-fetoprotein, des-gamma carboxyprothrombin, and lectin-bound alpha-fetoprotein in early hepatocellular carcinoma, Gastroenterol. Sci. 137 (2009) 110–118.
[33] S.J. Park, J.Y. Jang, S.W. Jeong, et al., Usefulness of AFP, AFP-L3, and PIVKA-II, and their combinations in diagnosing hepatocellular carcinoma, Med. (Baltimore) Sci. 96 (2017) e5811.
[34] I. Gentile, A.R. Buonomo, R. Scotto, et al., Diagnostic accuracy of PIVKA-II, alpha-fetoprotein and a combination of both in diagnosis of hepatocellular carcinoma in patients affected by chronic HCV infection, In Vivo Sci. 31 (2017) 695–700.
[35] K. Motohara, Y. Kuroki, H. Kan, et al., Detection of vitamin K deficiency by use of an enzyme-linked immunosorbent assay for circulating abnormal prothrombin, Pediatr. Res. Sci. 19 (1985) 354–357.
[36] T. Naraki, K. Watanabe, Y. Shimazuru, et al., Development and evaluation of the EIA kit for detection of PIVKA-II using double antibody sandwich system: monoclonal antibody to PIVKA-II and polyclonal antibody to prothrombin, Clin. Immunol. Sci. 18 (1986) 479–492.
[37] J.J. Chieh, K.W. Huang, C.P. Chuang, et al., Immunomagnetic Reduction Assay on des-gamma-carboxy prothrombin for screening of hepatocellular carcinoma, IEEE. Trans. Biomed. Eng. Sci. 63 (2016) 1681–1686.
[38] F. Auzel, Upconversion and anti-Stokes processes with f and d ions in solids, Chem. Rev. Sci. 104 (2004) 139–173.
[39] W. Zheng, D. Tu, P. Huang, et al., Time-resolved luminescent biosensing based on inorganic lanthanide-doped nanoprobes, Chem. Commun. Sci. 51 (2015) 4129–4143.
[40] L. Li, L. Zhou, Y. Yu, et al., Development of up-converting phosphor technology-based lateral-flow assay for rapidly quantitative detection of hepatitis B surface antibody, Diagn. Microbiol. Infect. Dis. Sci. 63 (2009) 165–172.
[41] P. Corstjens, M. Zuiderwijk, A. Brink, et al., Use of up-converting phosphor reporters in lateral-flow assays to detect specific nucleic acid sequences: a rapid, sensitive DNA test to identify human papillomavirus type 16 infection, Clin. Chem. Sci. 47 (2001) 1885–1893.