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Joint detection of tumor markers with imaging ellipsometry biosensor

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ABSTRACT

Tumor marker detection contributes to the early diagnosis of cancers. However, due to the lack of detection specificity, its results cannot act as a direct evidence to confirm cancer occurrence in clinic. Joint detection of tumor markers may improve the detection specificity. As a trial for clinical diagnosis of hepatocellular carcinoma, α -fetoprotein, α -L-fucosidase and ferritin have been combined and detected with a label-free, phase sensitive and high throughput imaging ellipsometry biosensor (IEB). Eighty-two sera have been quantitatively detected with IEB and the results are in agreement with the clinical standard approaches. Evaluated by receiver operating characteristic analysis, the specificity of joint detection improves remarkably with IEB for hepatocellular carcinoma. It can be foreseen that the joint detection of tumor markers with IEB has a potential for clinical cancer diagnosis.

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1. Introduction

Cancer is the uncontrolled growth and spread of cells [1] and it has become a serious danger to human beings [2]. However, its survival chance is still basically dependent on tumor types, time of diagnosis and of course, clinical therapies [3]. The detection of tumor markers has been widely used to screen cancers on a population basis [4]. Although it contributes to cancer diagnosis and clinical therapies, tumor marker detection results cannot act as a direct evidence to confirm cancer presence because of the lack of detection specificity. In order to increase the detection specificity, several tumor markers are combined to detect cancers and improved results are acquired. However, joint detection of tumor markers is much more complicated than single marker detection and raises the requirement to detection methods. Thus, the need for effective methods to detect tumor marker combinations rapidly, sensitively and reliably is consequently subjected to broad interest.

Imaging ellipsometry [5,6] is derived from the conventional ellipsometry by introducing an expanded beam and a CCD camera instead of the traditional narrow beam and the photodiode detector. These changes render the unique advantages of conventional ellipsometry and microscope to imaging ellipsometry. Imaging ellipsometry is not only a label-free characterization technique with high resolution but it also owns a large field of view, providing the capability to detect a considerable area at the same time [7,8]. The concept of imaging ellipsometry biosensor (IEB) for visualization of biomolecular interactions was proposed in 1995 [9,10]. With the developments these years, it has become automatic equipment [11,12] for protein interaction analysis. IEB is primarily composed of a microfluidic reactor [13] and an imaging ellipsometer

[10,14]. The microfluidic reactor is served to fabricate a patterned protein microarray; while the imaging ellipsometer is used to measure the surface mass concentration distribution of a protein microarray. So far, IEB has accumulated several application experiences in biological and clinical fields, for instance, five markers of hepatitis B virus [15], tumor markers [16,17], phage M₁₃KO₇ [18], severe acute respiratory syndrome virus [19], avian influenza virus [20] and ricin antibody identification [21].

Hepatocellular carcinoma is one of most common cancers in developing countries [2] and the average survival period is only about several months. Accurate diagnosis in an early stage has a potential to extend the survival period effectively. α -Fetoprotein (AFP) [22] and α -L-fucosidase (AFU) [23] are considered as two specific tumor markers for hepatocellular carcinoma; while ferritin [24] is commonly used to monitor lesion in liver tissue. In this investigation, the quantitative detection of these three tumor markers simultaneously has been performed with IEB as a trial.

2. Materials and methods

2.1. IEB and its detection principle

During the last decade, IEB has been updated and improved in our laboratory [11,12,14]. Now, it is mainly composed of a microfluidic reactor and an imaging ellipsometer.

The microfluidic reactor integrating 48 independent flow channels is used to fabricate protein microarrays by a series of continuous processes, including the surface patterning, the ligand immobilization, the surface blocking and the analyte solution delivery as well as the surface rinsing [12]. When a silicon wafer substrate is placed on the top of the reactor, it forms the 48 independent reaction cells and each cell has an independent inlet and outlet for the solution delivery. The inlet channels are connected to a sample reservoir and the outlet channels are connected with

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the pumps offering negative pressure. With the microfluidic reactor, different solutions can be delivered to the appointed reaction cells and afterwards a microarray can be fabricated in a precise pattern.

The imaging ellipsometer acts as the data acquisition of a protein microarray. Imaging ellipsometry is an enhancement of the standard single beam ellipsometry, which combines the power of ellipsometry with microscopy and is worked in the off-null mode. It can be utilized for the visualization of surface mass concentration distribution of protein layers. A slight variation of surface mass concentration can be remarkably distinguished by the imaging ellipsometer and the result is represented in gray-scale images. Upon the high sensitivity requirements to observe biomolecule interactions, the imaging ellipsometer has been improved by the introduction of a spectroscopic light source and a low noise imaging device [12] with the optimization of polarization settings.

The principle to detect protein interactions with IEB is shown in Fig. 1 [11]. A ligand is immobilized on a surface to form a biosensing surface to a receptor which exists in an analyte solution. When the bio-sensing surface is exposed to the analyte solution, ligands and receptors can interact specifically with each other to form complexes due to their affinity. The surface mass concentration of protein layers will change during this recognition process. With the visualization of imaging ellipsometry, the change can be determined quantitatively in the format of gray-scale images. In that case, the existence of the receptor in the analyte solution can be verified.

2.2. Chemicals, samples and substrates

Silicon wafers are provided by Beijing GRINM Materials Company. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), succinic anhydride and aminopropyl-triethoxysilane (APTES) are purchased from Acros Organics. N-Hydroxy-succinimide (NHS), blocking buffer, phosphate buffered saline containing 0.05% Tween-20 (PBST) and protein A from *Staphylococcus aureus* are bought from Sigma-Aldrich. AFP and AFP rabbit monoclonal antibody are purchased from Sigma-Aldrich. AFU and its mouse monoclonal antibody are obtained from Abcam. Recombinant ferritin and its goat monoclonal antibody are purchased from Sigma-Aldrich. 82 human serum samples including 41 healthy people and 41 hepatocellular carcinoma patients are collected in Shandong Academy of Medical Sciences. Their detailed clinical background and commercial immunoassay kit detection results are presented in Tables A.1 and A.2 in Appendix A. Deionized water is produced by ion exchange demineralization, followed by passing through a Milli-Q plus system from Millipore.

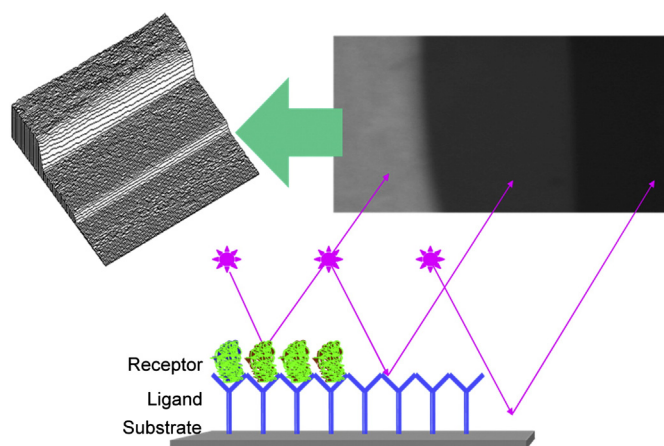


Fig. 1. IEB principle to detect protein interactions. The polarized light is incident on a protein layer and the reflective beam carries the information of the protein layer, for example its surface mass concentration. Ligand molecules can react with their receptors and form complexes on the biosensing interface, resulting in the change of layer surface mass concentration. This variation is embodied in reflection light intensity and can be quantitatively recorded in the format of gray-scale image [11].

2.3. Silicon wafer preparation and surface modification

Silicon wafer is used as a solid substrate for IEB. Due to the throughput need of 48 independent units, the silicon slides are cut into $25 \times 13 \text{ mm}^2$ rectangular pieces. Then, in order to wash out the organic and inorganic contamination, silicon wafers are cleaned by a fresh piranha solution for 30 min. After being thoroughly rinsed with deionized water and pure ethanol, silicon wafers are incubated in an ethanol solution of APTES (5% APTES and 95% pure ethanol) for 2 h at room

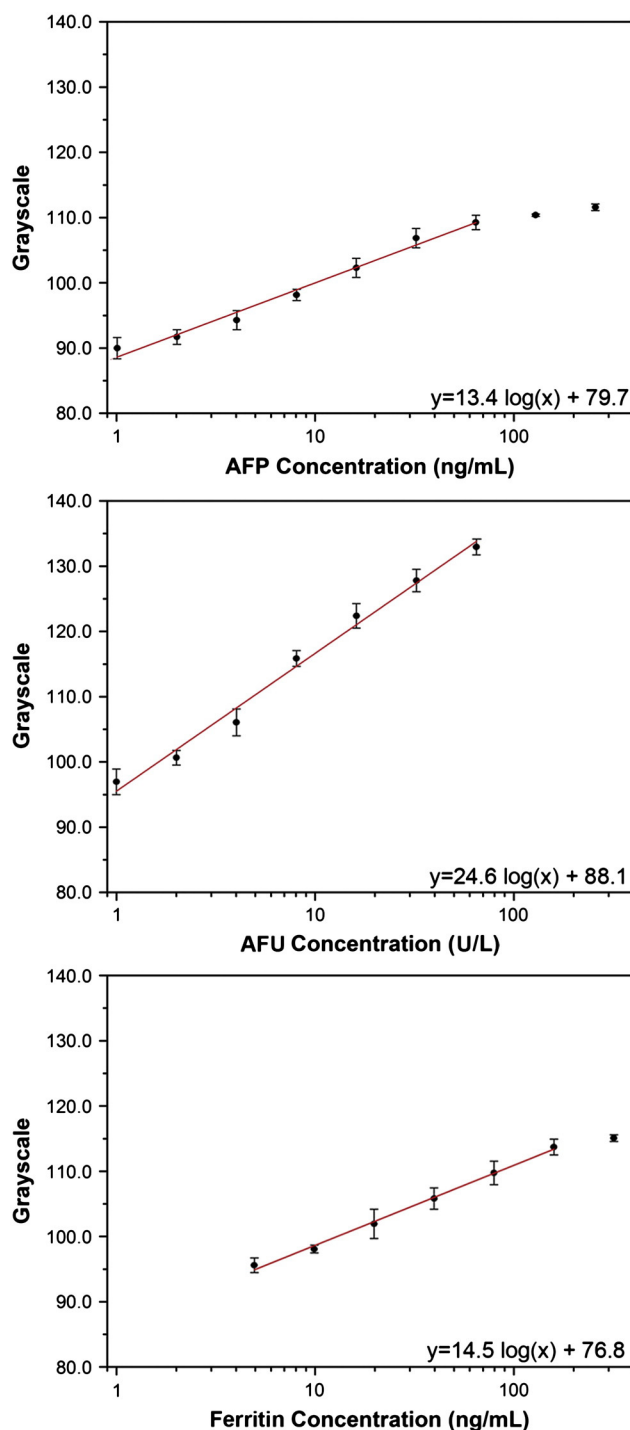


Fig. 2. The calibration curves for quantitative detection of AFP, AFU and ferritin. The calibration curves show that the relationship between IEB signals and the concentrations of AFP, AFU and ferritin is $y = 13.4 \log(x) + 79.7$, $y = 24.6 \log(x) + 88.1$, and $y = 14.5 \log(x) + 76.8$, respectively, and the detection limit of AFP, AFU and ferritin is content to the clinical test standard.

Table 1

Calibration curves for quantitative detection of AFP, AFU and ferritin.

	Regression equations	Linear detection range	Limit of detection
AFP	$y = 13.4 \log(x) + 79.7$	1–64 ng/mL	2 ng/mL
AFU	$y = 24.6 \log(x) + 88.1$	1–64 U/L	1 U/L
Ferritin	$y = 14.6 \log(x) + 76.8$	5–160 ng/mL	5 ng/mL

In the regression equations, y is IEB result in gray-scale value and x presents tumor marker concentration.

temperature. Followed by intensive rinsing in pure ethanol, silicon wafers silanized with APTES are treated with over-saturated succinic anhydride in ethanol for at least 3 h. After rinsing with pure ethanol, silicon wafers are stored in pure ethanol. The detailed surface modification process above has been described in a review article [25].

2.4. Establishing AFP, AFU and ferritin calibration curves

Quantitative detection of AFP, AFU and ferritin is based on establishing their calibration curves. Monoclonal antibodies of AFP, AFU and ferritin are used as the ligands to construct a sensing surface for the joint detection of AFP, AFU and ferritin. In order to preserve their bioactivity, protein A is applied for the oriented immobilization of AFP, AFU and ferritin antibodies, since protein A can bind the Fc portion of an antibody specifically and have the Fab part exposed outside the substrate. Antibody Fab part acts as the binding site to recognize against an antigen specifically. Maintaining antibody Fab part outward can lead to better performance to recognize and capture its corresponding antigen in the analyte solution.

Ligand surface concentration is a pivot to decide the amount of analyte to be captured, further influencing detection performance, for example, detection sensitivity and range. Less ligand results in the scarcity of analyte binding sites; whereas excess situation affects ligand bioactivity and increases the steric hindrance. In order to improve the detection performance, the concentrations of AFP, AFU and ferritin antibodies as the ligands have been optimized in advanced. A series of concentrations of AFP, AFU and ferritin antibodies are added to the protein A modification surface to fabricate AFP, AFU and ferritin sensing

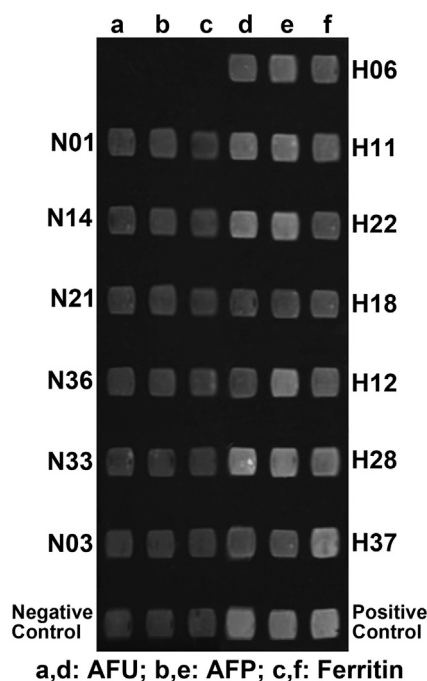


Fig. 3. An example of IEB result in gray-scale image. Seven sera from hepatocellular carcinoma patients, six sera from healthy persons as well as the negative and positive controls have been detected for three tumor markers AFP, AFU and ferritin in the same substrate.

surfaces and then tested by AFP, AFU and ferritin standard solutions. The ligand concentration obtained from the maximum increase of biosensor signal is chosen as the optimized value to construct the bio-sensing surfaces.

The detailed procedures to establish AFP, AFU and ferritin calibration curves are listed as follows. Firstly, a modified silicon wafer is placed into the microfluidic reactor. A mixture solution prepared with 0.05 mol/mL NHS and 0.2 mol/mL EDC in deionized water is passed through the surface at a flow rate of 5 μ L/min for 5 min. With NHS

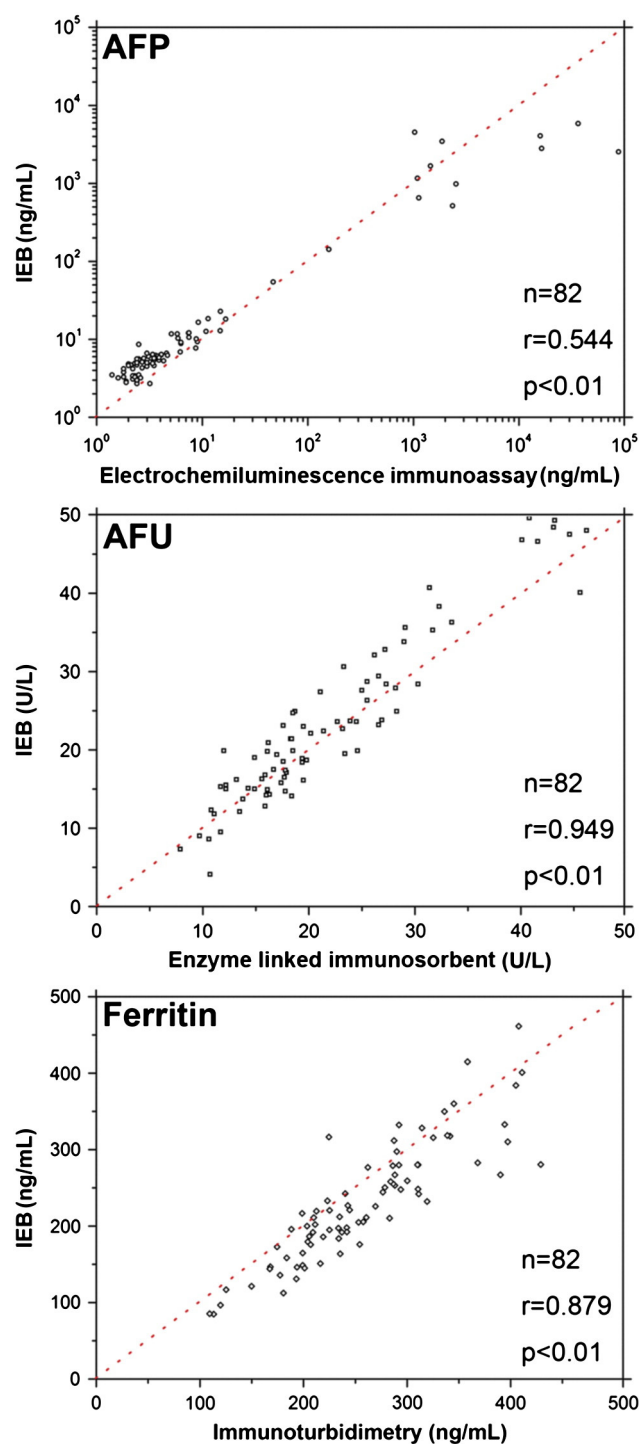


Fig. 4. Results of IEB and clinical methods for AFP, AFU and ferritin detection of eighty-two serum samples. The Pearson correlation coefficients for AFP, AFU and ferritin detection are 0.544, 0.949 and 0.879, respectively, suggesting IEB results are in good agreement with clinical methods.

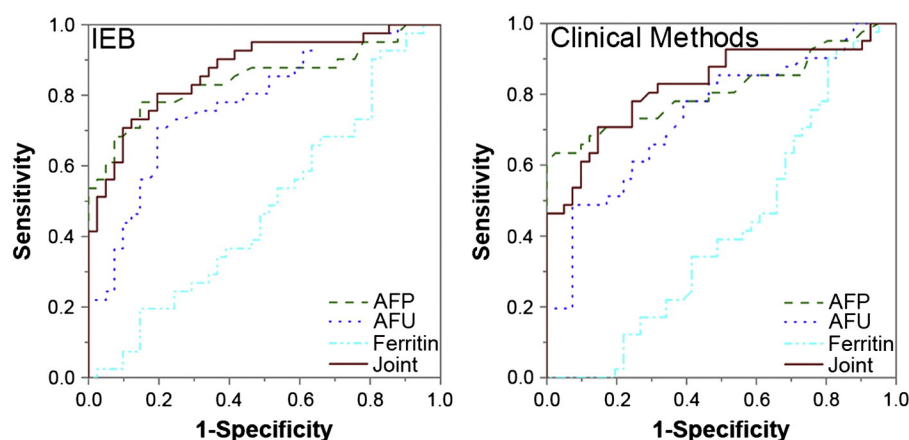


Fig. 5. The ROC curve for hepatocellular carcinoma detection with IEB and clinical methods.

and EDC, carboxyl groups on the substrate form sulfo-NHS-ester and are able to react with the amino groups of protein A. Then, protein A at 0.2 mg/mL is adsorbed on the substrate at a flow rate of 1 $\mu\text{L}/\text{min}$ for 15 min. AFP, AFU and ferritin antibodies are immobilized by protein A for 20 min at a flow rate of 1 $\mu\text{L}/\text{min}$. After that, the surface is blocked by IgG from the same species to the ligands and blocking buffer, respectively. Such IgG can be bound to protein A, but it cannot react with AFP, AFU, ferritin and human serum. It is used to occupy protein A left on the sensing surface and prevent the specific binding between protein A and antibody in the analyte solution. Blocking buffer can also bind to the bio-sensing surface and avoid the non-specific adsorption with the addition of analyte solution, especially, serum samples. After surface blocking, AFP, AFU and ferritin standard samples are delivered into their corresponding sensing surfaces at a flow rate of 1 $\mu\text{L}/\text{min}$ for 20 min. Finally, rinsed with plenty of PBST and completely dried under nitrogen, the microarray is sampled and stored into a gray-scale image by the imaging ellipsometer.

2.5. Quantitative detection of human serum samples

In terms of AFP, AFU and ferritin detection range determined by the calibration curves, serum samples are diluted properly before detections. If the result of a serum sample still exceeds the detection range, it will be diluted and then detected once more until its detection result is within the detection range. AFP, AFU and ferritin concentrations in one serum sample are detected in the same assay which can be used to accommodate up to 16 serum samples. The detection procedure is strictly followed to the process to establish AFP, AFU and ferritin calibration curves. Each serum sample is measured three times, and then AFP, AFU and ferritin concentrations in serum samples are calculated by their calibration curves.

3. Results

3.1. AFP, AFU and ferritin calibration curves

Optimized detection procedures are likely to result difference for AFP, AFU and ferritin. However, because the solution delivery process to fabricate a protein microarray in the microfluidic reactor is controlled by the same peristaltic pump, some parameters, for example detection rate and duration time for an assay, cannot be adjusted independently and has to be compromised to a unified value. By preliminary experiments, different detection procedure parameters have been compared and a compromised yet optimized condition has been utilized to establish AFP, AFU and ferritin calibration curves (shown in Section 2.4). Meanwhile, ligand concentration is also needed to be optimized in order to obtain good detection sensitivity and wide detection range. A series of concentrations

of AFP, AFU and ferritin antibodies have been screened to find out the optimized value to construct their sensing surface and the optimization concentration values for AFP, AFU and ferritin antibodies are 120 $\mu\text{g}/\text{mL}$, 80 $\mu\text{g}/\text{mL}$ and 60 $\mu\text{g}/\text{mL}$, respectively. In addition, for achieving the best image contrast for AFP, AFU and ferritin detection, the incidence angle in which the light beam is directed to the silicon substrate, the azimuth angles of polarizer, compensator and analyzer in ellipsometric setup are fixed at 75° [26], 82.2°, 45.0° and 10.8°, respectively.

AFP, AFU and ferritin standard samples at different concentrations have been added to their sensing surfaces for establishing their calibration curves (shown in Fig. 2). By multi-parameter fitting, the relationship between IEB signals in gray-scale value and tumor marker concentrations can be represented by the regression equations (listed in Table 1).

The limit of detection (LOD) value for AFP, AFU and ferritin is defined as their lowest detection concentrations while an increase caused by the antibody–antigen complex is over 3 times of standard deviation of the blank control [27,28]. Protein A is used to be the blank control and its standard deviation of 20 replicates is 1.0 in gray-scale value. Therefore, LOD values for AFP, AFU and ferritin detection achieve at 2 ng/mL, 1 U/L and 5 ng/mL, respectively, and meet the clinical detection requirements.

3.2. Serum sample detection

Eighty-two human serum samples gathered from 41 healthy persons and 41 hepatocellular carcinoma patients have been tested strictly according to the protocol in establishing the calibration curves for quantitative detection. Each serum has been detected three times and the result in gray-scale value is converted to tumor marker concentration in terms of the relationship obtained in the corresponding calibration curve. All the detection results for the eighty-two serum samples are listed in Tables A.3, A.4 and A.5 in Appendix A, and an example of detection result in gray-scale image for 13 serum samples is presented in Fig. 3.

IEB results are compared with those of the commercial approaches in clinical diagnosis and their differences are statistically evaluated by correlation analysis (shown in Fig. 4). The Pearson correlation

Table 2
AUC value of clinical method and IEB for hepatocellular carcinoma detection.

TM	Clinic method	IEB
AFP	0.811	0.845
AFU	0.740	0.780
Ferritin	0.419	0.481
AFP + AFU	0.827	0.972

coefficients [29] for AFP, AFU and ferritin detection are 0.544, 0.949 and 0.879, respectively. It is indicated that results of IEB and clinical tests have significant statistical relevance at the level of 0.01. In other words, IEB results are in good agreement with clinical methods.

3.3. Joint detection performance

Receiver operating characteristic (ROC) analysis [30] is used to estimate the detection specificity in clinical diagnosis by calculating the area under the ROC curve (AUC) value. If the AUC value is bigger than 0.5, the negative and the positive can be classified effectively by a detection method. Higher AUC value indicates better detection performance.

The ROC curve for hepatocellular carcinoma detection with IEB is shown in Fig. 5 and the AUC value is listed in Table 2. The AUC value of AFP and AFU is bigger than 0.5, so the two tumor markers play an effective role to distinguish the patients from the healthy people. However, AUC value of ferritin is less than 0.5, indicating that it is not related to hepatocellular carcinoma detection. In that case, only AFP and AFU are combined together to form a joint marker. Compared with the single marker detection, AUC value of the joint marker detection increases remarkably, suggesting the improvement on detection specificity for hepatocellular carcinoma diagnosis.

The ROC curve for hepatocellular carcinoma detection of the clinical methods is shown in Fig. 5. Its detection results show the same tendency to those of IEB. Only AFP and AFU are considered as specific tumor markers for hepatocellular carcinoma and contributed to the joint marker. However, no matter the single marker detection or the joint marker detection, the AUC value of the clinical method is slightly lower

than IEB, presenting IEB detection performance for hepatocellular carcinoma as having an advantage over the clinical methods.

4. Conclusions

IEB has been developed as a quantitative approach for the joint detection of hepatocellular carcinoma markers. By ROC curve analysis, AFP and AFU play a positive role to distinguish hepatocellular carcinoma patients from the healthy ones and are combined to the joint detection marker. The detection range and the detection limit are adequate for clinical diagnosis requirements. Eighty-two sera have been tested with IEB and their results are validated by commercial clinical methods. The joint detection is proved to improve the specificity for hepatocellular carcinoma detection.

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Appendix A

Table A.1

Clinical information and clinical detection results of 82 serum samples.

Sample	Age	Gender	AFP	AFU	Ferritin	Detection date	Clinic diagnosis
H01	42	F	2.5	19.5	262.2	2010/1/5	HCC +
H02	51	F	9.0	17.0	223.1	2010/1/5	HCC +
H03	69	M	5.1	17.9	199.3	2010/1/8	HCC +
H04	45	M	3.2	27.3	216.3	2010/1/8	HCC +
H05	73	M	4.6	29.0	242.8	2010/1/12	HCC +
H06	48	M	8.8	43.1	341.5	2010/1/19	HCC +
H07	48	F	1.8	18.7	234.1	2010/1/20	HCC +
H08	55	M	7.4	19.5	253.2	2010/1/20	HCC +
H09	40	M	8.7	12.2	286.3	2010/1/21	HCC +
H10	60	M	7.5	21.1	288.2	2010/1/25	HCC +
H11	55	M	16.7	40.8	311.3	2010/2/1	HCC +
H12	49	M	10.9	41.6	193.2	2010/2/2	HCC +
H13	51	M	9.2	21.4	339.0	2010/2/4	HCC +
H14	55	M	2.8	24.5	212.5	2010/2/4	HCC +
H15	66	F	1091.0	45.6	174.6	2010/2/5	HCC +
H16	38	F	2541.0	29.1	233.7	2010/2/8	HCC +
H17	64	M	3.6	28.2	203.6	2010/2/21	HCC +
H18	89	M	1.9	26.2	269.5	2010/3/2	HCC +
H19	29	M	2.3	12.0	183.9	2010/3/3	HCC +
H20	75	F	7.5	30.3	149.9	2010/3/3	HCC +
H21	41	M	2356.0	26.6	287.5	2010/3/3	HCC +
H22	44	M	1453.0	40.1	286.7	2010/3/5	HCC +
H23	66	F	16,380.0	23.2	209.9	2010/3/5	HCC +
H24	64	M	15,910.0	31.4	225.1	2010/3/5	HCC +
H25	70	M	88,130.0	26.6	205.7	2010/3/8	HCC +
H26	62	M	14.9	26.9	241.7	2010/3/8	HCC +
H27	72	M	2.5	17.6	234.9	2010/3/11	HCC +
H28	51	M	36,430.0	46.2	336.1	2010/3/15	HCC +
H29	62	M	158.3	18.4	292.2	2010/3/18	HCC +
H30	54	M	1125.0	44.6	358.4	2010/3/22	HCC +
H31	50	M	4.1	18.5	235.5	2010/3/23	HCC +
H32	46	M	1873.0	23.3	119.9	2010/3/29	HCC +
H33	35	F	11.4	18.3	198.8	2010/3/29	HCC +
H34	50	M	47.0	28.3	193.8	2010/3/29	HCC +

(continued on next page)

Table A.1 (continued)

Sample	Age	Gender	AFP	AFU	Ferritin	Detection date	Clinic diagnosis
H35	55	F	1029.0	43.2	199.3	2010/4/2	HCC +
H36	84	M	14.8	27.2	224.5	2010/4/2	HCC +
H37	56	M	2.3	11.1	325.3	2010/4/2	HCC +
H38	84	M	2.3	11.7	310.7	2010/4/6	HCC +
H39	61	F	2.2	17.6	207.0	2010/4/6	HCC +
H40	39	F	6.3	14.9	294.0	2010/4/6	HCC +
H41	48	M	3.4	15.9	284.1	2010/4/6	HCC +
N01	45	M	3.0	15.6	240.3	2009/11/6	Healthy
N02	45	M	1.8	10.6	314.4	2009/11/6	Healthy
N03	48	M	2.4	16.7	260.7	2009/11/6	Healthy
N04	40	M	6.3	14.9	292.1	2009/11/6	Healthy
N05	49	F	2.5	17.8	241.9	2009/11/6	Healthy
N06	41	M	5.8	32.3	368.3	2009/11/6	Healthy
N07	54	F	2.4	31.7	288.2	2009/11/6	Healthy
N08	40	F	2.2	17.7	283.2	2009/11/6	Healthy
N09	51	F	2.7	19.4	407.9	2009/11/6	Healthy
N10	58	M	2.6	18.4	300.2	2009/11/6	Healthy
N11	46	M	3.9	33.5	278.6	2009/11/6	Healthy
N12	46	F	2.4	25.0	390.2	2009/11/6	Healthy
N13	54	F	3.5	25.5	219.0	2009/11/6	Healthy
N14	46	F	1.6	19.4	168.1	2009/11/6	Healthy
N15	46	M	3.1	18.5	319.4	2009/11/6	Healthy
N16	50	M	3.5	17.8	254.3	2009/11/19	Healthy
N17	38	M	1.8	20.2	177.5	2009/11/20	Healthy
N18	49	M	2.0	16.1	310.1	2009/11/20	Healthy
N19	53	F	4.1	23.9	244.5	2009/12/9	Healthy
N20	62	M	2.2	16.1	290.1	2009/12/9	Healthy
N21	61	M	3.2	13.8	237.1	2009/12/9	Healthy
N22	63	M	3.0	19.8	257.7	2009/12/9	Healthy
N23	59	F	3.0	10.8	411.1	2009/12/9	Healthy
N24	55	F	2.5	16.1	345.2	2009/12/9	Healthy
N25	43	F	3.8	22.7	201.2	2009/12/11	Healthy
N26	51	F	3.9	13.5	405.2	2009/12/11	Healthy
N27	47	M	2.3	17.4	429.2	2009/12/11	Healthy
N28	53	M	3.5	16.2	397.2	2009/12/11	Healthy
N29	48	F	2.4	16.3	310.6	2009/12/11	Healthy
N30	40	M	2.4	16.0	204.2	2009/12/11	Healthy
N31	56	F	2.4	23.4	113.4	2009/12/11	Healthy
N32	57	M	6.2	24.6	167.5	2009/12/11	Healthy
N33	46	M	4.3	14.3	211.3	2009/12/14	Healthy
N34	55	M	2.1	15.9	188.4	2009/12/14	Healthy
N35	41	F	1.9	25.5	394.3	2009/12/14	Healthy
N36	54	F	2.0	13.2	125.4	2009/12/14	Healthy
N37	55	F	1.4	12.2	109.4	2009/12/17	Healthy
N38	69	M	5.9	10.7	276.7	2009/12/17	Healthy
N39	49	M	4.7	9.7	180.7	2009/12/17	Healthy
N40	61	M	2.7	7.9	225.2	2009/12/17	Healthy
N41	43	F	3.4	11.7	209.2	2009/12/17	Healthy

HCC +: hepatocellular carcinoma patients. The units of AFP, AFU and ferritin concentrations are mg/mL, U/L and mg/mL, respectively.

Table A.2

Description and ANOVA of 82 serum samples.

Tumor marker	Sample	Description					ANOVA	
		Number	Average	SD	Minimum	Maximum	F	Significance
AFP	Healthy	41	3.1	1.2	1.4	6.3	3.10	0.08
	Patient	41	4114.7	14,952.0	1.8	88,130.0		
AFU	Healthy	41	18.1	6.0	7.9	33.5	17.08	0.00
	Patient	41	25.8	10.2	11.1	46.2		
Ferritin	Healthy	41	269.5	85.7	109.4	429.2	2.15	0.15
	Patient	41	246.1	55.5	119.9	358.4		

ANOVA: analysis of variance. SD: standard deviation.

Table A.3

AFP detection result of 82 serum samples with IEB.

Sample	IEB results in gray-scale value					IEB results in concentration (ng/mL)	
	Repeat 1	Repeat 2	Repeat 3	Average	SD	Average	SD
H01	91.1	93.0	92.7	92.3	1.0	8.7	1.4
H02	91.3	92.6	94.1	92.7	1.4	9.4	2.2
H03	92.6	94.3	95.2	94.0	1.3	11.8	2.6
H04	88.8	90.5	88.3	89.2	1.2	5.1	1.1
H05	90.0	92.1	89.9	90.7	1.2	6.6	1.5
H06	93.0	92.8	93.9	93.2	0.6	10.1	1.0
H07	87.1	86.5	88.9	87.5	1.2	3.8	0.9
H08	92.5	93.9	96.0	94.1	1.8	12.2	3.7
H09	91.4	91.7	91.8	91.6	0.2	7.7	0.3
H10	92.5	93.6	94.4	93.5	1.0	10.7	1.7
H11	96.0	95.3	98.4	96.6	1.6	18.4	5.4
H12	93.9	94.9	94.7	94.5	0.5	12.6	1.1
H13	94.4	96.7	97.0	96.0	1.4	16.7	3.8
H14	89.9	90.6	89.0	89.8	0.8	5.7	0.8
H15	97.6	97.9	98.8	98.1	0.6	1169.1	127.8
H16	93.1	91.6	94.3	93.0	1.4	988.9	224.9
H17	88.7	90.3	91.9	90.3	1.6	6.3	1.7
H18	85.0	86.0	87.0	86.0	1.0	3.0	0.5
H19	86.4	85.3	88.4	86.7	1.6	3.4	0.9
H20	94.0	94.3	94.6	94.3	0.3	12.2	0.6
H21	90.0	88.3	89.4	89.2	0.9	513.0	74.1
H22	99.2	100.8	100.6	100.2	0.9	1681.3	240.3
H23	88.8	89.8	90.8	89.8	1.0	2834.3	484.1
H24	92.1	92.2	91.5	91.9	0.4	4051.7	258.3
H25	81.2	80.0	82.0	81.1	1.0	2536.8	428.8
H26	97.1	98.9	97.8	97.9	0.9	22.8	3.6
H27	71.4	73.3	72.2	72.3	1.0	5.6	0.9
H28	89.2	88.8	91.8	89.9	1.6	5901.1	1747.3
H29	95.6	95.0	95.0	95.2	0.3	141.8	8.6
H30	94.5	95.2	94.3	94.7	0.5	647.9	53.4
H31	90.4	89.6	91.2	90.4	0.8	6.3	0.9
H32	102.6	104.6	105.9	104.4	1.7	3501.0	959.0
H33	97.5	96.6	95.8	96.6	0.9	18.2	2.7
H34	101.2	103.6	104.1	103.0	1.6	54.9	13.5
H35	105.4	106.8	105.8	106.0	0.7	4535.1	572.6
H36	94.6	94.5	94.9	94.7	0.2	12.9	0.5
H37	88.0	89.6	89.5	89.0	0.9	5.0	0.7
H38	87.2	85.8	87.3	86.8	0.8	3.4	0.5
H39	85.8	86.1	87.1	86.3	0.7	3.1	0.4
H40	93.0	92.4	91.6	92.3	0.7	8.7	1.0
H41	88.7	91.7	90.9	90.4	1.6	6.4	1.6
N01	87.8	89.0	88.8	88.5	0.6	4.5	0.5
N02	86.3	86.9	87.1	86.8	0.4	3.3	0.2
N03	85.6	86.8	85.4	85.9	0.8	2.9	0.4
N04	92.9	91.3	93.4	92.5	1.1	9.1	1.6
N05	86.1	87.7	87.3	87.0	0.8	3.5	0.5
N06	95.2	93.6	93.1	94.0	1.1	11.6	2.3
N07	86.7	89.3	90.1	88.7	1.8	4.8	1.3
N08	88.4	86.6	89.2	88.1	1.3	4.2	0.9
N09	88.1	88.3	91.0	89.1	1.6	5.1	1.5
N10	85.6	86.4	87.4	86.5	0.9	3.2	0.5
N11	88.0	90.1	90.5	89.5	1.3	5.5	1.2
N12	85.2	85.8	85.5	85.5	0.3	2.7	0.1
N13	89.1	89.0	90.5	89.5	0.8	5.4	0.8
N14	87.0	85.1	87.1	86.4	1.1	3.2	0.6
N15	89.1	91.4	89.7	90.1	1.2	6.0	1.3
N16	88.2	88.1	89.7	88.7	0.9	4.7	0.7
N17	88.7	87.0	88.4	88.0	0.9	4.2	0.6
N18	88.7	88.8	89.1	88.9	0.2	4.8	0.2
N19	89.9	89.9	91.9	90.6	1.2	6.5	1.3
N20	85.4	88.5	86.2	86.7	1.6	3.4	1.0
N21	85.0	86.3	85.3	85.5	0.7	2.7	0.3
N22	88.1	89.1	90.0	89.1	1.0	5.0	0.8
N23	90.4	90.3	91.5	90.7	0.7	6.6	0.8
N24	89.5	89.8	89.6	89.6	0.2	5.5	0.1
N25	89.8	91.2	89.6	90.2	0.9	6.1	0.9
N26	88.3	89.5	91.9	89.9	1.8	5.9	1.9
N27	86.3	87.2	86.8	86.8	0.5	3.3	0.3
N28	89.3	90.3	89.9	89.8	0.5	5.7	0.5
N29	87.8	90.4	90.9	89.7	1.7	5.7	1.5
N30	88.4	89.2	89.7	89.1	0.7	5.0	0.6
N31	86.3	85.7	86.4	86.1	0.4	3.0	0.2

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Table A.3 (continued)

Sample	IEB results in gray-scale value					IEB results in concentration (ng/mL)	
	Repeat 1	Repeat 2	Repeat 3	Average	SD	Average	SD
N32	89.2	91.9	91.7	90.9	1.5	7.0	1.6
N33	88.7	88.8	90.8	89.4	1.2	5.3	1.1
N34	87.3	89.6	89.4	88.8	1.3	4.8	1.0
N35	84.7	86.5	86.0	85.7	0.9	2.8	0.4
N36	87.9	89.9	87.9	88.6	1.2	4.6	1.0
N37	87.4	86.7	87.2	87.1	0.4	3.5	0.2
N38	92.9	93.9	93.1	93.3	0.5	10.3	0.9
N39	88.4	91.6	90.8	90.3	1.7	6.2	1.7
N40	87.8	87.9	89.1	88.3	0.7	4.3	0.6
N41	88.7	87.7	90.4	88.9	1.4	4.9	1.2

SD: standard deviation.

Due to the detection range, samples H15, H16, H22, H23, H24, H25, H27, H28, H29, H30, H32 and H35 have been diluted before detection.

Table A.4

AFU detection result of 82 serum samples with IEB.

Sample	IEB results in gray-scale value					IEB results in concentration (U/L)	
	Repeat 1	Repeat 2	Repeat 3	Average	SD	Average	SD
H01	116.7	117.4	118.9	117.7	1.1	16.1	1.7
H02	119.0	121.0	118.9	119.6	1.2	19.4	2.2
H03	118.2	118.3	118.6	118.4	0.2	17.2	0.3
H04	123.9	123.6	123.9	123.8	0.2	28.4	0.5
H05	126.0	126.1	124.9	125.7	0.7	33.9	2.1
H06	128.5	130.8	129.1	129.5	1.2	48.3	5.5
H07	122.1	123.0	121.9	122.3	0.6	24.8	1.4
H08	122.1	121.7	120.6	121.5	0.8	22.9	1.6
H09	117.5	116.7	117.6	117.3	0.5	15.5	0.7
H10	123.4	123.8	123.0	123.4	0.4	27.4	1.0
H11	129.1	130.8	129.4	129.8	0.9	49.6	4.3
H12	128.1	128.6	130.5	129.1	1.3	46.6	5.6
H13	121.8	121.6	120.3	121.2	0.8	22.4	1.7
H14	122.4	122.8	120.0	121.7	1.5	23.6	3.2
H15	127.4	127.1	128.0	127.5	0.5	40.1	1.7
H16	126.9	126.3	125.4	126.2	0.8	35.6	2.5
H17	123.8	122.5	124.3	123.5	0.9	27.8	2.4
H18	124.2	126.3	124.7	125.1	1.1	32.1	3.3
H19	119.2	119.6	121.0	119.9	0.9	19.9	1.8
H20	122.8	124.1	124.4	123.8	0.9	28.4	2.2
H21	122.4	121.4	121.0	121.6	0.7	23.2	1.6
H22	127.8	129.9	129.7	129.1	1.2	46.9	4.9
H23	122.1	119.7	122.2	121.3	1.4	22.7	2.9
H24	127.6	127.8	127.5	127.6	0.2	40.6	0.6
H25	124.1	124.7	123.7	124.2	0.5	29.4	1.4
H26	122.5	120.6	122.4	121.8	1.1	23.8	2.3
H27	122.3	121.5	120.8	121.5	0.8	23.1	1.6
H28	130.9	128.3	128.9	129.4	1.4	48.0	6.2
H29	115.2	115.2	118.0	116.1	1.6	14.0	2.2
H30	129.4	128.8	129.8	129.3	0.5	47.6	2.2
H31	123.6	121.1	122.0	122.2	1.3	24.7	2.9
H32	125.2	125.4	123.0	124.5	1.3	30.6	3.6
H33	121.2	121.0	119.9	120.7	0.7	21.3	1.4
H34	122.6	122.1	122.4	122.4	0.3	24.9	0.6
H35	129.3	131.2	128.5	129.7	1.4	49.3	6.5
H36	126.3	124.0	125.6	125.3	1.2	32.8	3.5
H37	114.2	114.5	114.4	114.4	0.2	11.8	0.2
H38	115.2	117.7	118.1	117.0	1.6	15.2	2.1
H39	120.3	119.2	117.9	119.1	1.2	18.5	2.1
H40	116.2	118.2	116.3	116.9	1.1	15.0	1.6
H41	118.8	119.0	116.5	118.1	1.4	16.8	2.1
N01	117.5	117.5	118.4	117.8	0.5	16.3	0.8
N02	110.0	112.3	110.5	110.9	1.2	8.6	1.0
N03	118.8	118.5	118.5	118.6	0.2	17.5	0.3
N04	119.8	118.0	120.5	119.4	1.3	19.0	2.2
N05	117.3	118.3	119.8	118.5	1.3	17.4	2.1
N06	126.1	127.4	127.4	127.0	0.8	38.2	2.6
N07	126.4	126.0	126.1	126.2	0.2	35.4	0.7
N08	117.5	119.5	116.7	117.9	1.4	16.5	2.3
N09	117.7	119.6	119.8	119.0	1.2	18.3	1.9
N10	120.4	119.9	121.8	120.7	1.0	21.4	2.0
N11	126.4	127.2	125.6	126.4	0.8	36.3	2.7

Table A.4 (continued)

Sample	IEB results in gray-scale value					IEB results in concentration (U/L)	
	Repeat 1	Repeat 2	Repeat 3	Average	SD	Average	SD
N12	122.8	123.1	124.5	123.5	0.9	27.6	2.4
N13	124.4	122.9	124.3	123.9	0.8	28.7	2.2
N14	118.7	120.4	119.0	119.4	0.9	18.9	1.6
N15	120.8	118.9	120.1	119.9	1.0	19.9	1.8
N16	116.0	117.5	116.6	116.7	0.8	14.7	1.0
N17	121.5	120.2	121.5	121.1	0.8	22.1	1.5
N18	119.7	120.1	120.0	119.9	0.2	19.9	0.4
N19	122.7	121.3	121.5	121.8	0.8	23.7	1.7
N20	115.6	117.7	116.9	116.7	1.1	14.8	1.4
N21	115.7	115.8	116.3	115.9	0.3	13.7	0.4
N22	119.5	119.9	118.4	119.3	0.8	18.7	1.3
N23	114.8	115.6	114.0	114.8	0.8	12.3	0.9
N24	116.7	117.5	116.3	116.8	0.6	14.9	0.9
N25	121.9	122.2	121.2	121.8	0.5	23.6	1.1
N26	115.2	115.0	113.7	114.6	0.8	12.1	0.9
N27	118.5	116.6	117.2	117.4	1.0	15.8	1.4
N28	120.1	120.5	120.9	120.5	0.4	20.9	0.8
N29	115.5	117.2	116.4	116.4	0.9	14.3	1.1
N30	116.2	117.2	115.5	116.3	0.9	14.2	1.1
N31	119.9	119.2	120.2	119.8	0.5	19.6	0.9
N32	119.9	119.9	120.0	119.9	0.1	19.8	0.1
N33	115.7	116.6	118.5	116.9	1.4	15.1	2.0
N34	114.4	114.7	116.3	115.1	1.0	12.7	1.2
N35	122.1	123.1	123.5	122.9	0.7	26.2	1.7
N36	117.3	118.9	117.0	117.7	1.0	16.2	1.6
N37	116.4	116.9	117.4	116.9	0.5	15.0	0.7
N38	103.6	103.1	102.5	103.1	0.6	4.1	0.2
N39	111.0	111.5	111.6	111.4	0.3	9.0	0.3
N40	109.4	108.8	109.2	109.1	0.3	7.3	0.2
N41	111.7	110.8	113.5	112.0	1.4	9.5	1.2

SD: standard deviation.

Table A.5

Ferritin detection result of 82 serum samples with IEB.

Sample	IEB results in gray-scale value					IEB results in concentration (ng/mL)	
	Repeat 1	Repeat 2	Repeat 3	Average	SD	Average	SD
H01	102.9	103.3	104.5	103.6	0.8	277.9	37.6
H02	103.4	104.1	102.7	103.4	0.7	232.2	27.8
H03	98.9	101.7	101.6	100.7	1.6	149.8	37.2
H04	99.4	101.3	101.9	100.9	1.3	152.1	32.1
H05	103.3	102.4	104.1	103.3	0.9	227.5	32.9
H06	105.5	105.2	105.0	105.2	0.3	316.7	13.7
H07	102.8	101.4	101.8	102.0	0.7	182.8	23.1
H08	103.9	101.7	102.1	102.6	1.2	203.2	42.6
H09	104.2	103.3	105.8	104.4	1.3	280.4	62.2
H10	103.3	102.9	105.4	103.9	1.3	255.0	61.7
H11	102.0	104.5	104.3	103.6	1.4	243.5	53.7
H12	99.2	99.9	101.1	100.1	1.0	131.7	22.1
H13	104.7	104.5	106.5	105.2	1.1	320.4	63.3
H14	102.9	102.2	104.1	103.1	1.0	220.3	36.9
H15	99.9	102.2	102.8	101.6	1.5	174.5	42.5
H16	101.8	101.7	103.8	102.4	1.2	198.6	42.4
H17	102.1	103.2	102.3	102.5	0.6	199.9	20.6
H18	103.2	103.8	102.7	103.2	0.6	225.3	21.3
H19	100.3	102.0	101.2	101.2	0.9	158.7	23.0
H20	98.9	99.6	100.4	99.6	0.8	121.8	15.7
H21	104.7	104.2	106.4	105.1	1.2	313.5	64.4
H22	103.8	104.2	103.9	104.0	0.2	254.8	9.2
H23	102.7	103.5	102.3	102.8	0.6	210.5	22.4
H24	102.9	101.0	103.1	102.3	1.2	195.0	36.4
H25	101.8	102.6	102.0	102.1	0.4	186.3	13.5
H26	101.3	102.0	104.0	102.4	1.4	199.7	49.9
H27	101.7	102.3	104.5	102.8	1.5	214.4	56.8
H28	104.3	106.0	107.0	105.8	1.4	353.0	79.7
H29	103.3	106.1	106.8	105.4	1.9	336.1	97.2
H30	106.9	106.7	106.8	106.8	0.1	414.0	7.1
H31	102.1	100.5	101.4	101.3	0.8	163.2	22.2
H32	98.1	99.4	97.1	98.2	1.2	96.0	19.1
H33	101.9	103.4	103.7	103.0	1.0	217.7	34.3

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Table A.5 (continued)

Sample	IEB results in gray-scale value					IEB results in concentration (ng/mL)	
	Repeat 1	Repeat 2	Repeat 3	Average	SD	Average	SD
H34	100.1	101.8	100.1	100.7	1.0	146.1	25.6
H35	102.1	100.4	101.6	101.4	0.9	164.3	23.9
H36	103.5	105.6	106.4	105.2	1.5	319.6	77.2
H37	105.3	104.6	105.7	105.2	0.6	315.6	29.7
H38	103.7	105.2	104.6	104.5	0.8	280.6	35.8
H39	101.1	102.1	102.2	101.8	0.6	176.3	17.8
H40	103.0	104.7	103.6	103.8	0.9	247.9	37.3
H41	102.6	104.2	105.2	104.0	1.3	260.4	56.7
N01	102.0	104.8	104.0	103.6	1.4	243.9	57.0
N02	105.5	105.0	105.8	105.4	0.4	328.0	22.5
N03	102.3	102.6	103.7	102.9	0.7	212.1	27.5
N04	105.1	103.9	104.4	104.5	0.6	278.5	29.0
N05	102.6	102.3	102.0	102.3	0.3	191.6	9.8
N06	103.7	104.9	105.1	104.6	0.8	283.9	35.5
N07	104.0	104.5	104.2	104.2	0.3	266.8	11.6
N08	101.8	103.2	103.5	102.8	0.9	211.4	31.5
N09	107.5	105.8	108.7	107.3	1.5	463.0	112.4
N10	103.8	103.8	104.6	104.1	0.5	259.7	21.0
N11	104.2	103.0	104.3	103.8	0.7	250.2	29.9
N12	104.8	103.7	104.1	104.2	0.6	265.9	25.7
N13	100.7	103.2	102.3	102.1	1.3	186.7	39.1
N14	100.9	100.1	101.2	100.7	0.6	146.8	14.0
N15	102.0	102.5	105.4	103.3	1.8	235.2	78.8
N16	100.9	102.1	102.4	101.8	0.8	176.7	23.2
N17	99.0	99.9	101.8	100.2	1.4	137.1	34.6
N18	103.4	104.9	105.2	104.5	1.0	281.6	44.4
N19	102.6	102.9	103.9	103.1	0.7	221.8	26.5
N20	105.6	103.3	105.4	104.8	1.3	296.6	60.4
N21	101.6	102.0	103.3	102.3	0.9	192.9	30.3
N22	103.2	102.6	102.2	102.7	0.5	204.3	17.8
N23	105.1	107.8	106.7	106.5	1.4	402.6	91.1
N24	105.1	105.8	107.0	106.0	1.0	362.2	60.7
N25	100.7	100.0	101.3	100.7	0.7	145.3	16.1
N26	105.2	106.4	107.4	106.3	1.1	386.7	72.1
N27	105.7	103.6	103.9	104.4	1.1	278.0	56.6
N28	104.7	103.8	106.6	105.0	1.4	312.1	78.9
N29	104.2	104.5	102.4	103.7	1.1	246.3	45.2
N30	101.7	101.1	102.9	101.9	0.9	180.2	28.9
N31	97.2	98.1	97.3	97.5	0.5	84.7	7.3
N32	99.2	102.4	99.8	100.5	1.7	144.0	44.4
N33	103.0	100.8	103.7	102.5	1.5	202.4	49.1
N34	102.1	102.3	102.9	102.4	0.4	196.2	14.2
N35	104.5	105.3	106.7	105.5	1.1	335.4	65.3
N36	99.5	98.2	100.4	99.4	1.1	117.1	21.7
N37	97.2	98.6	96.8	97.5	0.9	85.3	14.3
N38	102.5	104.2	104.4	103.7	1.0	245.8	41.6
N39	98.8	99.2	99.6	99.2	0.4	112.7	7.7
N40	103.2	103.4	102.7	103.1	0.4	219.8	13.4
N41	101.4	102.8	102.7	102.3	0.8	192.5	24.7

SD: standard deviation.

Due to the detection range, all samples have been diluted to 4 times before detection.

References

- [1] P. Anand, A. Kunnumakara, C. Sundaram, K. Harikumar, S. Tharakan, O. Lai, B. Sung, B. Aggarwal, *Pharm. Res.* 25 (2008) 2097.
- [2] A. Jemal, F. Bray, M. Center, J. Ferlay, E. Ward, D. Forman, *CA Cancer J. Clin.* 61 (2011) 69.
- [3] A. Montazeri, *Health Qual. Life Outcomes* 7 (2009) 1.
- [4] D.H. Roukos, *Expert. Rev. Anticancer. Ther.* 9 (2009) 389.
- [5] M. Erman, J.B. Theeten, *J. Appl. Phys.* 60 (1986) 859.
- [6] D. Beaglehole, *Rev. Sci. Instrum.* 59 (1988) 2557.
- [7] C. Brinker, A. Hurd, G. Frye, K. Ward, C. Ashley, J. Non-Cryst. Solids 121 (1990) 294.
- [8] H. Rotermund, G. Haas, R.U. Franz, R.M. Tromp, G. Ertl, *Science* 270 (1995) 608.
- [9] G. Jin, P. Tengvall, I. Lundström, H. Arwin, *Anal. Biochem.* 232 (1995) 69.
- [10] G. Jin, R. Jansson, H. Arwin, *Rev. Sci. Instrum.* 67 (1996) 2930.
- [11] G. Jin, *Phys. Phys. Status Solidi A: Appl. Mat.* 205 (2008) 810.
- [12] G. Jin, Y.H. Meng, L. Liu, Y. Niu, S. Chen, Q. Cai, T.J. Jiang, *Thin Solid Films* 519 (2011) 2750.
- [13] Z.H. Wang, Y.H. Meng, P.Q. Ying, C. Qi, G. Jin, *Electrophoresis* 27 (2006) 4078.
- [14] Y. Niu, G. Jin, *Protein Cell* 2 (2011) 445.
- [15] C. Qi, W. Zhu, Y. Niu, H.G. Zhang, G.Y. Zhu, Y.H. Meng, S. Chen, G. Jin, *J. Viral Hepat.* 16 (2009) 822.
- [16] C.H. Huang, Y.Y. Chen, C.X. Wang, W. Zhu, H.W. Ma, G. Jin, *Thin Solid Films* 519 (2011) 2763.
- [17] C.H. Huang, Y.Y. Chen, G. Jin, *Ann. Biomed. Eng.* 39 (2011) 185.
- [18] C. Qi, Y. Lin, J. Feng, Z.H. Wang, C.F. Zhu, Y.H. Meng, X.Y. Yan, L.J. Wan, G. Jin, *Virus Res.* 140 (2009) 79.
- [19] C. Qi, J.Z. Duan, Z.H. Wang, Y.Y. Chen, P.H. Zhang, L. Zhan, X.Y. Yan, W.C. Cao, G. Jin, *Biomed. Microdevices* 8 (2006) 247.
- [20] C. Qi, X.S. Tian, S. Chen, J.H. Yan, Z. Cao, K.G. Tian, G.F. Gao, G. Jin, *Biosens. Bioelectron.* 25 (2010) 1530.
- [21] Y. Niu, J. Zhuang, L. Liu, X.Y. Yan, G. Jin, *Thin Solid Films* 519 (2011) 2768.
- [22] G.J. Mizejewski, *Exp. Biol. Med.* 226 (2001) 377.
- [23] K. Tanaka, T. Nakano, S. Noguchi, W. Pigman, *Arch. Biochem. Biophys.* 126 (1968) 624.
- [24] E.C. Theil, *Annu. Rev. Biochem.* 56 (1987) 289.
- [25] Y. Niu, G. Jin, *Appl. Surf. Sci.* 281 (2013) 84.
- [26] H. Arwin, I. Lundström, *Anal. Biochem.* 145 (1985) 106.
- [27] D. MacDougall, *Anal. Chem.* 52 (1980) 2242.
- [28] G.L. Long, J.D. Winefordner, *Anal. Chem.* 55 (1983) A712.
- [29] J. Lee Rodgers, W.A. Nicewander, *Am. Stat.* 42 (1988) 59.
- [30] T. Fawcett, *Pattern Recognit. Lett.* 27 (2006) 861.