

Determination of α -Fetoprotein in Human Serum by a Quartz Crystal Microbalance-based Immunosensor

SHU-FEN CHOU,^{1,2} WIN-LIN HSU,³ JING-MIN HWANG,³ and CHIEN-YUAN CHEN^{2*}

Background: Increased α -fetoprotein (AFP) in adult plasma is considered an early indication of hepatocellular carcinoma and teratoblastoma. The aim of this study was to develop a rapid method to measure AFP in human serum by use of a direct immunosensor based on a quartz crystal microbalance (QCM).

Methods: A self-assembled monolayer prepared by the cystamine method was applied to immobilize anti-AFP monoclonal antibodies on the gold surface of a quartz crystal. The frequency shifts of the QCM were measured and related to AFP concentrations. The piezoimmunosensor used no labeled reagent and no pretreatment of samples.

Results: Ten cycles of measurements could be performed on the gold surface of the same crystal regenerated with a solution of glycine-HCl. A linear relationship existed between the frequency shifts (Hz) and the log values of AFP concentrations from 0.1 to 100 $\mu\text{g/L}$ in buffer and human serum. When used for 15 days, the frequency shifts were all >95% of those on the response at the first day. The regression equation was $y = 1.03x - 0.06$ ($S_{y/x} = 3.92$; $r = 0.9987$) for this QCM method and RIA in 29 clinical human serum samples.

Conclusions: The QCM sensor can measure AFP in buffer and human serum and offers advantages of high specificity, reusability, low detection limit, no label or sample pretreatment, and low sample requirement. The assay format of the immunosensor was more rapid and simpler than conventional methods.

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α -Fetoprotein (AFP)⁴, a 65- to 70-kDa glycoprotein, was first identified by Bergstrand and Czar in 1956 as the X-component in human cord blood (1). In 1963, Abelev et al. (2) demonstrated that this embryonal α -globulin could be detected not only in normal pregnant mice and the sera of newborn mice, but also in mice bearing hepatocellular carcinomas. In 1964, Tatarinov (3) identified AFP in the sera of patients with primary liver tumors. It appears that AFP is first produced in the yolk sac and later in the liver of the embryo. The synthesis of AFP in the liver decreases after birth and can be found in only extremely low amounts in the adult except in the presence of hepatomas or teratomas. The AFP concentration in a healthy adult serum typically is <20 $\mu\text{g/L}$ (4).

The conventional methods for the determination of AFP are a RIA and a nonisotopic immunoassay. Although the RIA has advantages of high sensitivity and low cost, it requires special disposal and has stability problems (5). The nonisotopic immunoassay, based on enzymes or fluorescent molecules, has high sensitivity and is fully automated on many platforms (6). However, these methods must rely on the detection of labeled molecules and are complicated and time-consuming.

Immunosensors, which combine the inherent specificity of antigen-antibody (Ag-Ab) reactions with the high sensitivities of various physical transducers, have gained attention as methods for clinical diagnosis (7). This study focused on microbalances based on piezoelectric crystals, where a decrease of the resonance frequency is correlated to the mass accumulated on its surface. The potential of piezoelectric devices for chemical sensor applications was realized by Sauerbrey (8), who derived the following equation describing the frequency-to-mass relationship in the air phase:

⁴ Nonstandard abbreviations: AFP, α -fetoprotein; Ag, antigen; Ab, antibody; MAb, monoclonal antibody; SAM, self-assembled monolayer; QCM, quartz crystal microbalance; ConA, concanavalin A; and PBS, phosphate-buffered saline.

¹ Department of Food Health, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan, R.O.C.

² Department of Agricultural Chemistry, National Taiwan University, 1, Sec. 4, Roosevelt Road, Taipei, Taiwan, R.O.C.

³ Department of Radiation Oncology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan, R.O.C.

*Author for correspondence. Fax 886-2-23661696; e-mail chenyan@ccms.ntu.edu.tw.

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$$\Delta F = -2.3 \times 10^6 F^2 \Delta M / A$$

where ΔF is the measured frequency shift (Hz) of the coated crystal, F is the resonance frequency (MHz) of the crystal, A is the area coated, and ΔM is the change produced by the mass deposited. On the other hand, the relationship between the change in oscillation frequency of a quartz resonator in contact with liquid and accumulated mass was realized by Kanazawa and Gordon (9) and Hillier and Ward (10), who derived the following equation:

$$\Delta F = -f_0^{3/2}(\rho_l \eta_l / \pi \mu_q \rho_q)^{1/2}$$

where f_0 is the resonance frequency (MHz) of the crystal; ρ_l and η_l are the absolute density and viscosity of the solution, respectively; and μ_q and ρ_q are the shear stiffness and density of the quartz crystal, respectively. The terms ρ_q (2.648 g/cm³) and μ_q (2.947×10^{11} dyne/cm² for AT-cut quartz) are constant. The frequency response to the mass of the Ag accumulated on the electrode surface is related to the density and viscosity of the solution in the microenvironment, i.e., in the interface between the sample solution and the electrode surface.

In this study, immobilization of monoclonal antibodies (MAbs) on the gold surface of quartz is also discussed. The use of self-assembled monolayers (SAMs) in various fields of research is growing rapidly. In particular, many biomedical fields apply SAMs as an interface layer between a metal surface and a solution. The pioneers in the assembly of sulfur-containing molecules noticed that dialkane sulfides form highly ordered monolayers on metal surfaces. van der Waals forces between methylene groups orient and stabilize the monolayer. The structure of a SAM depends on the morphology of the metal. Au(III) is most often used for the formation of monolayers because it is reasonably inert (11). For example, if the amino- and sulfur-containing molecules are adsorbed on the gold surface and then activated by glutaraldehyde, the aldehyde groups of glutaraldehyde can bind to the amino groups of the protein to form Schiff bases (12, 13). Thus, the proteins (e.g., enzymes and Abs) can be immobilized on the gold surface.

Here we report methods for immobilizing anti-AFP MAbs on the surface of the gold electrode, the reusability of quartz coated with MAbs, and the operating stability of this quartz crystal microbalance (QCM) sensor. In addition, we measured AFP concentrations in buffer and human serum with this sensor and compared the QCM method with RIA in clinical human serum samples.

Materials and Methods

REAGENTS

AFP from human cell culture-derived (M_r 70 000; purity $\geq 95\%$ by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was obtained from Calbiochem-Novabiochem International. Anti-human AFP MAbs were produced previously in our laboratory (Chou SF, Chen CY.

Production of monoclonal antibodies against human α -fetoprotein, a hepatocellular tumor marker, and clinical detection, in preparation). Protein A, cystamine dihydrochloride, and concanavalin A (ConA) were purchased from Sigma. Bovine serum albumin was obtained from Chemicon International, Inc. All other chemicals used were of analytical grade.

MATERIALS

An AT-cut quartz crystal oscillator (10 MHz; $8 \times 8 \times 0.18$ mm; diameter of the gold electrode, 5 mm; Tai Tien Electric Co., LTD) was used throughout the work. Chromium and gold were successively deposited to give layers 20 and 2000 nm thick, respectively. Chromium was deposited to obtain good adhesion between the gold and the crystal (14).

APPARATUS

Shown in Fig. 1 is a quartz crystal oscillation circuit built in-house to amplify the signal of resonant frequency of the oscillator. The frequency of the oscillator was determined by a computer-controlled frequency counter (Universal counter; Hewlett Packard Model 53131A). The measurement set-up used for all experiments is shown in Fig. 2. The crystal installed in the poly(vinyl chloride) cell (not a flow cell) was connected to the oscillation circuit. The diameter of this poly(vinyl chloride) cell well was 5 mm.

MEASUREMENT OF RESONANCE FREQUENCY BY THE QCM

The crystals were cleaned by immersion in 1.2 mol/L NaOH for 10 min and in 1.2 mol/L HCl for 5 min; one drop of concentrated HCl was then added for 30 s (15). After each step, the crystal was thoroughly washed with distilled water. The crystals were dried at room temperature, and initial frequencies were then read. Only one side of the crystal was exposed to the solutions (3 μ L) added to the cell well, and a stable frequency value was

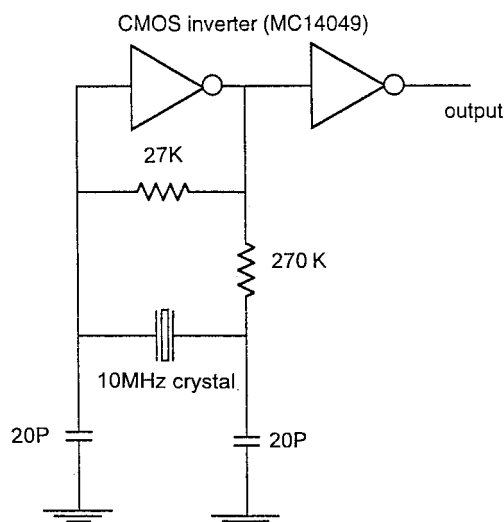


Fig. 1. Block diagram of the oscillator circuit.

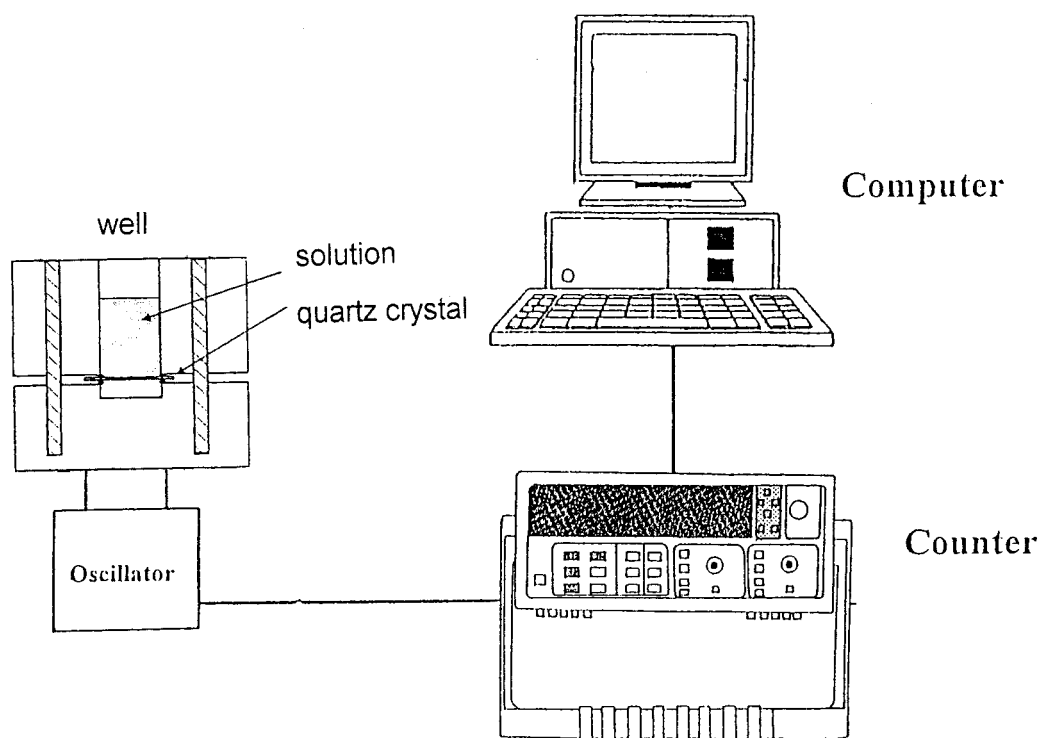


Fig. 2. Measurement setup of the QCM used.

reached and read. The 3- μ L drop was placed on exactly the same place on the electrode (marked on the gold surface) in the repeated measurement (sensor area, 3.14 mm²). All the frequencies were measured at room temperature at atmospheric pressure.

IMMOBILIZATION OF ANTIBODIES ON GOLD DISK

Adsorption method. In the adsorption method, the crystal was immersed in an Ab solution (10^6 μ g/L) for 1 h. Subsequently, the crystal was washed with phosphate-buffered saline (PBS; 5 mmol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.0) and distilled water, and then dried. Finally, a stable frequency value was reached and read (15). All procedures were performed at room temperature at atmospheric pressure.

Protein A method. In the protein A method, 5 μ L of a protein A solution [1 mg of protein A in 1 mL of 50 mmol/L PBS (pH 7.0) and 1 mL of 0.1 mol/L acetate buffer (pH 5.5)] was added to the electrodes on one side of the crystal. After drying, the crystal was immersed in distilled water for 30 min. Subsequently, 5 μ L of a 10^6 μ g/L Ab solution was spread over the electrode surface. After drying, the crystal was washed with PBS and distilled water, and dried (16, 17). Finally, a stable frequency value was reached and read. All procedures were performed at room temperature at atmospheric pressure.

Cystamine-glutaraldehyde method. For the cystamine-glutaraldehyde method, the crystal was immersed in a cysta-

mine solution (10 mmol/L cystamine, 50 mmol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.0) for 1 h, washed with distilled water, and dried. The crystal was dipped in 100 mL/L glutaraldehyde in water for 30 min and washed twice with distilled water. After drying, the crystal was immersed in a 10^6 μ g/L Ab solution for 1 h, washed with PBS and distilled water, and dried. The crystal was blocked with a glycine-PBS solution (0.1 mol/L glycine, 50 mmol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.0) for 30 min (13, 18), washed with PBS and distilled water, and dried (12). Finally, a stable frequency value was reached and read. All procedures were performed at room temperature at atmospheric pressure.

Cystamine method. For the cystamine method, the crystal was immersed in a cystamine solution (10 mmol/L cystamine, 50 mmol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.0) for 1 h, washed with distilled water, and dried. The crystal was then dipped into a 10^6 μ g/L Ab solution for 1 h, washed with PBS and distilled water, and dried. The crystal was blocked with a 0.1 mol/L glycine-PBS solution for 30 min, washed with PBS and distilled water, and dried. Finally, a stable frequency value was reached and read. All procedures were performed at room temperature at atmospheric pressure.

ConA method. For the ConA method, the crystal was dipped into a ConA solution (85 nmol/L ConA in PBS containing 100 μ mol/L Ca²⁺ and Mn²⁺, pH 6.8) for 1 min, washed with distilled water, and dried. Subsequently, the

crystal was immersed in a $10^6 \mu\text{g/L}$ Ab solution for 1 h, washed with PBS, and dried. The crystal was then blocked with a 120 nmol/L aqueous dextran solution for 30 min, washed with PBS and distilled water, and dried (19). Finally, a stable frequency value was reached and read. All procedures were performed at room temperature at atmospheric pressure.

Results and Discussion

MASS LOADING OF Abs ON ELECTRODE SURFACE OF QCM DEVICE BY DIFFERENT IMMOBILIZATION METHODS, AS CALCULATED BY THE SAUERBREY EQUATION
In this experiment, five immobilization methods, including adsorption, protein A, ConA, cystamine-glutaraldehyde, and cystamine, were used to immobilize anti-AFP MABs on the gold surface of quartz crystals. A calibration curve based on frequency shift (Hz; y) vs mass accumulated (ng; x) on the gold electrode surface of the quartz crystal in the dry state (as calculated by the Sauerbrey equation) was obtained (data not shown). The linear regression equation was: $y = 1.51x + 2.03$ ($R^2 = 0.9944$). The mass loadings of Abs on one side of electrode surface of the QCM device obtained from the linear regression equation using these five immobilization methods are shown in Table 1.

REUSABILITY OF QUARTZ CRYSTALS COATED WITH ANTI-HUMAN AFP MABs BY DIFFERENT IMMOBILIZATION METHODS

In this experiment, the above-mentioned immobilization methods were used to immobilize the MABs on the gold surface of the quartz. Three microliters of AFP solution ($10 \mu\text{g/L}$ AFP in PBS, pH 7.0) was applied to the gold surface in the poly(vinyl chloride) cell well for detection. The regeneration solution, 0.1 mol/L glycine-HCl buffer (pH 2.1), was used after each measurement (15). Fig. 3 shows that the reusability of quartz crystals prepared by the cystamine method developed in our laboratory was better than those of the other methods. The quartz could be used continuously >10 times, and the relative frequency shifts obtained were all >95% of the response obtained for the first cycle. Generally, the formation of the stable monolayers is based on the strong adsorption of

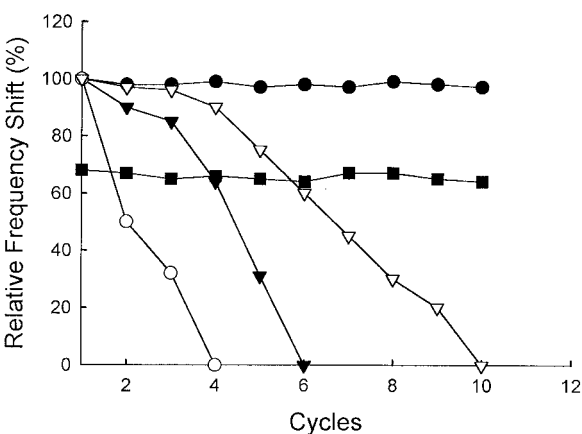


Fig. 3. Comparison of the reusability of quartz crystals coated with anti-human AFP MABs by different immobilization methods in the QCM system.

Anti-AFP MABs were immobilized on the gold surface of quartz crystals by the cystamine method (●), the adsorption method (○), the ConA method (▼), the protein A method (▽), or the cystamine-glutaraldehyde method (■). AFP ($10 \mu\text{g/L}$ in PBS, pH 7.0) was applied to the gold surface for detection. The regeneration buffer was 0.1 mol/L glycine-HCl (pH 2.1). The *Relative Frequency Shift (%)* is the frequency shift measured relative to the response for the first measurement.

disulfides (R-S-S-R), sulfides (R-S-R), and thiol (R-SH) on a metal (particularly gold) surface (11). In the cystamine and cystamine-glutaraldehyde methods, the disulfide bond of cystamine (2,2'-dithiobisethanamine; $\text{C}_4\text{H}_{12}\text{N}_2\text{S}_2$) is broken, and the compound strongly adsorbs to the gold surface. The isotype of the anti-AFP MABs used had been classified as IgG1 heavy chain in a previous study (Chou SF, Chen CY. Production of monoclonal antibodies against human α -fetoprotein, a hepatocellular tumor marker, and clinical detection, in preparation). In the cystamine-glutaraldehyde method, the aldehyde groups of glutaraldehyde bind to the amino groups of the Fab portion of the IgG molecule (i.e., the antigen binding site),

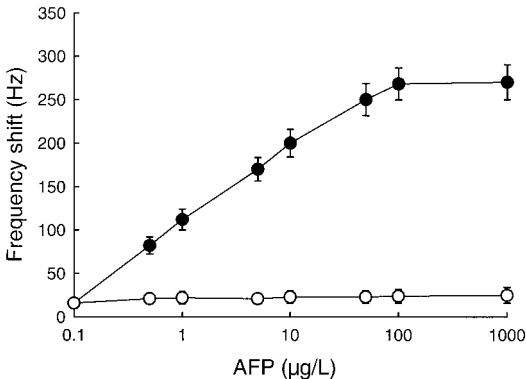


Fig. 4. Detection of AFP in human serum, using anti-human AFP MABs immobilized on the gold surface of a quartz crystal.

The concentrations of AFP diluted in human serum were 0.1, 0.5, 1, 5, 10, 50, 100, and 1000 $\mu\text{g/L}$. Immobilized antibodies used were monoclonal anti-AFP (●) and anti-bovine serum albumin (control; ○). The immobilization method used was the cystamine method. A linear relationship ($y = 84.36 \log x + 107.48$; $R^2 = 0.9957$) existed between the frequency shift (Hz) and the log values for AFP concentrations of 0.1–100 $\mu\text{g/L}$, determined using immobilized anti-AFP MABs. Bars, SD. Three replicates per specimen were measured.

Table 1. Mass loading of Abs on electrode surface of QCM device, obtained from the Sauerbrey equation, using different immobilization methods.

Immobilization method	Mean ^a (SD) mass loading of Abs on electrode surface of QCM device, ng/cm ²
Adsorption	13.04 (2.99)
Protein A	19.37 (2.87)
Cystamine-glutaraldehyde	10.28 (2.01)
Cystamine	19.11 (2.56)
ConA	12.56 (3.08)

^a Mean of three replicate analyses.

Table 2. Precision of the AFP determination by QCM immunoassay.

AFP concentration, $\mu\text{g/L}$	Frequency shift, Hz					
	In PBS (n = 3) ^a			In human serum (n = 3) ^a		
	Mean	SD	CV, %	Mean	SD	CV, %
0.1	15	2.45	16	16	6.12	34
0.5	86	6.12	7.1	82	9.79	12
1.0	110	7.34	6.7	112	12.2	11
5.0	160	11.0	6.9	170	13.5	7.9
10.0	195	11.0	5.6	200	15.9	8.0
50.0	260	12.2	4.7	250	18.4	7.3
100.0	280	14.7	5.2	268	18.4	6.9

^a Three replicates per specimen were measured.

so the binding of Ag to Ab might be partly hindered. Thus, the cystamine-glutaraldehyde method was unsuitable for immobilizing the IgG molecule (shown in Fig. 3). In the cystamine method, the amino groups of cystamine could easily bind to the carboxyl groups in the IgG molecule, presumably by electrostatic interaction or hydrogen bonding, but not bind to the amino groups of the antigen binding sites in the IgG molecule. The hydrogen bond was probably formed between C=O on the Ab and -NH on the SAM (20), so there were many hydrogen bonds in Ab-SAM. It had been demonstrated that extremes of pH can disrupt ion-pair interactions (electrostatic), but urea and guanidine hydrochloride interfere with hydrogen bonding (21). The Abs on the SAM should be random and partly multilayers. Many hydrogen bonds could also be formed within Ab layers. Because the amount of Ag measured was very low ($\mu\text{g/L}$), there were more hydrogen bonds in Ab-SAM than in Ab-Ag complexes. Therefore, the low pH of the regeneration buffer could easily disrupt Ag-Ab complexes, but not disrupt Ab-SAM complexes. However, there were enough Abs on the SAM to react with the Ags, even if the Abs had been partly disrupted at low pH. These experimental results demonstrate that the cystamine method is suitable for immobilizing the IgG molecule and that the reusability of the crystal was high.

CALIBRATION CURVES FOR HUMAN AFP IN BUFFER AND HUMAN SERUM

A calibration curve for human AFP in human serum is shown in Fig. 4. Human AFP in PBS and human serum was detected at concentrations of 0.1–1000 $\mu\text{g/L}$, using anti-AFP MAbs immobilized on the gold surface of a quartz crystal. A linear relationship existed between the frequency shifts (Hz) and the log values of AFP in human serum in the range 0.1–100 $\mu\text{g/L}$ (the samples containing high AFP could be diluted into this range). Although nonspecific binding of the proteins and/or lipids in the serum sample could occur, it did not interfere with the highly specific binding of the MAbs to the Ags. Therefore, the nonspecific binding did not affect the analytical data (as demonstrated by the experimental results). Similar

results were obtained in PBS (data not shown). The reaction time to reach equilibrium was 10 min in buffer and 20 min in human serum (data not shown).

PRECISION

Precision data for the determination of AFP (0.1–100 $\mu\text{g/L}$) in buffer and human serum by this QCM immunosensor are shown in Table 2. Three replicates per specimen were measured.

OPERATING STABILITY OF HUMAN AFP PIEZOIMMUNOSENSOR

We performed five cycles of measurements per day continuously for 15 days with this AFP piezoimmunosensor. The operating stability of this sensor was >15 days, and the relative frequency shifts were all >95% of those measured on the first day (data not shown).

CORRELATION BETWEEN THE QCM METHOD AND RIA

AFP concentrations in clinical human serum samples were simultaneously measured by the QCM immunoassay and RIA. The RIA results were obtained from the Department of Radiation Oncology of the Tri-Service General Hospital (Taipei, Taiwan). Mean values (SDs) by the QCM and RIA in 29 clinical human serum samples were 67.9 (96) and 67.0 (95) $\mu\text{g/L}$, respectively, with ranges of 2–335 and 3–328 $\mu\text{g/L}$. Deming regression yielded a slope of 1.03 (95% confidence interval, 0.96–1.09) and intercept of -0.06 (-0.76 to 0.64) $\mu\text{g/L}$ ($S_{y/x} = 3.92$; $r = 0.9987$).

In conclusion, in this study, AFP concentrations in buffer and human serum were measured by a new immunosensor based on a QCM. A SAM prepared by the cystamine method developed in our laboratory was applied to immobilize anti-human AFP MAbs on the gold surface of a quartz crystal. The reusability of the crystal coated with this SAM was better than the reusability of crystals prepared by the other immobilization methods. A linear relationship existed between the frequency shifts (Hz) and the log values of AFP in buffer and human serum in the range 0.1–100 $\mu\text{g/L}$. The correlation coefficient be-

tween QCM and RIA was 0.9987 for determination of AFP in the clinical human serum samples. The direct immunosensor system measured AFP without the need for a labeled reagent and pretreatment of samples. The assay format of the sensor was more rapid and simpler than conventional methods.

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