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ProteoChip-Based Immunoassay Method for Quantitative Determination of Serum Tumor Markers

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Abstract

Conventional biomarker assays are generally performed by an immunoassay system with several different detection methods. In this study, we have carried out validation experiments to verify performance of a novel chip-based immunoassay method for quantitative determination of tumor marker proteins by using "Well-on-a-Chip" (Well-Chip). The tumor markers, AFP and CEA, spiked in human serum matrix were analyzed by the fluorescence sandwich immunoassay method adopted for Well-Chip with arrayed mini-wells (1.5 mm diameter). Statistical analyses were carried out to validate precision, accuracy, and repeatability of the analytical data. Thus, the assay results obtained by the Well-Chip method were compared with those by a standard ELISA method to confirm reliability of the analytical data. It was demonstrated that quantitative assays of spiked AFP and CEA in human sera and buffer solution were possible in the linearity ranges with the Well-chip. The CV values were lower than 15% and recovery rates were within a range of ±10% in both AFP and CEA. The correlation coefficients of analytical values obtained by within-run and between-day were higher than 0.9. The assay values of the Well-Chip method were also well correlated with those of ELISA methods. The assay performance of the Well-Chip method was validated as a novel method for quantitative assays of tumor markers and shown to be highly dependable and reliable as much as a standard ELISA method. It is evident that the Well-Chip method can be useful for multiplex assays of biomarker proteins in human serum.

Keywords: ProteoChip, Tumor markers, Well-on-a-chip, ELISA

Introduction

Protein chip has become a useful tool for multiplex analysis of biomarkers and expressed proteins simultaneously with a small amount of samples. It has been widely applied for various studies on biomarker assay, new lead screening, proteome analysis and protein-protein interaction[1-4].

In order to make protein chips more practical as a novel bio-analytical tool for biomarker proteins, it is a critical task to improve surface performance of capture proteins immobilized on solid chip surface. For many years, several different methods of protein immobilization on solid substrates have been studied including physical bonding, covalent bond, biological affinity method, and self assembling linker molecules[5-7]. It has been, however, noted that there are some problems in these immobilization methods for protein chip[8]. It is an important strategy for constructing protein chip that proper orientation and activity must be maintained to attain interaction between the immobilized capture antibodies and target proteins such as biomarker proteins.

In the previous report, we had introduced ProteoChip, a protein chip base plate coated with ProLinker^{TM}, which was particularly designed for efficient immobilization of capture proteins such as antibodies[9,10]. ProLinker^{TM} is a novel molecular linker for immobilizing capture proteins tightly and densely with orderly orientation and intact activity on solid surface[11]. The surface performance of ProteoChip was verified by demonstrating that ProteoChip has high selectivity and sensitivity in protein-protein interaction[11,12]. ProteoChip has been suggested to be a useful bio-analytical tool for solid phase immunoassay of biomarkers, differential protein expression profile analysis and high-perform-ance lead screening[13-15].

In the various application areas for disease diagnosis or screening, chip-based biomarker assay system has been paid much attention since it may be able to provide an alternative method for multiplex and sensitive protein assays[16-20]. For the purpose of early cancer detection using biomarker assay, we have developed a 'Well-on-a-Chip' (Well-chip), which is a ProteoChip with mini-wells (1.5 mm diameter) that require 1 μL

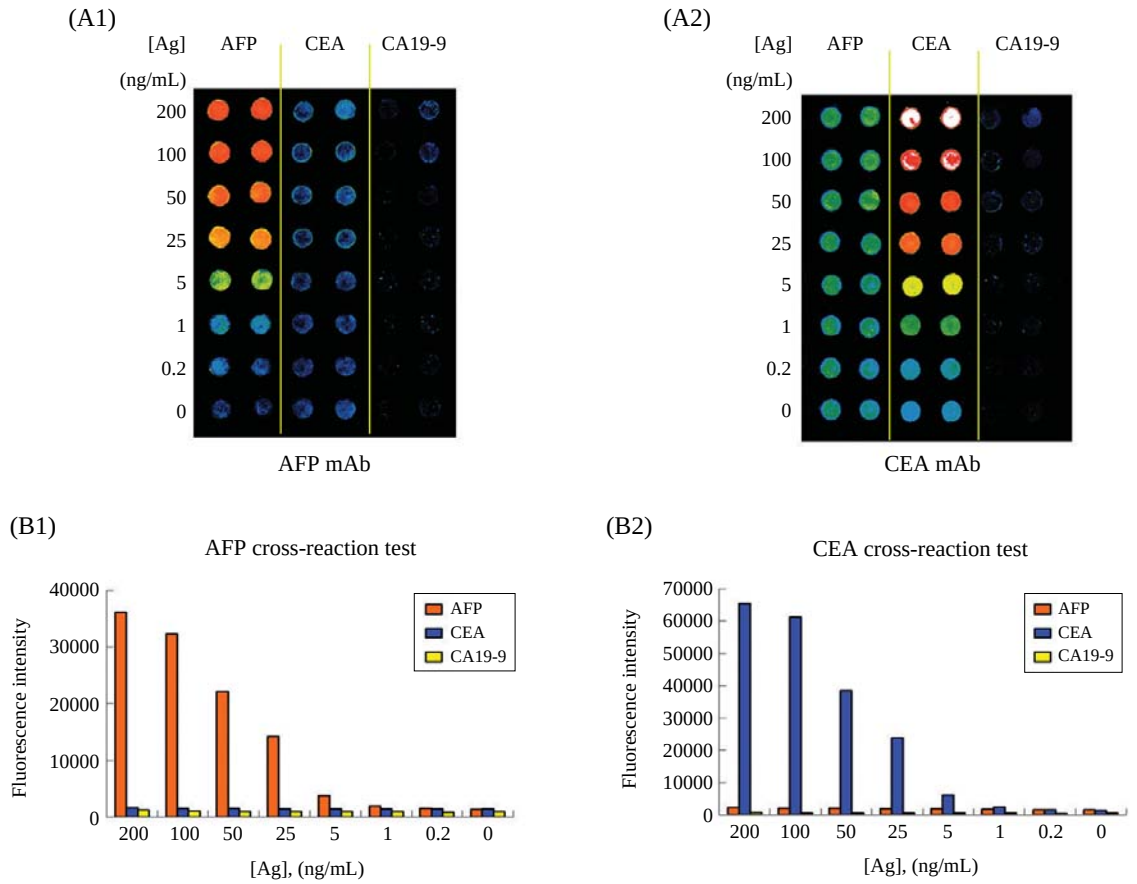

Figure 1. Cross-reactivity of antibody-antigen interaction on Well-Chip. Fluorescence images of tumor markers, AFP, CEA and CA19-9 detected by immobilized anti-AFP mAb and anti-CEA mAb, respectively (A1 and A2) and numerical bar-graphs of fluorescence intensity estimated from A1 and A2 (B1 and B2).

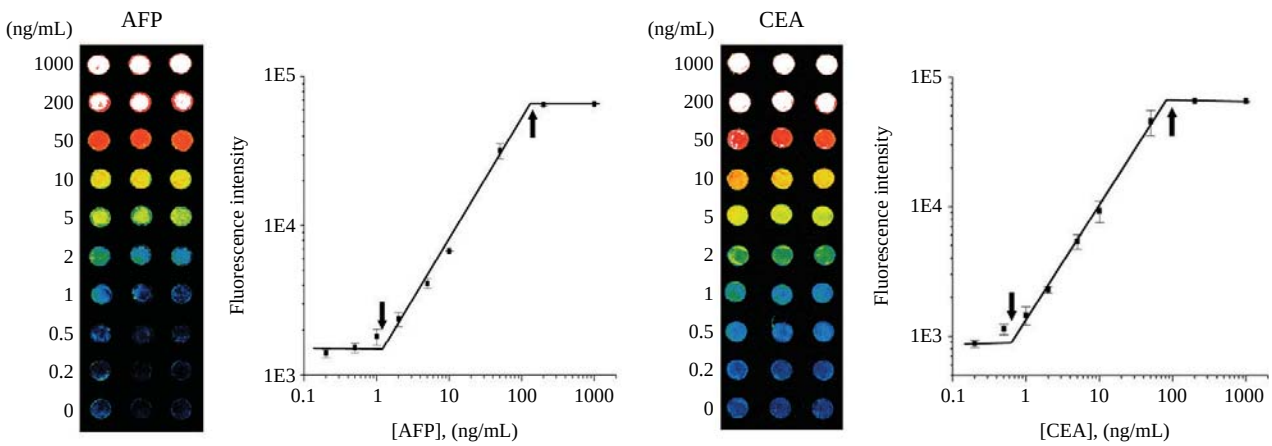

Figure 2. Fluorescence images and dose-response curves of AFP and CEA assays showing linear curves within the lower and upper detection limit.

application volume of reagents. The Well-Chip enabled accurate quantitative analysis of marker proteins with a small quantity of sample and reagent solutions to perform.

In this study, we have examined the analytical performance of the Well-Chip by applying serum tumor markers such as alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) as model biomarker proteins for quantitative assay. The assay results were statistically evaluated in terms of 1) specificity and sensitivity, 2) accuracy and precision, 3) repeatability and reliability by comparing them with a gold standard method such as ELISA to verify the analytical performance of the newly introduced chip-based assay method.

Results and Discussion

Cross Reactivity Tests for AFP and CEA on Well-Chip

We examined the cross reactivity between capture antibodies against AFP and CEA and the marker proteins on Well-Chip. The results demonstrated clearly specific interaction of immobilized anti-AFP and anti-CEA monoclonal antibodies (mAbs) with the respective antigen, AFP and CEA (Figure 1). It represented that the immobilized mAb on Well-Chip surface specifically interacts with the corresponding antigen. It was noted that background fluorescence signal by non-specific interaction of the immobilized different antibodies was negligible in this Well-Chip assay method.

Linearity for Quantitative Assays of AFP and CEA with Well-Chip

The linear standard curves were demonstrated in the experiments with the biomarkers spiked in PBS buffer solution as a carrier solution. Numerical plots of fluorescence intensities were proportional to the concentrations of proteins (Figure 2). The linearity was shown within the dynamic concentration ranges of 3 ng mL^{-1} -440 ng mL^{-1} and 1 ng mL^{-1}-210 ng mL^{-1} for AFP and CEA, respectively. Linear standard curve in log-log scale proved that the chip-based assay system can be suitable for quantitative analysis of tumor marker proteins with the Well-Chip-based fluorescence immunoassay method using minute volume (1 μL) of reagents.

Validation of Precision and Accuracy of the Chip-based Assay Results

In order to validate analytical performance of the chip-based assay method, precision and accuracy were determined by estimating CV values and recovery rates of analytical results obtained from the pre-set concentration levels of AFP and CEA spiked in test samples. The assay data were obtained from test experiments conducted three times in 5 days by the Well-Chip method and analyzed statistically to evaluate precision and accuracy. The fluorescence array images by the Well-Chip method were analyzed for quantification of AFP and CEA test samples prepared in PBS buffer solutions and human serum matrix (Figure 3).

The standard curve was plotted with 7 to 8 calibration points including blank as shown in Figure 3. The formula for estimating concentrations of target marker proteins in test samples from calibration standards was represented by a linear equation as Y (log value of fluorescence signal)=A+B∗X (log value of protein concentration), where the linear regression coefficient values (R) were in the range of 0.995-0.999 ($P < 0.001$) (Figure 3). Assay results for test samples at high, medium, low and LLOQ concentration levels were obtained by test experiments of within-run (three times) and between-day (three times in 5 days) for precision and accuracy. We have taken LLOQ value of 4 ng mL^{-1}, it is thought to be a practical level of the lower limit within the linear range of calibration curves for both marker proteins, in the following validation experiments for assay performance of the Well-Chip method. The LLOQ was defined as the lowest tumor marker concentration which can be determined with an accuracy between 100±20%.

As the precision of assay results, the CV values of test samples in low, medium, and high concentrations were found to be within ±15% of their each concentration, while those for LLOQ samples were also less than ±20% of their each concentration. The precision of all assay results determined by within-run and between-day for AFP and CEA prepared in both buffer solution and human serum matrix.

The accuracy of assay results obtained by the Well-Chip method was evaluated by the extent of recovery of the marker concentrations experimentally determined in comparison with the theoretical values, which are pre-set concentrations of the marker proteins. The values of accuracy tests were represented by the % recovery of analytical values for AFP and CEA in PBS buffer and human serum matrix (Tables 1 and 2). The recovery values for both AFP and CEA in four test samples of different concentration levels were found to be in a range from 90% to 110% with CV values lower than 15%. Also, it was observed that the CV values of LLOQ (4 ng mL^{-1}) were within 20%. The test results of both precision and accuracy for test sam-ples of AFP and CEA were not affected by the use of different matrices.

Correlation between Assay Results of Well-Chip and ELISA Method

To evaluate analytical performance of the Well-Chip method for tumor marker assay, the prepared calibra-

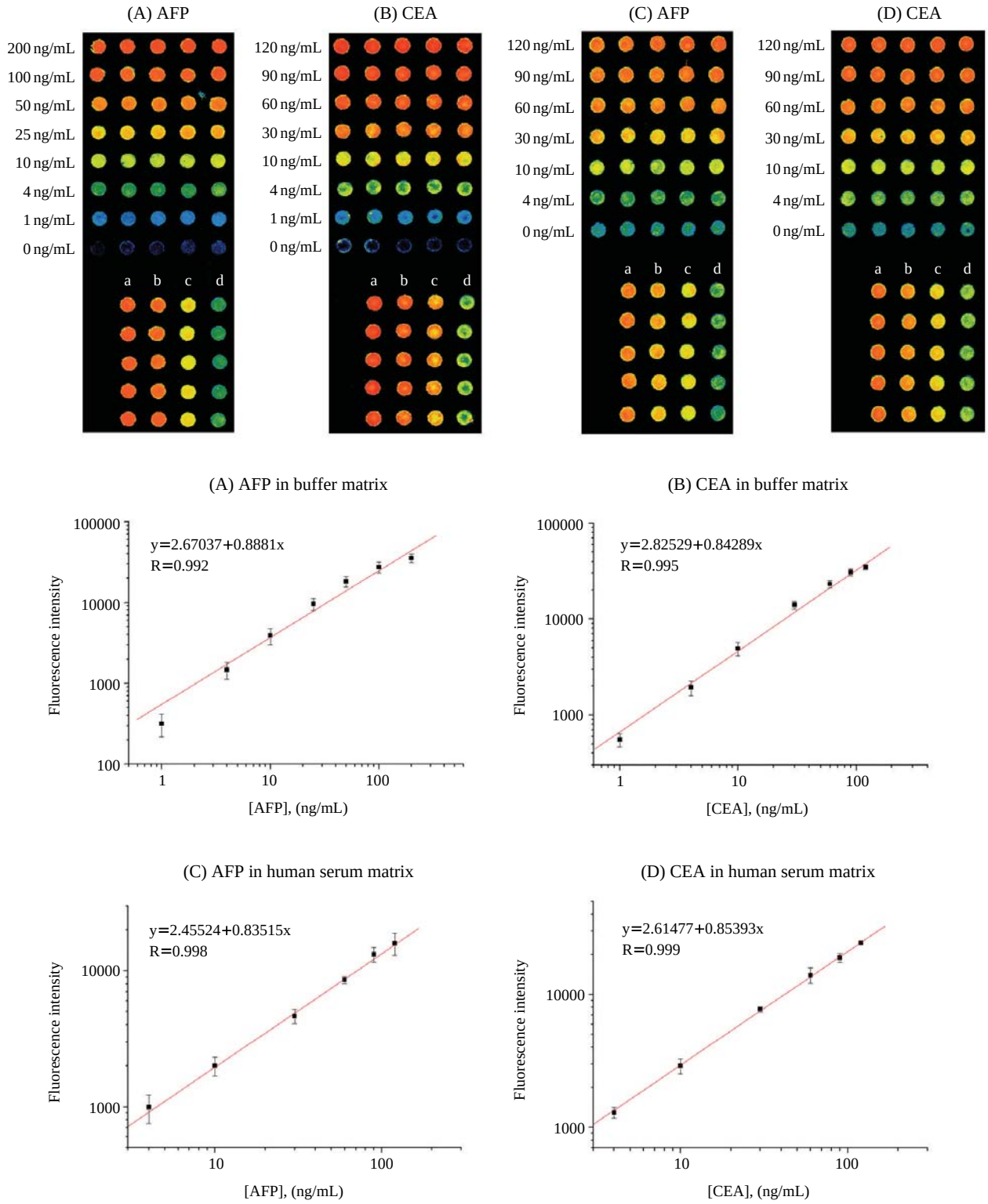

Figure 3. Fluorescence images and standard graphs of Well-Chip array for determinations of tumor markers in standard and test samples. (A) AFP and (B) CEA in PBS buffer matrix, (C) AFP and (D) CEA in serum matrix: a, b, c, and d represent high, medium, low, and LLOQ (Lower Limit Of Quantization) concentration levels.

Table 1. Precision and Accuracy of assay values for test samples of AFP and CEA spiked in PBS buffer matrix by Well-Chip method.

Buffer matrix		Within-run						Between-day					
AFP ($ng\ mL^{-1}$)	CEA ($ng\ mL^{-1}$)	Mean ± SD ($ng\ mL^{-1}$)		CV		% Recovery		Mean ± SD ($ng\ mL^{-1}$)		CV		% Recovery	
		AFP	CEA	AFP	CEA	AFP	CEA	AFP	CEA	AFP	CEA	AFP	CEA
120 (High)	80 (High)	120.06 ± 5.13	78.52 ± 5.48	4.28	6.98	100.05 ± 4.28	98.15 ± 6.85	105.27 ± 14.43	76.56 ± 5.69	13.71	7.44	89.50 ± 11.86	94.47 ± 7.90
80 (Medium)	40 (Medium)	83.41 ± 3.04	42.32 ± 2.88	3.65	6.80	104.26 ± 3.80	105.80 ± 7.20	75.00 ± 10.00	41.98 ± 2.78	13.33	6.62	92.98 ± 13.21	104.63 ± 7.39
20 (Low)	20 (Low)	20.03 ± 1.48	20.50 ± 0.45	7.41	2.21	100.17 ± 7.42	102.48 ± 2.26	20.60 ± 2.18	20.97 ± 1.33	10.60	6.34	100.58 ± 12.99	104.31 ± 7.05
4 (LLOQ)	4 (LLOQ)	4.12 ± 0.65	3.75 ± 0.31	15.88	8.27	103.00 ± 16.36	93.69 ± 7.75	4.01 ± 0.76	3.72 ± 0.43	18.84	11.56	99.21 ± 17.37	91.38 ± 8.37

Table 2. Precision and Accuracy of assay values for test samples of AFP and CEA spiked in human serum matrix by Well-Chip method.

Serum matrix	Within-run						Between-day					
AFP/CEA ($ng\ mL^{-1}$)	Mean ± SD ($ng\ mL^{-1}$)		CV		% Recovery		Mean ± SD ($ng\ mL^{-1}$)		CV		% Recovery	
	AFP	CEA	AFP	CEA	AFP	CEA	AFP	CEA	AFP	CEA	AFP	CEA
80 (High)	79.45 ± 6.74	80.76 ± 7.57	8.48	9.37	99.32 ± 8.42	104.95 ± 7.27	83.96 ± 5.82	80.93 ± 5.87	6.93	7.25	100.95 ± 9.46	101.16 ± 7.33
40 (Medium)	43.68 ± 2.07	41.58 ± 4.56	4.73	10.96	109.21 ± 5.17	110.84 ± 3.08	44.34 ± 1.23	42.91 ± 2.56	2.78	5.96	103.96 ± 11.39	107.27 ± 6.39
20 (Low)	18.74 ± 1.32	20.86 ± 2.19	7.03	10.48	93.71 ± 6.58	94.88 ± 9.80	18.98 ± 1.96	20.62 ± 1.76	10.33	8.55	104.30 ± 10.93	103.12 ± 8.82
4 (LLOQ)	3.70 ± 0.29	3.88 ± 0.54	7.78	13.91	92.61 ± 7.20	93.12 ± 11.19	3.72 ± 0.45	4.20 ± 0.56	12.02	13.37	97.10 ± 13.50	105.05 ± 14.04

tion standards and the test serum samples (n=40) were utilized for quantitative analysis with the Well-Chip method as well as ELISA method as a gold standard method. We evaluated correlations of analytical results determined by commercial ELISA and the Well-Chip method with AFP and CEA spiked in serum samples. As the comparison of Well-chip method with ELISA method, the correlation coefficient (R) values of assay values by the two different methods were estimated to be 0.964 and 0.982 for AFP and CEA, respectively (Figure 4A and 4D). It suggested that the assay results obtained by Well-Chip method with a minute amount of reagent volume (1 μL) showed high correlation with those determined with a well-established ELISA method. Repeatability of tumor marker assay by the Well-Chip method was found to be excellent through the correlation of assay results by within-run and between-day test. The R values were estimated to be 0.984 and 0.986 for AFP and CEA assayed by within-run test, respectively (Figure 4B and 4E). Also, in between-day test, the R values were 0.975 and 0.980 for AFP and CEA, respectively (Figure 4C and 4F). In conclusion, the overall outputs of validation tests by the Well-Chip method have met the criteria in precision, accuracy and repeatability of analytical results recommended for general application of a new bio-analytical tool.

Discussion

Protein microarray chip has been paid much attention by proteomics scientists as the next-generation bio-analytical tool for multiple quantitative and qualitative analyses for target proteins of interests and protein-protein interaction analysis[23-25]. There are a number of reports that protein microarray has been applied for disease diagnosis such as cancers and analyses of protein expression profiles for discovery of new disease markers[26-28]. It is thought that microarray tech-

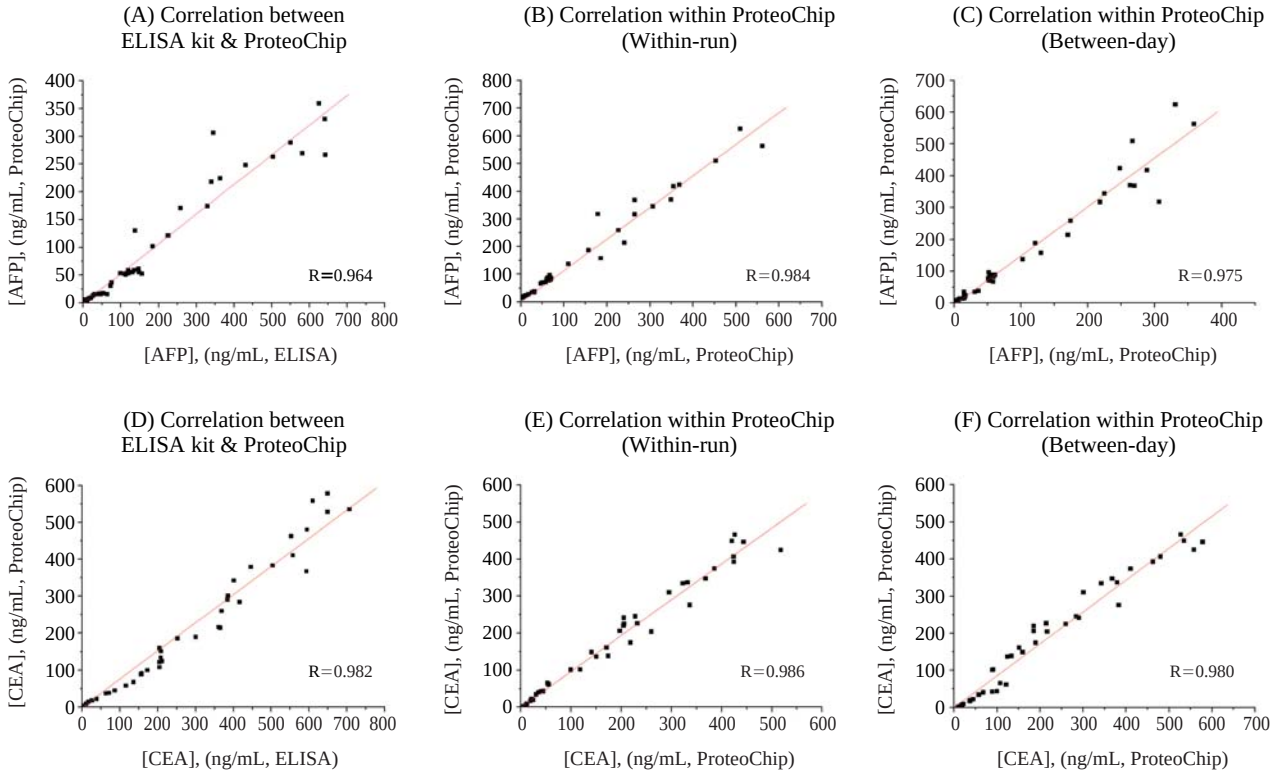

Figure 4. Correlation plots between assay values determined by ELISA kit and Well-Chip method (A and D), between assay values determined by the Well-Chip method in within-run test (B and E) and between-day test (C and F) for AFP and CEA in human serum matrix.

nology may provide advantages over conventional analytical tools by way of providing a high throughput system and handling a small quantity of protein in scale of micro to nano volume leading to reduction of analytical cost and time[29].

Various platforms of protein chip have been studied for detection and quantification of human serum proteins, which are present from high-abundance to low-abundance[30,31]. However, we have noted that there is intrinsic limitation in quantitative assays of marker proteins by protein microarray chip. It is that immobilization of capture antibody in a given spot is not well controlled and spot boundary is not clear enough to accommodate an exact volume of analyte proteins.

In this regard, we have developed Well-on-a-Chip (Well-Chip), which is a ProteoChip with arrayed mini-wells with 1.5 mm diameter and useful for quantitative detection of bio-marker proteins. ProteoChip is a slide glass coated with ProLinker^{TM}, which captures proteins such as antibody very efficiently and tightly. In the previous report, it was estimated by QCM method that ProLinker^{TM} surface can capture 5-6$*10^{12}$ antibody molecules per 1 cm^2 [11]. Indeed, ProteoChip has been proven to be well applicable for preparation of sensitive antibody microarray for antigen detection.

Regarding to the detection sensitivity, it has been emphasized that the detection sensitivity of an analytical method should not be miss-led as a criterion of the LLOQ, which is based on linearity in calibration curves for quantitative analysis[32-34]. According to our unpublished experiments, it has been observed that there is increased detection sensitivity in the chip-based biomarker detection due to avidity effect of antigen-antibody interaction. Such increased detection sensitivity at lower concentration range of analyte protein may be attributed to the increased number of antibody immobilized on chip surface[11]. However, it is stressed that the lower concentration ranges is not acceptable for quantitative analysis of biomarker proteins due to out-of-linearity range for calibration and increased variation of signal-to-noise ratio. Such an increase in detection sensitivity appears to be highly dependent not only on the performance of capture antibody immobilized on chip surface and consistency of assay processes, but also overcoming of intrinsic limitation of fluorescence detection systems and minimum detectable number of antigen molecules in a given volume for application.

From the assay values of 15 analytical points obtained from the present experiments, we have estimated the lower limit of detection (LLD) and the biological limit of detection (BLD), based on the standard deviation of the response and the slope[33,34], which were 0.49 ng mL^{-1} and 2.94 ng mL^{-1} for AFP and 0.28 ng mL^{-1} and 3.07 ng mL^{-1} for CEA, respectively. We concluded that the estimated value of BLD was reasonably well agreeable with LLOQ value. LLOQ, regardless of matrix components was reproducible at 4 ng mL^{-1}, which can be determined with an accuracy between $100 \pm 20\%$. Since the CV values of assay results for both CEA and AFP at LLOQ were found to be less than 20%, the concentration range may be considered to be a level of 'functional sensitivity' for the Well-Chip method.

In this report, we presented validated analytical performance of the Well-Chip method useful for tumor marker assays in terms of specificity, precision, accuracy, repeatability and reliability of assay results according to the recommendation in Guidance for Industry, Bio-analytical Tool Validation[22]. It was confirmed that the chip-based assay method is applicable for quantitative analysis of tumor marker proteins in human serum. The assay results obtained by the Well-Chip method showed high correlation with those obtained by different immunoassay methods such an ELISA. In correlation results, low concentration in serum samples were more corresponded than high concentration in serum sample above 200 ng mL^{-1}. Dilution as variable can influence the bias of correlation. But, as dose-response graph by chip-based assay showed that concentration above 200 ng mL^{-1} was saturated, serum samples of high concentration needed dilution. It has tested at various serial dilution rates with buffer solution in serum matrix. Up to 10 fold, dilution rate had no effect on result of quantified concentration. In this study, as focusing on the quantification of cut off level of tumor marker such as 4 ng mL^{-1} of AFP and 10 ng mL^{-1} of CEA, we evaluated test samples containing tumor markers within 2-480 ng mL^{-1}. According to our unpublished data for AFP assays in human serum, the analytical results of the tumor marker by the Well-Chip method were also remarkably well correlated with those obtained by three different assay methods such as ELISA, RIA and AxSYM autoanalyzer (Abbott Labs.). These test results suggested that the Well-Chip method for tumor marker assay is highly reliable in comparison with existing immunoassay methods. Thus, it is proposed that the Well-Chip method can be well applicable for quantitative determination of serum tumor markers in the concentration range higher than 1 ng mL^{-1}.

It should be noted that the Well-Chip method enables multiplex analysis of biomarkers with a small volume (1 μL) of reagents including capture antibodies and analyte sera, whereas the existing analytical methods requires a large quantity of reagents (50-100 μL). The use of a micro-quantity of human serum samples has particular advantage in that multiple assays can be performed for multiple target analytes with a given quantity of a same serum specimen from a patient. It is also expected that development of an automation system for the miniaturized chip-based immunoassay for serum marker proteins may reduce the assay costs and times in medical examination.

Conclusions

Well-Chip method enables multiplex analysis of biomarkers with a small volume (1 μL) of reagents including capture antibodies and analytes. It was validated that quantitative results of tumor markers in serum were high correlation between conventional method ELISA and chip based assay. Also, experimental reproducibility of assay was verified by repetitive analyses for within-run and between-day. The use of a micro-quantity of human serum samples has particular advantage in that multiple assays can be performed for multiple target analytes with a given quantity of a same serum specimen from a patient. It is also expected that development of an automation system for the miniaturized chip-based immunoassay for serum marker proteins may reduce the assay costs and times in medical examination. The overall validation results by the Well-Chip method was proven to be a useful tool for quantitative analyses of the tumor markers, which are further applicable for screening or monitoring of cancer patients in clinical practice.

Materials and Methods

Materials

AFP, CEA, and anti-CEA polyclonal antibody were purchased from Biodesign International (Kennebunk, ME, USA). Anti-AFP monoclonal antibody (clone 5H7) was supplied by Hytest (Turku, Finland). Anti-AFP polyclonal antibody (rabbit IgG) was purchased from Fitzgerald Industries International (Concord, MA, USA) and Goat anti-rabbit IgG-Cy5 conjugate from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin (BSA), Tween-20 and human sera (aseptically filled) were obtained from Sigma (St. Louis, MO, USA).

Preparation of Serum Sample

The biomarker assay with Well-Chip was carried out

by analysis of the selected tumor markers, AFP and CEA in given concentrations, which were spiked in normal human serum. AFP and CEA are well known biomarker proteins frequently found in patient sera with liver and colorectal cancers, respectively. The spiked test samples (n=40) were prepared in normal human serum pool for the marker proteins in several different concentrations from 2 ng mL^{-1} to 480 ng mL^{-1}. These spiked samples were used to test in both the Well-Chip and ELISA methods for comparison. As standard concentration was saturated above 200 ng mL^{-1}, we set up the high standard concentration of 120 and 200 ng mL^{-1}. In the case of test samples at low concentration below 120 ng mL^{-1} (high concentration of calibration standard), the neat serum was used for analysis without dilution. But tumor markers in neat serum samples of high concentration above 120 ng mL^{-1} were not accurately calculated with standard samples below 120 ng mL^{-1}. Thus, test samples containing a marker protein more than 120 ng mL^{-1} were diluted 10-fold with a dilution buffer, 10 mM phosphate buffered saline (PBS) solution containing 3% BSA. Dilution rate up to 10 fold had no influence in the serum matrix.

Sandwich Immunoassay for Detection of Tumor Markers

Chip-based Immunoassay

The Well-Chip used in this experiment was designed to array 96 mini-well spots with a dimension of 1.5 mm diameter (the well depth, 10 μm) as same as 96 well plate system of ELISA. The chip-based sandwich immunoassay was carried out by using manual spotting method as described in the previous report[10]. The 1 μL of application volume of all reagent solutions was used for the chip-based assay experiments. A capture protein, monoclonal antibody specific to a marker protein was immobilized by applying 1 μL of the 100 μg mL^{-1} antibody solution prepared in 10 mM PBS buffer containing 10% BSA and 30% glycerol on each spot. Subsequently, the chip was incubated for overnight in 4˚C chamber. The chip was then rinsed with PBS buffer containing 0.5% tween-20 to remove unbound proteins for 10 minutes and dried under nitrogen gas. The chip immobilized capture protein was blocked with PBS buffer containing 3% BSA for 1 hour at room temperature. The pre-fabricated chip with capture antibody was used for the marker protein assay by dispensing 1 μL of calibration standard solutions and test samples on each well spots. The chip thus prepared was incubated in a moisturized chamber for 1 hour at 37˚C. In the following step, the conventional sandwich immunoassay method was adopted for this chip-based assay system. After binding polyclonal antibody (10 μg mL^{-1}) against the target marker protein, the bound marker protein was detected by application of the secondary antibody, Cy5-labeled goat-anti rabbit IgG (1 μg mL^{-1}) as a signal generator. The concentration of the detection antibodies was optimized to generate the maximum signal. The chip was then incubated in a moisturized chamber at 37˚C for 30 minutes. It was rinsed with PBS buffer containing 0.5% tween-20 to remove unbound proteins for 10 minutes and dried under nitrogen gas. Finally, fluorescence intensity of each well spots on a chip was analyzed by a fluorescence scanner (Axon Instruments, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was carried out to compare with analytical results of the chip-based assay method. ELISA tests were performed as described in standard assay procedures with commercial microtiter plate kits (DRG, Inc.) with 96 wells, which were pre-coated with a capture antibody[21]. Calibration samples, enzyme conjugated reagent, 3,3′,5,5′-Tetramethyl-benzidine (TMB) reagent and stop solution (1 N HCl) were provided in kit. The optical density of each well was measured with an ELISA reader (Finstruments™ Microplate Reader) at 450 nm.

Validation Method for Analytical Results

In order to evaluate the validity of the analytical performance of Well-Chip for tumor marker assay as a novel bioanalytical tool applicable for clinical diagnosis or screening of cancer patients, we have carried out statistical analysis of assay results according to the recommendation in The Guidance for Industry, Bioanalytical Method Validation[22]. The validation tests for the chip-based assay method were carried out with two different tumor markers, AFP and CEA, spiked in two different carrier solutions, one in buffer matrix, PBS solution containing 3% BSA and the other in serum matrix, normal human serum pool. The 7 different final concentrations in the ranges of 1, 4, 10, 25, 50, 100, and 200 ng mL^{-1} were used as calibration standard, while test samples of the 4 different concentration levels of 4, 20, 80, and 120 ng mL^{-1} were used for validation experiments. The tumor marker assays were carried out three times in a day (within-run) and serially during 5 days (between-day) for repeatability tests of different Well-Chips and assay processes. The five determinants per concentration level were measured with a Well-Chip for each eight different calibration standard solutions and four test samples. To validate analytical values determined by

the Well-Chip method, accuracy and precision, repeatability, and reliability of the assay data were evaluated as a parameter for analytical performance of the chip-based assay method. Accuracy is the degree of conformity of a measured quantity to its nominal value, while precision is the closeness of degree of scatter between a series of values obtained from multiple sampling of the same sample. Precision of assay values was evaluated by CV values within ±15%. Likewise, accuracy was based on recovery rates at a given concentration within ±15% except for lower limit of quantification (LLOQ). The precision and accuracy of assay values at LLOQ have been recommended to be lower than 20% for CV values and in the range of 80-120% for recovery rate, respectively. All statistical analyses and graphs were automatically calculated with OriginPro 7.0.

Data Analysis

The tumor marker concentrations in the arrayed spots of a Well-Chip were determined by fluorescence intensities detected with a fluorescence scanner, GenePix 4000B (Axon Instruments, CA, USA). Fluorescence signal by Cyanine dye (Cy5) was analyzed in wavelength 635 nm of fluorescence scanner. The fluorescent image was visualized by a rainbow color display system installed with the scanner in such a way that signals show red color for a high concentration and blue color for a low concentration. The fluorescence signals detected by the scanner were converted into numerical values by GenePix Pro 3.0 software.

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