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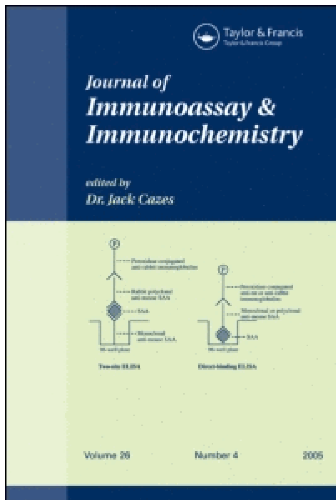

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A NEW MODEL ELISA, BASED ON TWO MONOCLONAL ANTIBODIES, FOR QUANTIFICATION OF FATTY ACID SYNTHASE

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A NEW MODEL ELISA, BASED ON TWO MONOCLONAL ANTIBODIES, FOR QUANTIFICATION OF FATTY ACID SYNTHASE

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ABSTRACT

A new model ELISA, based on two monoclonal antibodies, was developed for the quantification of fatty acid synthase (FAS). In this sandwich assay, a monoclonal antibody M6 was used as a capture on Nunc MaxiSorp ELISA/EIA

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Modules and another monoclonal antibody M3, labeled with biotin, was used as a detection antibody. More than 10 molecules of biotin were labeled on the anti-FAS monoclonal antibody using modified biotinylation conditions. The within- and between-run CVs were less than 10%, and the detection limit was 3.22 ng/mL. Recoveries were 98.54–121.95%, averaging 106.05%. The average FAS concentration obtained from the total 55 healthy volunteers blood was 4.07 ± 1.81 ng/mL, 4.25 ± 2.14 ng/mL in women ($n = 37$) and 3.70 ± 0.74 ng/mL in men ($n = 18$). When compared with the previously developed polyclonal–monoclonal ELISA, a different pattern of FAS levels was observed in the supernatant of two cultured breast cancer cell lines in a time course study and there was no linear correlation between the two assays using 215 human blood samples. Thus, this new model FAS–ELISA could be used as an independent assay in measuring clinical samples. In summary, this monoclonal–monoclonal FAS–ELISA is sensitive, accurate, and precise in quantification of fatty acid synthase and has potential as a complementary tool in testing clinical samples.

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INTRODUCTION

Overexpression of Fatty acid synthase (FAS EC 2.3.1.85) is associated with cancer and therefore is a putative tumor marker. Previous studies of human cancers and their precursor lesions have found surprisingly high levels of *de novo* fatty acid synthesis and FAS expression. Elevated levels of FAS have been identified immunohistochemically in cancers of the breast,[1–6] colon,[7,8] prostate,[9–11] ovaries,[12] oral tongue,[13] thyroid,[14] lung,[15] endometrium,[16] and mesothelioma.[17] Moreover, patients with FAS positive breast, prostate, or endometrial cancer, have a poorer prognosis than those with low or absent FAS expression. In studies of node-negative early breast cancer patients, those with high levels of FAS expression experienced both shortened disease-free and overall survival.[4,5]

The quantitative determination of tumor marker levels in blood is a simple, less invasive approach for cancer detection and treatment monitoring. We have successfully developed a two-site sandwich polyclonal–monoclonal enzyme linked immunosorbent assay (ELISA) for the quantitative determination of FAS.[18] Elevated levels of FAS were observed in the blood of breast, prostate, colon and ovarian cancer patients. We recently studied two human breast cancer cell lines, MCF-7 and ZR-75-1,

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along with a number of other cancer cell lines using this ELISA method and Western blotting analysis.[19] The data showed significantly elevated levels of FAS in the cell culture supernatant of the two breast cancer cell lines. Elevated levels of FAS in the circulation of breast cancer patients were also found in comparison with normal controls. In addition, blood FAS levels were significantly higher in breast cancer patients with advanced clinical stages.

In this paper, we present a new model ELISA for the quantification of FAS based on two monoclonal antibodies. We describe the assay development and a comparison with the previously developed polyclonal–monoclonal assay in the supernatant of cultured breast cancer cells and clinical samples.

EXPERIMENTAL

Reagents and Buffers

BSA was purchased from Sigma Chemical Company (St. Louis, MO); EZ-LinkTM Sulfo-NHS-LC-Biotinylation kit (Cat. No. 21430), AP conjugate (alkaline phosphatase-labeled streptavidin conjugate, Cat. No. 21324) and PNPP (*p*-nitrophenyl phosphate, disodium salt) substrate were purchased from Pierce (Rockford, IL). The following buffers were used in this study. They are TBS: 50 mmol/L Tris–HCl buffer, 150 mmol/L NaCl, pH 7.5; dilution buffer: TBS containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20; blocking solution: TBS containing 1% (w/v) BSA; wash solution: 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20.

Calibration Solution

The calibration solution was prepared from concentrated cell culture supernatant of ZR-75-1 breast cancer cells. When cells were confluent, the culture supernatant was collected and concentrated approximately ten fold with Centriprep® YM 10 (Millipore) and calibrated with purified FAS protein to determine the FAS level. The calibration solution was prepared in the dilution buffer and had concentrations of 0, 3, 6, 12, 24, 48, 96, and 192 ng/mL.

Antibody Biotinylation

Affinity-purified rabbit polyclonal and mouse monoclonal antibodies against FAS were produced from ZR-75-1 breast cancer cells. Specificity

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of these antibodies was previously demonstrated by Western blotting analysis.[5,18] Purified monoclonal (M3) and polyclonal (PcAb) antibodies were labeled with biotin using a modified procedure of the Sulfo-NHS-LC-Biotinylation kit consisting of performing the conjugation of the antibody and biotin overnight at 4°C in the dark with continuous stirring. Determination of the number of biotin molecules per IgG molecule was accomplished by the HABA (2-(4′-hydroxyazobenzene) benzoic acid) method as described in the kit instructions. The format of McAb M6 for coating and M3 for detection is designated C-M6/D-M3, monoclonal–monoclonal ELISA; PcAb for coating and McAb M6 for detection is designated C-PcAb/D-M6, polyclonal–monoclonal ELISA.

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Protocol for FAS–ELISA

Nunc MaxiSorp ELISA/EIA modules were coated with antibody (50 μg/mL in 100 mmol/L carbonate coating buffer, pH 9.6) and incubated overnight at 4°C. The assay was performed as follows with washing in between each step: 200 μL of blocking solution was added into each well and incubated for 30 min at 37°C; 100 μL of FAS calibration solution or sample was added and incubated overnight at 4°C; 100 μL of biotinylated antibody was added to each well and incubated for 2 h at room temperature, and 100 μL of AP conjugate (1 : 1250) was added and incubated for 1 h at room temperature. Finally 100 μL of PNPP (1 mg/mL of 10 mM Diethanolamine buffer, pH 9.5) solution was added to each well and incubated at room temperature for 30 min or the given time as indicated. Modules were read at 405 nm with the SPECTRAmax 190 ELISA reader (Molecular Devices, Sunnyvale, CA). The FAS–ELISA calibration curve was generated using a 4-parameter curve fit.

Assay Validation

The detection limit of the assay was determined by analyzing 20 replicates of the zero FAS calibration solution (dilution buffer only). The FAS concentration that corresponded to the absorbance of the zero calibration solution plus 2 SD was determined to be the detection limit of the assay. Within-run imprecision of the assay was evaluated by assaying 20 replicates of samples with three different concentrations of FAS. To evaluate between-run (day-to-day) imprecision, the same three samples were assayed on 20 runs with reagents from three different lots. Analytic recovery was assessed

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in three samples which were assayed before and after addition of 3, 12, and 48 ng/mL FAS. Recovery was calculated as the ratio of recovered to added FAS concentrations (expressed as a percentage). In addition, three samples with different concentrations of FAS were serially diluted in dilution buffer for the determination of assay linearity. The FAS concentration was determined for each dilution following the assay procedure.

Cell Cultures and Cell Lines

Two breast cancer cell lines, MCF-7 and ZR-75-1, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO_2.

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Blood Samples

A total of 270 blood specimens were analyzed in this study. Specimens were obtained from 55 healthy subjects for the determination of FAS concentrations. An additional 215 blood specimens (115 normal controls and 100 from breast cancer patients) were analyzed by both FAS assays in a method correlation study. Thawed samples were centrifuged to remove particulate material prior to analysis. All samples were analyzed in duplicate.

Statistical Analysis

Data were analyzed with SPSS 10.0 for windows. Linear regression analysis was used to examine the relationship between the two FAS–ELISAs. A probability level of less than 0.05 was chosen as a threshold for statistical significance.

RESULTS

Assay Development

In this new model monoclonal–monoclonal FAS–ELISA, a modified procedure was adopted, whereby the conjugation of biotin onto the detection antibody was carried out at 4°C overnight in the dark with continuous stirring instead of 2 h on ice or 30 min at room temperature as per the kit

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instructions. The modification enhances the conjugating efficiency with more than 10 biotin molecules per IgG molecule, compared with 2.1–2.8 using the standard approach.[18] The optimized concentration of the detection antibody (200 ng/well) used in the monoclonal–monoclonal model was 5 times lower than the concentration used in the previously developed polyclonal–monoclonal model due to the increased labeling efficiency, however assay sensitivity was not sacrificed.

A representative calibration curve of the new model monoclonal–monoclonal FAS–ELISA is shown in Fig. 1. It was also observed that the greatest amount of chromogen production resulted from development at room temperature for prolonged reaction times of 1 h or more. Sensitivity as a function of development times for the AP conjugate could be increased with longer reaction times, as shown in Fig. 2.

Assay Validation

The analytical performance of the new model FAS assay was evaluated based on analytical sensitivity, precision, recovery, and linearity. The analytical lower limit of detection of the FAS assay was estimated as the dose that was equivalent to the absorbance of the mean of 20 replicates

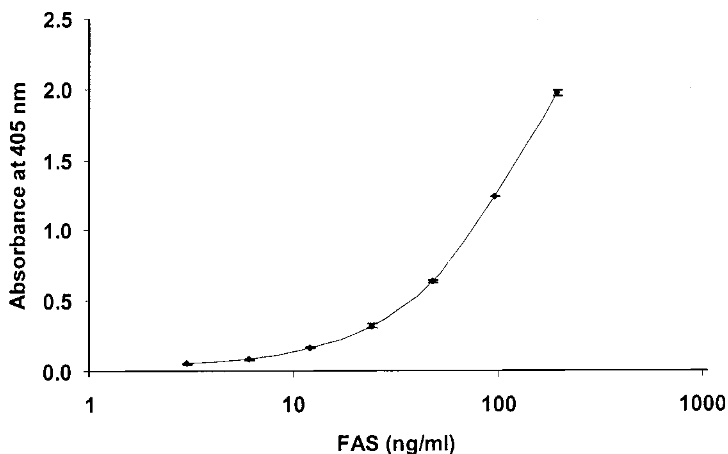

Figure 1. Representative calibration curve for the monoclonal–monoclonal FAS–ELISA. FAS was determined using a combination of a monoclonal antibody M6 for capture and another monoclonal antibody M3 for detection using Nunc MaxiSorp ELISA/EIA modules as described in the Experimental section.

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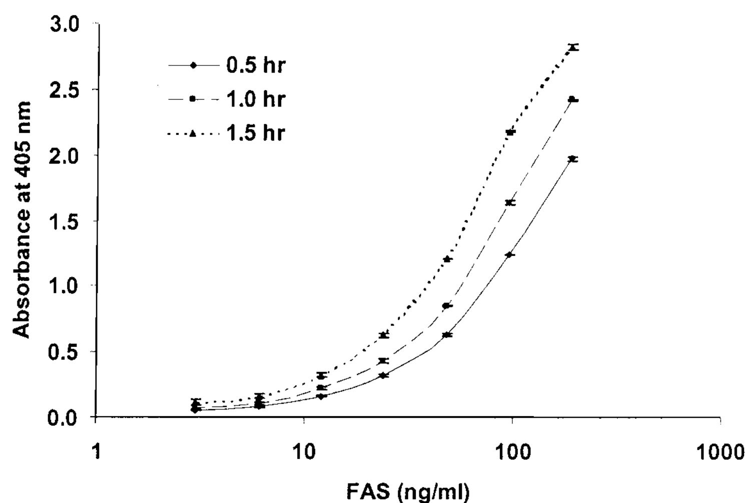

Figure 2. Development reactions of the monoclonal–monoclonal FAS–ELISA at different times. The time-dependent enzyme kinetics of the AP-conjugate with the enzyme substrate PNPP was measured at the following reaction times: Diamond, 0.5 h; Square, 1.0 h; Triangle, 1.5 h. The reactions were carried out at room temperature and the absorbance was measured without stopping the enzyme reaction.

Table 1. Precision of the Monoclonal–Monoclonal FAS–ELISA

	n	FAS ng/mL (Mean ± SEM)	CV (%)
		Within-run	
Sample 1	20	12.64 ± 0.56	4.39
Sample 2	20	41.31 ± 2.24	5.42
Sample 3	20	86.35 ± 0.99	1.14
		Between-run	
Sample 1	20	14.70 ± 1.45	9.84
Sample 2	20	52.54 ± 3.59	6.83
Sample 3	20	81.54 ± 3.48	4.26

of the zero calibrator plus 2 SD. The FAS concentration that corresponded to that signal was calculated to be 3.22 ng/mL. The reproducibility of the assay was estimated using three samples having different FAS concentrations. The within and between-run CVs were 1.14–5.42% ($n = 20$) and 4.26–9.84% ($n = 20$), respectively, as shown in Table 1. To evaluate the recovery of FAS, we added FAS in dilution buffer to cell culture super-

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Table 2. Recovery of the Monoclonal–Monoclonal FAS–ELISA

Cell Culture Supernatant	FAS (ng/mL)			
	Added	Measured	Recovered	Recovery (%)
A	0	0.10		
	3	3.66	3.55	118.38
	12	12.18	12.07	100.61
	48	47.93	47.82	99.63
B	0	0.12		
	3	3.78	3.66	121.95
	12	12.18	12.06	100.49
	48	47.42	47.30	98.54
C	0	0.14		
	3	3.59	3.45	115.00
	12	12.15	12.01	100.07
	48	48.05	47.91	99.82
Average				106.05

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natant to give final concentrations of 3, 12, 48 ng/mL. Recoveries are shown in Table 2. Recoveries ranged from 98.54–121.95%, averaging 106.05%. Three pooled samples were serially diluted with dilution buffer in a linearity study. Results are presented in Fig. 3. The calculated FAS values were linear with dilution. The correlation coefficients between expected and observed values averaged 0.96.

FAS Level in Healthy Subjects' Blood

FAS level in 55 healthy volunteers was determined using this new model monoclonal–monoclonal FAS–ELISA. Thirty seven women, ages: 26–64 years and 18 men, ages: 28–55 years. The average FAS concentration from the total 55 healthy volunteers was 4.07 ± 1.81 ng/mL, 4.25 ± 2.14 ng/mL in women and 3.70 ± 0.74 ng/mL in men.

Comparison of Two FAS–ELISA's

Comparison between the new model monoclonal–monoclonal FAS–ELISA and previously developed polyclonal–monoclonal FAS–ELISA was performed by parallel studies measuring FAS concentration in the super-

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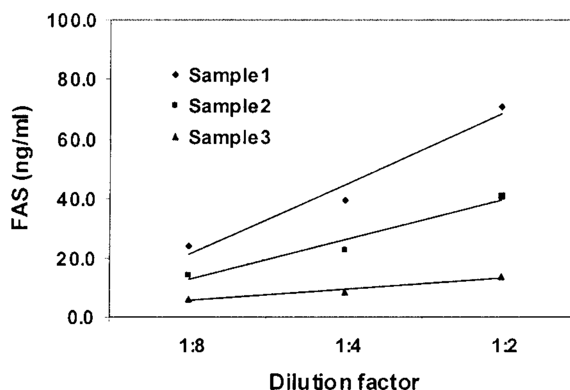

Figure 3. Evaluation of assay linearity over the range of 6–71 ng/mL FAS for the monoclonal–monoclonal FAS–ELISA. Data are based on the serial dilution of three samples with dilution buffer. The equations are: sample 1: $y_1 = 23.53(x_1) - 2.17$, $r_1 = 0.97$; sample 2: $y_2 = 13.42(x_2) - 0.86$, $r_2 = 0.95$; sample 3: $y_3 = 3.68(x_3) + 2.1$, $r_3 = 0.97$, respectively.

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natant of cultured cancer cells and human blood samples. A time course study was carried out by incubation of cell culture supernatant collected from two cultured breast cancer cell lines (MCF-7 and ZR-75-1) at 37°C with various incubation periods (0, 2, 4, and 8 days). As shown in Fig. 4, The pattern of FAS levels measured by the two assays differed. In the new model FAS–ELISA, FAS was detected throughout the whole 8 day time course. In contrast, in the polyclonal–monoclonal model, high levels of FAS were detected at the zero time point of the time course which is the original cell culture supernatant. FAS levels decreased dramatically after two days at 37°C. Measurement of FAS in 215 human blood samples using the two ELISAs is shown in Fig. 5. No correlation was found between the two models.

DISCUSSION

Although normal tissues have low levels of fatty acid synthesis, a number of studies have demonstrated surprisingly high levels of FAS expression in a wide variety of human malignancies and their precursor lesions, including carcinoma of the breast,[1-6] colon,[7,8] prostate,[9-11] ovaries,[12] oral tongue,[13] thyroid,[14] lung,[15] endometrium,[16] and mesothelioma[17] as determined by immunohistochemistry methods. Patients with tumors rich in FAS exhibit a significantly poorer clinical prognosis

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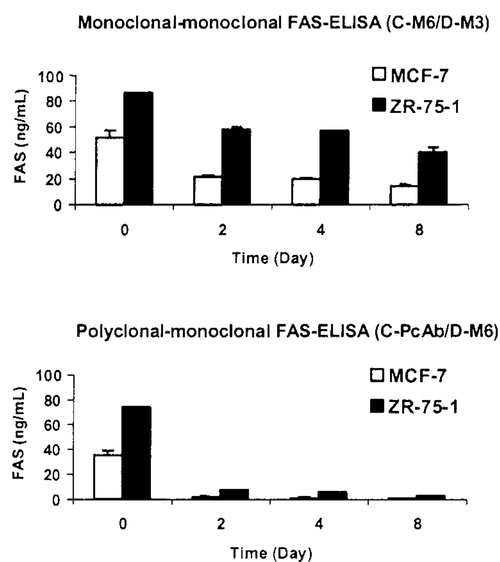

Figure 4. Comparison of two FAS–ELISAs in the supernatant of cultured breast cancer cells. FAS levels were measured by the new model monoclonal–monoclonal FAS–ELISA and previously developed polyclonal–monoclonal FAS–ELISA as described in Section 2 in the supernatant of cultured two breast cancer cells (MCF-7 and ZR-75-1) stored at 37°C in 0, 2, 4, and 8 days.

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than those with lower level or absent of this enzyme. The widespread overexpression of FAS in human cancers suggests that FAS could function as a tumor marker.

Recently, we successfully developed a polyclonal–monoclonal ELISA for the quantitative determination of FAS.[18] Using this assay, we have detected elevated levels of FAS in the supernatant of cultured breast cancer cell lines. In breast cancer patients, the blood FAS levels were significantly higher compared with healthy subjects.[19] FAS quantitation could offer a powerful tool to study the physiological behavior of this protein in tumorigenesis and has potential to be used in clinical chemistry for diagnosis and/or prognosis of cancers. In order to improve the assay to meet these purposes, a new model two-site sandwich ELISA using a combination of one monoclonal antibody (M6) for capture and another monoclonal antibody (M3) for detection has been developed. In this paper, we have described the assay development and analytical evaluation. With a modified biotin labeling procedure, more than 10 biotin molecules were successfully conjugated onto a single molecule of IgG (detection antibody).

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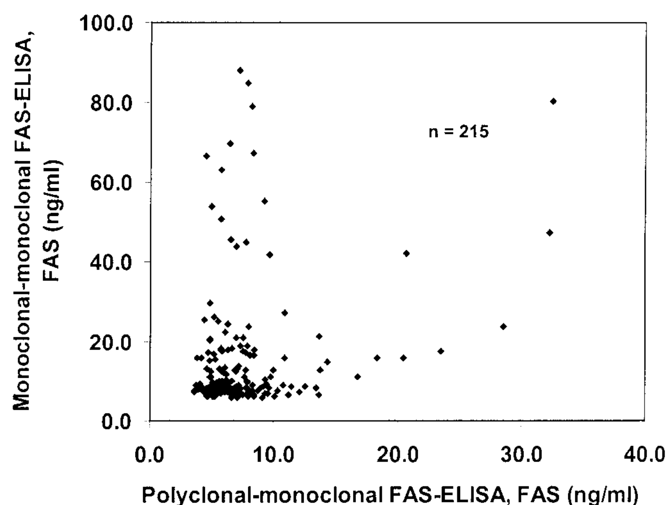

Figure 5. Correlation between the two FAS–ELISAs. FAS levels in 215 blood samples were measured by the new model monoclonal–monoclonal FAS–ELISA and previously developed polyclonal–monoclonal FAS–ELISA as described in Experimental.

The concentration of detection antibody required in the new model FAS–ELISA was 5 times less than the previously developed model without adversely affecting assay sensitivity. The performance of the new model has been evaluated and been shown to be reliable for the quantitation of FAS. The FAS concentration from the healthy volunteers blood was measured by this assay. The average of total 55 subjects was 4.07 ± 1.81 ng/mL, 4.25 ± 2.14 ng/mL in women ($n = 37$) and 3.70 ± 0.74 ng/mL in men ($n = 18$).

In the time course study, the detection pattern of FAS levels in the supernatant of cultured breast cancer cell lines varied between the two FAS–ELISAs, therefore these two models may detect different FAS molecules in the same samples based on antibody specificity for different epitopes on the FAS molecule. FAS levels were measured in 215 human blood samples with these two FAS–ELISAs and the relationship between the two assays determined using linear regression analysis. No linear correlation was found between the two models. Therefore, the new model FAS–ELISA could potentially serve as an independent assay and a complementary tool in determining FAS levels in patient blood.

In conclusion, we have developed a new model two-site sandwich ELISA based on two monoclonal antibodies for the quantification of

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FAS. This assay is sensitive, accurate, precise and able to detect immunoreactive FAS levels in cell culture supernatant and human blood samples. Further study is needed to determine the clinical applications for the new model monoclonal–monoclonal FAS–ELISA.

ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; FAS, fatty acid synthase; BSA, bovine serum albumin; NaCl, sodium chloride; HABA, 2-(4′-hydroxyazobenzene) benzoic acid; PNPP, *p*-nitrophenyl phosphate, disodium salt; AP conjugate, alkaline phosphatase-labeled streptavidin conjugate; FBS, fetal bovine serum; PcAb, polyclonal antibody; McAb, monoclonal antibody

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