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Dual Detection of Cancer Biomarker CA125 Using Absorbance and Electrochemical Methods

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Abstract

An enzyme-linked immunoassay based on the dual signal transduction mechanisms has been developed for detection of ovarian cancer biomarker CA125. The immunoassays use a nanoelectrode array (NEA) chip and absorbance methods for the dual detection. The NEA is used to confirm the optical detection of CA125 that is carried out in a high-binding 96-well plate. An alkaline phosphatase (AP) enzyme was used to label the detection antibody to allow for both the optical and electrochemical detection of CA125. Two kinds of substrates were catalyzed by the AP enzyme. Para-nitrophenylphosphate (PNPP) produces chromogenic para-nitrophenol (PNP), which can be optically detected at 405 nm. Para-aminophenylphosphate (PAPP) produces electroactive para-aminophenol (PAP), which can be detected amperometrically between -0.1 to 0.3 V. The linear ranges have been determined to be $5\text{--}1000$ U/mL and $5\text{--}1000$ U/mL for the optical and electrochemical immunoassays, respectively. The limit of detection of the optical immunoassay is 1.3 U/mL and 40 U/mL for the optical and electrochemical methods, respectively.

1. Introduction

Cancer takes 7.6 million lives worldwide every year.¹ In the US, about 20,000 women are diagnosed with ovarian cancer (OC) annually, 14,436 women lost their lives to this disease in 2009.² In women, ovarian cancer is the fifth leading cause of cancer death in the US compared with breast, colorectal, and pancreatic cancers.³ Globally, 225,500 in the world wide are affected with ovarian cancer, more than half of whom pass away every year.⁴ OC is a disease of the ovaries that does not present specific symptoms at the early onset of the disease. Its symptoms are vague until it is at a later stage at which time it has spread in organs beyond the ovaries. OC can be classified according to the origin of the abnormal cells within the ovarian tissue of which, 90% of new cases are diagnosed as epithelial carcinoma.⁵ In the advanced stage, abdominal pain, bloating, difficulty eating, and constipation are common symptoms which are not specific enough to identify ovarian cancer.⁶ Only at a later stage when symptoms such as vaginal bleeding, weight gain or weight loss, urinary frequency and/or urgency, nausea, and vomiting necessitates various

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scans for diagnosis. But at a late stage, survival rates are very low, making OC a “Silent Killer”.⁷ When symptoms begin to show positive indications of OC, diagnosis aside from pelvic examination such as computed tomography, transvaginal ultrasound, or MRI (magnetic resonance imaging) of the abdomen are among the tests that are used for detection in the ovary and its possible spread outside the pelvic area.⁸ Confirmation of the disease stage is carried out with colonoscopy, laparoscopy and biopsy are invasive and uncomfortable.⁹ Alternatively, protein based assays are choices of tests for cancer detection and monitoring using serum, blood, or urine samples.¹⁰ However, reports indicate that genetic and epigenetic changes have been observed in the different types and stages of OC.¹¹

One of the most commonly used protein assays that serves as initial testing for various types of cancers including ovarian cancer is based on the serum levels of CA125 cancer antigen which was described by Bast et al.¹² This protein is a glycoprotein that can be detected using monoclonal antibody, anti-CA125. It has been established that the levels of CA125 are elevated in the serum of more than 80–85% of women with epithelial ovarian cancer, but only in 50% of patients in the early stages of OC.¹³ Once the CA125 assay is positive, the level of disease malignancy is confirmed with a number of subsequent tests.

The biomarker CA125 is a very important tumor marker with normal levels at 35 U/mL.¹⁴ This level is elevated in the advanced-stage of ovarian cancer and in malignancies such as colon, breast, pancreas, pericardium, and other epithelial cell diseases.^{15,16} Today, screening of ovarian cancer using CA125 is carried out in combination with physical examination.^{17,18} Its early detection is important to achieve effective OC diagnosis in order that treatment regimen can be initiated at the onset of the disease.¹⁹

Herein sensitive, reliable, and rapid dual transduction methods have been developed for detection of low levels of CA125 that indicate early-stage OC. A sandwich-type enzyme linked immunoassays (ELISA) on a 96-well plate with optical and electrochemical methods of signal transduction was developed, as shown in Fig. 1. The assay was carried out on a high binding surface that was used to immobilize the capture antibody and to build the sandwich assay. The enzyme label was alkaline phosphatase (AP) that catalyzed the hydrolysis of orthophosphoric monoesters, para aminophenyl phosphate (PAPP) or para nitrophenyl phosphate (PNPP), releasing a substrate, para amino phenol (PAP) or paranitrophenol (PNP), that has electrochemical and optical properties, respectively.²⁰

Experimental Section

Chemicals and Materials

All chemicals were used without further purification. Mouse anti mouse CA125 IgG1, mouse anti mouse CA125 detection antibody IgG 1, human CA125 antigen and alkaline phosphatase kit were purchased from Genway Biotech (San Deigo, CA). Tris(hydroxymethyl), bovine serum albumin fraction powder (BSA), myoglobin from horse heart, cytochrome C from bovine heart, 10 m M phosphate buffer saline (PBS), p-nitrophenylphosphate (PNPP), paraaminophenylphosphate (PAPP), sodium hydroxide (NaOH), Hydrochloric acid (HCl), sodium chloride (NaCl) and Trizma base, sulfuric acid

(H₂SO₄), hydrogen peroxide (H₂O₂), ammonium hydroxide (NH₄OH) were obtained from Sigma-Aldrich (St. Louis, MO). (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution pH 7.2–7.5 Invitrogen (Washington, DC), dialysis cassette (Spectrum, Houston, TX). Aqueous solutions were prepared with deionized (DI) from a Millipore Milli-Q filtration system. The high binding 96 well plate (NUNC) clear plate and the polystyrene sphere for the NEA fabrication were obtained from Fisher Scientific (Pittsburgh, PA). The dialysis cassettes were from Spectrum (Houston, TX).

Buffers

Reaction buffer: 10 mM PBS, pH 7.4; Electrochemical Signal detection buffer: 4 mM PAPP in 10 mM Tris with 0.1 M KCl, pH 9.0; Optical Signal detection buffer: 4M PNPP in 0.1 M Tris buffer pH 9.0; Washing buffer: Tris buffered saline-Tween 20 (TBST) solution with detergent Tween 20 that contained 137 mM NaCl, 20 mM Tris, 0.05 % Tween-20, pH7.6; Coating buffer: 10 mM PBS pH 7.4.

Instruments

Optical absorbance measurements were performed with a Microplate reader (Synergy H4 Hybrid Reader, BioTek Instrument, Inc. Winooski, Vermont). A CH 1200B bipotentiostat was used for electrochemical detection (CH Instruments, Austin, Texas). Cleaning of the NEA were performed with O₂ plasma etcher (March Instruments Inc.). Gold deposition were carried out with an e-beam evaporator (Temescal BJD-2000).

Fabrication of the nanoelectrode array (NEA)

The nanoelectrode array (NEA) was fabricated by nanosphere lithography technique.^{21,22} Gold-coated glass slides were used as the substrates to fabricate the NEA. Initially the substrates were sequentially sonicated for 5 min each in acetone, methanol and deionized water. Then the substrates were cleaned in acid piranha bath (3H₂SO₄:1H₂O₂) at room temperature for 5 min and then in base piranha bath (5H₂O:1NH₄OH:1H₂O₂) at 65 °C for 2 h. A closely packed 1000 nm sized polystyrene sphere monolayer was formed on the substrates using the well-established method.^{21,22} The polystyrene spheres were etched to 150±30 nm in a diameter using O₂ plasma etcher at ~ 100 mTorr of pressure and 25 mW of power. A ~15 nm thick insulating layer (SiO₂) was deposited on the pattern using e-beam evaporator. The polystyrene spheres were removed by sonicating the substrates in ethanol for 5 min. Each nano-well acted as individual nanoelectrode.

Preparation of the alkaline phosphatase labeled antibody, Ab-AP

The ZipLink alkaline phosphatase conjugation kit was used to label the CA125 detection antibody with alkaline phosphatase, mAb-AP. Briefly, 10 µL of 8.9 mg/mL mAb was diluted in 90 µL of 40 mM HEPES buffer that was modified with 10 µL of modifier reagent. The mixture was incubated overnight at room temperature to allow the alkaline phosphatase to covalently link with the mAb. Subsequently, 10 µL of quencher buffer was added and incubated for additional 30 min to deactivate the chemicals from the ZipLink kit. Purification to eliminate unconjugated AP was carried out using a dialysis cassette. The

activity of the AP conjugated antibody was tested by exposing a 1 μ L of sample to 10 μ L of PNPP and PAPP.

Immobilization of the capture antibody, mAb CA125

Coating with the capture antibody, mAbCA125 was performed by physical adsorption. Individual wells were treated with 10 mM PBS, pH 7.4 for 24 h before introducing 50 μ L of 2 μ g/mL mAb CA125, the plate was covered with a sealer and kept in the refrigerator for 12–24 h at 40C. The coating solution was removed and the wells were washed three times with 200 μ L of 10 mM PBS pH 7.4. The solutions were removed by tapping these plates over a waste solution container and the remaining drops were removed by tapping the plate on paper towel.²³

Capture of the antigen, CA125

The mAb CA125 coated microplate was blocked with 50 μ L/well of blocking buffer containing 1% BSA in Tris at room temperature (RT) for 60 min. Between each step, the microplates were washed with washing buffer (TBST) three times. Then 50 μ L of serially diluted CA125 antigen were incubated at room temperature for one hour, washed and tapped to dry. The incomplete assay was composed of mAbCA125 + CA125 at this stage.

Immobilization of the detection antibody, Ab-AP

The wells containing the mAbCA125+CA125 was treated with 50 μ L of the 5 μ g/mL Ab-AP per well. The well was incubated for 1 h. Subsequently, the wells were washed with the TBST three times to remove all reagents that were not immobilized. The completed assay contained mAbCA125+CA125+Ab-AP.

Optical signal detection using para-nitrophenylphosphate (PNPP)

For optical detection, the enzyme substrate, para-nitrophenylphosphate (PNPP), is hydrolyzed to produce para-nitrophenol (PNP). The amount of PNP is directly proportional with the number of Ab-AP, which is a measure of the number of CA125 that is present in the sample. Thus, because PNP has maximum absorbance at 405 nm, the signal can be generated after incubating the PNPP in the wells over a fixed period of time.²⁰

The plate was tapped dried and 50 μ L of the 4 M PNPP in 0.1 M Tris at pH 9.0 was added in each well. The plate was incubated for at RT 30 min. 12.5 μ L of stop solution (3N NaOH) was then added and allowed to incubate further for 5 min. The signals were recorded at 405 nm. Both the PNPP and the PNP were protected from light to avoid oxidation.²⁴

Optimization of the concentration of the Enzyme substrate

Using the various optimized parameters, the concentration of the enzyme substrate, PNPP, was also optimized in order that there will be excess reagent during the assay transduction. Four concentrations of PNPP (0.004, 0.008, 1, 4, 4.5 mM) with 1000 U/mL CA125 antigen, 5 μ g/mL Ab-AP and 2 μ g/mL mAb CA125 were used to generate the absorbance signals after 30 min.

Electrochemical signal detection using para-aminophenylphosphate (PAPP)

Similar to PNPP, para-aminophenylphosphate (PAPP) is also hydrolyzed by AP releasing para-aminophenol, PAP. This enzymatic hydrolysis product is electrochemically active between -0.1 to 0.3 V. The current, i , recorded at this potential window is a measure of the amount of PAP, which is directly proportional to the amount of immobilized Ab-AP and this is proportional to the amount of CA125 in the sample. After the detection antibody was immobilized on the wells, the tapped dried plate was incubated with $50\text{ }\mu\text{L}$ of 4 mM PAPP in 10 mM Tris at pH 9.0. The plate was incubated at RT for 30 min. For detection of the electrochemical signal, $50\text{ }\mu\text{L}$ of samples were pipetted onto the NEA chip that was setup with Ag/AgCl as reference and counter electrodes. The signals were recorded at -0.1 to 0.3 V at a scan rate of 50 mV/s . Similar to the PNPP and the PNP, the PAPP and the PAP were protected from light to avoid oxidation.

Optimization of capture antibody, mAb CA125

To find the condition at which all signals were controlled only by the amount of antigen in the sample, the optimum capture antibody concentration was determined. Capture antibody was exposed to the wells on the 96-well plate in triplicate analysis at 1, 2, 10, and $20\text{ }\mu\text{g/mL}$ in 10 mM PBS (pH 7.4) coating buffer. The plate was incubated overnight at $4\text{ }^{\circ}\text{C}$ to allow maximum absorption of the capture antibody. The plate was washed with TBST three times. A 1000 U/mL CA 125 and $2\text{ }\mu\text{g/mL}$ Ab-AP was used for optimization.

Optimization of the concentration of detection antibody, Ab-AP

The optimum Ab-AP concentration within the linear range of detection was also established in order that the signals were controlled only by the amount of antigen in the sample. Using the optimum mAb CA125 established in the previous section, $2\text{ }\mu\text{g/mL}$, the Ab-AP was titrated to determine the concentration that would produce the best signal-to-noise ratio. The serial dilution of CA125 antigen was tested against five concentrations (2, 5, 25, 50, and $100\text{ }\mu\text{g/mL}$) of Ab-AP.

Dynamic range and standard calibration curve

To establish the dynamic range of concentrations at the optimal capture and detection antibody concentrations, the assay was performed with known concentrations of the standard sample (5, 50, 100, 200, 1000, and 5000 U/mL) in a physiologic buffer. The assay was performed at $2\text{ }\mu\text{g/mL}$ and $5\text{ }\mu\text{g/mL}$ capture and detection antibodies, respectively.²⁵ The optical density was recorded for each well at 405 nm and the linear range was established.

Using the established linear range, the assay was performed in triplicate at unoptimized incubation times. Signals were generated using PNPP to establish the optical signals. A separate set of triplicate analysis was performed using PAPP to generate the electrochemical signal using the NEA. The optical and electrochemical calibration curves were established to determine the relative signals for known concentrations of antigen in the presence of biological matrix.

Testing in the presence of human serum and other proteins

To validate the AP-mediated ELISA for CA125 detection in real samples, the assay was carried out in 50% of biological samples (human serum, plasma, bovine serum albumin, cytochrome C, and myoglobin). Preparations in real matrices were done by mixing 25 μ L of the antigen with 25 μ L of human serum (or the other matrices as given above). The lyophilized proteins were dissolved in 1 mL of sterile 10 mM PBS (pH 7.4) and diluted to 4 μ g/mL.

2. Results and discussion

The optical and electrochemical detection of CA125 was dependent on multiple parameters. These parameters had to be optimized in order that the ultimate biosensor signal was dependent only on the antigen concentrations and not on any of the other parameters in the assay. The results of this study showed that the optical detection can be confirmed with the NEA electrochemical methods of detection.

Preparation of the alkaline phosphatase labeled detection antibody, Ab-AP

Enzymes are the most common labels for immunoassay, such as horseradish peroxidase, alkaline phosphatase and *E. coli* β -D-galactosidase.²⁶ In this work alkaline phosphatase enzyme was used due to the following advantages:²⁷ (i) inexpensive;

(ii) high substrate turnover rate; (iii) stability at classic assay temperatures (4, 25, and 37 °C) for more than six months; (iv) commercially available in free and conjugate forms that can conjugate with antigen or antibody; and (v) good anti-interference from biological components. On the other hand, AP needs a specific buffer with pH >7 without chelating agents and inorganic phosphates, which can cause its inactivation.²⁸ Additionally, to determine the amount of active AP-labeled antibody in the dispersion, the mixture was reacted with its substrates PNPP. The reaction product was read at 405 nm,^{20–29} as shown in Fig. 1.

Optimization of the capture antibody, mAb CA125

One of the most important parameters that limit the reliability of an immunoassay is the capture antibody. In this system, the optimum concentration of the mAb CA125 controls the amount of the antigen that gets detected. Hence, it is very important to optimize the concentration of the mAb CA125, which are immobilized on the solid surface used for the assay.³⁰

To carry out this process, four concentrations of the mAb CA125 1, 2, 10, and 20 μ g/mL was used while the Ab-AP and the antigen concentration was set at a constant, 2 μ g/mL and 1000 U/mL, respectively, so that these would not interfere with the results. The signals showed that 2 μ g/mL mAb CA125 yielded the highest signals as shown in Fig. 2A. Thus, this mAb CA125 concentration was used for all succeeding studies unless otherwise specified.

Optimization of the concentration of detection antibody, Ab-AP

The detection antibody is the second important parameter that needed to be optimized in order to reflect the real amount of antigen in the sample without any effect from other parameters, thereby, getting a high signal to noise ratio. Un-optimized detection antibody leads to inaccurate measurements that may not reflect the actual antigen concentrations in the sample.³⁰

To perform this process, five concentrations of Ab-AP were tested with constant concentrations of CA125 antigen at 1000 U/mL and 2 µg/mL of mAb CA125. The results in Fig. 2B showed that 5 µg/mL was the optimal concentration of the Ab-AP, which exhibited the highest signal for the chosen CA125 concentration. This Ab-AP concentration was used for all succeeding studies unless otherwise specified.

Optimization of the concentration of the Enzyme substrate

Using the various optimized parameters, the optimum concentration of the enzyme substrate, PNPP, was also optimized. Out of the four concentrations of PNPP that was tested (0.004, 0.008, 1, 4, 4.5 mM) the results indicated that 4 mM PNPP and higher gave the optimum signals. Thus, this 4 mM PNPP concentration as well as PAPP were used in all the studies.

Electrochemical behavior of NEA

Fig. 3A shows the scanning electron microscope (SEM) image of the NEA used in the study. The average size of the nano-well was 150 ± 30 nm with an average distance of 970 ± 20 nm (well center to well center). The distance between the nano-well center-to-center was about 13 times the radius of the nano-well. Fig. 3B shows the cyclic voltammetry of NEA in different concentrations (0.01, 0.2 and 1 mM) of $\text{Fe}^{2+}/\text{Fe}^{3+}$ in 0.1 M KCl at 50 mV/s with Ag/AgCl as the reference/counter electrode. The average charging current ($N=15$) was recorded at 8 ± 2 nA that was recorded in 0.1 M KCl at a scan rate of 50 mV/s.

Dynamic range of concentration and calibration curve

A set of CA125 concentrations from 5 U/mL to 5000 U/mL using the optimized concentrations of mAb CA125 and Ab-AP was tested to establish the dynamic range of concentration for the optical assay. The assay showed that the linear range was between 5 and 1000 U/mL (Fig. 4). This range was used to establish the standard calibration curve for the assay. The average of three sets of experiments was used to obtain the linear curve, shown in Fig. 5A. The regression equation for the linear curve is given, $y = 0.0005x + 0.13$ with $R^2 = 0.99$, where y represents absorbance (absorbance intensity) and x represents the concentration of CA125 in units per milliliter, U/mL. The standard deviation that was calculated from the blank signal (at least 20 measurements) of 4 M PNPP (all components without antigen) was 0.019. The calculated limit of detection (LOD) using the equation

$$OD \text{ at LOD} = OD_{\text{mean blk}} + 1.645 SD_{\text{blk}}$$

The LOD was estimated to be 1.3 U/mL. This LOD is comparable with the currently available commercial method for CA125.³¹ This indicated the ability of this assay to detect CA125 in a wide range of concentrations that is applicable for early detection of cancer.

Similarly, the standard calibration curve was also established for the NEA-based electrochemical assay. The electrochemical assay showed that the linear range was between 50 and 1000 U/mL for CA125. Concentrations lower than 50 U/mL gave signals that were comparable to the signals for the blank, which indicated that such concentrations were not experimentally detectable with the NEA. This may be attributed to the high charging current that was observed during the characterization of the NEA electrodes (Fig. 3B). The linear range was used to establish the standard calibration curve for the assay. The average of three sets of experiments shown in Fig. 5B gave a regression equation given, $y = 0.15x + 12$ with $R^2 = 0.99$, where y represents the current (nA) and x represents the concentration of CA125 in U/mL. The standard deviation was calculated from the signal (at least 20 measurements) that was obtained from the blank (10 mM Tris, pH 9.0) of 4 M PAPP (all components without antigen). The calculated limit of detection (LOD) ($N=20$, $i = 13 \pm 3$ nA) using the equation

$$i \text{ at LOD} = i_{\text{mean blk}} + 1.645 SD_{\text{blk}}$$

The value of i was estimated to be 13 nA, which corresponded to the LOD of 40 U/mL.

As far as we know this is the first time to use the NEA to confirm the optical detection of cancer biomarker, CA125. Although optical detection has known advantages to detect CA125 in sample, diagnosis always need to be confirmed by other procedures in order to obtain accuracy especially for screening and early detection. In this study, the AP-enzymatic label presented the ability to perform both optical and electrochemical methods of detection in an assay for CA125. For optical detection, the AP substrate PNPP generated the optically active hydrolysis product PNP that was detected at 405 nm. For electrochemical detection, the AP substrate was changed to PAPP which generated the electrochemically active product PAP that was amperometrically detected between -0.1 to 0.3 V.

The results showed that the LOD of optical assay is lower than that the NEA-based electrochemical assay. Although the NEA was theoretically expected to reach a current signal that is as low as 1 nA (when the background is eliminated) corresponding to 6 U/mL, a charging current of 12 nA was recorded, which hindered the ability of this assay to detect CA125 in concentrations lower than 50 U/mL. Nevertheless, NEA-based detection with an LOD of 40 U/mL is significant enough for early detection of cancer because the system can be used to detect very close to the physiological level of CA125 in normal healthy individuals.¹⁴ In addition, the NEA system offers the possibility of detection in a disposable chip format, which offers portability and ease of use. Disposable microfluidic chip based biosensors are now used for diseases such as diabetes,³² which do not require skilled personnel and can be performed in point-of-care (POC) fashion. Similarly, a NEA disposable microfluidic chip is now being envisioned and developed for fully automated electrochemical CA125 assay for early cancer detection.

Testing in biological matrix and other proteins

The assay was tested to examine the possibility of interferences of human serum and proteins such as bovine serum albumin, cytochrome C and myoglobin. Interference studies were performed in human serum and other components that may be present in real matrix.³³

Human serum, bovine serum albumin (BSA), cytochrome C and myoglobin were mixed with known amount of antigen to evaluate the detection in their presence. The data in Table 1 indicated that there was no significant difference in the signals in the presence of these biological materials and proteins. Hence, the assay can be performed in diluted matrix without significant interferences to the signals.

3. Conclusions

The ovarian cancer biomarker (CA125) was successfully measured by the alkaline phosphatase enzyme-linked immunoassays. When PNPP was used as the substrate, the enzyme generated product of PNP, which was detected by optical absorbance at 405 nm. When PAPP was used as the substrate, the enzyme generated electroactive PAP, which was measured amperometrically between -0.1 to 0.3 V. Both the optical and electrochemical immunoassays showed the wide dynamic range of concentration for CA125. The LODs of the optical and electrochemical immunoassays were 1.3 U/mL and 40 U/mL, respectively. The higher LOD for the NEA assay was attributed to a relatively high charging current. In addition, further assay time optimization may allow CA125 detection within less than an hour while the patient is in the waiting room. Nevertheless, the assay performed reasonably well without significant changes in signals in the presence of different biological materials that are present in real samples. Thus, the 96-well plate ELISA for CA125 with AP as an enzyme label with dual transduction using optical and electrochemical methods holds great promise for early detection of ovarian cancer.

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Fig. 1.
Schematic of the sandwich immunoassay for CA125. (mAb-capture antibody; Ab-AP-alkaline phosphatase labeled detection antibody; antigen-CA125)

Fig. 2. Optimization of (A) capture antibody (1, 2, 10, and 20 $\mu\text{g/mL}$) using 1000 U/mL CA125 antigen and 2 $\mu\text{g/mL}$ Ab-AP; and (B) Ab-AP (1, 5, 25, 50 and 100 $\mu\text{g/mL}$) with 1000 U/mL using 2 $\mu\text{g/mL}$ capture antibody.

Fig. 3. (A) SEM of the NEA chip; (B) Cyclic voltammetry curves of NEA in various concentrations (0.01, 0.2, 1 mM) of Fe²⁺/Fe³⁺ in 0.1 M KCl at 50 mV/s with the Ag|AgCl reference electrode.

Fig. 4.

Dynamic range of concentration (0, 5, 50, 100, 1000, 2000, 5000 U/mL) of the optical assay with 50 μ L of 4 M PNPP in 0.1 M Tris, pH 9.0 after 30 min of incubation.

Fig.5. Standard calibration curve using CA125 antigen with 2 μ g/mL mAb CA125, 5 μ g/mL Ab-AP. (A) Absorbance based detection with 50 μ L of 4 M PNPP in 0.1M Tris, pH 9.0 after 30 min of incubation; (B) NEA-based detection using 50 μ L of 4 M PAPP in 0.1M Tris, pH 9.0 after 30 min of incubation.

Table 1

Effect of human serum and other proteins on the optical signal.

CA125 U/mL	OD ₄₀₅ in buffer	OD ₄₀₅ with biological samples		%Difference
50	0.139	50% human serum	0.135	3
100	0.213		0.190	11
200	0.235	2 µg/mL BSA	0.214	9
	0.235	2 µg/mL Cytochrome C	0.223	5
	0.235	2 µg/mL Myoglobin	0.205	13