Highly Sensitive Fluoro-Immunosensing for Biomarker Detection Using an Automatic Pipette Tip-Type Biosensing System

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# Highly Sensitive Fluoro-Immunosensing for Biomarker Detection Using an Automatic Pipette Tip-Type Biosensing System

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S *Supporting Information*

ABSTRACT: An automatic fluorescence detection system based on a pipette tip-type biosensor was developed to detect a prostate-specific antigen (PSA), a biomarker for prostate cancer. Fluorescence-based immunosensing for PSA detection was performed by a sandwich assay with a fluorescence-labeled antibody or its fragments. A small protein A-immobilized reaction plate was placed on a plastic pipette tip (referred to as a Biologi tip). For automatic PSA detection, a programmable dispensing robot equipped with a photodetector was used to suction the samples (i.e., capture antibody, antigen, primary antibody, and reporter molecule), incubation, and migration of the pipette tip to the detection port to measure fluorescence. The use of Fab-fragments and $F(ab')_2$-fragments as fluorescently labeled detection antibodies in a sandwich assay resulted in higher signal-to-noise ratios than those for whole IgG. The PSA detection in normal human serum using an Alexa Fluor 647-labeled $F(ab')_2$-fragment secondary antibody showed that the fluorescence intensity increased proportionally with PSA concentration, with an estimated detection limit of 1.2 ng/mL. Moreover, 10 pg/mL PSA was detected using an enzymatic reaction with a peroxidase-conjugated $F(ab')_2$-fragment secondary antibody and Amplex Red as a substrate. These data indicate that this system is capable of highly sensitive fluorescence-based detection of PSA and suggests the potential for early diagnosis of disease or applications with other biomarkers.
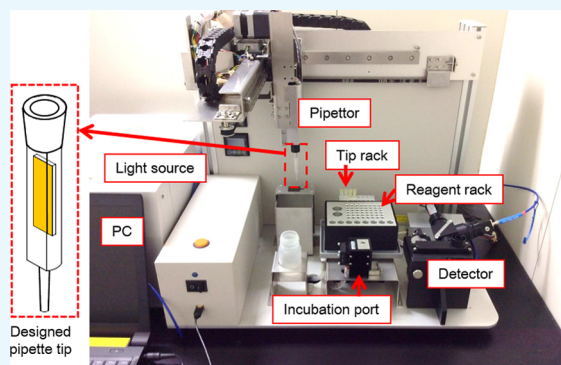
INTRODUCTION

A biomarker is an indicator of a biological process or state, such as a disease or disease progression.[1] Biomarkers are used to determine the most effective treatment regimen for a specific cancer type and predict the long-term susceptibility to cancer. Prostate-specific antigen (PSA) is a biomarker of prostate cancer,[2,3] which shows the highest incidence among cancers in the United States.[4,5] Therefore, simple, rapid, and sensitive detection of PSA is of very important for the early diagnosis of prostate cancer and for preventing disease recurrence. Normal levels of PSA in the blood are <4.0 ng/mL, whereas 4.0−10 ng/mL PSA is considered as the gray zone.[6−10] Moreover, a continuous increase in the PSA level over time may be a sign of prostate cancer. A recent study estimated that >70% of prostate cancer cases were successfully detected using PSA, which is a 2-fold increase compared to detection before the use of PSA.[11] Moreover, PSA screening has led to a sustained decline in prostate cancer mortality.[12]

Several methods have been developed for highly sensitive PSA detection,[13−15] such as surface plasmon resonance,[16] electrochemical methods,[17−19] and enzyme-linked immunosorbent assays. The latter method shows high sensitivity but is complex and time-consuming. Additionally, this method produces large amounts of waste derived from repeated dispensing and washing processes that require exchanges of the pipette tip, and accordingly is not ideal from a green chemistry or economical perspective. Therefore, we developed an analytical device that minimizes the use of disposable materials.

In a previous study, we developed pipette tip biosensors for bacterial double-stranded (ds) DNA using bioluminescence induced by zinc finger (ZF)-conjugated luciferase.[20] This system used a newly designed plastic pipette tip known as a "Biologi tip" equipped with a reaction plate inside the tip. In this sensing system, the tip was combined with an automatic analyzer. The ZF protein bound to a specific DNA sequence, and the target dsDNA was detected by monitoring the enzymatic activity of ZF luciferase. The emission intensity increased proportionally as the concentration of synthetic dsDNA increased in the concentration range of 0−200 pM. Additionally, an actual genomic DNA sample from *Escherichia*
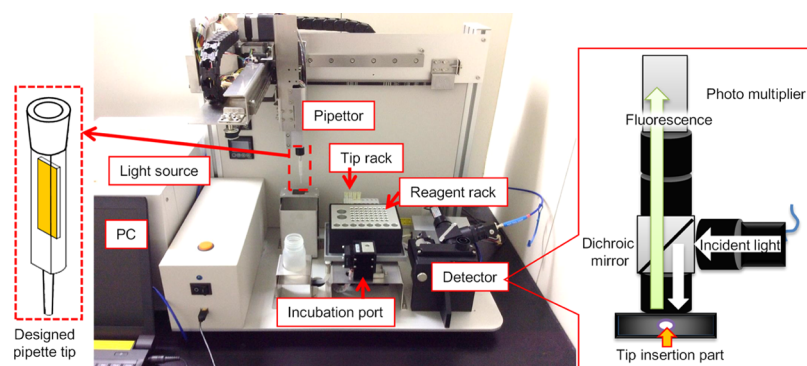
Figure 1. Illustration and photograph of the automatic detection set up for the tip-type biosensing system. Biologi tip with a reaction plate (left), automatic analyzer (middle), and fluorescent detection port (right).

coli O157 was successfully detected at 1.2×10^2 to 1.2×10^5 copies in a polymerase chain reaction sample.

In this study, we developed a highly sensitive fluorescence-based immunosensing method for PSA using our previously developed pipette tip-type biosensing system and a newly designed detection port to enable fluorescence measurement (Figure 1). The entire procedure for sandwich assay-based biosensing, which involves immobilization of a capture antibody, capture of the analyte (PSA), and introduction of a reporter molecule (e.g., Alexa Fluor 647-labeled secondary antibody) for detection, was performed successively and automatically using a single pipette tip. Sensitive detection using this biosensing system was investigated using whole IgG, Fab-fragments, and $F(ab')_2$-fragments as fluorescently labeled detection antibodies in a sandwich assay. Furthermore, detection methods based on the avidin−biotin complex or enzymatic reactions were developed, and approximately 10 pg/mL PSA was successfully detected. This fluorescence-based automatic biosensing system can be applied for diagnosing a wide range of diseases.

■ RESULTS & DISCUSSION

Effective Immobilization of Anti-PSA Capture Antibody. To effectively immobilize antiPSA on the reaction plate, two different methods were applied: antiPSA was immobilized on an NHS-modified reaction plate (Figure 2a) and on a
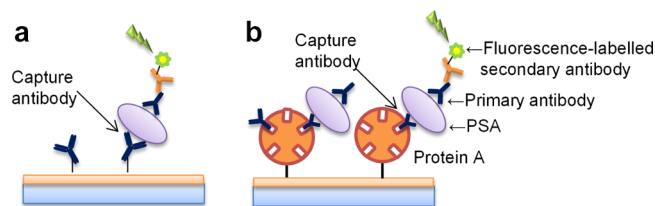

Figure 2. Schematic illustration of capture antibody immobilization on an NHS-modified reaction plate (a) and on a protein A-immobilized reaction plate (b).

protein A-immobilized reaction plate (Figure 2b); protein A had five binding sites for the Fc domain of the IgG antibody. Using these reaction plates, a fluorescence-based PSA sandwich assay was performed using the automatic analyzer with an Alexa Fluor 647-labeled secondary antibody (Figure 3).

When the antiPSA antibody was immobilized on the NHS-modified reaction plate, the fluorescence intensity at 0 and 1
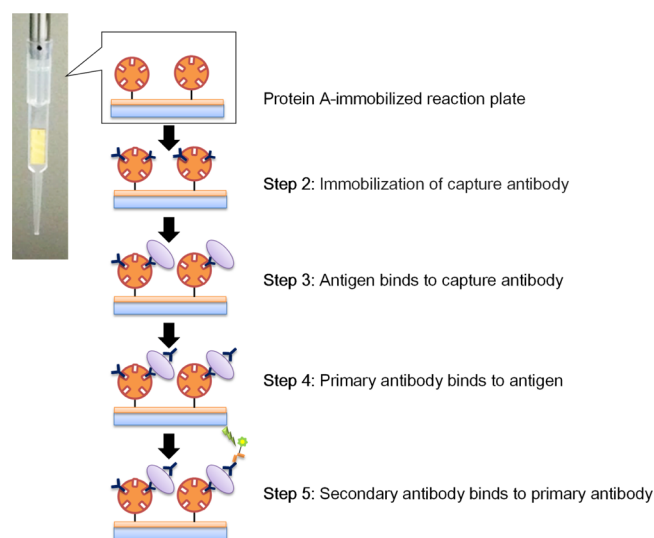

Figure 3. Schematic illustration of the workflow of the PSA sandwich assay.

ng/mL PSA exhibited little difference, indicating that 1 ng/mL PSA cannot be detected using the reaction plate. In contrast, using the protein A-immobilized reaction plate, the fluorescence intensity was clearly greater at 1 ng/mL PSA than at 0 ng/mL (Figure 4). These results indicate that protein A contributes to efficient immobilization of the antiPSA capture
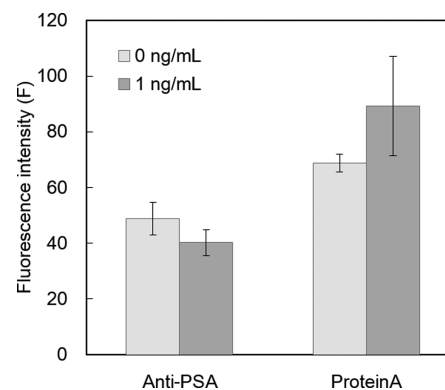

Figure 4. Effect of capture antibody immobilization. Fluorescence intensity of the PSA sandwich assay using the direct antiPSA capture antibody-modified reaction plate and the protein A-modified reaction plate for a PSA concentration of 0 or 1 ng/mL ($n = 3$).

antibody on the reaction plate, thus enabling an efficient PSA sandwich assay.

Detection Antibody Selection. Protein A led to the effective immobilization of the antiPSA capture antibody, as shown in Figure 4. However, it may also lead to nonspecific binding of primary and secondary detection antibodies during the sandwich assay, resulting in low reproducibility of the automatic detection system. To address this issue, we investigated whether the use of Fab fragments and $F(ab')_2$ fragments without the Fc region as detection antibodies resulted in highly reproducible PSA detection. Three secondary antibodies, that is, Alexa Fluor 647-labeled Fab-fragment, $F(ab')_2$-fragment, and whole IgG, were used for the fluorescence-based sandwich assay for PSA detection.

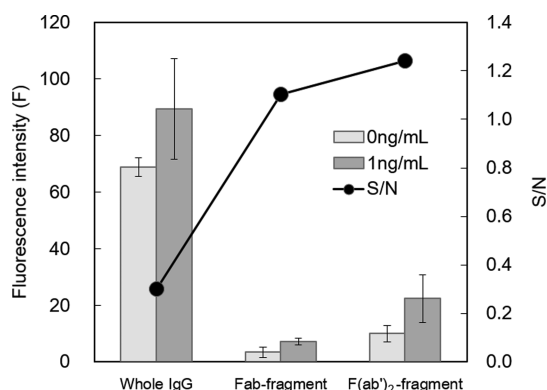Figure 5 shows the fluorescence intensity of the PSA sandwich assay at a PSA concentration of 0 or 1 ng/mL using

Figure 5. Fluorescence intensity of the PSA sandwich assay using the Alexa Fluor 647-labeled whole anti IgG, Fab-fragment, or $F(ab')_2$-fragment at a PSA concentration of 0 or 1 ng/mL ($n = 3$). The signal-to-noise ratio was estimated as follows: $S/N = (F - F_0)/F_0$.

these detection antibodies. At 1 ng/mL PSA, the fluorescence intensity using whole IgG showed the highest value. However, the reproducibility was quite low (large error bar), leading to a low signal-to-noise (S/N: $(F - F_0)/F_0$) ratio (approximately 0.3). Although, the use of the Fab fragment and $F(ab')_2$ fragment resulted in lower fluorescent intensities than those obtained for whole IgG, the S/N values for Fab or $F(ab')_2$ were higher than those for whole IgG (1.10 and 1.24, respectively). Among these antibodies, $F(ab')_2$ showed the highest S/N ratio. Fab and $F(ab')_2$ fragment antibodies do not possess the Fc fragment; therefore, there was low nonspecific absorption, explaining the high S/N ratio.

Shortening Each Measurement Time. Shortening the measurement time for each sample is important for practical applications. The protocol demonstrated above includes 30 min of incubation for each modification step, resulting in a long total measurement time (approximately 200 min) for one sample. To shorten the detection time, the incubation protocol was altered to include suction/discharge, which was repeated five times at each step. The detector was changed from a CMOS camera to PMT.

In the first step, the sensitivity of PMT was evaluated by fluorescence measurements of Alexa Fluor 647-labeled anti-IgG $F(ab')_2$ using the automatic detection system. Various concentrations of Alexa Fluor 647-labeled anti-IgG $F(ab')_2$-fragment [phosphate-buffered saline (PBS), 100 μL] were withdrawn into the Biologi tip without a reaction plate using

the automatic pipetting device. The fluorescence intensity increased as the concentration of Alexa Fluor anti-IgG $F(ab')_2$ increased from 0 to 200 pg/mL (Figure S1), and the detection limit was 76 pg/mL, defined as three times the standard deviation of the blank.

Next, the PSA sandwich assay was performed with the repeated suction/discharge process to shorten each measurement time. The fluorescence intensity of the PSA sandwich assay was determined without modification (incubation procedure) and with the modified protocol (suction/discharge procedure) (Figure S2). Although the fluorescence intensity decreased drastically, the S/N ratio increased from 0.13 (incubation procedure) to 0.93 (suction/discharge procedure) because nonspecific adsorption was effectively suppressed. It is possible that weak nonspecific adsorption of Alexa Fluor anti-IgG $F(ab')_2$ was effectively suppressed. This result indicates that the suction/discharge step leads to a higher S/N ratio and shorter measurement time for PSA sandwich assays, and the total measurement time for each sample was dramatically shortened from approximately 200 min to 16 min.

PSA Detection in Human Serum with the Sandwich Assay. Next, 0−10 ng/mL PSA dissolved in PBS [containing 15 mg/mL bovine serum albumin (BSA)] or normal human serum was analyzed with the modified protocol (suction/discharge procedure). The fluorescence intensity increased linearly as the concentration of PSA increased in the range of 0−10 ng/mL (Figure S3). The estimated detection limit defined as three times the standard deviation of the blank was 4.1 ng/mL (in PBS containing 15 mg/mL BSA) (Figure S3a) and 1.2 ng/mL (in human serum) (Figure S3b), respectively. Furthermore, in the PSA concentration range of 1.5−50 ng/mL, the fluorescence intensity increased as the concentration of PSA increased according to a logarithmic plot (Figure 6).
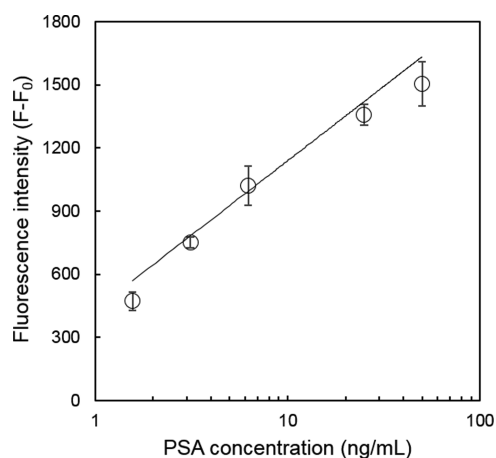

Figure 6. Plot of fluorescence intensity vs log concentration of PSA in normal human serum ($n = 3$).

PSA Detection with the Sandwich Assay Using Avidin−Biotin Interactions. To improve the sensitivity of the method, the PSA sandwich assay was performed using an avidin−biotin complex method, with PSA detection performed using the automatic analyzer with a biotinylated $F(ab')_2$-fragment secondary antibody and Alexa Fluor 647-labeled streptavidin. To determine the optimal concentration of Alexa Fluor 647-labeled streptavidin for detection, 100, 250, or 500 ng/mL Alexa Fluor 647-labeled streptavidin was used in the sandwich assay. At 1 ng/mL PSA, the S/N ratio was highest at

a concentration of 250 ng/mL (Figure S4). At 100 ng/mL, the concentration of streptavidin was not sufficient for the reaction; in contrast, high background fluorescence was observed because of nonspecific adsorption of the Alexa Fluor 647-labeled streptavidin in the case of 500 ng/mL. Based on these results, the optimal concentration of Alexa Fluor 647-labeled streptavidin was 250 ng/mL.

Using 250 ng/mL Alexa Fluor 647-labeled streptavidin, 0, 10, 100, and 1000 pg/mL PSA were analyzed with the avidin–biotin complex method using the automatic analyzer. The fluorescence intensity increased linearly as the concentration of PSA increased (Figure S5), and fluorescence was observed at 100 pg/mL PSA. The estimated detection limit was 223 pg/mL, defined as three times the standard deviation of the blank.

PSA Detection with the Sandwich Assay Using an Enzymatic Reaction. Finally, fluorescence-based PSA detection was conducted utilizing HRP-F(ab′)$_2$ and Amplex Red as a substrate. The Amplex Red reagent is a colorless substrate that reacts with hydrogen peroxide (H_2O_2) in the presence of horseradish peroxidase. This reaction generates resorufin (excitation/emission maximum wavelength, 570/585 nm). The excitation/emission filter was changed according to the wavelength. Figure S6 shows the fluorescence intensity of the PSA sandwich assay at PSA concentrations of 0 or 10 pg/mL in PBS containing 15 mg/mL BSA; the fluorescence intensity was clearly higher at 10 pg/mL PSA than at 0 ng/mL. In the PSA sandwich assay, the S/N ratio decreased as the enzymatic reaction time proceeded. However, the calculated p-values were 0.12, 0.11, and 0.02 for 10, 20, and 30 min, respectively. On the basis of these results, 10 pg/mL PSA can be detected over an enzyme reaction time of 30 min.

To determine an optimal concentration of HRP-F(ab′)$_2$ for the detection of PSA in human serum, 100, 250, or 500 ng/mL of HRP-F(ab′)$_2$ was used for sandwich assay. At 10 pg/mL PSA, the S/N ratio was highest at a concentration of 250 ng/mL (Figure S7). At 100 ng/mL, the concentration of HRP-F(ab′)$_2$ was not sufficient for the reaction. In contrast, high background fluorescence was observed because of nonspecific adsorption of HRP-F(ab′)$_2$ in the case of 500 ng/mL. Using 250 ng/mL HRP-F(ab′)$_2$, 10 pg/mL PSA-spiked human serum was assessed to confirm the pg/mL-level sensitivity of this assay. The fluorescence intensity was significantly higher at 10 pg/mL PSA than at 0 ng/mL (Figure 7; $p < 0.05$). These results suggest that 10 pg/mL PSA in human serum can be detected during an enzyme reaction. However, this requires more time because enzymatic amplification is required. Among the previously reported detection methods for PSA, the sensitivity of pipette tip-type immunoassay was better than those for ELISA (LOD: approximately 100 pg/mL),[21] microfluorometric assay (LOD: approximately 462 pg/mL),[22] and metal-enhanced fluorescence assay (LOD: approximately 27 pg/mL),[23] although the various ultrasensitive detection methods such as surface plasmon fluorescence immunoassay (LOD: approximately 2 pg/mL)[24] and bio-electronic immunosensing (LOD: approximately 75 fg/mL)[25] have also been developed. Furthermore, this pipette tip-type biosensing system is highly advantageous from an environmental perspective because it minimizes the waste of disposable materials, as PSA detection can be performed using only one pipette tip.
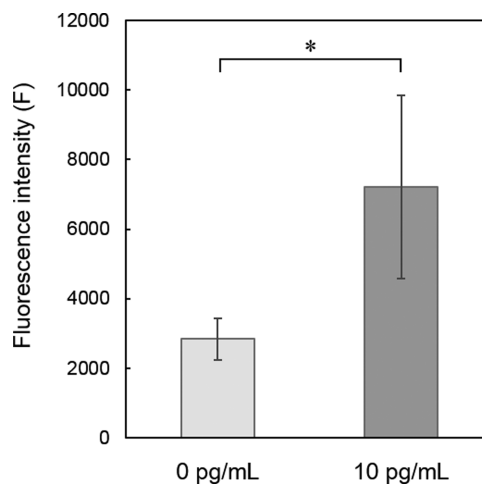

Figure 7. Fluorescence intensity of PSA sandwich assay using the HRP-F(ab′)$_2$ and Amplex Red at a PSA concentration of 0 or 10 pg/mL in human serum. ($n = 3$). One asterisk (*) indicates $p < 0.05$.

◼ CONCLUSIONS

We demonstrated a highly sensitive fluorescence-based immunosensing method for PSA, an important prostate cancer biomarker, using a pipette tip-type biosensing system with sandwich immunoassay. The protein A-immobilized reaction plate allowed for efficient immobilization of antiPSA capture antibodies, enabling efficient PSA sandwich assay. Additionally, using a fragment antibody lacking the Fc region for detection, background fluorescence was successfully reduced, resulting in a high S/N ratio. When PSA detection was performed using an Alexa Fluor 647-labeled F(ab′)$_2$-fragment secondary antibody, the estimated detection limit was 1.2 ng/mL; when PSA detection was performed using the avidin–biotin complex method, the estimated detection limit was 223 pg/mL. Furthermore, the use of the enzyme reaction with sandwich assay led to a high sensitivity, that is, 10 pg/mL PSA was successfully detected. These results indicate the successful, rapid, and sensitive detection of PSA using the automatic analyzer equipped with a Biologi tip. It may be possible to detect different types of target molecules by changing only the reaction plates in conjunction with other specific antibodies, aptamers, ligands, and artificial recognition elements.

◼ EXPERIMENTAL SECTION

Automatic Detection System. An automatic fluorescent detection system based on a pipette tip-type biosensor was developed. The automatic analyzer consisted of an automatic pipetting device, tip rack, reagent rack, incubation port, detector, and computer. The plastic pipette tip (Biologi tip) was specifically designed for the automatic detection system, wherein a very small glass substrate (reaction plate, 4.3 × 9.8 mm) was settled, and a Biologi tip was set in the tip rack. The analyzer automatically performed reagent pipetting, incubation with the reaction solution, washing, and detection as programmed in the computer. The temperatures in the reagent rack and incubation port were controllable. After completing all reactions, the pipettor moved to the fluorescent detection port and directly detected a fluorescent signal in the Biologi tip. The light from a xenon lamp passed through a band-pass filter (624 ± 20 nm; Edmund Optics, Barrington, NJ, USA) and fluorescence was measured using a photomultiplier (PMT, H10721-20; Hamamatsu Photonics K.K., Shizuoka, Japan)
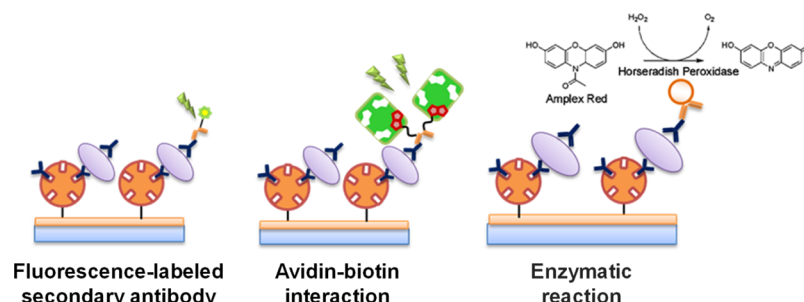
Figure 8. Schematic illustration of the fluorescence-based PSA detection method.

passed through an emission filter (694 ± 20 nm; Edmund Optics) inserted in front of the PMT.

Fluorescent Detection Test for Automatic Detection System. Various concentrations of Alexa Fluor 647-labeled anti-IgG F(ab′)$_2$ [0−200 pg/mL in PBS (7.75 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 15 mg/mL BSA, 100 µL] were withdrawn into the Biologi tip without a reaction plate using the automatic pipetting device. Fluorescence was detected using the automatic detection system with a photomultiplier [1 s (1 ms × 1000 times)].

Protein A-Modified Reaction Plate. A glass reaction plate (4.3 × 9.8 mm) was coated with Ti and Au by radio-frequency sputtering. The Ti layer functioned as an adhesive layer, and the film thickness of Au was approximately 130 nm. The gold-sputtered reaction plate was treated with UV−O$_3$ (UV Ozone Cleaner; BioForce Nanosciences, Inc., Salt Lake City, UT, USA) for 20 min, and then dipped in 1 mM 11-amino-1-undecanethiol hydrochloride in ethanol (3 mL) for 24 h at 30 °C to form a self-assembled monolayer. After the reaction, the reaction plate was washed with ethanol and water. The self-assembled monolayer-modified reaction plate was immersed in 5 mM bis-dPEG$_4$-NHS ester or bis-dPEG$_4$-TFP ester solution [dimethyl sulfoxide (DMSO), 3 mL] for 1 h at 25 °C. After rinsing with DMSO and water, 5 µL of recombinant protein A solution (150 µg/mL, PBS) was dropped onto the NHS or TFP-modified reaction plate and covered with a cover glass. After incubation for 3 h at 25 °C, the reaction plate was washed with PBS and water and then dried with nitrogen gas.

Direct Immobilization of the Anti-PSA Capture Antibody on the Reaction Plate without Protein A. An antiPSA capture antibody-modified reaction plate was prepared in same manner as the protein A-immobilized reaction plate. AntiPSA capture antibody solution (150 µg/mL, PBS) was dropped onto the NHS-modified reaction plate and covered with a cover glass. After incubation for 3 h at 25 °C, the reaction plate was washed with PBS and water and then dried with nitrogen gas.

PSA Sandwich Assay Using Fluorescent-Labeled Secondary Antibody with Incubation. A Biologi tip containing a protein A-modified reaction plate was settled on the automatic analyzer. After attaching the Biologi tip to the pipette, the automatic analyzer was used to perform the following steps. (1) The blocking buffer (2 wt % skim milk in PBS, 100 µL) was pipetted, and the plate was incubated (10 min at 25 °C) and washed (PBS with 0.05 wt % Tween 20, 150 µL, four times). (2) AntiPSA capture antibody (10 µg/mL in PBS, 100 µL) was pipetted, followed by incubation (30 min at 25 °C) and washing (PBS with 0.05 wt % Tween 20, 150 µL,

four times). (3) PSA solution (0 or 1 ng/mL, PBS containing 15 mg/mL BSA, 100 µL) was pipetted, followed by incubation (60 min at 25 °C) and washing (PBS with 0.05 wt % Tween 20, 150 µL, four times). (4) AntiPSA primary antibody (2 µg/mL in PBS, 100 µL), incubation (30 min at 25 °C) was pipetted, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 µL, four times). (5) Alexa Fluor 647-labeled anti-IgG secondary antibody (2 µg/mL in PBS containing 15 mg/mL BSA, 100 µL) was pipetted, followed by incubation (30 min at 25 °C) and washing of the plate (PBS with 0.05 wt % Tween 20, 150 µL, twice and PBS, 150 µL, twice). (6) Finally, fluorescence intensity was measured. Fluorescence was measured using a detection apparatus combining a fluorescence microscope unit (BX3-URA; Olympus, Tokyo, Japan) and sCMOS camera (ZYLA-5.5-USB3; Olympus), rather than PMT. Light at the excitation wavelength was focused on the reaction plate through the objective lens, and fluoresced light was transmitted to the sCMOS camera. Fluorescence images on the reaction plate were captured using the camera. For fluorescence detection using the antiPSA directly modified reaction plate without protein A, the PSA sandwich assay was performed following the same procedure, without step (2).

The same procedure was carried out for the PSA sandwich assay using Alexa Fluor 647-labeled Fab-fragments or F(ab′)$_2$-fragment antimouse IgG as the detection antibody. At step (5), 0.67 µg/mL Alexa Fluor 647-labeled Fab-fragment or 1.33 µg/mL Alexa Fluor 647-labeled F(ab′)$_2$-fragment was used. Fab-fragment antimouse IgG was labeled using the Alexa Fluor 647 Monoclonal Antibody Labeling Kit.

PSA Sandwich Assay Using a Fluorescence-Labeled Secondary Antibody Utilizing the Suction/Discharge Process. The following modified incubation method was applied. (1) Blocking buffer (2 wt % skim milk in PBS, 100 µL) was pipetted, suction/discharge was repeated five times, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 µL, four times). (2) AntiPSA capture antibody (10 µg/mL in PBS, 100 µL) was pipetted, suction/discharge was repeated five times, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 µL, four times). (3) Various concentrations of PSA solution were pipetted (PBS containing 15 mg/mL BSA, or human serum, 100 µL), suction/discharge was repeated 10 times, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 µL, four times). (4) The antiPSA primary antibody (2 µg/mL in PBS, 100 µL) was pipetted, suction/discharge was repeated five times, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 µL, four times). (5) Alexa Fluor 647-labeled F(ab′)$_2$-fragment antimouse IgG secondary antibody (1.33 µg/mL in PBS containing 15 mg/mL BSA, 100 µL) was pipetted, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 µL, twice + PBS, 150 µL, twice). (6) Finally,

fluorescence intensity was measured by PMT [1 s (1 ms × 1000 times)] (Figure 8).

PSA Sandwich Assay Utilizing the Biotin–Avidin Complex Method. A biotinylated secondary antibody and fluorescent-labeled streptavidin were used for PSA sandwich assay. The procedure was same as above, with a time-reduction procedure for steps (1−4). After step (4), the following steps were performed. (5) Biotinylated F(ab′)$_2$-fragment antimouse IgG secondary antibody (1.33 μg/mL in PBS containing 15 mg/mL BSA, 100 μL) was pipetted, suction/discharge was repeated five times, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 μL, four times). (6) Alexa Fluor 647-labeled streptavidin (100, 250, or 500 ng/mL in PBS containing 15 mg/mL BSA, 100 μL) was pipetted, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 μL, twice + PBS, 150 μL, twice). (7) Fluorescence intensity was measured by PMT [1 s (1 ms × 1000 times)] (Figure 8).

PSA Sandwich Assay Utilizing an Enzymatic Reaction. A peroxidase-conjugated secondary antibody and Amplex Red, a fluorescent reagent, were used. The procedure was the same as above, with a time-reduction procedure at steps (1−4). After step (4), the procedure was as follows. (5) Peroxidase-conjugated F(ab′)$_2$-fragment antimouse IgG secondary antibody (HRP-F(ab′)$_2$; 100, 250, or 500 ng/mL in PBS containing 15 mg/mL BSA, 100 μL) was pipetted, suction/discharge was repeated five times, and the plate was washed (PBS with 0.05 wt % Tween 20, 150 μL, four times). (6) Amplex Red (50 μM in reaction buffer containing 1 mM hydrogen peroxide, 100 μL) was pipetted, followed by incubation (10, 20, or 30 min at 25 °C). (7) Fluorescence intensity was measured by PMT [1 s (1 ms × 1000 times)]. The light from a xenon lamp was passed through a band-pass filter (520 ± 20 nm; Edmund Optics) and absorptive neutral density (ND) filter (OD 0.3; Edmund Optics). Fluorescence was measured with a PMT passed through an emission filter (590 ± 20 nm, Edmund Optics) inserted in front of the PMT (Figure 8).

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02850.

> Materials, Standard curve of the Alexa Fluor 647-labeled anti-IgG F(ab′)$_2$ fragment. Fluorescence intensity of PSA sandwich assay for the long and short methods. Signal-to-noise ratio of PSA sandwich assay using various concentrations of Alexa Fluor 647-labelled streptavidin. PSA calibration curve for sandwich assay using avidin−biotin interactions. PSA sandwich assay using the horseradish peroxidase-labeled F(ab′)$_2$-fragment anti mouse IgG and Amplex Red in PBS containing 15 mg/mL BSA. Signal-to-noise ratio of PSA sandwich assay using various concentrations of horseradish peroxidase-labeled F(ab′)$_2$-fragment anti mouse IgG in human serum (PDF)

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