BioChip J. (2018) 12(2): 154-162
DOI 10.1007/s13206-017-2208-6

Original Article

Immunoreaction-based Microfluidic Diagnostic Device for the Detection of Prostate-Specific Antigen

Hyung Jin Kim[1], Byeong Hee Kim[1] & Young Ho Seo[1,*]

Received: 24 August, 2017 / Accepted: 22 September, 2017 / Published online: 23 May, 2018
© The Korean BioChip Society and Springer 2018

Abstract This paper addresses an immunoreaction period-independent microfluidic diagnostic method for the detection of prostate-specific antigen. Immunoreaction-based biosensors generally use sandwich binding of a capture antibody, followed by an antigen, and then a detection antibody with a fluorescent label. Fluorescent intensity is proportional not only to the antigen density in a sample but also to the immunoreaction period. Generally, immunoreaction-based sensors are dependent on the immunoreaction period because the amount of antigen bound to fluorescent labels increases as the immunoreaction period increases. A wash-out step is suggested to eliminate dependence on a specific reaction period. Further, the optimal time to start the wash-out step was investigated. For the conventional detection process, fluorescent intensity increases constantly as the immunoreaction period increases, even though the antigen concentration in the sample has not changed. After applying the wash-out process, however, the fluorescent intensity was maintained over a 15-min period. Consequently, even though a user may miss the optimal reading time for the assay, an accurate result can be reported because the wash-out step enables microfluidic diagnostic devices to maintain a constant fluorescent intensity.

Keywords: Immunoreaction, Microfluidic, Diagnostic device, Prostate-specific antigen, Wash-out

[1]Advanced Mechanical Engineering, Kangwon National University, Chuncheon, Gangwondo, Republic of Korea
*Correspondence and requests for materials should be addressed to Y.H. Seo (✉ mems@kangwon.ac.kr)

Introduction

Millions of people face the risk of malignancies, one of the leading causes of mortality worldwide. Reliable, sensitive tests for tumor markers and a reliable testing system are important for early clinical diagnosis and evaluating the recovery of patients with certain tumor-associated diseases[1]. Among various cancers, prostate cancer accounts for approximately 10% of all deaths, and it is the third most common cancer in men[2-5]. Early detection of this cancer is crucial to reducing the mortality rate associated with this disease. Prostate-specific antigen (PSA) is a valuable biomarker for the detection of prostate cancer[5]. Most of the current PSA assays are variants of an enzyme-linked immunosorbent assay (ELISA), which use enzymatic, fluorescent, or chemiluminescent labels to detect PSA. Because microfluidic diagnostic assays require only about 10-20 min to provide a result, they offer a point-of-care tool to diagnosis specific diseases[6-8].

ELISAs have been the main technique for detecting tumor markers. The most popular format involves sandwich binding of a capture antibody (cAb), followed by an antigen (Ag), and then a detection antibody (dAb) with a tracer label. Since a detectable signal is difficult to obtain from an Ag-Ab reaction, a tracer is generally bound with dAb to produce an analytical signal to indicate immunoreaction. Figure 1 presents a test procedure with sandwich binding of cAb-Ag-dAb with a fluorescent label. When the test sample includes target Ag mixed with aAb with a fluorescent label, Ag and then the dAb with fluorescent label are specifically bound, as shown in step 1 of Figure 1. Next, a mixed sample having specific binding to the Ag, which binds to the dAb with a fluorescent label, was applied to

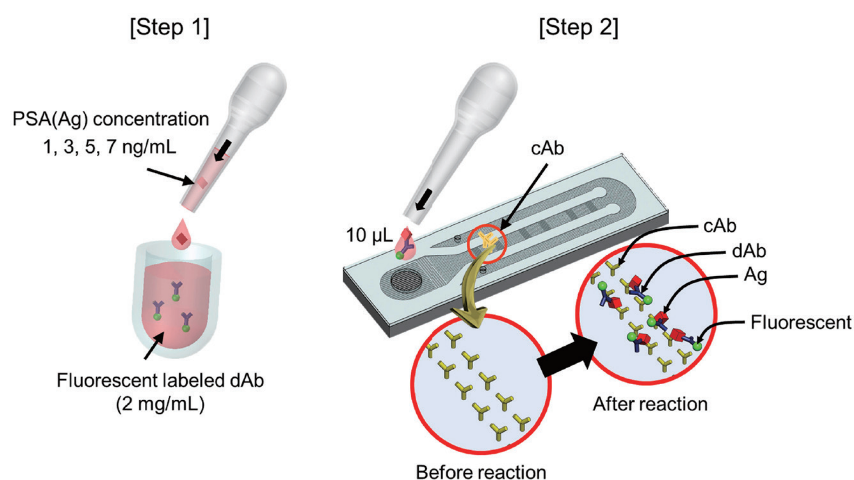

Figure 1. Test procedure of sandwich binding of antibody-antigen-antibody (cAb: capture antibody, dAb: detection antibody, Ag: Antigen).

immunosensors where cAb was immobilized on the detection region of the device (step 2 in Figure 1). Eventually, sandwich binding of cAb-Ag-dAb with a fluorescent label is achieved. Sandwich assays have the advantage of high specificity and high sensitivity because of the use of two matched antibodies[1,9-12]. Fluorescence analyte detection methods offer the advantages of sensitivity and selectivity in the bio-analytical field. In addition, one of the most exciting aspects of fluorescence technology is that the sample volume can be as little as a single molecule. Thus, fluorescence technology provides an opportunity for miniaturization of devices and improvements in high-throughput screening. The detection of biomarkers, specific Ags, is increasingly used in the diagnosis of disease[13,14].

To increase the assay sensitivity, several amplification methods such as dendritic amplification[15], and rolling-circle amplification[16] have been proposed to modify dAb. However, amplification methods required the modification of test protocol. On the other hand, enriching the concentration of cAb can enhance the sensitivity while still using the same assay protocol. An increased concentration of immobilized cAb in each well of the test plate can improve the capturing capacity of target Ag bound to a dAb-tracer, and, consequently, more sandwich pairs are present at the end of the assay, leading to increased signals for detection[10]. Detection signals such as fluorescent intensity are constantly increased as the immunoreaction period increases as shown in Figure 2(a), even though the Ag concentration in a sample has not changed[2]. That means user must read the signal at the appointed time, if not incorrect result could be measured. Reading time in conventional methods is no fixed reference and used according to instructions of manufacturer. Usually reading time for PSA detection requires a time of 18 to 60 minutes[17]. Therefore, we propose a method in which non-specifically bound Ags or remaining Ags are removed by a pipette-based wash process, as shown in Figure 2(b).

Conventional immunoreaction-based microfluidic sensors are immunoreaction period-dependent. Thus, this work suggests a wash-out step to eliminate the dependence of results on the reaction period. As shown in Figure 2, when the wash-out step was applied to microfluidic diagnostic devices, both an increase in detection signals and an independency from the immunoreaction period can be expected because the background signal is removed. In addition, an optimal starting time for the wash-out step was determined.

Results and Discussion

Three different experimental sets were performed. In the first experimental set, conventional approach having no wash-out step was performed. Fluorescent intensity was measured over reaction-period for four different concentration of PSA of 10 μL. The volume of test sample of 10 μL was similar amount of capillary pump in microfluidic diagnosis device. In the second experimental set, wash-out solution of phosphate buffered saline (PBS) was applied into the sample inlet hole after test sample was loaded in order to determine the proper starting time of wash-out step. After 5, 10 and 15 min., to supply test sample 5 μL in inlet hole, the

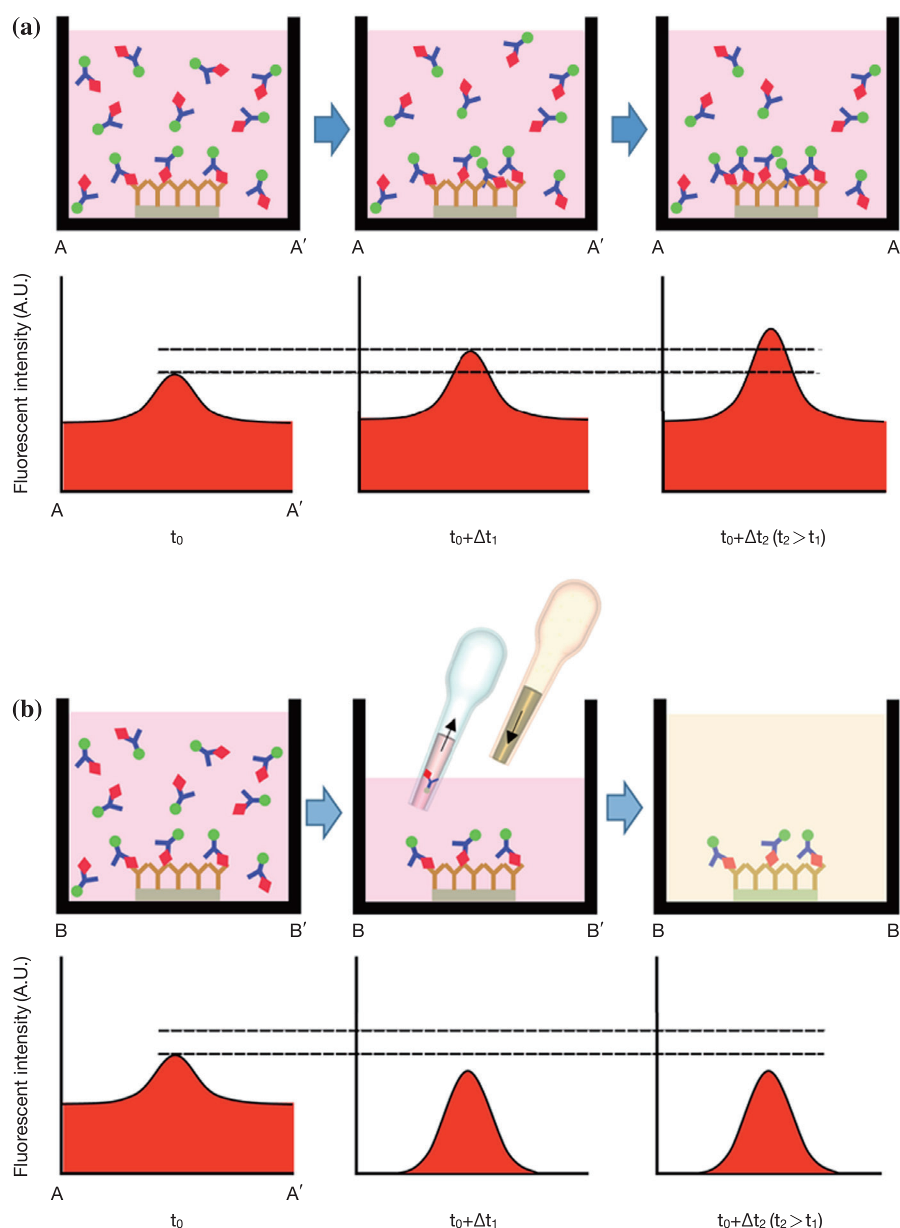

Figure 2. Effect of wash-out process in fluorescent intensity; (a) without wash-out step, (b) with wash-out step.

wash-out solution of 5 μL was dropped in the same inlet hole. Fluorescent intensity was measured over reaction-period for the PSA concentration of 7 ng/mL. In the third experiment, the suggested immunoreaction-period independent approach based on wash-out step was performed for four different concentration of PSA. In this experiment, wash-out starting time determined by the second experiment was used.

In order to investigate the reaction-period dependency of immunoreaction sensor, fluorescent intensity of sandwich binding of (cAb)-(Ag)-(dAb with fluorescent label) was measured without wash-out step. Test sample volume of 10 μL was supplied in sample inlet hole, and after 5 min., fluorescent intensity was measured using confocal microscope every 1 min. Figure 3(a) presents the fluorescent images got from PSA concentration of 7 ng/mL every 5 min. As shown in Figure 3(a), fluorescent intensity in cAb immobilized area was increased as reaction-period was longer. Also background fluorescent intensity was observed, thus it was slightly hard to distinguish between detection line and background. Figure 3(b) shows quantative fluorescent

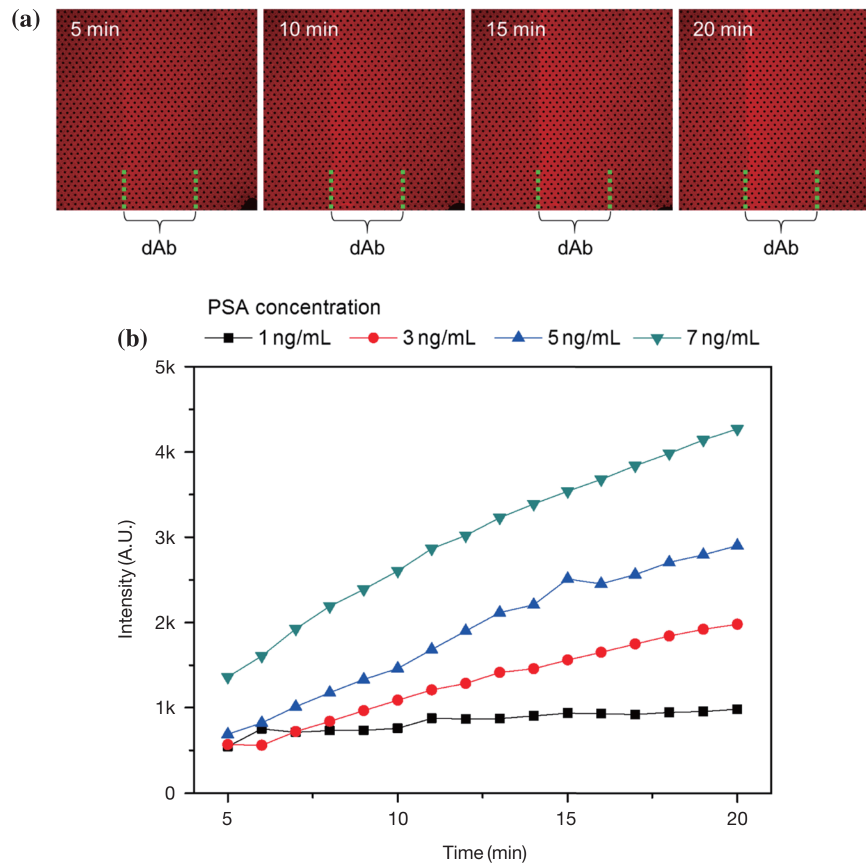

Figure 3. Experimental results obtained by conventional approach without wash-out step; (a) fluorescent images every 5 min. for the concentration of prostate specific antigen of 7 ng/mL, (b) average fluorescent intensities every 1 min. for four different concentration of prostate specific antigen.

intensities that were average intensity difference in detection line and surrounding area, every 1 min. For all PSA concentration, average fluorescent intensity in detection line was gradually increased as immunoreaction-period increased. Therefore, if user missed the appointed reading time-point of fluorescent intensity, wrong experiment result might be reported.

The second experiment was carried out in order to determine a proper applying time of PBS wash-out solution. After 5, 10, and 15 min. to load test sample of 5 μL, PBS wash-out solution of 5 μL was dropped in the same inlet hole. In this experiment, PSA concentration of 7 ng/mL was used. First experiment without wash-out process used 10 μL sample, but 5 μL sample and 5 μL wash-out solution was used in the second experiment because the volume of capillary pump was around 10 μL. Figures 4(a), 4(b) and 4(c) show the fluorescent images and quantative fluorescent intensity. As shown in fluorescent images above each graph in Figure 4, clear distinction between detection line and surrounding area could be observed after about 5 min. to apply wash-out solution. When wash-out step was started after 5 min. to load test sample, fluorescent intensity was gradually increased during 3 min. as shown in Figure 4(a). Because the wash-out solution pushed remained test sample inside microchannel toward the immobilized cAb again, fluorescent intensity in the detection line was increased. However after 5 min. to start wash-out process, fluorescent intensity was maintained steady over 20 min. When wash-out step was started after 10 min. to load test sample, fluorescent intensity was increased during 3 min., and then maintained steady over 20 min. as shown in Figure 4(b). After 25 min., however, final fluorescent intensity in Figure 4(b) was 1.36 times higher than that in Figure 4(a).

Because wash-out process in Figure 4(a) carried out in a state where the specific binding is not sufficient. On the other hand, wash-out process in Figure 4(b) was carried out after the specific binding progressed sufficiently. The final fluorescent intensity in Figure 4(b) was similar to that in Figure 4(c). In case of Figure 4(c), this procedure is time consuming. That is, 10

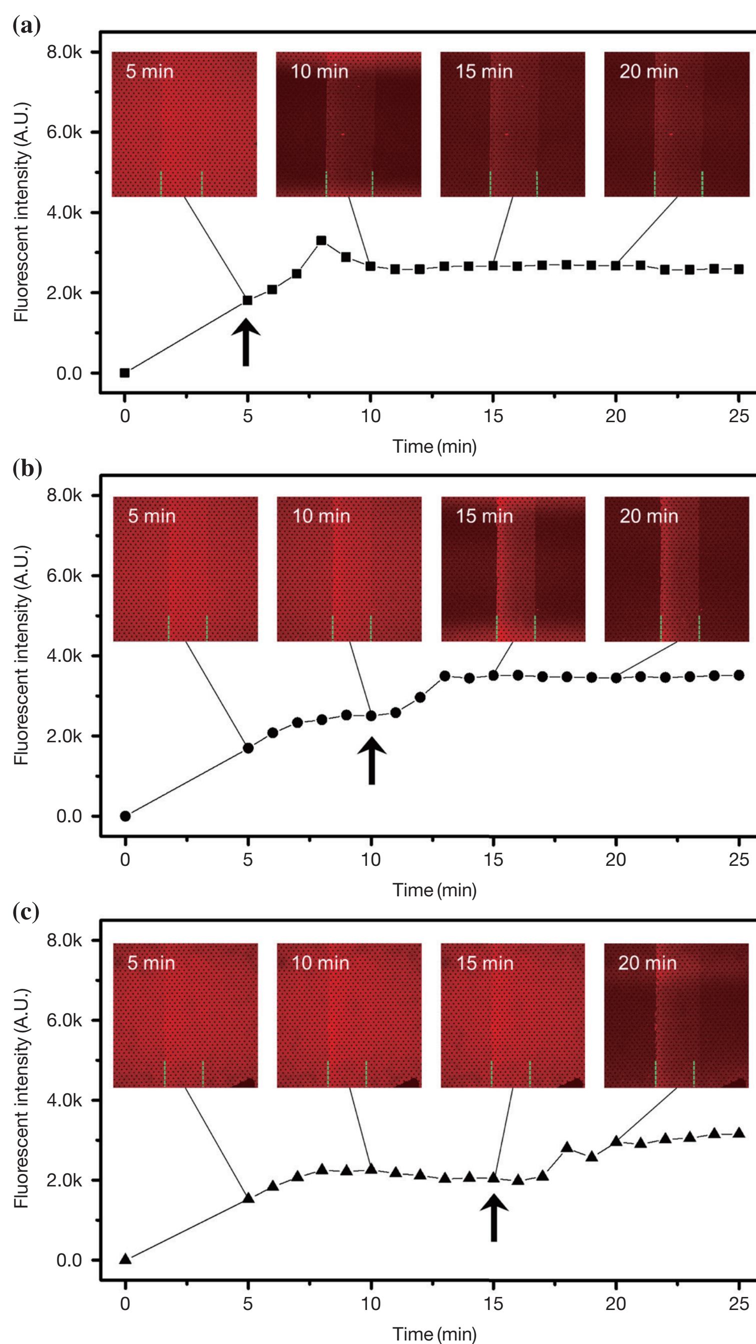

Figure 4. Experimental results obtained by different starting point of wash-out step for the concentration of prostate specific antigen of 7 ng/mL; (a) after 5 min. to load sample, (b) after 10 min. to load sample, (c) after 15 min. to load sample (↑: wash-out start point).

min. after loading the test sample in the microfluidic diagnostic device was the optimal point to begin the wash-out process.

Importantly, 5 μL of test sample and 5 μL of wash-out solution were used in the second experiment, while 10 μL of test sample was used in the first experiment due to the limitation of the capillary pump. However, the fluorescent intensity in the second experiment was almost same as that in the first experiment. That is, when the wash-out step was adopted in the microfluidic diagnostic assay, the test sample could be reduced by half. Because antibodies and fluorescent molecules

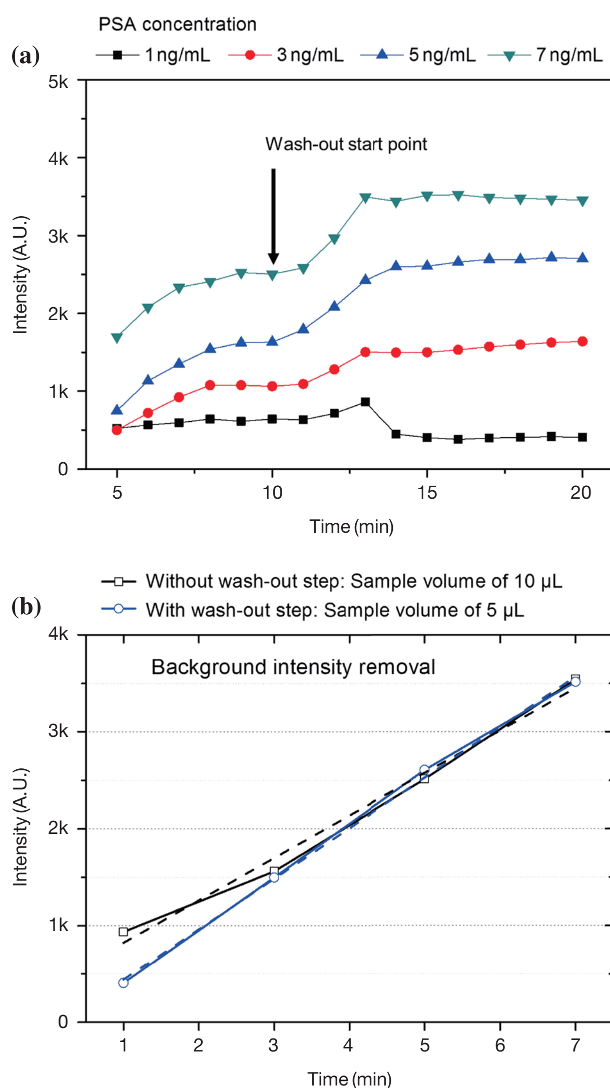

Figure 5. Experimental results obtained by reaction-period independent method with wash-out step; (a) average fluorescent intensities every 1 min. for four different concentration of prostate specific antigen, (b) sensitivities against the concentration of prostate specific antigen (Normal range <4 ng/mL, Risk range : ≥ 4 ng/mL).

are expensive, the ability to use a reduced test sample volume is a great advantage in biomedical diagnosis.

The third experiment was performed to measure fluorescence intensities after adopting the wash-out step. The wash-out starting point was fixed at 10 min. after the test sample was loaded. In this experiment, a test sample volume of 5 μL and a wash-out solution volume of 5 μL were used. Figure 5(a) presents the fluorescence intensities at four different concentrations, 1, 3, 5, and 7 ng/mL PSA. As shown in Figure 5(a), fluorescence intensities were step-up after 3-4 min. to supply wash-out solution, and then fluorescence intensities were maintained at a content level for 20 min. Even if the user missed the optimal reading time for the assay, the result could be reported because the wash-out step enabled constant fluorescent intensity to be maintained in assays performed on the microfluidic diagnostic devices. Figure 5(b) compares the average fluorescence intensities of the detection line obtained from the reaction period-independent method with the wash-out step with that obtained from the conventional approach without the wash-out step. As a result of comparing the R-square values after linear fitting the graphs shown in Figure 5(b), the R-square values of the experiments with and without wash-out step were 0.998 and 0.988, respectively. Even though the volume of test sample in the reaction period-independent method was only half of that used in the conventional method, the fluorescence intensities were almost the same, and more linear sensitivities against the PSA concentration.

Conclusion

This study evaluated an immunoreaction period-independent microfluidic diagnostic assay for the detection of prostate-specific Ag. Immunoreaction-based sensors are typically dependent on the immunoreaction period, with the amount of fluorescence increasing as the immunoreaction period increases. The suggested wash-out step effectively eliminated the dependence of the assay results on the reaction period. The optimal starting point for the wash-out step was determined to be about 10 min. after the test sample was loaded. The wash-out step also reduced the amount of sample required by moving any remaining sample inside the microchannel toward the dAb. From these experimental results, we conclude that the addition of a wash-out step in microfluidic diagnostic assays provides a stable detection signal and allows for the reduction of the test sample volume used. Also, the result of this paper is limited to the particular case where an antigen is detected in the closed microchannels of a microfluidic diagnostic device.

Materials and Methods

Design and Fabrication of Microfluidic Device

The microfluidic diagnostic device consisted of upper and lower plates as shown in Figure 6. The inlet hole for sample loading, the air vent hole for the capillary pump and immobilized cAb region were prepared in

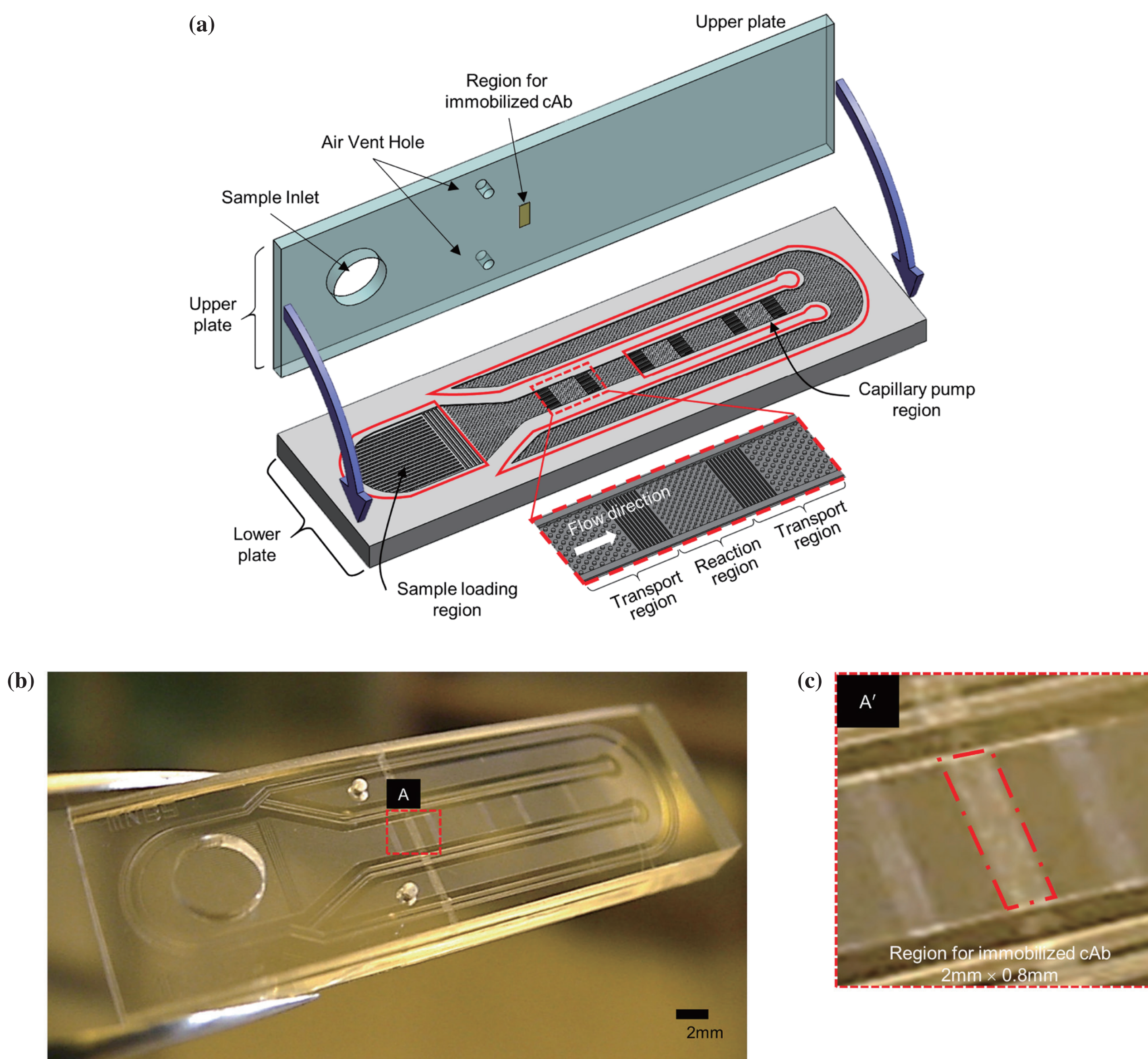

Figure 6. Design and fabrication of microfluidic diagnosis device; (a) design of capillary-driven microfluidic diagnosis device, (b) photograph of overall device, (c) enlarged photograph of the region for immobilized capture antibodies.

the upper plate, and the capillary microchannel was fabricated on the lower plate. Microposts inside the microchannel were formed for the prevention of roof-collapse during the bonding process of the upper and lower plates. The capillary microchannel on the lower plate was sectioned into four different regions, including the sample loading region, sample transport region, reaction region, and capillary pump region, as shown in Figure 6. The depth and width of the capillary microchannel were 20 μm and 2 mm, respectively. The diameters of the microposts in the transport and capillary pump regions were 50 μm and in the reaction region were 20 μm. The area of the immobilized cAb region on the upper plate was 0.8 mm × 2 mm.

The lower plates of the microfluidic diagnostic device were fabricated by a polydimethylsiloxane (PDMS, Sylgard-184, Dow Corning Co.)-casting process on a silicon mold. The silicon mold was prepared by a typical silicon-reactive ion etching process where photoresistance of the AZ1512 was used as an etch mask. The silicon mold was chemically treated with trichlorosilane (Sigma-Aldrich Inc.) to reduce the surface energy for easy detachment of PDMS from the silicon mold. Trichlorosilane was deposited by a vacuum va-

Table 1. Experiment condition for antigen-antibody reaction.

Items		Condition
Capture antibody (cAb) in PBS	Concentration Used volume cAb immobilized area	2 mg/mL 0.2 μL 0.8 mm × 2 mm
Fluorescent labeled detection antibody (dAb) in PBS	Concentration Volume	2 mg/mL 10 μL
Antigen (PSA)	Concentration	1, 3, 5, 7 ng/mL
Phosphate buffered saline (PBS)	pH	7.3-7.5

por deposition process for 1 hr. A typical PDMS has a contact angle of around 100° and shows hydrophobic characteristics; thus, capillary flow was not expected. To generate capillary flow through the PDMS microchannel, a 1.0 wt% hydrophilic surfactant, Silwet-L77 (Momentive Inc.), was added to the PDMS pre-polymer[18]. The contact angle of the hydrophilically treated PDMS was about 66°[17]. The PDMS pre-polymer, curing agent, and hydrophilic surfactant were mixed together at a mixing ratio of 100 : 10 : 1, and then the mixed PDMS was poured into the silicon mold. After curing at a temperature of 85°C for 2 hr, the lower plate was detached from the silicon mold. The upper plates were prepared by a simple PDMS casting process on a chemically treated glass wafer, and then the sample inlet holes and air vent holes were formed by punching. A 0.2-μL volume of cAb at a concentration of 2 mg/mL was immobilized on the predetermined area by oxygen plasma. The immobilization method was a simple drying and adsorption process. The immobilization process was naturally dried for 20 minutes at a temperature of about 20°C. and a humidity of about 40%, and then the upper and lower plate were assembled. Figures 6(b) and 6(c) show a photograph of the microfluidic diagnostic device and an enlarged photograph of the immobilized cAb region, respectively.

Sample Preparation and Test Procedure

Fluorescent molecule of Alexa Fluor 647 (Molecular Probes, Inc.) labeled in dAb was used as the tracer for the detection of antigen-antibody reaction. Table 1 shows the experimental conditions for antigen and antibody. According to the experiment procedure of immunosensors as shown in Figure 1, test sample of target antigen of PSA was mixed with fluorescent-labeled dAb whose concentration was 2 mg/mL. Excitation and emission wavelengths of Alexa Fluor 647 were 650 nm and 665 nm, respectively. The cAb and dAb used in this study were supplied by the boditech Med with the prostate-specific antigens as the same prostate-specific mouse monoclonal antibody. Ag of four different concentrations of 1, 3, 5 and 7 ng/mL were prepared as experimental samples, because the normal concentration range of PSA was 4 ng in 1 mL blood.

For each concentration of PSA sample, test sample was applied to the fabricated microfluidic diagnosis device, and then fluorescent intensity was measured by the confocal microscope of LSM 510 META (Carl Zeiss, Inc.).

Acknowledgements This work was supported by the Human Resources Development program (No. 201540302 00950) of the Korea Institute of Energy Technology Evaluation and Planning (KETEP) grant funded by the Korea government Ministry of Trade, Industry and Energy Also, This research was supported by Industry and Energy and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A1B03034696).

References

1. Wu, J., Fu, Z., Yan, F. & Ju, H. Biomedical and clinical applications of immunoassays and immunosensors for tumor markers. *Trends Analyt. Chem.* **26**, 679-688 (2007).
2. Okada, H., Hosokawa, K. & Maeda, M. Power-free microchip immunoassay of PSA in human serum for point-of-care testing. *Anal. Sci.* **27**, 237 (2011).
3. Saad, F. The treatment continuum in the management of prostate cancer patients: What's new? *Eur. Urol.* **5**, 539-542 (2006).
4. Grönberg, H. Prostate cancer epidemiology. *The Lancet* **361**, 859-864 (2003).
5. Ertürk, G., Hedström, M., Tümer, M.A., Denizli, A. & Mattiasson, B. Real-time prostate-specific antigen detection with prostate-specific antigen imprinted capacitive biosensors. *Anal. Chim. Acta* **891**, 120-129 (2015).
6. D'Orazio, P. Biosensors in clinical chemistry – 2011 update. *Clin. Chim. Acta* **412**, 1749-1761 (2011).
7. Lee, T.M.-H. Over-the-counter biosensors: Past, present, and future. *Sensors* **8**, 5535-5559 (2008).

8. Robinson, G. The commercial development of planar optical biosensors. *Sens. Actuators B Chem.* **29**, 31-36 (1995).
9. Barbosa, A.I., Castanheira, A.P., Edwards, A.D. & Reis, N.M. A lab-in-a-briefcase for rapid prostate specific antigen (PSA) screening from whole blood. *Lab Chip* **14**, 2918-2928 (2014).
10. Lee, S. *et al.* Improved porous silicon microarray based prostate specific antigen immunoassay by optimized surface density of the capture antibody. *Anal. Chim. Acta* **796**, 108-114 (2013).
11. Gembitsky, D.S., Lawlor, K., Jacovina, A., Yaneva, M. & Tempst, P. A prototype antibody microarray platform to monitor changes in protein tyrosine phosphorylation. *Mol. Cell. Proteomics* **3**, 1102-1118 (2004).
12. Saviranta, P. *et al.* Evaluating sandwich immunoassays in microarray format in terms of the ambient analyte regime. *Clin. Chem.* **50**, 1907-1920 (2004).
13. Yan, M. *et al.* Fluorescence immunosensor based on p-acid-encapsulated silica nanoparticles for tumor marker detection. *The Analyst* **137**, 2834-2839 (2012).
14. Lin, P., Wang, Y.L., Feng, J.J., Zou, Z.H. & Yang, Q. Competitive enzymatic fluorescence immunoassay for human IgG by using a temperature-sensitive phase separating polymer with regulated phase transition temperature. *Chinese J. Chem.* **26**, 794-798 (2008).
15. Hosokawa, K., Omata, M. & Maeda, M. Immunoassay on a power-free microchip with laminar flow-assisted dendritic amplification. *Anal. Chem.* **79**, 6000-6004 (2007).
16. Schweitzer, B. *et al.* Immunoassays with rolling circle DNA amplification: a versatile platform for ultrasensitive antigen detection. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 10113-10119 (2000).
17. Healy, D.A., Hayes, C.J., Leonard, P., McKenna, L. & O'Kennedy, R. Biosensor developments: application to prostate-specific antigen detection. *Trends Biotechnol.* **25**, 125-131 (2007).
18. Kim, Y.C., Kim, S.-H., Kim, D., Park, S.-J. & Park, J.-K. Plasma extraction in a capillary-driven microfluidic device using surfactant-added poly (dimethylsiloxane). *Sens. Actuators B Chem.* **145**, 861-868 (2010).

Copyright for the published papers belongs to the Korean BioChip Society. pISSN 1976-0280. eISSN 2092-7843