Korean J Biol Sci 7: 89-92, 2003

Simultaneous Quantitative Determination of Multiple Analytes with Fluorescence-Tagged Probes by Immunochromatography

Dong Seok Jeong and Eui Yul Choi[1]*

Central Research Institute of Boditechnology, Bioventure Inovation Center, Chuncheon 200-160, Korea;
[1]Department of Genetic Engineering, College of Natural Sciences, Hallym University, Chuncheon 200-702, Korea

Key Words:

Immunoassay
Microarray
Fluorescence
Antibody
Immunochromatography

Immunoassays have become indispensable tools and achieved great importance in scientific and medical research. However, typical immunoassays are time-consuming and use complex, multi-step procedures. In this study, we introduce a new immunoassay system for the quantification of several analytes at a time without any washing steps. It is comprised of a detector solution with fluorescence-labeled antibodies and a test strip with immobilized capture antibodies. Using a micro-array scanner, the antigen-antibody complex was quantitatively determined by measuring the intensities of fluorescence on the capture lines or dots of nitrocellulose membrane. This method demonstrated its rapid quantitative determination of analytes without many processing steps as well as specific identification of multiple analytes in biological specimens.

Since their first introduction in 1950s, immunoassays have become indispensable tools and have achieved great importance in the field of scientific and medical research (Ishikawa and Kohno, 1989; Oellerich, 1984). Immunoassay is rapidly evolving toward more simple approaches for the quick identification and diagnosis of biological specimens (Westermann et al., 2002). To quantify an analyte in immunoassays, there are too many steps for the completion of the immunoassays utilizing enzymes or radioisotope as signal generators. The assays require several washing steps and at least one more step for a substrate reagent, therefore the reading time is very long. Recently, there is great interest in the development of protein microarray capable of monitoring hundreds or thousands of proteins simultaneously (Kodadet, 2002). Likewise, the protein microarray also needs extensive washing steps and other extra steps to get the final result. Thus, there is a need for a general method that can provide a rapid, quantitative measurement of analytes present in samples and that is sufficiently simple to carry out without use of a laboratory or individuals trained in chemical analysis.

The rapid test kits based on immunochromatography have been popularized to access for diagnosis of various infectious diseases and cancers with whole blood, serum, and urine (Lou et al., 1993; Panteghini and Pagani, 1996). The result of these tests can be displayed as quickly as in 15 min. In a lateral-flow type of the sandwich or competitive immunochromatographic assay, a detector reagent is often the antigen or antibody coupled to gold-particle as a tracer molecule, remains unbound form onto a releasing pad, and releases along the rest strip during separation (Millipore, 1996). In this study, we introduce a new immunoassay system for rapid quantification of multiple analytes without several processing steps. The new assay takes advantage of the inherent simplicity of lateral-flow immunochromatography, while providing rapid quantification of analytes in samples. While the conventional immunochromatographic method usually measures one analyte in a fluid sample, we modified and applied it to use in multiple analytes using the microarray-type configuration for high-throughput testing.

*To whom correspondence should be addressed.
Tel: 82-33-240-1465, Fax: 82-33-258-6888
E-mail: euichoi@hallym.ac.kr

Materials and Methods

Chemicals

Bovine serum albumin (BSA), rabbit IgG, anti-rabbit IgG, poly ethylene glycol (PEG), sodium azide, and sodium bicarbonate were purchased from Sigma (St Louis, MO, USA). Sandwich ELISA pairs for CEA, AFP, and CRP were purchased for Fitzerald (USA). Sephadex G25 and Cy-3/Cy-5 were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and Molecular Probes (Eugene, OR, USA), respectively. The nitrocellulose membrane (NC) HF180 was from Millipore Corp.

(Watertown, MA, USA) and the absorption pads were obtained from Schleicher & Schuell Corp. (Keene, NH, USA). The sample pad and Bio-spin column were purchased from Pall (New York, USA) and Bio-Rad (Hercules, CA, USA), respectively.

Enzyme-linked ImmunoSorbent Assay (ELISA)

For direct ELISA, 96-well microtiter plates were incubated with 50 μl of the antigen at the concentration of 2 μg/ml overnight at 4°C. After washing with PBS-Tween 20 (PBST) extensively, the plates were treated with culture supernatant for 1 h, and then for additional 1 h incubation with HRP-conjugated goat anti-mouse IgG. Following the final rinse with PBS, the color reaction was initiated by adding 50 μl of TMB substrate solution (KEM EN TEC, Denmark) and stopped by adding 10 mM H_2SO_4. For sandwich ELISA of antigen in serum, each well was coated with 50 μl of capture antibody at the concentration of 2 μg/ml overnight and blocked with 5 mg/ml fish gelatin in PBST for 1 h. Then, 50 μl of serum was applied into each well and incubated for 2 h. After washing three times with PBST, 100 μl of biotinylated capture antibody (1 μg/ml) was added to each well and incubated for 2 h. Washing with PBST three times was followed by incubating with 100 μl of HRP-conjugated streptavidin (1 μg/ml) for 1 h. After the final washing with PBST, the substrate solution was added and the absorbance was read at 450 nm.

Fluorescence conjugation to proteins

For labeling of antibodies with fluorescence, 10 μl of 1 M sodium carbonate buffer (pH 8.3) was mixed well with 100 μl of protein (1 mg/ml) in PBS, and followed by addition of 1 μl of Cy-5 or Cy-3 (10 mg/ml) to the mixture prior to overnight incubation at 4°C. The mixture was applied onto a Sephadex G-25 chromatography column to separate free fluorescence dye from conjugates, and the antibody-fluorescence conjugate was collected as elutes after centrifugation of the column at 2,500 rpm for 2 min.

Preparation of assay strip

Assay strip consists of a nitrocellulose membrane, a sample pad, an absorption pad, and a backing card (Fig. 1A & B). On the adhesive side of the backing polystyrene card are laid the nitrocellulose membrane, the sample pad, and the absorption pad. The nitrocellulose membrane (Millipore HF 180) is the place where a detection zone is located on, and the bottom side of membrane is covered with a thin plastic film. The width of dispensing line was 0.5-1 mm and the dispensing volume was 0.5-1 μl/cm. The diameter of dispensing dot was 0.5 mm. Dispensing was performed by a microarray dispenser (Proteogen, Korea) for multiple dots and a microdispenser for multiple lines (Bio-Dot, USA). It was critical to keep the humidity between 50% and 60% while drying after dispensing of the antibody or antigen. Before sitting on nitrocellulose membrane, the sample pad was completely soaked in PBS containing 1% BSA and 0.05% Tween 20, and vacuum-dried at 50°C for 1 h. The absorption pad was set up at the end of the nitrocellulose membrane.

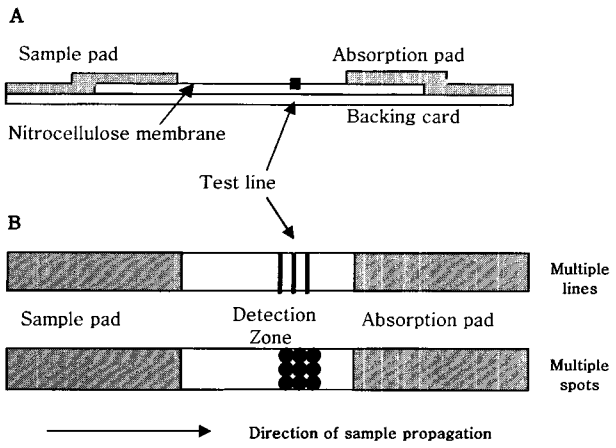

Fig. 1. A schematic diagram of fluorescence immunochromatographic assay strip. (A) and represents the side and the upper view of the strip (B), respectively. Antibodies or antigens are immobilized at the test line by a microdispenser as described in Material Methods.

Results and Discussion

We adapted the principle of immunochromatographic assay for the analysis of multiple analytes in this study. A well-known example of the method is the pregnancy test for women. As shown in Fig. 1, the test strip is composed of the same key components used in the conventional immunochromatographic strips. These include a sample pad for sample application, a nitrocellulose membrane for separation and detection of analytes, an absorption pad for the generation of capillary action, and a plastic backing card for the protection of the strip. A capture antibody is dispended and immobilized on the nitrocellulose membrane. Instead of 40-nm gold particles in the rapid test of the conventional immunochromatography, we used a fluorescence dye as a signaling molecule, which is conjugated to the detector antibody. When an analyte in a sample is first mixed with the detector antibody and then applied into a sample pad, the analyte-detector antibody complex moves from the sample pad toward absorption pad. During the propagation, the complex encounters a capture antibody immobilized on the detection zone of the nitrocellulose membrane and forms a sandwich-type detector-analyte-capture antibody complex. Since the capture antibody is fixed on the membrane as a test line, the analyte-antibody triple complexes continuously accumulate on the line as the

sample solution moves toward the absorption pad. In the meantime, free detector antibody passes over the test line. The higher the concentration of an analyte in the sample, the more accumulation of the detector antibody on the test line, and thus the stronger fluorescence signal appears. The peak of fluorescence intensity reflects the amount of fluorescence conjugates accumulated on the test line. In this kind of sandwich test by lateral-flow assays, the test line shows no positive signal if the anlayte is not present in the sample. Another antibody irrelevant to the analyte is usually fixed at the control line to confirm that the sample goes through the detection zone and the test works properly. One major advantage of immunochromatography over ELISA is that the method does not need any washing steps (Brooks et al., 1999). In ELISA, several washing steps are required to remove unbound analyte and detector antibody.

We first tested the assay system with a prostate-specific antigen (PSA). PSA is a 30-kDa glycoprotein and is known to be a tumor marker for prostate cancer (Duffy, 1996). Capture monoclonal antibody was immobilized on the test zone and detector antibody was labeled with fluorochrome Cy-5. Production and characterization of the PSA mAbs were described previously (Cho et al., 2003). The serum for testing was obtained from a patient with prostate cancer (SacredHeart Hosptital, Chuncheon). Concentration of the serum from the patient was confirmed with a PSA ELISA kit (MEGA Corp, USA). Samples were prepared by a serial dilution of the high-titer serum from the patient with a normal serum (0, 4, 8, 16, 32 ng/ml). 200 μl of the diluted sample was applied onto the sample pad of the strip, processed for 15 min for immune reaction, and scanned with a microarray analysis system (ScanArray Lite, GSI Lumonics, USA). As shown in the actual fluorescence images of Fig. 2A, the fluorescence intensity at the test line was increased as a concentration-dependent manner. For quantification, the scanned image was converted into a relative fluorescence intensity using Quantity Program. The intensity of PSA arrested on the test zone was calculated and the values were plotted as a function of the concentration. The equation showed a good linearity in Fig 3B. The result suggested that the antibody-immobilized assay system adapted for detection of PSA met the previous assumption and worked accordingly.

We next tested the assay system composed of the membrane fabricated with multiple lines. For a positive control, we used a biotin-streptavidin complex, which is known to show a strong affinity between the components (Santora, 2000). To immobilize capture antibodies on the reaction zone, we dispensed BSA-biotin conjugate on the first line and then four mAbs to C-reactive protein (CRP), carcinogenic embryonic antigen (CEA), alpha-feto protein (AFP), and PSA in order (de Ferranti and Rifai, 2002; Adinolfi et al., 1975; Guinan et al., 1975). The detector solution contained straptavitin, anti-PSA mAb,

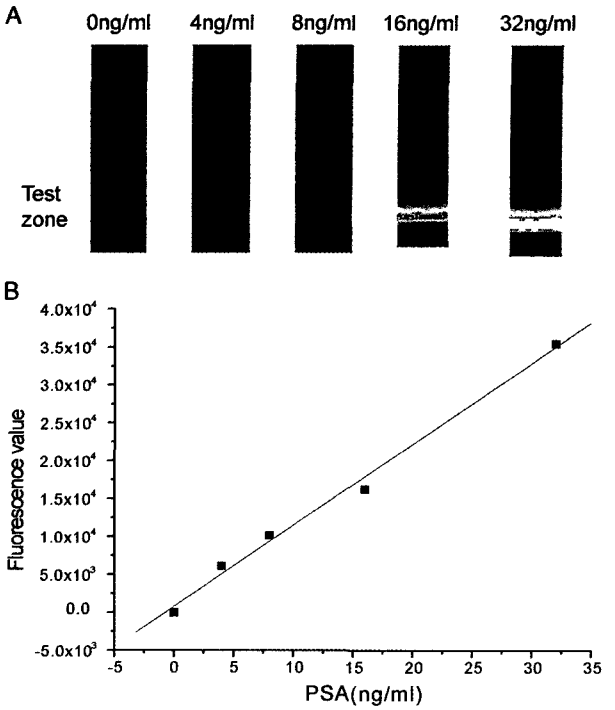

Fig. 2. The fluorescent scanning images of strip prepared for PSA antibody-immobilized system (A) and standard calibration curve prepared by calculating the relative fluorescence intensity on the detection zone (B).

anti-AFP mAb, anti-CEA mAb, anti-CRP mAb. Streptavidin and all of the antibodies were labeled with Cy-5. Sample solution was prepared by spiking the four proteins into a normal serum. The sample was added to the detector solution and the mixture was applied onto the sample pad. After 15 min, the strip was scanned with the ScanArray and the image was converted to a relative intensity of

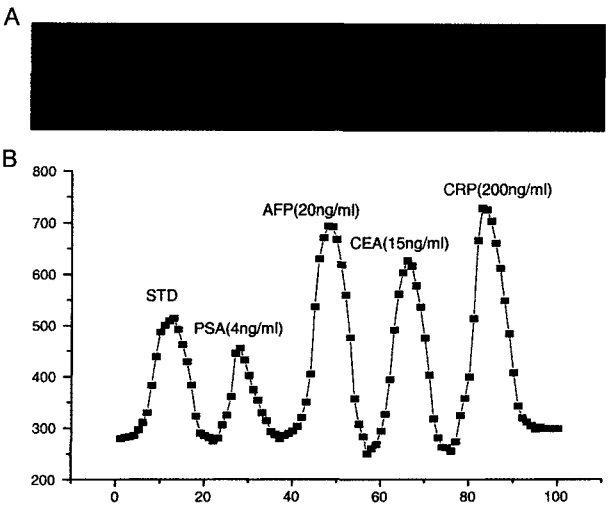

Fig. 3. The fluorescent scanning images of strip of multiple lines prepared in antibody-immobilized system (A) and the relative fluorescence intensity on the test converted from the scanned image (B).

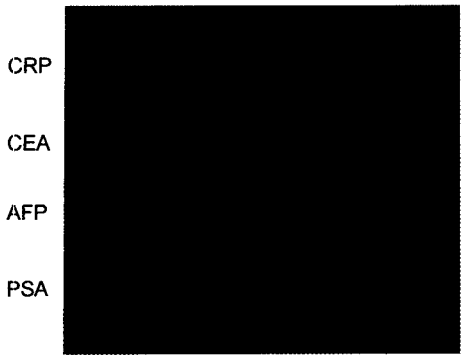

Fig. 4. The fluorescent scanning images of a low-density antibody microarray. Capture antibodies were dispensed at the concentration of 1.5 mg/ml (first column), 0.75 mg/ml (second), 0.3 mg/ml (third), 0.15 mg/ml (fourth), and 0.07 mg/ml (fifth). The concentration of detector antibodies and antigens were 10 μg/ml and 10 ng/ml, respectively. While PSA pairs showed the highest signal at the condition, the fluorescence intensity of CRP was quite weak.

fluorescence (Fig. 3 A & B). The fluorescence intensities of different proteins did not coincident with the concentration of each protein. That is because each antibody has different affinity to the antigen. From the observation that five distinctive bands were detected, the specific antigen-antibody reactions appear to occur on the reaction zone.

To fabricate protein microarrays strip for immuno-chromatography assay, we deliver nanoliter volumes of the antibodies with different concentration onto the membrane with a micro-array machine (Proteogen, Korea) and yielded 20 spots per quarter square centimeter. In this case, we dispensed different concentration of antibodies onto the membrane. Compared to the previous spot, next spot contained half amount of antibody. The spotted antibodies were the same mAbs as used above, antibodies to CRP, CEA, AFP, and PSA. For distinction, we labeled the detector CRP antibody and CEA antibody with the fluorocheome Cy-3, and AFP antibody and PSA antibody with Cy-5, respectively. The known amount of protein analytes in normal serum was mixed with the detector solution and applied onto the sample pad. After reaction, the strip was scanned with the ScanArray (Fig. 4). The fluorescence intensity on each spot was increased as a concentration-dependent manner. The spot immobilized with less amount of the capture antibody showed a weaker fluorescence signal. The result suggested the possibility that we could quantify the target protein by adjusting amount of capture antibody.

We tested a wash-free immunoassay to measure the level of a soluble, immunogenic analyte in samples. Particularly, the assay showed a potential application for the measurement of multiple analytes fabricated on antibody microarray for high-throughput test. Like DNA microarray, the protein microarray need extensive washing steps and an extra probing step. Thus, the whole process takes at least several hours, sometimes longer than overnight. The assay we developed is simple, rapid and usually do not require addition of extra reagents other than a fluid sample containing the analyte. We expect that the assay can facilitate common use of antibody microarray in medical and scientific field for the detection of a wide variety of analytes.

Acknowledgements

We thank Dr Cho Jin Seon for kindly providing PSA sera and normal sera for this study. This work was supported by a grant from the National Research Laboratory Program (M1-0104-00-0164) of the Korean Ministry of Science and Technology.

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[Received January 17, 2003; accepted February 11, 2003]