

AUTOMATIC pH CHANGING SYSTEM FOR SENSITIVITY IMPROVEMENT OF ELISA ON LAB-ON-PAPER

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ABSTRACT

In this work, automatic pH changing system on lab-on-paper devices for enzyme-linked immunosorbent (ELISA) has been developed in order to improve their sensitivity and make the technique much more applicable to real samples. The most widely used enzyme in immunoassay are alkaline phosphatase (ALP) and peroxidase. ALP-conjugated secondary detection with BCIP/NBT substrate provides high sensitivity, however, the enzymatic reaction require alkaline medium. By automatic pH changing system, the immunoreaction is allowed to undergo first in the test zone at the neutral pH. Subsequently, enzymatic reaction occurs at the alkaline pH due to switching of pH from neutral to alkaline.

KEYWORDS: automatic pH changing, lab-on-paper devices, ELISA

INTRODUCTION

In recent years, there is a clear need for a simple, rapid, highly sensitive and selective analytical method for application in a variety of areas such as clinical diagnosis. ELISA is commonly used method in clinical fields. Despite of high sensitivity of ELISA technique, it takes relatively long analysis time due to multistage procedure. Lab-on-paper have recently received considerable interest for the development of point-of-care diagnostics because its affordability and low cost [1, 2]. In previous work, we developed automated paper-based devices for automatic multistep sequencing of sandwich ELISA [3]. The devices with the new concept of multistep flow control were obtained by fabrication of sequence control pattern on a piece of nitrocellulose (NC) membrane and by separating areas of pre-deposited reagents. The fabricated device is practical and can be employed ELISA using alkaline phosphatase (ALP)-conjugated secondary detection systems to detect hCG with a single-step sample application. The optimal pH for the enzymatic reaction of this enzyme and their substrate (BCIP/NBT) were pH 9. However, pH values is one of the factor affecting antigen-antibody reactions, which optimal pH is range from 6.5 to 8.4. Thus, alkaline may reduce antigen-antibody binding capability [4].

Herein, automatic pH changing system has been developed on automated devices. The alkaline condition was prepared at pre-spotted region on devices. Only a single-step application of the sample solution at neutral pH to device, the sandwich ELISA is run automatically. At the test zone, the sandwich immunoreactions occur at neutral pH and enzymatic reactions is followed at alkaline pH.

EXPERIMENTAL

The delaying pattern of lab-on-paper device was fabricated in the NC membrane (HF135MC100; Millipore) by dipropylene glycol methyl ether acetate containing 20% acrylic polymer using an SLT0505-HKF inkjet printing machine (SIJ Technology; Tsukuba, Japan). The outsize rims of the device were cut by following the print line.

Figure 1 shows schematics of the automated paper-based device with pH changing system for the sandwich ELISA. For detection of hCG by ELISA, the anti-mouse IgG pAb and anti-hCG α mAb solutions (0.5 μ L at 1 mg/mL) were immobilized by application to the NC membrane in the control and test zones, respectively. The ALP-anti-hCG mAb (0.5 μ L at 25 μ g/mL), the BCIP/NBT substrate (0.5 μ L at 1:10 dilution) and the alkaline reagents (2 μ L of BCIP/NBT substrate dilution buffer (Dojindo, Japan) with 0.1 μ L of 0.5 M Tris buffer + 0.5 mM MgCl₂ pH 11.3) were spotted by hand onto the NC membrane and allowed to dry. hCG were prepared in 50 mM of Tris buffer pH 7.4.

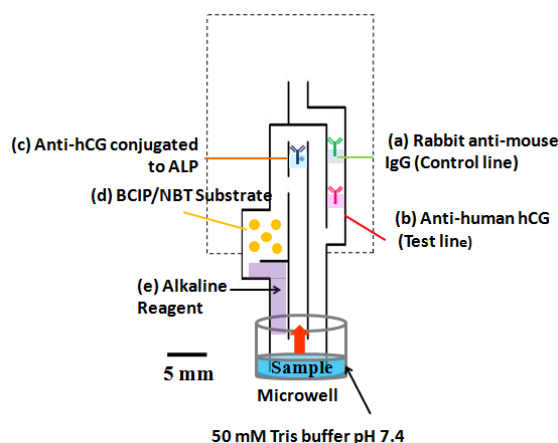


Figure 1: Schematic illustration of the automated paper-based device with pH changing system for the sandwich ELISA. The prepared substances composed on the devices of (a) the control zone, which contains the immobilized Ab to confirm that the test has operated correctly, (b) the test zone, which contains the immobilized specific Abs to the target Ag (forming a sandwich ELISA) and shows a colored band for positive test samples, (c) enzyme-linked detection antibody (the second antibody) that is allowed to bind to the antigen, (d) substrate mixture that reacts with the enzyme label to generate an insoluble colored product, and (e) alkaline reagent.

RESULTS AND DISCUSSION

Previous work, we already demonstrated the utility of “automated lab-on-paper” for hCG ELISA. ALP and BCIP/NBT were selected to use as enzyme and substrate because it provides high sensitivity and generates signal color that can be easily identified by naked eye. The enzymatic reaction was optimal at the substrate kit buffer of pH 8.7. To improve the performance and the sensitivity of device for detection of real sample at neutral pH, automatic pH switching system in lab-on-paper device has been proposed.

Initially, the pH for sandwich immunoreaction and enzymatic reaction was optimized using simple test strip (consist of control line, test line and pre spotted ALP-Ab2) with two-step hand operation including sample loading and substrate loading. The buffer and pH used to prepare hCG sample and substrate were shown in Table 1. The best result of 500 ng/mL hCG detection obtained by using 50 mM Tris buffer pH 7.4 and 50 mM Tris buffer pH 8.7 in sample loading and substrate loading steps, respectively (Figure 2). In addition, the advantage of the paper-based device is that the reagent could be prepared on their surface before testing. Therefore, for automatic pH changing system, the substrate region was prepared with alkaline reagents and the sample was loading with neutral pH by 50 mM Tris pH 7.4.

Table 1. The buffer and pH used to prepare hCG sample and substrate for hCG detection.

	Step 1: Sample loading (40µL of 500 ng/mL hCG in buffer)		Step 2:Substrate loading (BCIP/NBT 1:10 dilution with buffer)	
	Buffer	pH	Buffer	pH
I	HEPES	pH 7.4	HEPES	pH 7.4
II	HEPES	pH 7.4	Tris	pH 8.7
III	Tris	pH 7.4	Tris	pH 7.4
IV	Tris	pH 7.4	Tris	pH 8.7
V	Tris	pH 8.7	Tris	pH 8.7
VI	PBS	pH 7.4	PBS	pH 7.4
VII	PBS	pH 7.4	Tris	pH 8.7

*HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
 PBS = Phosphate buffered saline
 Tris = 2-Amino-2-hydroxymethyl-propane-1,3-diol

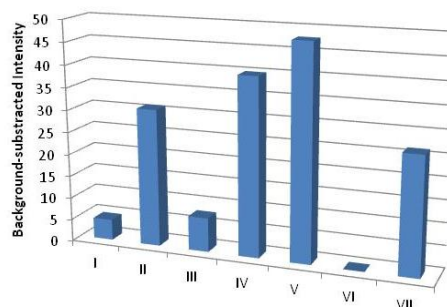


Figure 2: The effect of buffer condition (corresponding to Table 1) for hCG ELISA using ALP-conjugated secondary detection systems on immunoreactions and enzymatic reaction. (To realize the development of automatic pH changing system, Tris pH 7.4 was selected to prepare sample.)

Then, the alkaline reagents for adjusting pH at the test zone was optimized by varying the buffer type, pH and volume. The reagents were pre-spotted below the pre-spotted substrate region on the devices. The results were shown that 0.5 µL of substrate dilution buffer with 0.5 µL of 0.5 M Tris + 0.5 mM MgCl₂ pH 11.3 provided the best performance for detection of 500 ng/mL hCG in 50 mM Tris buffer pH 7.4. The color intensity at the test zone obtained by this system is 10% higher than obtained by the automatic paper-based device without pH changing system with loading of hCG in substrate dilution buffer pH 8.7. This is due to the immunoreactions and enzymatic reaction were preformed under optimized condition including pH value. Therefore, this condition was chosen for subsequent experiment.

Next, the pH gradients in the automated paper-based device with the new system were investigated using cresol red in 50 mM Tris buffer pH 7.4, which changes the color from yellow to pink red in range (7.2-8.8). The results were shown in Figure 3.

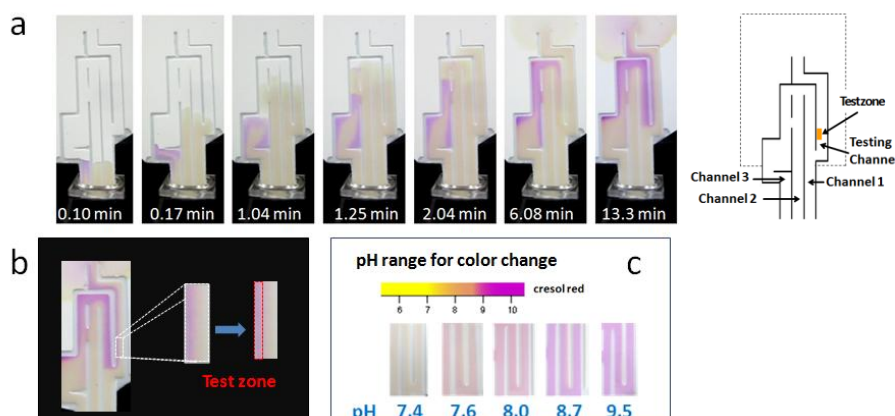


Figure 3: Image of (a) pH gradient in device with automatic pH changing system was observed by cresol red in 50 mM Tris buffer pH 7.4. (b) Focus at test zone, the violet color was observed. (c) The calibration chart of pH changing color for the screening test.

By automatic pH changing procedure, the alkaline reagent is automatic delayed by delaying pattern on the device. After the sample solution is applied to the device (Figure 3A), the sample fluid flow in channel 1 passes through test area with pH 7.4. While, the analyte solution migrates to the pre-spotted region of the alkaline reagents in channel 3. The alkaline reagent is rehydrated and the front move to test area with pH about 9. After alkaline reagent arrive at test zone, the pH is switched from 7.4 to 9 (Figure 3B). These results indicated that the pH is automatically switched at test zone for ELISA, after immunoreactions at neutral pH, which allows enzymatic reaction at alkaline condition. The changing of pH at the test zone is obtained by the delaying pattern on the device allowing the alkaline pH start to migrate pass through test zone after sandwich immunoreactions occurred.

Figure 4 shows the calibration curve of hCG detection in 50 mM Tris buffer pH 7.4 using pH changing system on automated paper-based device. The detection limit for hCG were 25.9 ng/mL (3.7 mIU/mL), calculated from separate devices using three times the standard deviation (3σ) of the intensity of zero concentration ($n = 3$); which is lower than previous work (8.7 mIU/mL). This increase in sensitivity is due to improvement of antigen-antibody binding capability by pH 7.4, and also optimization of pH condition of immunoreactions and enzymatic reaction.

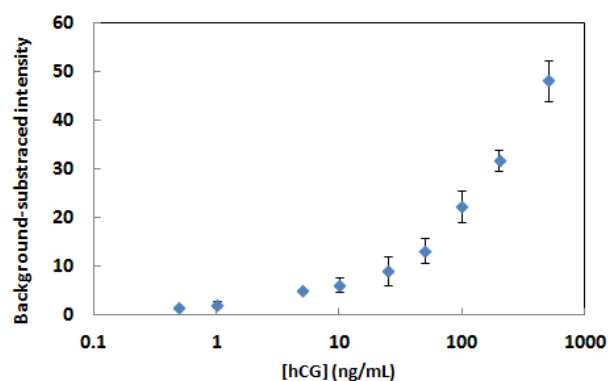


Figure 4: Calibration plot of the hCG ELISA results from 1–1000 ng/mL hCG in Tris buffer solution pH 7.4 on the automated paper-based device with pH changing system. The mean intensity values of each data point were obtained by using a digital camera (IXY200F, Canon, Japan, operated in automatic mode with no flash and using macro imaging setting) capture the results image on the paper-based device. Then, the mean color intensity of the image at the selected area was quantified using the histogram function with the RGB channel in Adobe Photoshop CS3.

CONCLUSION

In our previous work, an automatic paper based device for one-step ELISA with a single-step sample application were demonstrated. Here, we successfully made the automatic pH changing system in the devices. This system provided the optimized pH condition for immunoassay and enzyme reaction independently, which had an advantage especially for ELISA using ALP-conjugated secondary detection systems. Samples were loading at neutral pH into the device. The multi steps required for ELISA and pH change were automatically executed by the delaying pattern and the pre-spotted reagents at different locations in the devices. The pH was switched after the antigen-antibody binding. Finally, at the test zone, the ALP-Ab and BCIP/NBT reaction occurs at alkaline condition. This technique was successfully applied for simple analysis of hCG, and a detection limit of 3.7 ng/mL was obtained. The simple analysis using automatic paper based device with the automatic pH changing system makes the approach especially attractive for a wide range of clinical or environment sample.

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