Pressed Paper-Based Dipstick for Detection of Foodborne Pathogens with Multistep Reactions

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Supporting Information

**ABSTRACT:** This paper presents a pressed paper-based dipstick that enables detection of foodborne pathogens with multistep reactions by exploiting the delayed fluid flow and channel partition formation on nitrocellulose (NC) membrane. Fluid behaviors are easily modified by controlling the amount of pressure and the position of pressed region on the NC membrane. Detection region of the dipstick is optimized by controlling flow rate and delayed time based on Darcy’s law. All the reagents required for assay are dried on the NC membrane and they are sequentially rehydrated at the prepartitioned regions when the device is dipped into sample solution. In this manner, multistep reactions can be facilitated by one-step dipping of the dipstick into the sample solution. As a proof of concept, we performed detection of two fatal foodborne pathogens (e.g., *Escherichia coli* O157:H7 and *Salmonella typhimurium*) with signal enhancement. In addition, we expanded the utilization of channel partitions by developing a pressed paper-based dipstick into dual detection format.

Lateral flow tests (LFTs), also known as lateral flow immunochromatographic assays, have been widely used in various forms to detect a wide range of targets. LFTs offer many advantages as a rapid detection platform, including portability, user-friendliness, and simple operation principle that utilizes capillary forces. However, conventional strip-type format is not adequate to carry out sequential delivery of multiple reagents, which is essential to perform multistep reactions such as sandwich immunoassay and a number of colorimetric reactions for enhancing test results.

To overcome this limitation, a number of paper-based analytical devices that can perform multistep reactions have been reported. Some of the earlier approaches include cross-flow chromatography and bidirectional LFTs. However, they achieved multistep reactions manually by changing the location of fluid source pads or switching the dipping orientation of the test strip and not by controlling fluid flow in the paper channel. Therefore, with the goal of performing multistep reactions, they still need to be improved in many ways. As a widely available method, geometry modification of paper fluidic channel requires an increase in the channel length for controlling fluid flow. It means that the method is spatially limited, and the resulting channel would require larger sample volume to wet the channel, which is not suitable for treating a small volume sample like blood. Also, programming of fluid delivery time in such devices still requires redesigning of fluidic channels, which can be time-consuming and complicated. On the other hand, some methods for controlling fluid flow are not easily adjustable for desirable differentiated fluid flow. In addition, several methods require a complicated fabrication process, including chemical treatment and various use of a large amount of equipment. Finally complicated fabrication process increases time and cost, which is not suitable for large scale manufacturing. Therefore, a more simple method for programming fluid flow in paper fluidic channel is desirable.

To date, many foodborne illnesses caused by pathogenic bacteria such as *Escherichia coli* O157:H7, *Staphylococcus aureus*, and *Salmonella* species have been reported. Because above pathogenic bacteria show a high fatality rate with severe symptoms, pathogenic bacteria require rapid on-site detection. Therefore, a number of detection methods for foodborne pathogens have been developed using polymerase chain reaction (PCR), magnetic force based methods, and other complicated methods. However, they require external equipment and complicated operation principle that is not suitable for on-site detection. LFTs which are suitable for on-site detection for foodborne pathogens have been presented in various forms, including conventional LFTs, triple-
branched strip,24 multiple test lines in single strip,25 and manual multistep assays.26−28 Although foodborne pathogens have been detected with higher performance in manual multistep assays that overcome limitation of conventional LFTs, they did not program fluid flow that results in requirements for manual loading of multiple reagents sequentially. Finally, manual loading of multiple reagents results in complicated multistep operation principle which is not suitable for on-site detection. Therefore, detection of foodborne pathogens with one-step operated multistep reactions by programming fluid flow is in demand for higher performance of test and convenient use by untrained end-users.

Here, we present a detection method of foodborne pathogens with multistep reactions in pressed paper-based dipstick. Generally, to achieve multistep reactions without multiple reagent loading in paper-based microfluidic device, channel partitions for dividing multiple channels as well as a method for programming fluid transport are required. In addition, all the reagents required for assay should be preloaded on the device. It is highly desirable that one-step operation of multistep reaction is achieved by dipping the device into the sample solution. Previously, we reported a simple method for controlling fluid flow on pressed polypropylene sheet.29 Unlike previous work, one-step operation of multistep reactions is achieved by utilizing both delayed fluid flow and channel partitions which are made by pressing the nitrocellulose (NC) membrane using only a press machine. Particularly, the delayed fluid flow can be simply modified by controlling the amount of pressure as well as the position of pressed region. After optimizing the device design by controlling the flow rate and delayed fluid flow, we finally demonstrate detection of fatal foodborne pathogens (e.g., Escherichia coli O157:H7 and Salmonella typhimurium), which enables one-step operated multistep reactions. Furthermore, by utilizing multiple channel partitions, we developed multiple channels that each channel shows different flow velocity. Then we adapted this strategy into integration for dual detection format.

■ EXPERIMENTAL SECTION

Operation Principle. A pressed paper-based dipstick consists of three channels divided by two channel partitions. Channels 1 and 3 are partially pressed to cause a delay in fluid flow. There exist two tests, which consists of capture antibody (Ab) for each genus of pathogen. Two kinds of Ab conjugated gold nanoparticles (AuNPs) (for each genus of pathogen) and three components of gold enhancers (i, enhancer; ii, activator; and iii, initiator) are preloaded and dried on the NC membrane. For the enhancer to work properly, components i and ii need to be mixed first, before component iii is added. Therefore, we made a gap between ii and iii for incubation time between i and ii. The device operates by just one-step dipping into a sample solution (Figure 1). Two kinds of analytes in a sample solution flow into channel 2 (the first flow) and they rehydrate Ab conjugated AuNPs on the NC membrane. Each analyte reacts with its Ab conjugated AuNPs and corresponding immunocomplexes are captured by capture antibodies at the test line. Then colorimetric signals for each analyte are developed and are attached to the surface of Ab conjugated AuNPs so that they amplify colorimetric signals at each test line.12 Visualization of the fluid flow in the pressed paper-based dipstick with food dyes confirms that fluid flowing through channel 2 reaches the test line first, followed by delayed fluid flow through channels 1 and 3 (Figure S1 of the Supporting Information). Finally, enhanced signals are analyzed and used for the determination of concentration of each analyte.

Materials. Anti-E. coli O157 Ab and anti-S. typhimurium Ab were purchased from Abcam (Cambridge, U.K.). Antimouse IgG Ab produced in goat, AuNPs (diameter = 20 nm), succrose, Tween-20, sodium chloride (NaCl), potassium carbonate (K₂CO₃), tartrazine used as a yellow dye, and erioglaucine used as a blue dye were purchased from Sigma-Aldrich (St. Louis, MO). An absorbent pad (grade 222) was purchased from Alshorm (Finland).

A glass fiber pad (GFCP103000) was purchased from Millipore (Billerica, MA). A NC membrane (CNPC-SS12, 15 μm) which pore size is large and sufficient for penetration of pathogens was purchased from Advanced Microdevices (India). Gold enhancer solution was purchased from Nanoprobes (Yaphank, NY). Luria–Bertani (LB) broth and nutrient broth were purchased from BD Difco (Sparks, MD).

S. typhimurium (ATCC 14028) was cultured in nutrient broth, while E. coli O157:H7 (ATCC 35150) and Staphylococcus aureus (ATCC 25923) were cultured in LB broth. The concentration of cultured bacteria was determined by measuring optical density at 600 nm (OD₆₀₀). Bacteria were diluted into desirable concentration with phosphate-buffered saline (PBS) and were used in the experiments.
Calibration of Pressed NC Membrane Strip. A NC membrane was cut into strip (4 mm × 60 mm) with a knife. The NC membrane strip was placed on a load cell (CLS-1 T; Curiosity Technology, Paju, Korea). After acrylic stamp was placed over desirable position, the NC membrane strip was pressed by a hand press machine (SWP-HP180-120S; Sam Woo, Siheung, Korea). Pressure was controlled by monitoring the indicator connected to the load cell. For calibration, 500 μL of distilled water was dropped into the loading region (stacked glass fiber over the pressed NC membrane strip) of pressed NC membrane strip and the position of the fluid front was measured with respect to the time.

Assessment of Channel Partition Formation. A metal wire was amputated from the pin by removing the sharp and blunt part. Two cm long metal wire whose diameter was 0.75 mm was placed on desirable position of the NC membrane and pressed by a hand press machine. Because we cannot figure out the exact area of channel partition, we assessed a channel partition formation according to the applied pressing force. A volume of 3 μL of NaCl solution were loaded into a 96-well plate. A volume of 150 μL of AuNPs solution were loaded into a 96-well plate. A volume of 3 μL of K2CO3 solution was loaded into each well and pH to be adjusted to ~9 (isoelectric point of Abs). A volume of 10 μL of each Ab at different concentrations (e.g., 10, 20, 50, 100, 200, and 500 μg/mL) was loaded into each well and was incubated at room temperature for 20 min. Then 20 μL of NaCl solution was loaded into each well. AuNPs are not aggregated when the amount of Ab is sufficient for conjugation to AuNPs. In contrast, there is aggregation among AuNPs when the surface of AuNPs is insufficiently conjugated with Ab. After 20 min incubation at room temperature, a spectrum (350 to 750 nm) was obtained by spectrometer (SpectraMax 250; Molecular Devices, Sunnyvale, CA, USA). When AuNPs are aggregated, colors are changed from red to purple, due to the peak absorbance shifts from 520 to 580 nm. To analyze the saturated point, absorbance difference between 520 and 580 nm was plotted according to the increased concentration of Abs. From the plotted data, we determined the optimum concentration for anti-E. coli O157 Ab and anti-S. typhimurium Ab to be 200 μg/mL (Figure S2 of the Supporting Information).

Preparation of Ab-Conjugated AuNPs. A volume of 15 μL of K2CO3 solution was mixed with 750 μL of 20 nm AuNPs solution. In total, 50 μL of optimized concentration of Ab obtained by gold aggregation test was loaded. After 20 min incubation at room temperature, 100 μL of 3% bovine serum albumin (BSA) in distilled water was loaded for blocking. After 20 min incubation, unbound Abs were washed by removing supernatant after 30 min centrifugation at 8700 rpm at 4 °C. A couple of tubes obtained after the above processes were resuspended together into 50 μL of 3% BSA, 0.05% Tween-20 in PBS.

Fabrication of Pressed Paper-Based Dipstick. The NC membrane was cut with a laser cutter (C40-60W; Coryart, Anyang, Korea) to pattern the device. Channel partitions were formed on the prearranged position of the NC membrane by pressing over metal wire. Then predefined regions in the NC membrane were pressed for delayed fluid flow by pressing over an acrylic stamp. A pressed paper-based dipstick includes two pressed regions for delayed fluid flow and two channel partitions for dividing channels (Figure 2). As shown in Figure 2A, each region exhibits different pore sizes. When the NC membrane is exposed to pressure, decreased pore size plays a role as a fluid resistance that causes delayed fluid flow. In addition, when the NC membrane is exposed to a high pressure, pores are collapsed to an extent sufficient to prevent fluid flow. Consequently, fluid cannot easily invade the pressed region that works as a channel partition. An absorbent pad was attached by overlapping with the top part of NC membrane. The 1 mg/mL anti-E. coli O157 Ab, 1 mg/mL anti-S. typhimurium Ab, and 1.1 mg/mL antimois IgG Ab were dispersed on the NC membrane with dispenser (LPM-02; Advanced Microdevices, India). After drying in a desiccator at room temperature, modified papers were partially blocked with 7.5 μL of 3% BSA, 0.25% Tween-20, and 10% sucrose in PBS. After the modified papers were dried in a desiccator at room temperature, 0.5 μL of Ab conjugated AuNPs were dispensed on the dual fixed positions of the modified papers and were dried in the desiccator again at room temperature. This process was repeated once more. Finally three components of gold enhancer i, ii, and iii were dispensed on the three bottom regions of the NC membrane and the modified papers were dried in the desiccator. After the modified papers were cut into one by one, it can be utilized as a pressed paper-based dipstick.

A homemade cartridge for pressed paper-based dipstick was fabricated using a 3D printer (3Dison Multi; Rokit, Korea), which was utilized for one-step dipping of sample solution (Figure 2B). After developing colorimetric signals, dual analytes can be detected through the detection windows (Figure 2C).

Image Analysis. The colorimetric signals at the detection regions were captured with stereoscope (SZX16; Olympus, Japan). On the basis of flow demonstration with yellow and blue dyes in the device, the length of the left and right regions of interest (ROI) were determined. On the basis of the length of ROIs, signal intensities were analyzed with ImageJ software.
Signal intensities of ROIs were normalized by subtracting signal intensity of 0.7 mm downstream from ROIs.

**RESULTS AND DISCUSSION**

Delayed Fluid Flow in the Pressed NC Membrane Strip. The NC membrane was partially pressed with different amount of applied pressure after placing an acrylic stamp on the position that need to be pressed. As shown in Figure 3A, the pore of pressed region in the NC membrane was collapsed and the height of pressed region was also decreased. We found that the porous networks were mechanically collapsed in paper and did not recover their porosity over the time due to inelastic property of NC membrane. Over the applied pressure of 19.6 MPa, thickness decrease in the NC membrane was almost saturated at a certain thickness (Figure S3 of the Supporting Information). To investigate an effect of pressed region on the fluid flow, we conducted wicking experiments using distilled water. When the equal amount of water was applied to the membrane, the wicking distance of fluid was plotted with respect to the time (Figure S4 of the Supporting Information). Delayed fluid flow was observable after the pressed region, showing better repeatability than that of polypropylene. To measure the minimum force required for channel partition in the NC membrane, a metal wire was placed on the prearranged position of NC membrane. Then pressure much higher than that for the delayed fluid flow that is sufficient to prevent fluid flow was applied over the metal wire. When the channel partition was successfully formed on the NC membrane, pores were collapsed to an extent sufficient to prevent fluid flow compared to pores in the unpressed region (Figure 4A–D). The width of channel partition was around 300 μm. To measure the minimum force required for channel partition, a different amount of force over the metal wire was also assessed. The role of channel partition is that fluid flowing through the left channel should not invade into the right channel because dried reagents in the right channel should be rehydrated by delayed fluid flow. To estimate the feasibility of channel partition, we designed a pressed paper-based dipstick that consisted of two channels whose width were the same and one channel had a pressed region for delayed fluid flow. The pressed region was pressed with 14.7 MPa, which is sufficient pressure for delaying fluid flow. As shown in Figure 4E, the channel partition was well developed at 250 kgf (∼400 MPa). However, the NC membrane was torn when the applied force was over 350 kgf (∼570 MPa; data not shown). Therefore, the
pressed channel partition on the NC membrane was formed when a pressing force was between 250 and 350 kgf.

Optimization of Pressed Paper-Based Dipstick and Detection of *E. coli* O157:H7 and *S. typhimurium*. To test the feasibility of pressed paper-based dipstick as a multistep assay platform, we designed a pressed paper-based dipstick with two channels. It has a single detection zone and dual channels, whose right channel includes a pressed region for delayed fluid flow (Figure 5A). The ratio of w1 to w2 was 1:1 and the pressed region in the right channel was only pressed with 9.8 MPa. (B) The picture of pressed paper-based dipstick that detected 10^5 CFU/mL of *S. typhimurium*. Its format was the same with that of panel A. (C) The picture of the test line in the pressed paper-based dipstick indicated by the red dotted box in panel B (unit: CFU/mL). (D) Signal intensity of the left and right ROIs was analyzed according to increasing concentration of *S. typhimurium*. The error bars represent the standard deviation of three replicates of the assay. (E) Flow demonstration at equilibrium in an optimized pressed paper-based dipstick that the ratio of w1 to w2 is 3:5. The pressed region was pressed with 4.9 MPa. (F) The picture of the optimized pressed paper-based dipstick that detected 10^6 CFU/mL of *S. typhimurium*. Its format was the same with that of panel E. (G) *E. coli* O157:H7 and *S. typhimurium* were detected in an optimized pressed paper-based dipstick with respect to increasing concentration. The error bars represent the standard deviation of three replicates of the assay.

Figure 5. Determination and optimization of detection region. (A) Flow demonstration at equilibrium in pressed paper-based dipstick that the ratio of w1 to w2 is 1:1. The pressed region was pressed with 9.8 MPa. (B) The picture of pressed paper-based dipstick that detected 10^4 CFU/mL of *S. typhimurium*. Its format was the same with that of panel A. (C) The picture of the test line in the pressed paper-based dipstick indicated by the red dotted box in panel B (unit: CFU/mL). (D) Signal intensity of the left and right ROIs was analyzed according to increasing concentration of *S. typhimurium*. The error bars represent the standard deviation of three replicates of the assay. (E) Flow demonstration at equilibrium in an optimized pressed paper-based dipstick that the ratio of w1 to w2 is 3:5. The pressed region was pressed with 4.9 MPa. (F) The picture of the optimized pressed paper-based dipstick that detected 10^5 CFU/mL of *S. typhimurium*. Its format was the same with that of panel E. (G) *E. coli* O157:H7 and *S. typhimurium* were detected in an optimized pressed paper-based dipstick with respect to increasing concentration. The error bars represent the standard deviation of three replicates of the assay.

pressed region in the right channel was only pressed with 9.8 MPa, which is the minimum applied pressure that allows signal enhancement which requires multistep reactions. For the signal enhancement, Ab conjugated AuNPs should pass through the test line before gold enhancer reaches the test line. The minimum pressure was chosen from the tested pressures in Figure 3B. Under 9.8 MPa, a time interval between fluid flow along the left and right channels was insufficient for multistep reactions. It means that Ab conjugated AuNPs cannot reach at the right ROI and results in no signal generation in the right ROI (Figure S5 of the Supporting Information). To estimate the performance of the pressed paper-based dipstick, we detected 10^5, 10^6, and 10^7 CFU/mL of *S. typhimurium*. As shown in Figure 5A, two distinct regions (left and right ROIs) were visualized at the test line due to the laminar flow profile caused by continuous flow supply. Usually a laminar flow profile is developed when flows along different paper channels are merged and reach equilibrium.31

The width of each color dye is inversely proportional to the applied pressure at the pressed region.29 Therefore, the length of left and right ROIs at the test line is different. To investigate the length of left and right ROIs, we measured the width of blue and yellow dyes’ portion at the test line. It was shown that the width of left and right ROIs were 2.54 and 1.46 mm, respectively. Signals were amplified only in the right ROI because rehydrated gold enhancer flows only through the right ROI (Figure 5B,C). On the basis of the length of ROIs, we analyzed the signal intensity of the left and right ROIs. Right ROI showed higher signal intensity than the left ROI because of signal amplification (Figure 5D). Moreover, it is worth noting that invisible signal at the left ROI becomes visible at the right ROI with signal amplification for the detection of 10^6 CFU/mL *S. typhimurium*. This indicates that the AuNPs signal alone, which could have suggested a negative test result, can be corrected with enhancement to prevent a false negative result. In other words, false negative signal in the left ROI was distinguished by signal amplification in the right ROI. Also it can be understood that the detection limit of *S. typhimurium* was improved about 10 times with signal enhancement. Accordingly, right ROI should be utilized as the detection region. However, the length of right ROI was too short to identify with the naked eye. The reason why the right ROI is shorter than the left ROI is that the flow rate right channel is decreased by the pressed region. It can be understood based on Darcy’s law. When sample volume is continuously applied at source pad with enough capacity of absorbent pad, flow rate can be represented by Darcy’s law in the case of fully wetted flow.

$$Q = -\frac{\kappa WH}{\mu L} \Delta P$$

where Q is the volumetric flow rate, κ is the permeability of the porous network, W is the width of the channel, H is the thickness of the paper, μ is the viscosity of fluid, L is the length of paper channel, and ΔP is the pressure difference. When the NC membrane is pressed, the thickness of the paper and permeability of the porous network are decreased, resulting in a decrease in flow rate.

To extend visibility at the right ROI, we controlled three components of the pressed paper-based dipstick: (1) the position of channel partition, (2) the amount of pressure for pressed region, and (3) the position of pressed region. We increased the flow rate along right channel based on Darcy’s law and behavior of delayed fluid flow was controlled in the right channel. First we increased the term W by increasing the width of right channel by 0.1 cm. Second, we increased the terms H and κ by decreasing the amount of applied pressure for the pressed region into 4.9 MPa. However, mentioned above, 4.9 MPa is an insufficient applied pressure for sequential delivery. On the other hand, based on the calibration of pressed NC membrane strip, delayed fluid flow occurs after the pressed region. As the flow rate is not also affected by the position of...
pressed region, we moved the position of the pressed region downstream 0.5 cm to supplement a decrease in delayed time caused by decreasing applied pressure into 4.9 MPa. As a result, we increased the length of the right ROI from 1.46 ± 0.02 mm to 2.26 ± 0.09 mm.

Increased length of the right ROI was identified by flow demonstration with blue and yellow dyes (Figure 5E), which was also confirmed by the increased length of the amplified signal (Figure 5F). Nonuniform signal in test line is caused by rehydration of dried Ab conjugated AuNPs from the NC membrane. This phenomenon has also been presented in LFTs with rehydration of dried reagents on the NC membrane. In this optimized design of the pressed paper-based dipstick, we detected two kinds of foodborne pathogens E. coli O157:H7 and S. typhimurium (Figure 5G). Compared with the results in Figure 5D, a background signal was also developed. However, it does not affect the detection limit dramatically and the overall signal was increased. Therefore, we concluded that the optimized design can be utilized despite the background signal.

Although assay results indicated that bacteria pass through the test line, there would be a little interference between bacteria and the porous network of the NC membrane. To minimize this kind of interference, different compositions of running buffer or blocking solution have been investigated. Accordingly, after adjusting the optimum composition of running buffer or blocking solution, it is also expected to improve sensitivity by increasing the total number of bacteria that pass through the test line. The detection limit of E. coli O157:H7 was around 10⁵ CFU/mL while the detection limit of S. typhimurium was around 10⁶ CFU/mL. The detection limit is not as low as that of manual multistep assays; it would be improved after further optimization procedures, including concentration of capture antibodies, rehydration condition of dried reagents, and adjustment of device dimension. Another way to increase the sensitivity is to adjust the size of the AuNPs. For example, 40 nm is the most commonly used size of AuNPs for LFTs because it results in maximum visibility with the least steric hindrance when conjugated with antibodies.

In addition, for foodborne pathogen detection, the sample volume is large and sufficient for preconcentration procedures unlike clinical samples. Therefore, weakness in the detection limit would be also improved by preconcentration procedures such as immunomagnetic separation and centrifugation. Commercial LFTs require pretreatment as well. Furthermore, aside from signal enhancement of AuNPs-based colorimetric signal, we expect this platform can also be applied to perform other multistep reactions for a wide range of analysis and diagnosis purposes.

Utilization of Multiple Channel Partitions and Its Application for Dual Detection Format. To demonstrate an availability of a number of pressed channel partitions for dividing multiple channels that each channel has different fluid velocity, we utilized two channel partitions for dividing three channels. By applying the optimized design of pressed paper-based dipstick, we newly designed a pressed paper-based dipstick for dual detection E. coli O157:H7 and S. typhimurium. The left detection zone is assigned for E. coli O157:H7 and the right detection zone is assigned for S. typhimurium. We detected a mixture of 10⁷ CFU/mL E. coli O157:H7 and 10⁷ CFU/mL S. typhimurium as well as 10⁸ CFU/mL E. coli O157:H7 and 10⁵ CFU/mL S. typhimurium. To confirm negative control and cross-reactivity with other foodborne pathogens, we also tested 10⁸ CFU/mL S. aureus which was the highest concentration that we detected with target pathogens. As shown in Figure 6A, colorimetric signals were generated at the test and control lines.

In addition, colorimetric signal was developed according to the corresponding sample solution (Figure 6B). The enhanced signal at each ROI indicated by red arrows was regarded as a detection signal of the analyte. Control line indicates that device worked properly and the signal at the test line is reliable. On the basis of the signal intensities at the two detection regions, we compared dual signals of two analytes (Figure 6C). The analyzed signal intensity showed similar appearances with the results in Figure 5G. Also, there was no interruption between two kinds of pathogens by comparing signal intensities of the mixture of two kinds of pathogens and that of individual pathogens. No cross-reactivity with other foodborne pathogen was confirmed with S. aureus. Therefore, applicability of multiple channel partitions was confirmed by dual detection of foodborne pathogens.

Furthermore, as different application, one-step operation of many kinds of multistep reactions that require more reaction steps than signal enhancement is expected to be performed by forming multiple channels that each channel shows differentiated fluid flow (Figure S6 of the Supporting Information). Differentiated fluid flow along each channel in four-partitioned NC membrane was simply developed by controlling the amount of applied pressure as well as the position of the pressed region. Also, because we have been confirmed applicability for multiple use of channel partitions by...
demonstrating dual detection format, multiple detection of target analytes with multistep reactions can also be simply achieved by integrating pressed paper-based dipstick side-by-side with channel partitions.

**CONCLUSIONS**

In summary, we developed a pressed paper-based dipstick for detection of foodborne pathogens with one-step operated multistep reactions by utilizing delayed fluid flow and channel partition formation. Both delayed fluid flow and channel partitions were demonstrated by pressing a NC membrane. It is worth noting that the formation of both partitioning and the pressed region for delayed fluid flow can be realized by the same method. In particular, the delayed time was easily controlled by varying the amount of pressure as well as the position of the pressed region. By simply controlling the flow rate and programming delayed fluid flow along each channel, we achieved an optimum design of the pressed paper-based dipstick. In this manner, a whole process required to fabricate the pressed paper-based dipstick for multistep reactions was achieved by simple pressing. Finally, we performed detection of two kinds of foodborne pathogens *E. coli* O157:H7 and *S. typhimurium*. As a kind of multistep reactions, we performed signal enhancement procedures. All assay procedures do not require multiple reagent loading step and the measurement is simply operated by one-step dipping the device into the sample solution. In this sense, our method would be widely applicable on-site detection that requires multistep reactions. In addition, untrained people are expected to easily utilize pressed paper-based dipstick for on-site detection of foodborne pathogens.

For the wide use of the pressed paper-based dipstick, manufacturing repeatability should be accompanied by different pieces of pressed papers. There was no significant difference in average wicking time regardless of the date of device production as well as the date of measurement for pressed paper which were fabricated on the same day (Figure S7 of the Supporting Information). On the basis of these results and the repeatability of the manufacturing process of the pressed papers, it is improbable that the deviation in the fluid flow between devices contributed to the deviation in the average signal intensity of bacteria detection. It is assumed that the deviation in the signal intensity of bacteria detection would be derived from reagent dispensing procedures. Meanwhile, the sensitivity and selectivity of detection would depend largely on the quality and specificity of antibodies used. Therefore, high-quality antibodies and high-end dispensing instruments as well as further optimization of the dispensing method are expected to increase the performance of the devices on the mass production scale.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsaanalchem.5b04743.

Details of analysis, and additional information (Figures S1−S7) (PDF)

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Notes

The authors declare no competing financial interest.

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