Multiplexed Detection of Foodborne Pathogens from Contaminated Lettuces Using a Handheld Multistep Lateral Flow Assay Device

Joong Ho Shin,† Jisoo Hong,‡ Juhwan Park,† Minsuk Kong,§ Sangryeol Ryu,§ Kwang-Pyo Kim,|| Eunjung Roh,†∥ and Je-Kyun Park*†‡§

†Department of Bio and Brain Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea
‡Microbial Safety Team, National Institute of Agricultural Sciences, Rural Development Administration, Wanju-gun 55365, Republic of Korea
§Department of Agricultural Biotechnology, Seoul National University, Seoul 08826, Republic of Korea
||Department of Food Science and Technology, Chonbuk National University, Jeonju 54896, Republic of Korea

Supporting Information

ABSTRACT: This paper presents a handheld device that is capable of simplifying multistep assays to perform sensitive detection of foodborne pathogens. The device is capable of multiplexed detection of Escherichia coli (E. coli) O157:H7, Salmonella Typhimurium (S. Typhimurium), Staphylococcus aureus, and Bacillus cereus. The limit of detection for each bacterium was characterized, and then, the detection of bacteria from contaminated fresh lettuces was demonstrated for two representative foodborne pathogens. We employed a sample pretreatment protocol to recover and concentrate target bacteria from contaminated lettuces, which can detect $1.87 \times 10^4$ CFU of E. coli O157:H7 and $1.47 \times 10^4$ CFU of S. Typhimurium/1 g of lettuce without an enrichment process. Lastly, we demonstrated that the limit of detection can be reduced to 1 CFU of E. coli O157:H7 and 1 CFU of S. Typhimurium/1 g of lettuce by including a 6 h enrichment of contaminated lettuces in growth media before pretreatment.

KEYWORDS: contaminated lettuces, foodborne pathogens, handheld device, lateral flow assay, multiplexed detection

INTRODUCTION

Foodborne illness is a serious problem that causes a number of problems such as nausea, diarrhea, hospitalization, and even death. In the United States, estimates of more than 9 million foodborne illnesses are reported each year. 1 Although it is known that the use of proper heat in meat and poultry cooking prevents infection, such a heating process is skipped if fresh products such as fruits and vegetables are to be consumed fresh. With the increased consumption of fresh products for the prevention of chronic diseases, the number of foodborne outbreaks from the consumption of fresh products has also increased. 5,6 Furthermore, globalization and increasing trade along with a faster distribution of food products also increase the risk of outbreaks. 4 Thus, a rapid detection process needs to be developed that can be applied to the food industry in order to prevent future outbreaks.

The current detection method for fresh products, which is considered as a “gold standard”, is a culture-based method. 5,6 The method requires a series of processes that involves stomaching, enrichment, bacteria culture, and identification of target bacteria. First, to remove bacteria present on the surface of and also deep within the food sample, the sample is put in a sterile plastic bag with bacteria growth medium and put into a stomacher machine. The machine vigorously pounds the outer surface of the plastic bag, repeatedly and violently compressing and shearing the food sample. 7 Then, the extracts are collected into sterile tubes and are put in a 37 °C incubator over a long period of time, usually between 18 and 24 h to enrich and increase the number of bacteria. This process usually ends up containing nontarget bacteria, and it is impossible to identify the presence of target bacteria in this state. The enriched sample is then streaked and incubated for another 24 h on an agar plate for colony analysis. Then, a colony has to be isolated to ensure purity and an additional incubation is required. The purified colonies can be identified visually in this state; however, a combination of assays such as agglutination assay, additional culture on special media, enzyme/biochemical tests, and target-specific selective gram staining are required to confirm the identity of the colony. This whole process is labor intensive, requires proper lab equipment, and takes several days (3−5 days) for the analysis result.

Many methods of rapid detection have been attempted as an alternative to the time-consuming culture-based method. Some of the methods include the use of enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), 8 bioluminescence signal, 9 flow cytometry, 10 and latex bead agglutination test. 11 ELISA is reported to detect bacteria in the order of $10^4$ colony-forming unit (CFU)/mL, 12 and PCR provides highly sensitive and highly specific detection results due to the amplification of the target fragment. Although both ELISA and PCR can provide sensitive detection results, they
are both complicated, require a trained technician, as well as expensive and bulky equipment, which hinders them from being applied for on-site detection. For on-site analysis purposes, a lateral flow test (LFT) is one of the most promising methods due to its convenience, quick readout (10–20 min), and the signal that can be interpreted with the naked eye. The operation starts by loading a liquid sample into the sample pad. The sample flows forward and rehydrates predried labeling agents and forms immunocomplexes. The immunocomplexes continue to flow through the strip by capillary force, and they are captured by antibodies on the test line. The flow is sustained by the absorbent pad, and the result appears as a visible line with the naked eye. LFT kits for various pathogens exist on the market today, but there is still room for improvement in terms of the limit of detection.

One way to simplify and improve the detection limit of LFT is to add an additional reagent loading step and perform a multistep assay on LFT. By increasing the size of the gold nanoparticles (AuNPs) as signaling probes with enhancers after completion of an assay, the signal intensity can be increased and a 100-fold improvement of the detection limit was demonstrated. Enzyme-linked immunoreactions were also performed on LFTs by first applying a sample along the length of the strip and then flowing a substrate across the width of the strip for enzyme–substrate-based signal generation. The processes however require manual reconfiguration of multiple pads and wait for the primary reaction to finish before performing a secondary reaction. Although such requirements are not suitable for on-site detection purposes, the multistep assays have demonstrated sensitive analyte detection, and in order to simplify the assay process, customized packaging technologies are being developed to perform the sequential tasks with ease.

With the recent advancement in paper-based fluidics and by utilizing fluid delay phenomena, an engineered paper network with multiple channels of different fluidic path lengths was developed. Fluids travel a longer time in the longer channel, thus arriving at the test zone much later as compared to fluids that flow through a shorter channel. The device exploits this mechanism to perform the sequential flow of samples, buffers, and enhancing solution to increase the sensitivity of an assay. However, such a format requires up to 1 h due to the fluidic resistance that increases with increasing channel length, and the use of single wicking pad, whose absorption rate saturates over time.

Our group recently developed a rotary type device that offers a solution to the above-mentioned problem. It was used to simplify the process required for ELISA on LFT and was able to detect *Escherichia coli* (E. coli) O157:H7 in 22 min. In this paper, we modified the design of the handheld device and expanded the number of test strips for multiplexed detection. In order to reduce and simplify the assay steps, we utilized AuNP and enhancer instead of enzyme–substrate pair for colorimetric signal. The device consists of four test lines that indicate the presence of *E. coli* O157:H7, *Staphylococcus aureus* (S. aureus), *Salmonella* Typhimurium (S. Typhimurium), and *Bacillus cereus* (B. cereus), thereby allowing multiplexed detection. We used the device to detect bacteria from contaminated lettuce, and then demonstrated increased detection sensitivity by incorporating a bacteria enrichment step.

### MATERIALS AND METHODS

**Design and Fabrication.** The proposed device is designed to simplify the complicated and laborious multistep assays while simultaneously detecting up to four pathogenic bacteria with high sensitivity. As shown in Figure 1A, it is equipped with two test strips: the left strip is used to detect *E. coli* O157:H7 (ATCC 35150) and *S. aureus* (ATCC 25923); the right strip is used to detect *S. Typhimurium* (ATCC 14028) and *B. cereus* (ATCC 10987). EC, SA, ST, and BC stand for *E. coli*, *S. aureus*, *S. Typhimurium*, and *B. cereus*, respectively. The device consists of a stationary part (bottom piece) and a rotating part (top piece). Sample pads (S1–S3) and their corresponding absorbent pads are contained in the top piece, and the nitrocellulose (NC) test strip is contained in the bottom piece. S2 contains a mixture of AuNPs that label *E. coli* O157 and *S. aureus* antibodies, while S2 contains a mixture of AuNPs that label *S. Typhimurium*, as well as AuNPs for *B. cereus*.

A patterned OHP transparency film, which has through-holes, is attached to the bottom of the top piece (Figure 1B). This enables
direct contact between the sample pad and the test strip as well as between the test strip and the absorbent pad. The top and bottom pieces were made of 3 mm thick poly(methyl methacrylate) (PMMA) plates, which were patterned and etched with a laser cutter (C40-60W; Coryart, Anyang, Korea). The diameter of the handheld device was 8 cm. The through-holes were patterned on the OHP film with a laser cutter, and then adhered to the top piece with a double-sided tape.

One mg/mL anti-S. aureus antibody (ab20002; Abcam) and 1 mg/mL anti-E. coli O157 antibody (ab20976; Abcam) were immobilized parallel to each other on a 15 μm pore NC strip as two test lines (SA, EC), respectively, and 1 mg/mL antimouse IgG antibody (M4155; Sigma-Aldrich, MO, USA) was dispensed as a control line (CL 1) for the left strip. After the antibodies were dried, the NC strip was cut into 4 × 25 mm² strips. Ten μL blocking buffer (2% skim milk and 0.15% Tween-20 in phosphate buffered saline (PBS)) was dropped on the center of each strip and dried at room temperature. This strip was used as a left strip for S. aureus and E. coli O157:H7 detection. A cell wall binding domain (CBD) of an endolysins produced by a B. cereus phage was prepared for B. cereus detection as previously described.28 One mg/mL anti-S. Typhimurium antibody (ab8274; Abcam) and 1.5 mg/mL CBD against B. cereus were immobilized on a 15 μm pore NC strip as two test lines (ST, BC) for the right strip, respectively. CBD was diluted with PBS containing 5% BSA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) prior to immobilization to minimize the nonspecific signal and increase the detection signal-to-noise ratio.27 One mg/mL antimouse IgG antibody was dispensed as the first control line (CL 1), and 1 mg/mL anti-glutathione-S-transferase (GST) antibody (A190-122A; Bethyl) was dispensed as the second control line (CL 2).

**Gold Nanoparticle Preparation.** AuNPs for the labeling of E. coli O157:H7, S. aureus, and S. typhimurium are conjugated with antibodies. For the AuNP—antibody conjugation, the pH of the AuNP (20 nm diameter) solution was adjusted to 9 by adding 3 μL of K₂CO₃ (0.2 M)/150 μL of AuNP (753610; Sigma-Aldrich). The stock concentration (1x) was 6.54 × 10¹⁶ particles/mL. Then, 1 μg of antibody/150 μL was added to the AuNP and incubated at room temperature for 20 min. Thereafter, to block the surfaces of antibody conjugated AuNPs, 100 μL of PBS containing 3% BSA was added and incubated for 20 min. The AuNP was then centrifuged at 7000 × g for 30 min to remove unbound antibodies. The supernatant was removed, and the AuNP was resuspended in a PBS solution containing 3% BSA and 0.05% Tween-20.

AuNPs for the labeling of B. cereus are conjugated with CBD proteins. For the AuNP—CBD conjugation, 10 μL of 1 M Tris—Cl buffer (pH 9.0) was added to 1 mL of a AuNP (20 nm diameter) solution suspended in citrate buffer (741965; Sigma-Aldrich). Five μL of Cys-GST-tagged CBD protein solution was added to the AuNP solution and incubated for 1 h at room temperature. To block the surfaces of the CBD-conjugated AuNP, 100 μL of 2 mM PEG (dissolved in 10 mM Tris—Cl, pH 9) (SunBio, Anyang, Korea) was added and incubated for 1 h at room temperature. The solution was then centrifuged at 8000 × g for 30 min to remove unbound proteins. The supernatant was removed, and the AuNP pellet was resuspended in a PBS solution containing 3% BSA and 0.05% Tween-20.

**Device Operation.** Prior to starting the detection process, 40 μL of AuNP mixtures was loaded into their respective AuNP pads: AuNP-E. coli O157 antibody mixed with AuNP-S. aureus antibody was loaded into S2, and AuNP-S. Typhimurium antibody mixed with AuNP-CBD is loaded into S2. Then, 80 μL of enhancer was loaded into S3. To begin the assay, 260 μL of bacteria sample suspended in 2% BSA containing PBS was loaded into S1. As shown in the scheme in Figure 1C, the assay first requires delivery of the sample to the NC strip for the bacteria capture step. After 10 min of sample flow, the top piece was rotated counterclockwise to deliver AuNPs to label the captured bacteria. After 6 min of AuNP labeling, an enhancer solution (GoldEnhance EM; Nanoprobes, Yaphank, NY, USA) was delivered to amplify the AuNP signal intensity. The gold enhancer solution consists of an enhancer, activator, initiator, and buffer, which are mixed prior to use. As mentioned in previous publications,28,29 the gold enhancer has the ability to decrease the limit of detection and help distinguish unclear signals, which can prevent false negative detection results. After 3 min of enhancement, the top layer was detached from the device and pictures of the test lines were taken and analyzed. The sequential delivery for the aforementioned multistep assay was easily performed simply by incrementally rotating the top piece of the device with the hands.

**Detection of Bacteria from Contaminated Lettuce.** To determine whether the developed device can be applied for bacteria detection from contaminated fresh vegetable samples, fresh lettuce leaves were inoculated with different amounts of E. coli O157:H7 and S. Typhimurium. Bacteria were grown in tryptic soy broth (TSB) (BD Difco, Sparks, MD, USA), centrifuged at 3000 × g for 20 min and resuspended in PBS, and then diluted to the target inoculation number by measuring the optical density (OD) of the bacteria suspension. The target inoculation amounts were 10⁵, 10⁶, and 10⁷ CFU/10 g of lettuce. However, it is technically difficult to measure the exact target CFU of the bacteria sample and dilute it to the exact target concentrations based on the OD measurement alone, a portion of the diluted samples were spotted (10 μL of samples was spotted on the agar plate after further log-dilution) on an agar plate. The actual number of inoculated bacteria was calculated by counting the number of bacteria from the agar plate the next day when the colonies were visible. Portions of 100 μL of the diluted bacteria solutions were dropped on different spots of the lettuces, which were then dried for 5 min, and put into a plastic bag. Portions of 90 mL of PBS were poured into each plastic bag, and then, the bags were put into a stomacher machine (BagMixer 400; Interscience, Saint Nom, France) for 1 min to homogenize the solution and retrieve the bacteria from the lettuce. The solution from each bag was poured into two 50 mL tubes and centrifuged at 3000 × g for 20 min. The supernatants were decanted, and the samples were resuspended in 150 μL of PBS. From the suspension, 10 μL was log-diluted and spotted on an agar plate to measure the retrieved number of bacteria, which was counted the next day. From the remaining solution, 130 μL was taken and diluted to a final volume of 260 μL containing 2% BSA in PBS, which was loaded into the device for detection.

**Bacteria Enrichment.** Fewer number of bacteria (in the order of 10⁵, 10⁶, 10⁷ CFU/10 g of lettuce) were inoculated on the lettuce. After the contaminated lettuces were put in the bag, 90 mL of growth medium were poured into the bag instead of pouring PBS. The bag was then incubated in an incubator at 37 °C for 6 h. After enrichment, the sample was put into a stomacher machine, centrifuged, and concentrated as described previously.

## RESULTS AND DISCUSSION

**Optimization of Gold Nanoparticle Concentration and Duration.** The concentration of AuNP-Ab used to label the captured bacteria is related to the total amount of AuNP and antibody used, which is directly related to the total cost of an assay. Moreover, the duration of AuNP flow is related to the total time required to perform each assay so that both the duration of AuNP flow and the concentration of AuNP need to be optimized in order to reduce the total cost and time. Antigen—antibody binding is a reversible interaction with the association and dissociation of antigen—antibody complexes. The total number of antigen—antibody complex, which determines the signal intensity, depends on both the concentration of bacteria and the concentration of AuNP. Theoretically, using a higher concentration of AuNP would use the lowest AuNP concentration that allows the signal to appear at the lowest bacteria concentration as possible.

The test line’s intensity using several concentrations of AuNP was screened by captured S. Typhimurium (10⁷ CFU/mL flowed for 10 min). The concentrations of antibody-conjugated AuNPs were 2X, 1X, 0.50X, and 0.25X with 1X being 6.54 ×
10^{11} \text{ particles/mL}. As shown in Figure 2A, the intensity of the detection signal increases over time, and the detection signal generated with a higher concentration of AuNPs saturates at a higher intensity (Figure 2B). According to the graph, the use of 2× AuNP and the flow duration for 14 min would result in a highest signal intensity. Before reducing the AuNP concentration and the flow duration, the limit of detection of S. Typhimurium with 2× AuNP and 14 min flow duration was determined to be 10^5 \text{ CFU/mL} with an enhancement step (Figure S1 of the Supporting Information). Thus, additional experiments were performed to reduce the AuNP concentration and the flow duration while maintaining the limit of detection.

To find an optimum condition, the AuNP flow duration was varied with a fixed AuNP concentration (2×). 6, 10, and 14 min of AuNP flow durations were tested, and the pictures show that 6 min is sufficient to generate a detection signal of 10^5 \text{ CFU/mL} S. Typhimurium (Figure 2C). Although the intensity of the detection signal is stronger after flowing for 10 min, a prolonged AuNP flow is not necessary to generate a visible signal. It is interesting to note that the flowing for 14 min results in a reduced signal intensity. The reason behind the decreased signal intensity is unclear. It is assumed that a long duration causes dissociation of bacteria from the capture antibodies, thereby resulting in a reduction in the total number of captured bacteria, which would also reduce the signal intensity.

Lastly, the AuNP flow duration was fixed at 6 min, while the AuNP concentration was varied. The detection signals of 10^5 \text{ CFU/mL} S. Typhimurium show that no signal is visible below 1X AuNP concentration and 1X AuNP is sufficient to generate a visible signal (Figure 2D). This data indicates that the minimum AuNP concentration required to generate a visible signal of lowest bacteria concentration is 1X. Thus, the optimal labeling condition for the rest of the experiments in this study was determined to be 6 min AuNP flow at 6.54 \times 10^{11} \text{ particles/mL}.

**Double-Strip Device Detection Specificity.** The specificity of the device was tested while being equipped with two strips. First, a PBS sample containing only 2% BSA (negative control) was tested and showed no signal from the test lines (Figure 3A). This is very important for the negative control to avoid any background noise because a higher background noise results in a low signal-to-noise ratio and can potentially increase the limit of detection. Then, a sample containing all four species of bacteria (each species was 10^5 \text{ CFU/mL}) was loaded into the device. As shown in Figure 3B, detection signals from all test lines indicate no significant problem caused by clogging or interference among the bacteria. One of the most crucial features of the multiple detection sensor is that signals must appear only when the target bacterium exists. Thus, a sample containing 10^7 \text{ CFU/mL} of each species results in appearance of their respective test line, indicating detection specificity. Red arrows indicate positive test lines.

![Figure 3](image-url)
detection signal increases as the bacteria concentration increases.

Detection of Bacteria Recovered from Contaminated Lettuce. Experiments were performed to validate that the device can actually be used to detect bacteria from fresh vegetables. As a demonstration, lettuce was chosen and contaminated with varying concentrations of *E. coli* O157:H7 and *S. Typhimurium* separately. Bacteria were recovered from the contaminated lettuces and concentrated into a small volume so that it can be loaded into the device. The amount of recovered bacteria has a significant impact on the detection result. If the majority of bacteria are lost during recovery, the detection result may show a false negative despite the sample contaminated with a sufficient amount of pathogens for detection. Thus, the bacteria count before and after the recovery process was measured. Figure 5 shows the relationship between the recovered bacteria from 10 g of lettuce versus the initial inoculation amount. The trend of both *E. coli* O157:H7 and *S. Typhimurium* shows linear relationships between the inoculated and recovered bacteria (*R*² values were 0.99 for both). The average bacteria recovery rate from contaminated lettuce was 41.80 ± 3.61% for *E. coli* O157:H7 and 24.47 ± 4.10% for *S. Typhimurium*.

The recovered samples were loaded into the sample pad, and the detection was performed with lettuces contaminated with three different concentrations of bacteria. The amount of bacteria used for contamination, recovered bacteria count, loaded sample concentration, and signal appearance are shown in Table S1 of the Supporting Information. As shown in Figure 5B–E, the rotary device is able to detect lettuces contaminated with *E. coli* O157:H7 above $1.87 \times 10^5$ CFU/10 g and *S. Typhimurium* above $1.47 \times 10^5$ CFU/10 g. When the bacteria from these samples are concentrated, their concentrations are $2.88 \times 10^5$ and $1.13 \times 10^5$ CFU/mL, respectively. These concentrations are within the same order of magnitude of the previously characterized limit of detection from spiked samples in PBS (Figure 4). The device, however, cannot detect bacteria if the lettuces are contaminated with fewer number of bacteria. It is difficult to conclude whether the detection limits are meaningful for practical uses for all target bacteria because the infective doses are different for each species. For example, for *B. cereus*, the infective dose is about $10^5$−$10^8$ cells/1 g of food in order to produce sufficient toxins, which means that the current system has no problem to detect the bacteria. On the other hand, as few as 10 CFU of *E. coli* O157:H7 can cause infection in human, which means any *E. coli* O157:H7 that exists below the current limit of detection can cause harm even if the detection result is negative. For bacteria whose infective dose is below the limit of detection in the rotary device, the bacteria need to be enriched in order to increase their number and also to increase the concentration for successful detection.

Detection of Bacteria after Enrichment. In order to detect bacteria that exist below the limit of detection, the contaminated lettuce sample needs to be enriched with growth media and the bacteria count must be increased. According to the previous data, the recovered and concentrated sample needs to be at least $10^5$ CFU/mL before loading into the device. Considering the final volume of the concentrated sample that is loaded into the device (260 μL), the total number of recovered bacteria from 10 g of lettuce needs to be at least $2.6 \times 10^4$ CFU,
or $2.6 \times 10^3$ CFU/g. This means that, regardless of the initial contaminated amount, as long as at least $2.6 \times 10^6$ CFU bacteria can be recovered from 10 g of contaminated lettuce after enrichment, the device can theoretically detect the contamination. For practical applications, it is important to determine the minimum time required to enrich the bacteria to reach the sufficient amount required for detection.

Experiments were performed to measure the bacteria growth over time. The lettuces were contaminated with a varying number of *E. coli* O157:H7 and *S. Typhimurium* that range from $10^1$ to $10^3$ CFU/1 g of lettuce, and the number of recovered bacteria was measured immediately after, 3 h after, and 6 h after enrichment in growth media. As expected, the graphs in Figure 6A,B show that recovering *E. coli* O157:H7 and *S. Typhimurium* from the lettuce immediately after the contamination results in a loss, which is also below the limit of detection (indicated by the red dotted line). After 3 h, the lettuces contaminated with $10^1$ and $10^2$ CFU per gram do not reach the minimum number of bacteria required for detection, while the initial contamination of $10^3$ CFU/g does reach the minimum number of bacteria for both species. Enrichment of 6 h is enough to enrich all levels of contamination to reach the minimum threshold for detection. On the basis of these results, it is concluded that as few as $10^3$ CFU/g of contamination can be theoretically detected after 6 h of enrichment.

To verify the enrichment process allowing for more sensitive detection of bacteria, bacteria contaminations were also tested in the following ranges: 0, $10^1$–$10^2$, $10^2$–$10^3$ CFU/g for *E. coli* O157:H7 and 0, $10^1$, $10^1$–$10^2$ CFU/g for *S. Typhimurium* (Table S2 of the Supporting Information). As shown in Figure 6C,D, the detection signals were visible for all tested concentrations except for the negative control. It is worth noting that even the lettuces contaminated with as low as 1 CFU/1 g show a positive detection result for both *E. coli* O157:H7 and *S. Typhimurium* after 6 h of enrichment. This can be explained by the fact that the concentrations of the concentrated samples loaded into the device for detection were all above $10^5$ CFU/mL, which was above the characterized limit of detection for both species. This result indicates that 6 h of enrichment is enough for detection of as few as 1 bacterium in 1 g of lettuce, which is equivalent to about 5 bacteria per single lettuce leaf.

In this paper, a handheld device capable of simplifying multistep assays was used to perform sensitive detection of foodborne pathogens. The result shows that contamination can

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**Figure 5.** Bacteria recovery rate and corresponding signal intensity from contaminated lettuce. (A) Graph showing the recovered bacteria counts versus the number of inoculated bacteria per 10 g of lettuce ($n = 3$). (B) Photos and (C) quantified signal intensity of recovered *E. coli* O157:H7 from contaminated lettuce ($n = 3$). (D) Photos and (E) quantified signal intensity of recovered *S. Typhimurium* from contaminated lettuce ($n = 3$). The data points and error bars represent the mean and standard deviation of the mean.

**Figure 6.** Graph showing the number of bacteria after varying enrichment time for (A) *E. coli* O157:H7 ($n = 3$) and (B) *S. Typhimurium* ($n = 3$) with varying bacteria count of initial contamination. The red dotted line indicates the minimum amount of recovered bacteria required for detection ($2.6 \times 10^3$ CFU/g). Pictures showing the detection results of (C) *E. coli* O157:H7 and (D) *S. Typhimurium* after enrichment with different amounts of initial bacteria inoculation. The data points and error bars represent the mean and standard deviation of the mean.

DOI: 10.1021/acs.jafc.7b03582

be detected as long as the concentrated sample contains bacteria above $10^5$ CFU/mL, which translates to the initial contamination amount of $2.6 \times 10^5$ CFU/1 g of lettuce. To further improve the limit of detection, the contaminated samples were incubated in growth media and as few as 1 CFU of \textit{E. coli} O157:H7 or \textit{S. Typhimurium}/1 g of lettuce was able to be detected after 6 h of enrichment. Such sensitivity is comparable to that of more expensive and complicated molecular detection methods such as PCR, which often involves a 24 h enrichment. 

The recovery and enrichment experiments with lettuce confirm that the device can actually be applied to detect pathogens from contaminated fresh vegetable samples. One of the biggest problems in the detection of food products is the debris and chunks that exist within the sample solution after the pretreatment process, which are known to interfere with assays. In our study, although food dye and some debris were observed between the sample pad and the NC strip (at the contact zone), they had no detrimental effect on the assay results.

The combination of the sample enrichment process along with the use of a rotary device can determine the presence of target bacteria much more quickly than previous methods. In general, pathogen identification by selective growth media, PCR, or LFT requires 18–24 h of enrichment and sample pretreatment prior to testing. After enrichment, identification by selective growth media along with biochemical tests requires an additional 2–3 days; PCR needs 2–3 h for amplification; and LFT takes about 20 min to complete the test. After enrichment and sample treatment, our device takes about 19 min to perform a multiplexed detection, which is much shorter than the time required for PCR amplification and biochemical tests. Furthermore, we were able to demonstrate that 6 h enrichment is enough for detection of as low as 1 CFU of \textit{pathogen}/1 g of sample. Considering the time required for sample enrichment, pretreatment, and detection, the entire test can be performed within 7 h using our protocol, which is a realistic time frame that can be applied for fresh product distribution. The proposed detection protocol can possibly be used in food processing facilities where raw materials need to be screened prior to processing and packaging. Such processes may include the packaging for ready-to-eat salads and assorted fresh vegetables. Furthermore, it is expected to be used for regular screening of new vegetables before distribution and help prevent food poisoning and foodborne pathogen outbreaks before they occur, potentially saving many lives.

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b03582.

Assessment of limit of detection (Figure S1), the average number of contaminated and recovered bacteria, and loaded sample concentration for the recovery experiment (Table S1), and the number of contaminated and recovered bacteria after enrichment, and loaded sample concentration for the enrichment experiment (Table S2) (PDF)

### AUTHOR INFORMATION

**Corresponding Authors**

* Fax: +82-42-350-4310. Phone: +82-42-350-4315. E-mail: jekyun@kaist.ac.kr.

**ORCID**

Je-Kyun Park: 0000-0003-4522-2574

**Author Contributions**

*J.H.S. and J.H. contributed equally to this work.

**Funding**

This research was supported by the Rural Development Administration of Korea (Grant No. PJ009842) and the National Research Foundation of Korea (Grant No. NRF-2016R1A2B3015986) funded by the Ministry of Science and ICT.

**Notes**

The authors declare no competing financial interest.

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