Ferrowax microvalve-utilized centrifugal microfluidic system for a fully automated serial dilution

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March 12, 2021

Abstract

We herein describe an ingenious centrifugal microfluidic system to accomplish a fully automated serial dilution. The liquid flow on the disc was automatically regulated by utilizing ferrowax microvalves systematically integrated into the channels within the specially designed metering structure. By opening the differently positioned microvalves through irradiation of IR laser to allow metering, the same amount of diluent was serially eluted to the dilution chamber from the same diluent chamber. After dilution, the diluted samples were automatically delivered to the respective final product chambers by appropriately opening or closing the microvalves in the connecting channels, followed by rotating the disc. Based on this unique design principle, six consecutive two-fold and ten-fold dilutions were successfully achieved, yielding excellent accuracy in a wide dynamic range up to six orders of magnitude. Very importantly, the overall serial dilution process, including the diluent addition, mixing, and product transfer steps, was completed very rapidly within five minutes, due to the minimized procedures enabled by the automated actuation of the ferrowax microvalves at the rationally designed positions. The centrifugal microfluidic system would serve as a powerful elemental tool to realize the fully automated diagnostic microsystem involving the serial dilution process.

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Abstract

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1. Introduction

Serial dilution is one of the most essential unit operations in numerous biological and chemical experiments, which currently normally involves tedious and time-consuming manual pipetting. The serial dilution by the labor-intensive manual pipetting, however, is quite influenced by the user's expertise and the accurate pipetting is not ensured, particularly when the sample volume is very small down to microliter scale. Therefore, there is a great incentive existing for the development of an automated serial dilution technique to minimize the manual efforts and eliminate the user influence.

Currently, two strategies are being mainly exploited to achieve automated serial dilutions, which basically rely on automated dispensing using pipetting machines [1-5] and microfluidic systems [6-12]. The pipetting machine can eliminate user influence and easily control the number, factor, and volume of dilutions. However, it is relatively expensive and many pipetting errors are still inevitable as in the conventional manual pipetting, particularly when handling highly viscous samples such as general biological specimens or PCR master mixes.

As an alternative approach, the microfluidic systems have been extensively exploited to achieve an automatic serial dilution by employing various elaborately designed microchannels and metering structures. A major advantage of the microfluidic systems is the ability to precisely manipulate small volume samples, thus reducing reagent and/or sample loss. The microfluidic systems, however, normally require device integration with the macroscale environment and essentially involve external pumps and connecting tubes between the channels and pumps for fluid control, which would make the system expensive and complicated. Furthermore, the control of the sample volume or dilution ratio in the microfluidic systems is quite intricated due to the continuous propensity of the fluidic flow within the indiscrete structure [9-12]. In this regard, the centrifugal microfluidic system could greatly simplify the microfluidic process by manipulating liquid flow by rotating the disc at various speeds and in different spinning directions, and eliminating the requirements for the pumps, tubings, and associated connectors [13-20].

Based on this background, centrifugal microfluidic devices have been intensively developed to achieve automatic serial dilutions. Most representatively, *Kim et al.* [21] developed a centrifugal microfluidic device capable of automatically conducting arbitrary serial dilution by utilizing reversible diaphragm valves and specially designed two metering zones. They demonstrated that the device was able to accomplish wide dynamic range serial dilutions with excellent accuracy ($R^2 > 0.97$). Juelg *et al.* [22] also reported the centrifugal microfluidic system for automated serial dilutions by utilizing fill-level-coupled valving as a key element. They successfully accomplished the automated serial dilutions required for the on-disc construction of qPCR standard curve, verifying the capability of the system for the serial dilution in a wide dynamic range with high accuracy and precision.

In this paper, we present another advanced centrifugal microfluidic system equipped with individually addressable laser-irradiated ferrowax microvalves (LIFM) [23], which enables fully automated serial dilutions in an accurate, ultrafast, and loss-free manner. To this end, the capability of the system was successfully

verified by accomplishing six consecutive two-fold and ten-fold dilutions with excellent accuracy.

2. Material and methods

2.1. Design and fabrication of the centrifugal microfluidic disc

The serial dilution disc was designed using computer-aided design software (AutoCAD, Autodesk, Inc., Sausalito, CA, USA). The disc was fabricated by assembling three separate layers: a 5 mm polycarbonate bottom plate (IDEL Corp., Chilgok, Korea), a 1 mm polycarbonate top plate (IDEL Corp., Chilgok, Korea), and a 0.1 mm middle layer of double-sided polyimide tape (930H, Coretech, Gimpo, Korea). Figure 1(a) shows an expanded view of this assembly, while its assembled form is shown in Figure 1(b). The top and bottom layers were milled by a computer numerical control machining center (HI-M1300, Hwacheon, Seoul, Korea). The bottom layer was further treated with a diluted chloroform solution to obtain a smooth and transparent milled-surface, and both the top and bottom layers were cleaned with 70% ethanol and distilled water (DW). The layers were additionally autoclaved when they were used to dilute the *Escherichia Coli* O157:H7 (Shigatoxigenic*Escherichia coli* (STEC)) samples. The bottom layer was mainly composed of six identical sections containing two diluent chambers, one dilution chamber, seven product chambers, and associated deep channels, while the top layer was designed to consist of the appropriately positioned ferrowax grooves, venting holes, inlet holes, and outlet holes. A double-sided polyimide tape for the middle layer was fabricated using a cutting plotter (Silhouette Cameo 3, Silhouette America® Inc., Orem, UT, USA). The middle layer was used to bind the top and bottom layers after the areas corresponding to the chambers and channels are peel off.

To facilitate the convenient and accurate assembly of the three layers, we employed 3D printed jig fabricated by 3D printing (3DWOX 2X, Sindoh Co., Ltd., Seoul, Korea) of a polylactic acid filament (Figure S1), which enabled the accurate alignment of the three layers having a perforated structure of the central axis and six alignment holes. Prior to the assembly, the ferrowax prepared by mixing the same volumes of paraffin wax (T_m = 50–52°C, Fluka Chemie GmbH, Buchs, Switzerland) and ferrofluid (APG 314, Ferrotec Inc., Santa Clara, CA, USA) was loaded onto the ferrowax grooves of the top layer. The assembled disc was finally laminated using a steel roller to eliminate any gaps or air bubbles between the tape and polycarbonate plates. A pressure-sensitive adhesive tape (HJ-Bioanalytik GmbH, Mönchengladbach, Germany) was used to seal the inlet and outlet holes during the dilution operation.

2.2. Rotor and optical systems for the centrifugal microfluidic disc

The rotor system and its operating software were purchased from Hanra Precision Engineering (Incheon, Korea) and modified by our lab. The system was composed of an 808 nm laser diode (TTG50TEC, Laser Solution Korea, Ansan, Korea) for the ferrowax microvalve operation, a camera (BFS-U3-16S2C-CS, FLIR Systems, Inc., Boston, MA, USA), and stroboscopic lights (BUB 0641 (G), PerkinElmer, Inc., Waltham, MA, USA) for the imaging of the disc. The laser module was automatically controlled by a one-dimensional step motor (EzM-42S-A, Fastech, Bucheon, Korea), and a polar coordinate system was used to precisely control the laser irradiation position on the disc based on the rotational angle and radial distance from the edge. A rotary servo motor (SGM7J-02A, Yaskawa Electric Corp., Kitakyushu, Japan) was used to rotate the disc. Home and trigger sensors (EE-SX671A, Omron Corp., Kyoto, Japan) were used to accurately adjust the rotational position. A detailed illustration of the rotor system is provided in Figure S2.

2.3. Materials and sample preparation

Ultrapure DNase/RNase-free DW (Bioneer®, Daejeon, Korea) was used in all experiments. Crystal violet and other chemicals for the reaction buffer were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) and used without further purification. *Bst*polymerase and RNase inhibitor were purchased from New England Biolabs Inc. (Beverly, MA, USA). Thermostable RNase H was purchased from NanoHelix Co., Ltd. (Daejeon, Korea). All oligonucleotides used in the study (Table S1) were synthesized and purified by Integrated DNA Technologies, Inc. (Coralville, IA, USA). Outer primers were purified by polyacrylamide gel electrophoresis (PAGE), while inner primers and cycling probe technology (CPT) probes were purified by high-performance

liquid chromatography (HPLC). The target bacteria, *Escherichia Coli* O157:H7 were provided by Dxgene, Inc. (Seoul, Korea). The bacteria were first cultivated at 37ºC for 16 h, and the colony-forming unit (CFU) was measured using plate count agar (PCA) (NaraeBiotech, Inc., Gunpo, Korea). Based on the determined CFU, the culture solution was diluted to 2.0×10^8 CFU/mL, which was then centrifuged at 11,000 rpm for 5 min (Centrifuge 5424, Eppendorf, Hamburg, Germany). The resulting precipitant was resuspended in the same volume of Tris-HCl buffer (10 mM, pH 8.8), which was then subjected to the serial dilution on the centrifugal microfluidic disc.

2.4. Optical analysis and isothermal amplification

For the absorbance analysis of crystal violet, 40 μL of the finally diluted crystal violet solutions were collected from the product chambers and mixed with 120 μL of DW, which was then analyzed by measuring the absorbance at 588 nm with a microplate reader (Infinite M200 Pro, Tecan Group Ltd., Maennedorf, Switzerland).

For the real-time isothermal amplification, 50 μL of serially diluted each bacterial sample was thermally lysed at 100 °C for 10 min using a heat block (MS-100, Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China). Ten μL of the lysed bacterial sample was then mixed with 10 μL of isothermal target and probe amplification (iTPA) [24, 25] reagent solution to prepare the final reaction solution (20 μL) containing inner primers (1 μM each), outer primers (0.1 μM each), dNTPs (1.6 mM each), 10 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 0.8 mM spermine, 6 mM dithiothreitol, 5 ng/μL bovine serum albumin, *Bst* polymerase large fragment (0.15 U/μL), RNase inhibitor (0.3 U/μL), thermostable RNase H (0.3 U/μL), and a 50 nM CPT probe. The reaction solution was subjected to the iTPA reaction at 65°C for 90 min using a CFX Connect Real-Time System (Bio-Rad Laboratories, Inc., Richmond, CA, USA). A detail description of the iTPA reaction is provided in Figure S3, or our previous reports [24, 25].

3. Results and discussion

3.1. Design and operational principle of the centrifugal microfluidic system

The centrifugal microfluidic disc designed in this work is composed of six identical sections to allow six simultaneous dilution processes. Each section mainly consists of two diluent chambers, a single dilution chamber, seven product chambers, and connecting channels between them, as shown in Figure 2(a). The diluent needs to be loaded to the two identical diluent chambers prior to the operation, and the user needs to apply only the target analyte solution to the dilution chamber to start the serial dilution process. After the diluent and analyte solutions are loaded through the green-colored inlet holes above the respective chambers, the inlet and outlet holes are all sealed using a pressure-sensitive adhesive tape. Each diluent chamber has three outlet channels at different levels, such that the diluent chamber is radially occupied by three equivalent volumes of the diluent. The six outlet channels were initially all closed by the ferrowax microvalves (DV1 ~ DV6, diluent valve) to isolate the dilution chamber from the diluent chambers. The main channel to elute the diluted sample from the dilution chamber to the product chamber has seven initially open ferrowax microvalves (EV1 ~ EV7, elution valve), which is disconnected to the seven final product chambers by the closed ferrowax microvalves (FV1 ~ FV7, final valve) located at the entry of the seven respective branch channels.

The process begins by eluting the original 1 X analyte solution by rotating the disc at 2,000 rpm after opening FV1. Then, predetermined volume of 1 X solution was eluted to the first product chamber, while the rest of the 1 X solution remained in the dilution chamber. For the first dilution, EV1 was closed to isolate the 1 X product chamber from the main channel, and DV1 was opened, followed by centrifugation at 2,000 rpm. Then, one third the initial volume of diluent was eluted to the dilution chamber. For the complete mixing of the analyte solution and the diluent, the disc was shaken by repeatedly rotating at 60 degrees clockwise and counterclockwise. After shaking, the first diluted solution was eluted to the second product chamber by opening the FV2 and rotating the disc at 2,000 rpm. The second and more consecutive dilutions can be accomplished by repeating the same dilution procedure but appropriately changing the microvalves to be opened and closed (Figure 2(b)).

As illustrated in Figure S4, the ferrowax was loaded in the groove of the top layer, which was then integrated with the bottom layer having discontinuous deep channel and the middle layer having the channel part peeled off. The ferrowax microvalves within the fabricated channels on the disc were initially all in an open state, but the DVs and FVs were made to be closed prior to the dilution process. The valve-closing process was automatically executed by irradiating the laser to the groove part to melt the ferrowax. Due to the capillary force, the melted ferrowax would be guided to the nearby shallow channel, where the ferrowax was solidified, consequently blocking the channel. Whenever needed, the closed channels were reopened by irradiating the laser to the shallow channel blocked by the ferrowax. The melted ferrowax was removed away by flowing in the same direction with the solution.

3.2. Six consecutive serial two-fold dilutions for crystal violet solution

To prove the serial dilution capability of the centrifugal microfluidic system, we first fabricated the disc capable of conducting six consecutive two-told serial dilutions and applied it for the serial dilution of crystal violet solution using DW as a diluent. We used 256 μM solution of the crystal violet dye as an input 1 X concentration. To start the dilution process, 100 μL of 1 X crystal violet solution and 150 μL of DW (300 μL for two chambers) were loaded to the dilution and diluent chambers, respectively. To fully accommodate the solutions during the dilution procedure, the three types of chambers were fabricated to be slightly larger than the volumes actually needed by the solutions (Figure 3(a)).

As presented in Figure 3(b), a single diluent chamber is supposed to be radially occupied by the three equivalent 50 μL volumes of the 150 μL DW diluent. As explained earlier, the six outlet channels from the diluent chambers were initially all closed, which are serially opened to release each 50 μL of the diluent from the diluent chamber to the dilution chamber. The dilution chamber was configured to elute half the volume (50 μL) of the initial solution or the diluted solutions by opening and closing the appropriate ferrowax microvalves within the connecting channels, followed by rotating the disc, while retaining the rest half the volume (50 μL) in the dilution chamber. After the elution of the initial 1 X sample solution, the first two-fold dilution can be performed by the consecutive process of the diluent addition, mixing, and elution. By repeating these dilution procedures six times (Figure 3(c)), we completed the six consecutive serial dilutions for the crystal violet solution. Actual images of the initial and final disc are shown in Figure 4, and step-by-step images of this process are provided in Figure S5. A video is also available in the supporting information (Movie S1).

After the completion of the serial dilutions, we collected the diluted crystal violet solutions (50 μL each) from the final seven product chambers, which range from 1 X (256 μM) to 2^{-6} X (4 μM), and analyzed them by spectrophotometer. Based on the spectrum from the samples, (Figure 4(c)), the absorbances at 588 nm were obtained and plotted against the concentrations of the diluted samples (Figure 4(d), red data) using the least square method. As a result, the curve showed excellent linearity (R^2 [?] 0.9910), quite comparable with that from the manual pipetting (Figure 4(d), black data), clearly verifying the accurate dilution capability of this centrifugal microfluidic system.

3.3. Six consecutive serial ten-fold dilutions for bacterial sample

To further verify the versatile capability of the system for the dilution in a wide dynamic range, we next fabricated the centrifugal microfluidic disc capable of accomplishing six consecutive ten-fold serial dilutions and applied it for the dilutions of the biological sample containing *Escherichia coli* O157:H7. As presented in Figure 5(a), the overall structural elements were all the same with the two-fold dilution system, and the same top layer used for two-fold dilution was used for ten-fold dilution. The principal design of the diluent chamber in the bottom layer was also almost same with that for two-fold dilution, and the each diluent chamber was designed to be radially occupied by three one third the volumes of the diluent (Figure 5(b)). However, the volumetric structures of the chambers in the bottom layer were modified to suit ten-fold dilutions. More specifically, the serially added diluent volume was changed to 90 μL from 50 μL in two-fold dilution, and the volume of the diluent chamber was accordingly larger to accommodate the larger volume of the diluent. The volumetric design of the dilution chamber was also quite modified, such that 90 % of the

diluted samples (90 μL) were eluted to the product chambers while leaving only the rest 10 % of the samples (10 μL).

In this manner, the dilution ratio could be manipulated simply by changing the serially added diluent volume from the diluent chamber and the remaining analyte volume in the dilution chamber. To start the ten-fold dilution process, 100 μL of the target bacterial sample and 270 μL of Tris-HCl buffer diluent (540 μL for two chambers) were loaded into the respective dilution and diluent chambers. Again, to conveniently accommodate the solutions during the dilution procedure, the three types of chambers were fabricated to be slightly larger than the volumes actually needed by the solutions. *Escherichia coli* O157:H7 sample of 2.0 $\times$ 10^8 CFU/mL was set as a 1 X sample, and it was ten-fold serially diluted down to 2.0 $\times$ 10^2 CFU/mL following the same procedures for two-fold dilutions (Figure 5(c)). Actual images of the initial and final disc are shown in Figure 6, and step-by-step images of this process are provided in Figure S6.

After the completion of the six consecutive serial dilutions, the diluted bacterial samples were collected from the final seven product chambers, and mixed with iTPA reagent solution to prepare the final seven reaction solutions ranging from 1.0 $\times$ 10^2 to 1.0 $\times$ 10^8 CFU/mL. To validate the accuracy of the automated serial dilutions in such a wide dynamic range, we performed iTPA reactions for the seven samples and obtained the real-time fluorescence curves from the amplified bacterial DNAs (Figure 6(c)). Based on the curves, we determined threshold time (T_t), defined as the reaction time at which the fluorescence signal exceeds the threshold line (fluorescence intensity = 120) for the seven target concentrations, which was then plotted against the log of target DNA concentration (Figure 6(d), red data) using the least square method. As a result, the curve showed excellent linearity (R^2 [?] 0.9818) in a range from 1.0 x 10^2 to 1.0 x 10^7 CFU/mL, confirming the accurate dilution capability of the centrifugal microfluidic system, which is almost the same with that (R^2 [?] 0.9815) of the conventional manual pipetting (Figure 6(d), black data). Unfortunately, the T_t value from the sample of 1.0 x 10^8CFU/mL deviated from the line. We assume that the lysed cell debris and other inhibiting components from the bacterial sample at such a high concentration of 1.0 x 10^8 CFU/mL might have impeded the iTPA reaction because we conducted the iTPA reaction directly using the lysed samples without any prior purification of nucleic acids. However, this deviation of the initial sample is just due to the limitation of the dynamic range of the iTPA technique but not associated with dilution process. All these results confirm that the developed centrifugal microfluidic system is quite capable of accurately diluting the samples in a very wide dynamic range, up to six orders of magnitude.

4. Conclusion

In this study, we developed a new centrifugal microfluidic system capable of accomplishing fully automated serial dilutions, by utilizing ferrowax microvalves. Using the centrifugal microfluidic system, we successfully achieved six consecutive two-fold and ten-fold dilutions for the crystal violet solution and the *Escherichia coli* O157:H7 sample, respectively, in a very accurate and loss-free manner. Very importantly, this system was fully automated by taking advantage of the automated actuation of the ferrowax microvalves such that any handling step was not required after the initial loading of the analyte. Moreover, the overall process was completed very rapidly within five minutes due to the elegantly designed minimized procedures. We believe that the fully automated system developed herein has a great potential for the development of diverse point-of-care diagnostic systems involving the serial dilution process.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by the Industrial Technology Innovation Program (20005957) funded by the Ministry of Trade, Industry & Energy (MI, Korea), the Technology Development Program (S2735682) funded by the Ministry of SMEs and Startups (MSS, Korea), and the BioNano Health-Guard Research Center funded by the Ministry of Science and ICT (MSIT) of Korea as Global Frontier Project (H-GUARD_-2013M3A6B2078964).

Conflict of interest

The authors declare no conflict of interest.

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