Thesis for the Degree of Master of Engineering

A Disposable, Pressurized, and Self-contained Cartridge for Performing Automated Bacterial Nucleic Acid Extraction

by

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Pukyong National University

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자동 박테리아 핵산 추출을 수행하기 위한 일회용, 압력 구동 및 자체 포함 카트리지

Advisor: Prof. Joong Ho Shin

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LIST OF ABBREVIATIONS

3D	Three-dimensional
CAD	Computer-aided design
CFU	Colony-forming unit
Ct	Cycle threshold
DLP	Digital light processing
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
EP	Tube
EtOH	Ethanol
FDM	Fused deposition modelling
IPA	Isopropanol
LAMP	Loop-mediated isothermal amplification
LB	Luria Bertani
LOD	Limit of detection
NPs	Nanoparticles
NTC	No-template control
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
PMMA	Polymethyl Methacrylate
POC	Point-of-care
POCT	Point-of-care testing

PP	Polypropylene
qPCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SLA	Stereolithography
SPE	Solid-phase extraction
TE	Tris-EDTA

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자동 박테리아 핵산 추출을 수행하기 위한 일회용, 압력 구동 및 자체 포함 카트리지

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요 약

분자 진단은 유해한 병원체를 신속하고 정확하게 검출하기 위하여 사용되는 중요한 접근법 중 하나로 이를 수행하기 위해서는 핵산인 DNA 또는 RNA 를 샘플에서 분리 및 정제해야 합니다. 널리 사용되는 방법 중 하나인 컬럼 기반 핵산 추출은 사용이 안전하고 용이하며 다양한 출발 물질의 처리가 가능하고 결과의 재현성을 지닙니다. 그러나 이 방법은 각 과정 사이 여러 pipetting 단계가 존재하며 탁상형 원심분리기를 사용해야 하기에 숙련된 기술자가 필요합니다. 따라서 본 연구에서는 closed-system 방식을 사용하여 미리 저장된 시약으로 세포 용해 및 자동 핵산 정제를 수행할 수 있는 가압 카트리지를 제안합니다. 카트리지에는 시약이 미리 저장된 3D 인쇄 튜브 홀더 4 세트와 silica membrane 이 장착된 미세 유체 칩 및 DNA 농축에 사용되는 바늘이 포함되어 있으며 용해 buffer 를 포함하는 구획화 3D 인쇄 튜브는 튜브 어레이에서 분리될 수 있습니다. Closed-system 을 유지하고 교차 오염을 방지하기 위해 외부 pumping 이나 복잡한 밸브에 의존하지 않으며 시약과 시료는 튜브 내부에 저장된 가압 공기로 인해 미세 유체 칩의 실리카 막을 통하여 입구 튜브에서 출구로 이동합니다. Closed-system 을 유지하고 교차 오염을 방지하기 위해 시약과 시료는 튜브 내부에 저장된 가압 공기로 미세 유체 칩의 실리카 막을 통해 입구 튜브에서 출구로 이동하며 외부 pumping 이나 복잡한 밸브에 의존하지 않습니다. 이 장치는 용해시간 33 분과 자동 핵산 정제 시간 3 분을 포함하여 36 분 이내에 고도로 정제된 대장균(E. coli) O157:H7 DNA 을 추출할 수 있습니다. Cartridge 를 이용하여 추출한 DNA 는 *rfbE* 유전자를 대상으로 실시간 중합효소연쇄반응(qPCR)을 수행하였을 경우 E. coli DNA 에 대하여 10^3 CFU 의 검출한계를 나타내며, 이는 상용 핵산추출키트와 유사한 농도, 순도 및 유사한

검출한계를 나타냅니다. 본 연구에서 제안된 카트리지는 현장 진단을 위한 solid-phase 핵산 추출을 자동화하는 새로운 방법을 보여줍니다.

A DISPOSABLE, PRESSURIZED, AND SELF-CONTAINED CARTRIDGE FOR PERFORMING AUTOMATED BACTERIAL NUCLEIC ACID EXTRACTION

Le Tran Huy Thang

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ABSTRACT

Molecular diagnostics is one of the most important approaches to rapidly and precisely detect harmful pathogens. To perform the test, nucleic acid, either DNA or RNA, needs to be extracted and purified from the sample. One of the most popularly used methods is column-based nucleic acid extraction. Compare to liquid-phase extraction, it is safer, easier to handle, reproducible, can extract process different kind of samples (water, plant, blood, etc.), and can be done within 1 hr. However, this method still requires bulky equipment such as centrifuge, a trained technician to perform, and multiple pipetting in between. Here, we propose a pressurized cartridge with pre-stored reagents that can perform cell lysis and automated DNA purification in a closed-system manner. The cartridge contains a 3D-printed tube holder consisting of four sets of tubes with pre-loaded reagents and a microfluidic chip equipped with silica membrane and needles is used to concentrate DNA. The compartmentalized 3D-printed tube containing lysis buffers can be separated from the tube array. To maintain a closed system and prevent cross-contamination, reagents and sample are moved from one tube to another through the silica matrix in the microfluidic chip with the help of air compressed inside the tubes instead of reliance on peripheral pumping system or intricate valving system. The device can extract highly purified *Escherichia coli* (*E. coli*) O157:H7 DNA within 36 min, including 33 min of lysis and 3 min of automated nucleic acid purification. Extracted DNA using the

cartridge shows similar concentration, purity, and comparable limit of detection, which is 10^3 CFU for *E. coli* DNA when performing real-time PCR targeting *rfbE* gene compared to that of conventional bacterial DNA extraction protocol. The proposed cartridge demonstrates a new method to automate the solid-phase DNA extraction for point-of-care testing.

CHAPTER 1: INTRODUCTION

1.1 Research Background

Nucleic acid extraction is a method to isolate, concentrate and purify nucleic acid, either DNA or RNA, from samples. It is a vital step for downstream molecular analysis applications such as genome sequencing, forensic analysis, or disease detection. The outbreak of Covid-19 pandemic has accentuated the important of qPCR, LAMP and rapid POC molecular testing [1]. Low quality nucleic acid due to not performing DNA extraction properly would lead to false results when carrying out analysis. Furthermore, skipping extraction and concentration of DNA would severely reduce the sensitivity of the assay [2].

Generally, nucleic acid isolation and purification methods could be categorized into two groups. The first group is solvent-based extraction, where a phase separation is created and nucleic acid can be recovered by alcohol precipitation. The second group is SPE, where DNA or RNA will bind to a solid material under specific conditions, such as under the presence of alcohol and chaotropic agents [3]. Compared to liquid-phase extraction, it is safer, easier to handle, reproducible, can extract nucleic acid from different kinds of samples (ex: blood, plants, water, etc.) and the whole extraction process can be done within 1 hr [4]. Some SPE methods include silica-based such as silica membrane, silica-coated magnetic beads, or diatomaceous earth, metal-based for example Au/AgNPs, polymer-based materials such as cellulose matrix, and non-metallic materials such as carbon [5]. However, conventional nucleic acid extraction process required a trained technician and bulky equipment such as centrifuge to perform. In order to prevent disease outbreak and enabling rapid detection of pathogen at POC, many research groups have been

working on a sample-in-answer out testing device for POCT by utilizing microfluidic technology.

1.2 Related Research Trend

Researcher have proposed different approaches for point-of-care nucleic acid extraction devices using microfluidics as shown in Table 1 [6,7]. Microfluidics is the study of fluid behavior at a micrometer and below scale and the technology that enable precise manipulation of the fluid. By utilizing microfluidics, amount of reagents consumption is reduced and the speed of the reaction and manipulation can be significantly increased which make it a promising candidate for POC applications including POC nucleic acid extraction devices [8].

Previous POC sample preparation devices were designed to operate by multiple syringe pumps. Sample and reagents are load back-to-back into the microfluidic chips. C. M. Klapperich group proposed a centrifugation-free room temperature sample preparation platform with pre-stored reagents. The extraction method was based on conventional SPE and syringes was used to replace the standard centrifuge for low-resourced settings. The total turnaround time is approximately 48 min which is comparable to conventional column-based extraction method. The limit of detection of the system is 10^3 copies/mL when extracting RNA from *HIV* samples [9]. Mahalanabis et al. proposed a microfluidic nucleic acid extraction platform by combining chemical and mechanical lysis to lyse bacteria and microscale silica bead and polymer composite to capture the nucleic acid [10]. The device is made of thermoplastic and has small footprint which enable parallelization the nucleic acid extraction process. The turnaround time for the extraction process is roughly 1 hr. Reagents are introduced to the chip by a syringe pump and users need to manually changing the reagents manually during the extraction process. Van Heirstraeten et al. proposed an automated nucleic acid extraction and purification cassette for diagnosis

of respiratory tract infections [11]. The extraction process is automated by using a syringe pump and two active turning valves. The sample was mixed with lysis buffer and incubate together with continuously mixing for 3 min. The extraction process of the device was adapted from conventional SPE. DNA and RNA of gram-positive bacteria, gram-negative bacteria and virus extracted from this device showed higher concentration then that of conventional method. In general, these devices require painstaking efforts to fill of the syringes with buffers manually, connect the tubes to the inlet and outlet of the chip, and monitor the tests which are not suitable for untrained users.

Other research groups proposed nucleic acid purification devices that tried to eliminate bulky peripheral hardware such as centrifuge and syringe pumps so they could be mass-manufactured at low cost and used at poor-resourced regions. Typically, reagents and sample are loaded to these devices by pipetting and the operation process need to be done manually. One approach is to use magnetic bead-based method. Lim et al. developed a modular chip-based DNA extraction device that can perform magnetic bead-based DNA extraction [12]. The device contains two separate modules, a vibration jig and a magnet jig. DNA was extracted by moving the extraction core chamber between these modules together with manually reagents loading & removing. Shi et al. proposed a platform that can extract RNA from up to 16 samples simultaneously through parallelization [13]. 1 μL of sample was used for each extraction. mRNA was extracted from 10 marine diatom cells within 5 min by moving magnetic beads through an array of droplets consisting of nucleic acid extraction reagents. However, this platform required loading of the reagents prior to the experiment and manually moving the magnet bar multiple time which need a trained technician to operate. M. Valiadi et al. proposed a novel method for sample preparation on a microfluidic chip by using anion exchange diethylaminomethyl magnetic microparticles [14]. The sample was introduced to the chip by an electronic

pipette. The device was designed so that eight 6.5 μL samples could be processed in parallel. To shorten the lysis time, the bacteria was incubated at 95°C for 5 min on the chip using a resistive heater. After the extraction, the DNA was amplified by RPA and the LOD was 1000 CFU. Other approach is to use silica-based method. Yoon et al. proposed a simple approach to perform DNA extraction by using a single syringe [15]. The bacterial cell lysates needed to be prepared off-chip. The reagents required for DNA extraction was sequentially loaded into the compartmentalized syringe chamber. After that, the lysate, which had been prepared off-chip, was sucked to the syringe. Then, the column containing silica membrane was mounted to the inlet of the syringe. DNA can be purified by manually pushing the plunger of the syringe. Paper-based approach is also utilized by many research groups. Byrnes et al. proposed a low-cost paper-based device that integrated lateral flow assay with chitosan that can capture and concentrate nucleic acid by anion exchange chromatography [16]. Purified sample can be collected by centrifuging the membrane in a centrifugal filter tube and used for downstream applications like qPCR. Y. Seok et al. developed a lateral-based nucleic acid extraction device [17]. The lysis buffer was pre-stored on the strip. After loading the sample, wash buffer was applied to the strip. Finally, 2 μL of elution buffer was used to collect the DNA using the micropipette. The limit of detection of the device is 10^4 CFU when extracting Staphylococcus aureus cells. Sullivan et al. developed a paper-based microfluidic device that could perform sample preparation from whole blood using isotachophoresis [18]. R. Tang proposed a paper-based sample preparation device that store reagents on sponges [19]. The distance between each reagent and the reaction chamber was optimized so that when the user pushed the sponge, reagents would be introduced to the reaction chamber sequentially. Nucleic acid can be extracted within 2 min from 30 μL samples. The limit of detection for *Hepatitis B* Virus DNA from whole blood samples was 10^4 copies/mL.

These developments have led to the integration of nucleic acid extraction, amplification and detection into one device [20]. K. T. L. Trinh et al. proposed a slidable device that integrated room temperature SPE-based sample preparation, LAMP and colorimetric detection using triphenylmethane dye [21]. The device was able to detect foodborne pathogens including gram-negative bacteria such as *E. coli* O157:H7 and gram-positive bacteria such as *S. aureus* in 75 min. Yoo et al. proposed an integrated microsystem that can perform magnetic bead-based nucleic acid extraction and LAMP to detect dengue virus in blood [22]. The authors simplified the lysis reagent and utilized heating and bead beating to perform sample preparation. Dengue virus in whole blood can be detect within 1 hours with this system. However, sample loading and fluid transferring process still need to be done manually by a syringe. N. Y. Lee group reported an origami paper-based tests that could perform nucleic acid purification, LAMP and colorimetric detection [23]. By treating the samples with propidium monoazide, only live bacterial cells were able to amplified. The capability of the device was demonstrated by detecting *E. coli* O157:H7 and *Salmonella*. Two years later, the same group proposed a foldable pop-up paper-based device that integrated extraction and LAMP and detection [24]. The extraction was done by incubating 5μL of sample and lysis reagents for 60 min at 25 °C. The device was folded and the wash buffer was applied. After the washing step, the device was folded so that the DNA spot contacted the LAMP reagents region. By utilizing pH-dependent colorimetric LAMP, the amplification result could be observed with naked eye. Z. Yang et al. developed an origami paper-based device for detecting harmful pathogens [25]. The samples were lysed off-chip and then loaded into the device for purification, amplified by LAMP and detected by measuring fluorescent signal using torch or smartphone camera. The origami device showed great sensitivity and specificity when detecting bacteria and virus in semen samples. P. Chen reported a low-cost, simple and fully integrated paper-based device for detecting gene mutations [26]. After loading 1.5 μL of sample to the device, reagents were introduced to the

FTA paper by manually rotating and folding the devices. The detection was done by LFA-based assay. The whole process took approximately 90 min. G. Whitesides research group developed a prototype “paper machine” that integrated nucleic acid extraction, LAMP, and end point detection using a mobile phenhone and portable UV source [27]. The paper strip in the middle layer of the device can be slide in and out the device. This allows the introduction of sample, wash buffers, LAMP reagents and detection reagent (SYBR Green I) to the device through pipetting. The lysis was done by heating up 30 μL of sample at 65 °C for 5 min without purification step. The device was able to detect *E. coli* DNA with a limit of detection of 5 cells. X. Jiang et al. introduced a platform that integrated nucleic acid extraction, RT-LAMP and colorimetric detection for Zika virus detection in human samples [28]. The sample preparation includes ball-based valve-enabled lysis, enrichment and purification. By manually sliding the mixing unit, extraction reagents were introduced from the buffer unit to the mixing unit and the detection unit which contains a paper filter for capturing nucleic acid. After enrichment step, the detection unit was separated from the device and place in a coffee mug for LAMP. Y. T. Kim et al. proposed a simple low-costed device for bacteria detection [29]. The device consists of 3 modules, which are SPE-based purification, PCR and LFA-based detection. The device consists of a movable chamber. After cell lysis of 12 μL of samples off-chip, the whole process can be done by slide the reaction chamber from one working station to another. P. Chen et al. reported a finger-actuated microfluidic chip that integrated sample preparation, gel-based LAMP and smartphone-based detection [30]. After sample preparation, extracted bacterial DNA was introduced to gelified LAMP reagents by finger-actuation. The device can detect as low as 1.6 copies of Salmonella. It also can perform multiplex detection by having 4 separated wells, each contains primers targeting different pathogens. These proposed devices are promising solutions for poor-resourced pathogen detection (Table 2). However, they include multiple steps that need to be performed manually by the users such as re-organizing

the device shape, actuation or pipetting. Furthermore, these devices are not closed, rather open systems which endanger users with infectious disease and increase the probability of contamination due to carry-over contaminants. In general, these approaches are still not optimal for POC usage by untrained users and have stimulated other groups to work toward an automated enclosed platform for nucleic acid testing.

Table 1. Comparison of previous nucleic acid extraction devices in the aspect of turnaround time, limit of detection, whether they require manual operation and pipetting, whether they are closed systems, and their maximum sample volume.

Reference	Turnaround Time	LOD	Require Manual Operation	Require Pipetting	Closed System	Maximum Sample Volume
[31]	20 min	Not mentioned	No	No	Yes	200 μL
[10]	1 h	10^2 CFU/mL for gram-negative and 10^4 CFU/mL for gram-positive bacteria	Lysis, syringes replacing	No	Yes	100 μL
[11]	< 1h	Not mentioned	No	No	Yes	100 μL
[19]	2 min	10^4 copies/mL Hepatitis B Virus	Button pushing	Yes	No	30 μL
[9]	35 min	10^3 copies/mL HIV-1	In syringe mixing, syringes pushing	No	Yes	70 μL
[15]	35 min	2 fg/μL *E. coli*	Lysis, syringe filling with reagents and sample, and device rotating	No	No	50 μL
[32]	Not mentioned	10^2 CFU/mL *E. coli*	Reagents loading and waste removing by pipetting, moving the core chamber between modular modules	Yes	No	200 μL

[18]	30 min	3×10^3 copies/mL HIV-1	Reagents loading by pipetting, separating the filter	Yes	No	33 μL
[16]	Not mentioned	Not mentioned	Lysis, reagents loading by pipetting	Yes	No	100 μL
[17]	3 min	10^4 CFU *S. aureus*	Reagents loading by pipetting	Yes	No	100 μL
[33]	< 40 min	30 IU/mL Hepatitis C Virus	Reagents and liquid wax loading, sample and lysis reagent mixing by pipetting	Yes	Yes	200 μL
[34]	12 min	10^4 copies/mL Hepatitis B Virus	No	No	Yes	100 μL
[35]	1.5 h	33 CFU/mL *E. coli*	Reagents loading by pipetting, device setting up and folding	Yes	No	300 μL
[13]	5 min	10^3 CFU/mL *Thalassiosira pseudonana*	Reagents loading by pipetting, mixing, magnet moving	Yes	No	1 μL
[14]	15 min	10^3 CFU/mL *Klebsiella pneumoniae*	Reagents loading by pipetting	Yes	Yes	6.5 μL
Our system	36 min	10^3 CFU *E. coli* O157:H7	Rotate the screw to release lysis buffer	No	Yes	200 μL

Previously, researchers' goals were to automate and combine the nucleic acid extraction, amplification and detection on a single device by using microfluidic (Table 3). This results in complicated peripheral system to operate the device. Bau et al. proposed a multifunctional microfluidic cassette with reagents pre-stored in flexible pouches that could perform cell lysis, nucleic acid extraction, PCR, and nucleic acid detection [36]. The reagents were manipulated by using 10 external actuators to push the pouches and the valves. A micro vacuum pump was also used to aid this process. Despite being a minimized and fully integrated device, the microfluidic cassette still requires a complex analyzer to manipulate fluid flow inside the microfluidic chip. Kwon et al. proposed a novel cartridge design with multiple syringe chambers to perform magnetic bead-based nucleic acid extraction [31,37,38]. A system of actuators is used to introduced the reagents inside the syringe to the reaction chamber, create negative pressure for precisely control the fluid flow from the reaction chamber to the waste and elution chambers and control the valving system. Ten actuators are used in total including two for reagents loading, four for flow control, one for valve control, and one for magnet control. P. Liu et al. proposed a fully integrated microfluidic chip for forensic short tandem repeat analysis using 6 external pumps [39]. The device can perform DNA purification, PCR, and capillary electrophoresis for separation and detection. The whole process can be done in 3h. Despite achieving automation of "sample-in-answer-out" [40–42], using multiple external peripheral hardware results in higher cost, larger instrument's size, and prone-to-error.

On the other hand, some designs have direct connection between the chip and the device's pressure source or valving system in order to manipulate reagents inside the microfluidic chip (Table 3). J. H. Wang et al. proposed a microfluidic chip that integrated sample preparation, and real-time PCR for detecting HIV-1 within 95 min [43]. A valving system and a micro suction pump were used to manipulated the

liquid inside the chip. By having four amplification chambers, multiplex detection of four different targets were achieved. H. Yang et al. reported a cartridge with pre-stored reagents that integrated sample preparation and RPA for nucleic acid analysis from body fluid [44]. Automated pipetting module was used for reagents transfer. The sensitivity and specificity of the device were tested by analyzing DNA from mycobacterium tuberculosis. B. Zhuang reported a microfluidic chip that combined SPE and amplification for whole blood analysis [45]. The fluid flow inside the chip was control by 3 diaphragm microvalves. Reagents were introduced to the chip by a syringe pump combined with a modular valve positioner. The device successfully analyzed 2 μL of whole blood within 100 min. Nevertheless, direct connection between the chip and the system could potentially contaminate the machine and cause cross-contamination when performing future tests [46–48]. Therefore, developing a closed system cartridge which doesn't have any direct connection between the pressure source from the machine and the reaction chamber is utmost need.

Table 2. Comparison of "sample-in-answer-out" nucleic acid extraction, amplification, and detection paper-based devices in the aspect of whether they have open reaction chamber and self-contained cartridge, require any related manual action for operation, whether on-chip lysis is included and their limit of detection.

Reference	Open Reaction Chamber	Self-contained Cartridge	Require Manual Operation	On-chip Lysis	LOD
[23]	Yes	No	Device folding and unfolding, reagents loading by pipetting	No	10^3 CFU/mL *E. coli* O157:H7& *Salmonella* spp.
[25]	Yes	No	Device folding and sealing, reagents loading by pipetting	No	50 CFU *Leptospira &* Brucella
[24]	Yes	No	Device folding and unfolding,	Yes	10^2 CFU/mL *Enterococcus*

			reagents loading by pipetting, seal removing		
[27]	Yes	No	Strip sliding, reagents loading by pipetting	Yes	5 CFU *E. coli*
[21]	No	No	Device sliding, reagents loading by pipetting	Yes	10^4 CFU/mL *E. coli* & *Salmonella*, 10^5 CFU/mL *S. aureus*
[49]	Yes	No	Reagents loading by pipetting, captured RNA migrating from extraction chip toLAMP chip	Yes	1 copies of Zika Virus
[26]	Yes	No	Device folding and rotating, reagents loading by pipetting	Yes	3 CFU NCI-H1975 cells
[29]	No	No	Chamber sliding, reagents loading by pipetting, reagents mixing by pushing vacuum chamber	No	5 CFU *E. coli and S. aureus*

Table 3. Comparison of fully integrated and automated nucleic acid purification, amplification and detection systems in the aspect of number of actuators (including motors, pumps and external active valves), whether the systems consist of self-contained cartridge with pre-loaded reagents, whether there is any direct connection

between the chip or cartridge and the machine to control the fluid flow and limit of detection.

Reference	Number of Actuators	Self-contained Cartridge	Direct Connection between Cartridge and Machine to Manipulate Fluid	LOD
[36]	10 actuators, 1 vacuum pump	Yes	Yes (Pump)	1.4×10^4 CFU/mL *B. Cereus*
[39]	6 pumps	No	Yes (Pump)	2.5 ng Standard genomic DNA 9947A
[44]	7 actuators, 1 pump	Yes	Not mentioned	$6.9x10^4$ copies/µL *Mycobacterium tuberculosis*
[43]	1 vacuum pump, 1 suction-type micro pump	No	Yes (Pump)	6.2x10 copies HIV-1
[40]	4 motors	No	Yes (Magnet rod)	12 copies Zika virus
[41]	air supply system, syringe pump, 8 pneumatic pistons	Yes	Yes (Air supply system)	Not mentioned
[45]	1 syringe pump, modular valve positioner	Yes	Yes (Pump)	Not mentioned
[42]	Pneumatic system (solenoid valves and pump)	No	Yes (Pneumatic system)	50 TU/mL Zika virus

1.3 Research Purpose

To achieve automation of "sample-in-answer-out" and streamline the nucleic acid extraction process, we've developed an enclosed single-use cartridge with pre-loaded reagents for sample preparation of bacterial DNA without the use of any external hardware or pipette to steer the fluid motion. The setup includes a set of tubes with pre-stored reagents that are held together by a 3D-printed tube holder and a 5-layers microfluidic chip with needles at the inlet and outlet. This setup allows ease of use for end users and protection of users from exposure to contagious diseases. A custom-made lysis tube with separated regions for storing reagents required for the lysis step was proposed and the lysis reagents can be easily released from the chamber by rotating the screw-type plunger. After cell lysis, the lysate is mixed with EtOH and chaotropic salt. The mixture is then delivered from the inlet tube to the outlet tube through the silica matrix inside the microfluidic chip by pressurized air. By using compressed air instead of external pressure sources, the machine only requires as low as two motors to operate which means a smaller carbon footprint. Moreover, it doesn't have any direct connection between the machine and the chip which minimizes the chance of carry-over contamination. For the fast-prototyping purpose, we used 3D printed technology, PMMA, and PDMS to build the cartridge and the machine. For commercializing, a mass-production method such as injection molding can be used to manufacture a large number of cartridges in a short amount of time and lower the cost of the cartridge. We also designed the cartridge so that it can be used with most of the available reagents in a commercial column-based nucleic acid extraction kit. We also performed extraction of *E. coli* O157:H7 DNA using the proposed design to examine the performance and usability of the cartridge. We believe with further development, our device will be a promising candidate for molecular diagnostics at point-of-care.

CHAPTER 2: CONCEPTS AND THEORY

2.1 Device Concept

In order to achieve a closed system and avoid cross-contamination, we decided to use one-time used tubes with pre-stored air pressure to steer the flow of liquid through the reaction chamber. Since silica-based SPE have demonstrated its superior performance for nucleic acid extraction, we utililized silica matrix to extract DNA. For activation of the reactions, we fixed needles at the inlet and outlet of the chip. When the rubber septums of the tubes are stabbed by the needles, the cartridge is in working state as shown in Figure 1. Solution will be steered from one tube to another tube by air pressure. When the needles are withdrawed from the stoppers, the reagents are stored inside the tubes which are sealed by the rubber cap. By having all reagents required for gram-negative bacterial DNA stored inside the tubes (lysis, bind, wash, elution), the automation of column-based SPE was succesffuly be achieved. Figure 2 illustrates the concept of our design. BThe whole process can be automated by using two axes of motion, which are moving the array of tubes up and down, and rotating the array of tubes to align the set of tubes that contains the desired reagent with the needles. In addition, our self-contained cartridge is a closed system by using compressed air to introduce the solution to the silica matrix.

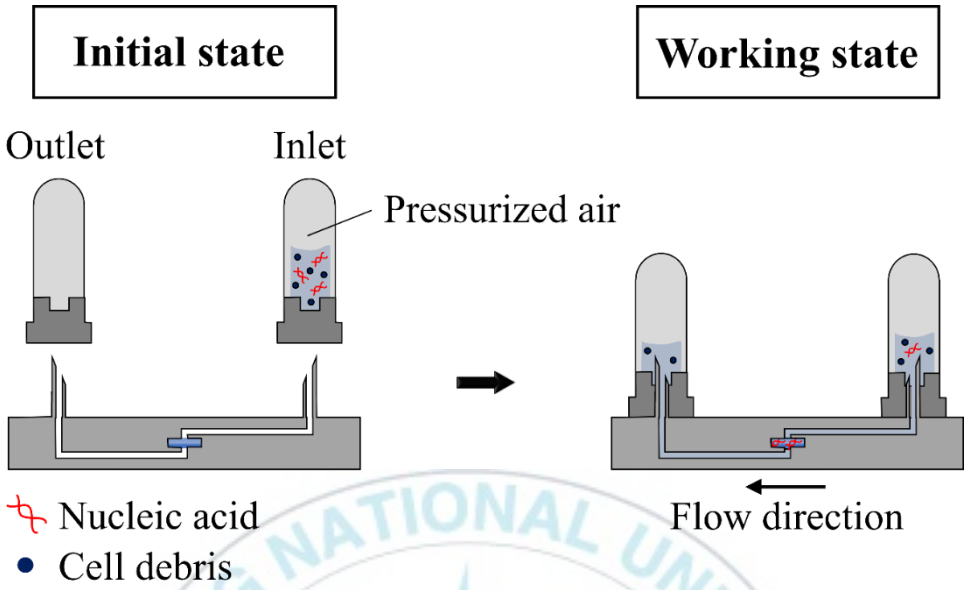

Figure 1. Schematic illustration of the working concept of the chip. At the initial state, the reagent is pre-loaded. At the working state, the tubes' septum are stabbed by the needles of the microfluidic chip which will allow pressurized air to transfer the reagent from the inlet to the outlet without using any external hardware to manipulate the fluid flow.

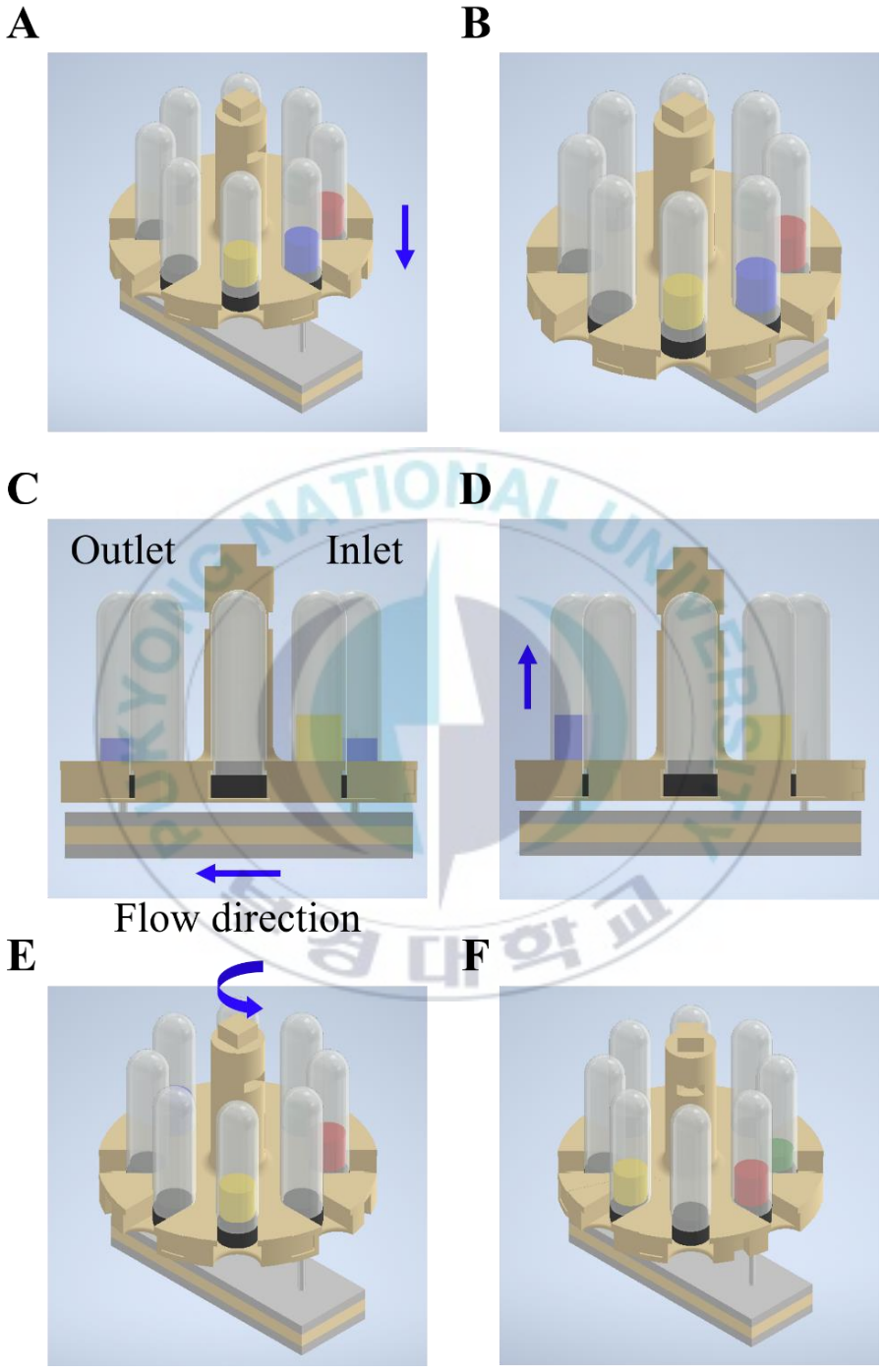
A
B
C
Outlet
Inlet
D
Flow direction
E
F

Figure 2: 3D drawing demonstrating how the cartridge work: (A) The tube's holder moves down from the initial position. (B) The rubber septums were punctured. (C) The blue reagent is steered from one tube to another. (D) After delivering the blue reagent to outlet, the array is lifted up. (E) The tube holder is at initial position. (F) After the tube array reaches its initial position, the tube array is rotated 45° to align another set of tubes with the needles.

2.2 Nucleic Acid Extraction

Nucleic acid extraction is a method to isolate and purify nucleic acid (DNA and RNA), which is essential for further downstream applications. Typically, sample will go through lysis step to destroy and break open the cell membrane and nucleus to release genetic materials. This process can be done by using chemical lysis techniques such as osmotic shock[50], enzymatic digestion [51], detergent-based lysis [52], or alkali treatment [53], or mechanical lysis techniques such as homogenization (blade or pestle) [54], and ultrasonication [33]. Then, nucleic acid can be separated from the cell debris through solvent-based extraction methods, for example by using acid guanidinium thiocyanate-phenol-choloform extraction [55] or SPE-based methods such as column-based extraction or magnetic bead-based extraction as shown in Figure 3.

Liquid-phase extraction process, for example acid guanidinium thiocyanate-phenol-chloroform extraction allow users to separate RNA or DNA from cell debris and denature proteins by using acidic solution and centrifugation to create phase separation including the upper phase and an organic phase located the bottom. The aqueous part consists of nucleic acid, either RNA or DNA depending on the pH of the solution, while proteins are positioned at the interphase and the lower region. The nucleic acid is captured through isopropanol precipitation [56]. Despite resulting

in higher purity and efficiency when capturing RNA, this method normally consumes more time than SPE methods and requires using hazardous and toxic reagents [4].

SPE methods are popular due to its ease of use, safer than solvent-based method, and could process different type of samples [4]. Instead of using phase separation, a solid material is used to capture nucleic acid. This can be silica membrane [15], magnetic bead [57], diatomaceous earth [58], glass particles [59], or cellulose matrix [60]. These methods normally involve four main steps: cell lysis to destroy the cell membrane and expose the DNA and RNA, binding in order to capture nucleic acid, washing to eliminate the remaining cell debris and impurities, and elution to recover the nucleic acid.

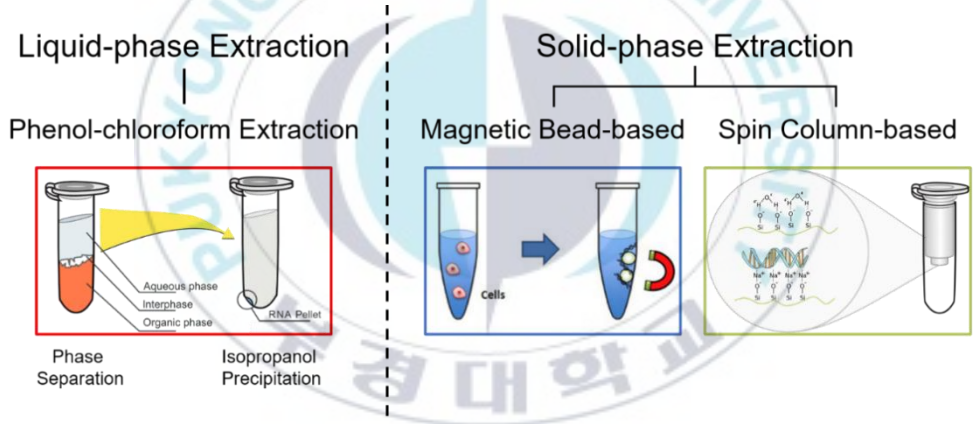

Figure 3: Popular methods for extracting DNA and RNA, which can categorized as liquid-liquid solvent-based extraction and SPE.

2.3 Spin Column-based Nucleic Acid Extraction

Amid these previously mentioned methods, spin column-based extraction is one of the most widely used methods [61]. The mechanism behind it is that nucleic acid will bind to silica membrane under certain condition. This method is user-friendly, not containing toxic solvents, fast, and can process different type of samples.

The spin column-based DNA extraction process includes cell lysis to release DNA and RNA, binding nucleic acid to silica membrane under a specific condition, washing the cell debris and elution to recover the nucleic acid as shown in Figure 4.

Firstly, the sample is normally treated with detergent such as sodium dodecyl sulphate (SDS) or Triton X-100 and chaotropic salt such as guanidine thiocyanate and guanidine HCL to break the cell membrane by solubilizing lipid and protein, and disrupting their hydrophobic interactions, and expose the nucleic acid [62]. Some enzymes such as proteinase K, RNase A and Lysozyme are also used to enhance the lysis process. Lysozyme is normally used to break the cell membrane of gram-positive bacteria since it triggers the hydrolysis of peptidoglycan [63]. Proteinase K is added to the lysis mixture to digest proteins and nucleases such as RNases and DNases by breaking the peptide bond in these proteins [64]. The optimal working temperature of proteinase K is 55 °C and remain more than 90% of its activity between 40-70 °C [65,66]. When extracting DNA from the samples, RNase A is also added to the lysis mixture to digest RNA. Complex biological samples may require additional treatment to break the cell membrane such as heating to higher temperature for longer period of time or mechanical treatment such as bead beating, or homogenizer [67].

Secondly, chaotropic salts and alcohol such as EtOH or isopropanol are added to the lysate and the binding step is performed. The mixture is loaded into the silica column and centrifuge at high speed for 1 to 2 minutes to let the lysate flow pass the silica matrix. DNA or RNA will selectively bind to the silica membrane through hydrogen-binding interaction in the presence of chaotropic agents and EtOH while other cell debris such as proteins flow through the silica membrane [68].

Thirdly, wash buffer is added to perform the washing step. This step will wash away impurities and salt that still remain on the silica membrane. Normally,

washing step is performed twice. The first wash solution is used to wash away proteins and cell debris that remain on the silica membrane. It normally consists of chaotropic agent such as Guanidine Hydrochloride and alcohol such as EtOH or isopropanol. The second wash, normally only consists of alcohol, is used to eliminate the remaining chaotropic salts [4]. Skipping the washing steps or the lysis step would result in low yields and low purity of extracted nucleic acid [69]. After the washing step, a drying step is normally performed to remove the remaining alcohol that is absorbed by the silica membrane by centrifugation.

Lastly, elution step is performed to release the nucleic acid from the silica membrane. Tris-EDTA (TE) buffer or DI water are used as lysis buffer. However, TE buffer is normally preferred since it prevents nucleic acid from degradation [70]. Elution buffer is loaded into the silica column and incubate at room temperature for 1 to 2 minutes so that the silica membrane is hydrated and release the nucleic acid. Then, the column is centrifuged at high speed for 1.5 min to collect the eluate in an EP tube. The eluate should be stored in a –20 °C freezer for long term storage or at 4 °C to use immediately for downstream applications.

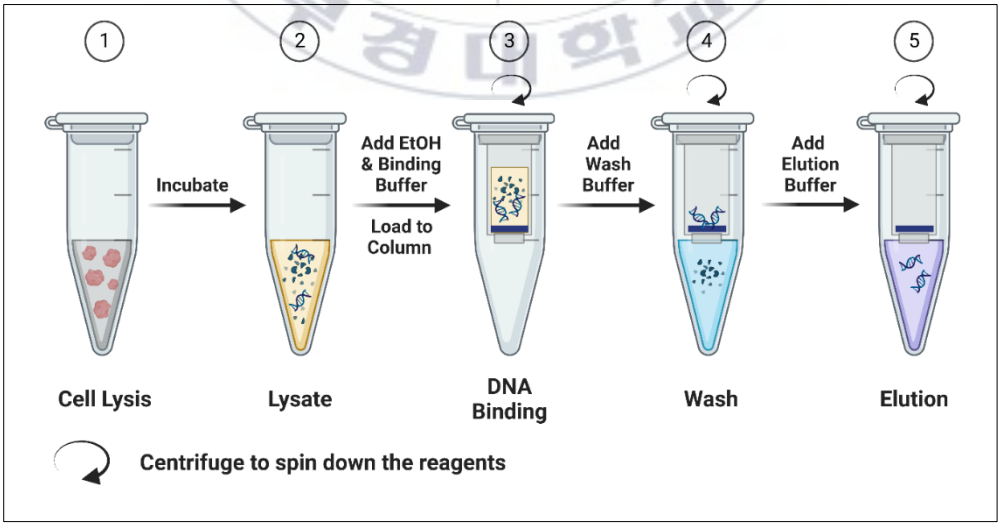

Figure 4: Illustration of spin column-based DNA extraction process. (1) Cell lysis: mix sample with lysis buffer and enzymes and incubate. (2) After incubating, EtOH and binding buffer are added to the lysate and the mixture is loaded to the spin-column for DNA binding (3). After centrifugation, the waste is removed, and wash buffer is added to perform the washing step. (4) After centrifugation, cell debris are washed away while the nucleic acid is captured by the silica membrane. (5) After adding elution buffer and centrifuge, DNA is recovered.

CHAPTER 3: MATERIAL AND EXPERIMENTAL

3.1 Chemical and Reagents

The PureLink Genomic DNA Mini Kit was purchased from Thermo Fisher Scientific, Inc. (USA). Polydimethylsiloxane (PDMS) base and curing agent were purchased from Sewang Hitech (Korea). The gel electrophoresis reagents were purchased from Daejung Chemicals (Seoul, Korea). The DNA ladder, gel loading dye, and Safe-pinky DNA gel staining solution were obtained from GenDEPOT (USA). Power SYBR™ Green PCR Master Mix was obtained from Thermo Fisher Scientific Inc. (USA). Primers targeting the *rfbE* gene of *Escherichia coli* (*E. coli)* O157:H7 (Table 4) were synthesized by Bioneer (Daejeon, Korea) (Paton and Paton, 1998). The amplicon size was 259 bp.

Table 4. qPCR primer sets for amplifying the target gene of bacteria. The amplification target's size is 259 base pairs.

Target	Target gene	Primer	Sequences (5'to 3')	Base pair (bp)
Escherichia coli O157:H7 (ATCC 35150)	*rfbE*	F	CGGACATCCA TGTGATATGG	20
		R	TTGCCTATGTA CAGCTAATCC	21

3.2 Bacterial Culture and Preparation

To demonstrate the capability of our device, gram-negative bacteria, namely *Escherichia coli* O157:H7 (ATCC 35150) was chosen for the assay. The bacteria were incubated in culture media (LB broth) at optimal temperature for bacteria's growth (37 °C) for 16 h to 20h and kept under continuous vibration at 200

rounds per minute (rpm). After overnight incubating, the cultured bacteria were measured for optical density (OD) 600 value and serial diluted. 100 μL of the diluted bacteria were loaded on to an agar plate and culture overnight for quantification and constructing the growth curve. The harvested bacteria were mixed with appropriate amount of LB broth to achieve desired working concentration.

3.3 Fabrication of Microfluidic Chip

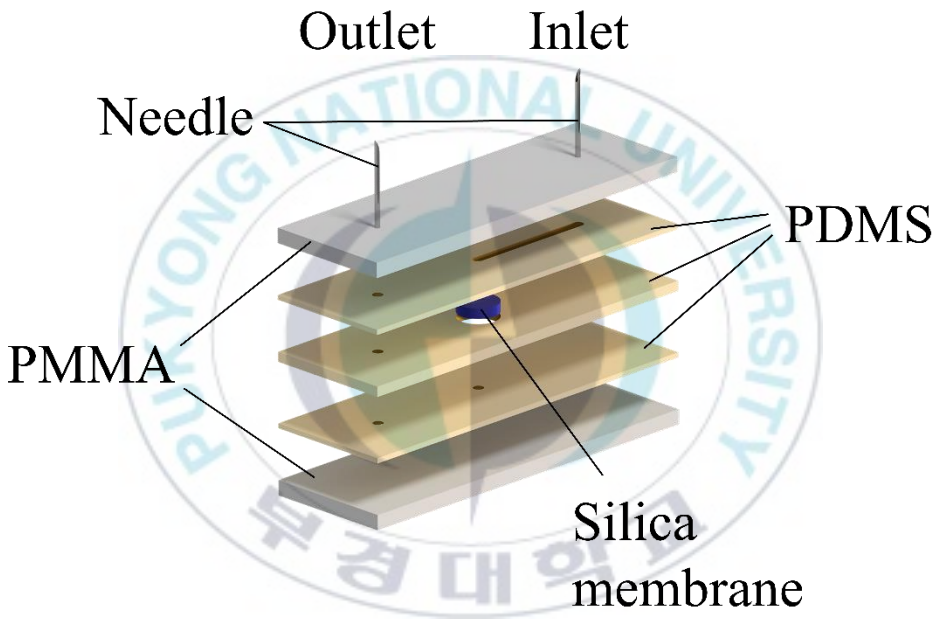

Figure 5. 3D CAD design split view of the microfluidic chip. The top and bottom of the chip are made of PMMA, and the middle contains of three PDMS pieces. The silica membrane is compressed between two PDMS pieces to capture nucleic acid. Two needles are glued to the chip's entrance and exit.

To automate the DNA purification process without using centrifuge, a new mechanism to push the liquid through the silica matrix was proposed. The microfluidic chip houses the silica filter and a plastic circular piece keep the filter in

place as shown in Figure 6. The compression ring is protruded from the chamber (in the second PDMS layer) when the silica membranes are not compressed (inset of Figure 6B). When the device is assembled and bolted, the membranes are compressed firmly as the compression ring is pushed downward. The filter and the compression circular piece were adapted from conventional DNA extraction kit. The chip also has two needles at inlet and outlet which act as a mechanism to activate the working state. By stabbing the vacutainer cap, pressurized air inside the EP tube will deliver the solution from the entrance of the chip to the exit. Therefore, the automation of the DNA purification process is achieved. As shown in Figure 5, the microfluidic prototype contains five pieces that can be bonded together by 8 bolts and nuts as shown in Figure 8A. The size of each pieces is described in Figure 7 and the overall size is 10 x 25 x 75 mm. The outer layers are laser-cut PMMA while the middle is PDMS. The 22mm needles are attached to the entrance and exit of the chip by superglue.

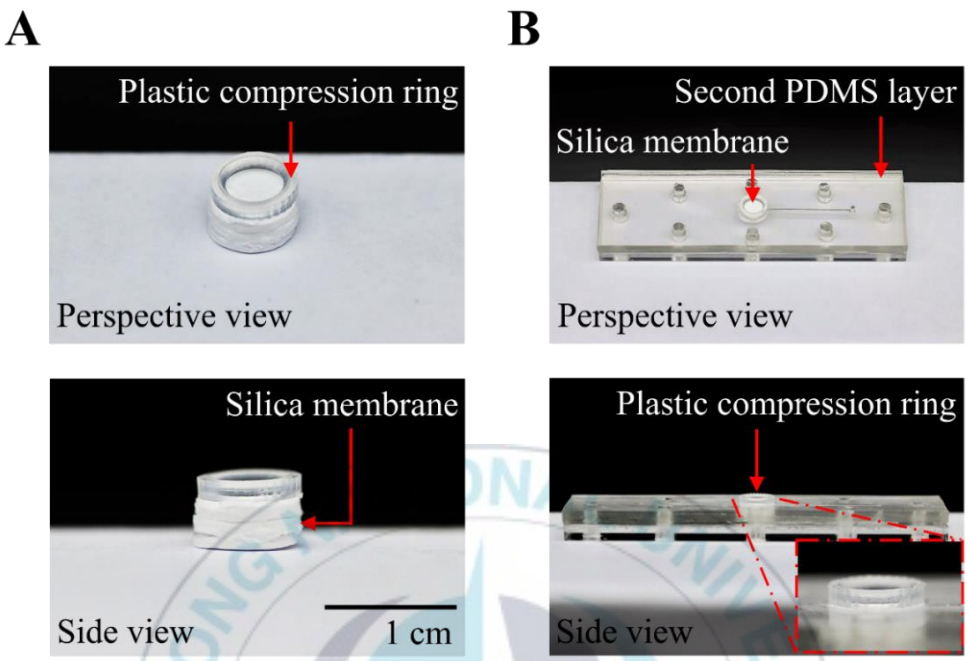

Figure 6. Digital images of (A) the plastic compression ring and the silica membrane showing the perspective view (top) and the side view (bottom). (B) Photos of compression ring-silica membrane stack positioned inside the second layer of the microfluidic chip showing the perspective view (top) and the side view (bottom). The compression is protruded from the chamber (in the second PDMS layer) when the silica membranes are not compressed (inset of Figure 6B). When the device is assembled and bolted, the membranes are compressed firmly as the compression ring is pushed downward.

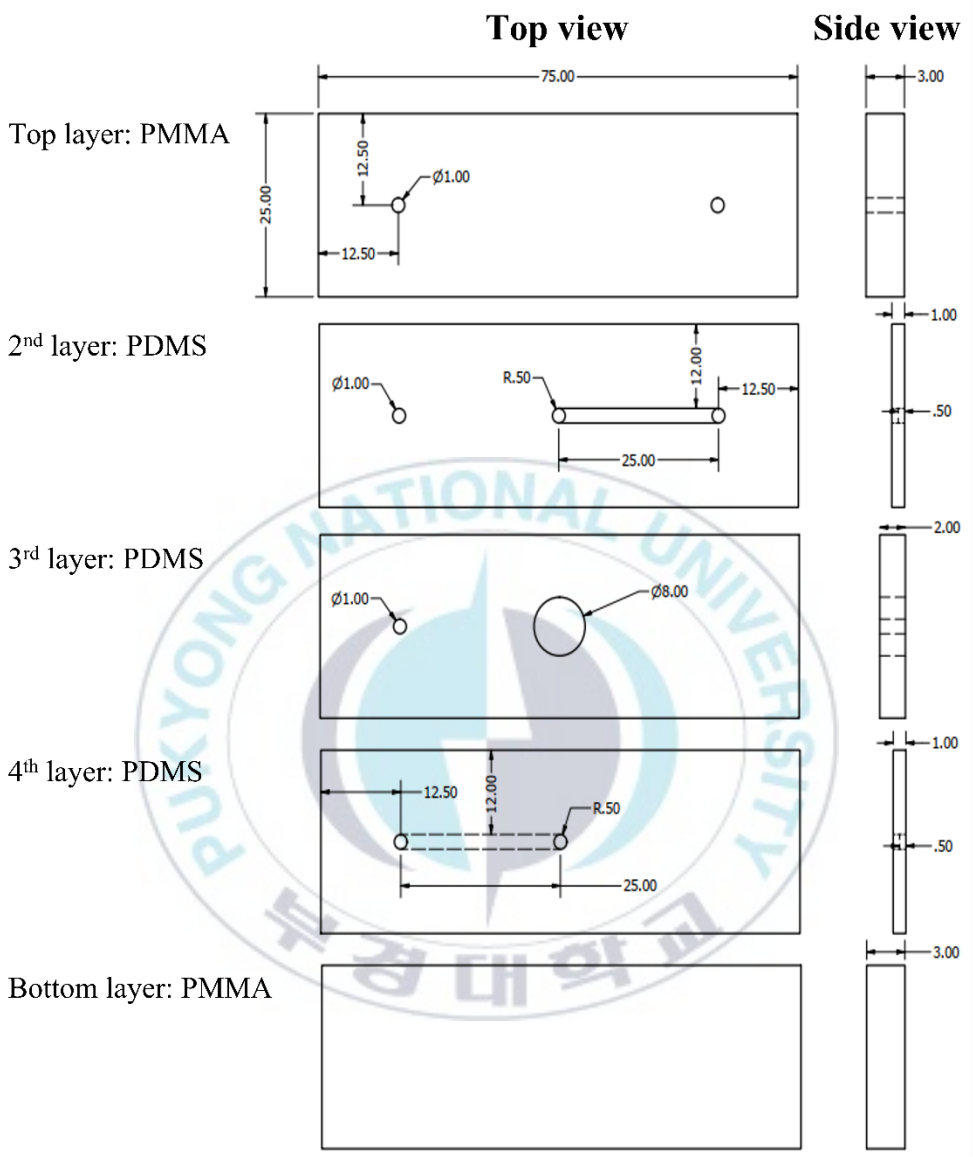

Figure 7. Drawing view describing the dimension of each part of the chip. The length and width of all layers are 75 and 25 mm respectively.

To make the desired PDMS pieces, CAD software, such as Solidwork 2022, can be used to design the molds. Then, Form 3 3D resin printer was used to print the molds. After 3D printing, the results were rinsed with IPA and then treated with ultrasonic machine for 15 minutes. Then, they were treated with UV under curing machine for 30 min and heated at 120 °C for 1 h. During that process, PDMS pre-polymer was mixed with its cross-linker and degassed for 30 min to 1h by vacuum pump. Then, the solution is poured into the negative mold, and heated for 2 h at 80 °C. In case the mold is deformed during the curing process, the curing temperature can be reduced while the curing time should be increased accordingly. For better alignment and prevent leakage from happening, the PDMS pieces could be bonded by plasma treatment and curing at 80 °C for 1 h. Covers were used to protect the needle from contamination as shown in Figure 8B and a chip holder was used to lock the chip inside the proposed cartridge as shown in Figure 8C. The wall of the 3D-printed cartridge is removed for better visualization. For mass manufacturing of the chip, either chemical bonding method or injection molding can be applied to create a monolithic disposable chip and eliminate the usage of bolts and nuts.

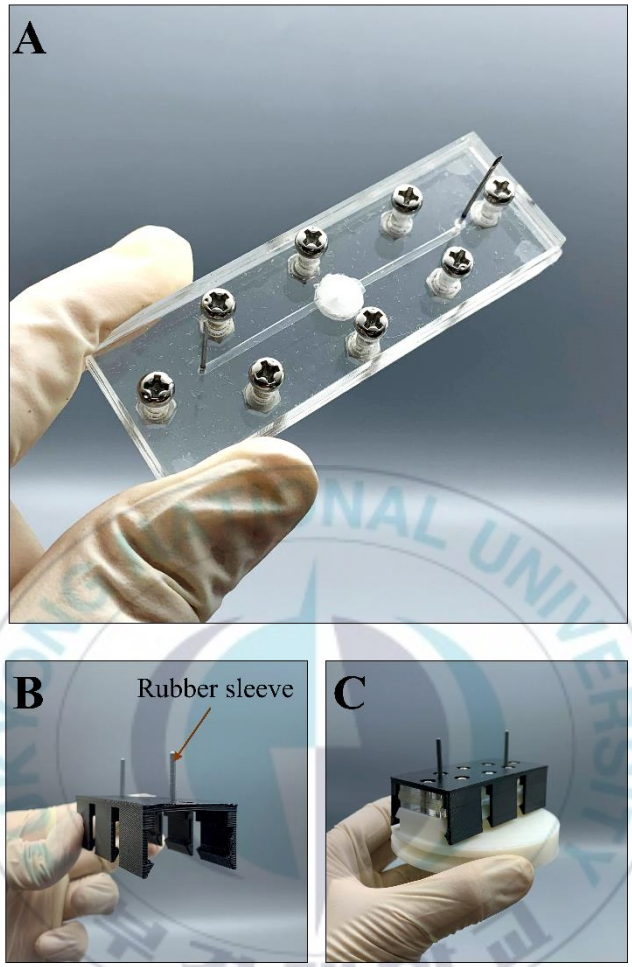

Figure 8. Digital images of the microfluidic chip and how it is fastened to the cartridge using the chip's holder. (A) The prototype of the microfluidic chip. (B) The 3D-printed chip's holder with covers to protect the needles. (C) The microfluidic chip is loaded into the proposed cartridge and fastened using its holder by snap-fit mechanism. The wall of the 3D-printed cartridge is removed for better visualization.

3.4 Fabrication of Lysis Tube

The sectional view of the 3D resin printed compartmentalized tube for cell lysis is illustrated in Figure 9. Since it is important to separate lysis buffers in different chambers and perform each step of the lysis process sequentially, we designed the lysis tube in commercial CAD design software with 5 separated chambers, each chamber comprises a buffer required for cell lysis. Then, Form 3 SLA printer (Form Lab, America) and transparent resin provided by the same entity was used for fabricating the tube. The remaining tubes of the cartridge were adapted from arbitrary column-based SPE kit such as Biofact™ (Korea) or Purelink™ (Thermofisher, America). Beside the 3D-printed body, the custom-made tube comprises the cap obtained from vacutainer, 2 plungers, and 6 stoppers adapted from 1 mL syringes to create sectionalized chambers as shown in Figure 9. The size of the resin printed tube is approximately 15 mm in diameter and 65 mm in height. The main reactor consists of digestion buffer while others comprise proteinase K, Rnase A, EtOH and binding buffer. All the buffers were utilized from the commercial kit. In order to release the buffers, users need to rotate the plunger to move the stoppers and buffers toward the reactor. A screw like mechanism was used for plunger movement in order to enable accurate position control and circumvent backlash since the tube contains pressurized air.

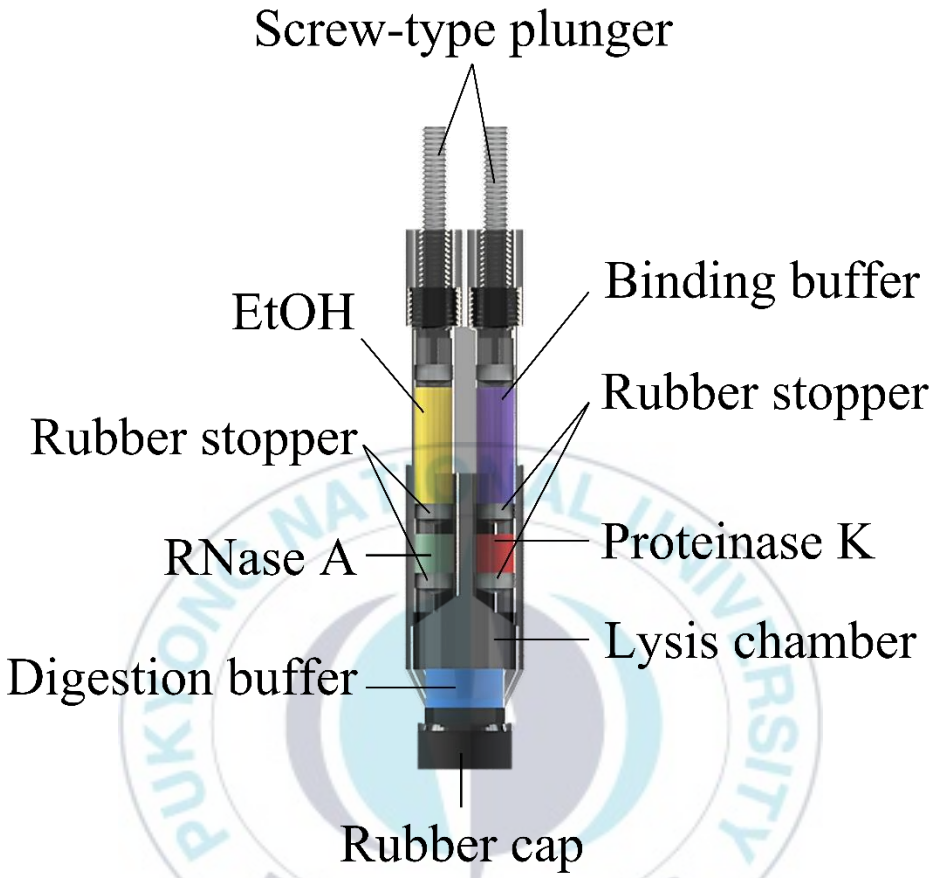

Figure 9. CAD design showing sectional view of the compartmentalized tube consisting of lysis reagents including digestion buffer, proteinase K, Rnase A, EtOH and binding buffer. These buffers are pre-loaded in compartmentalized chambers and can be released by rotating the screw-type plungers.

3.5 Design of the Tube Array and Cartridge

The 3D CAD design of the tube holder is shown in Figure 10. After designing, the model was saved as .stl file and converted to g.code format by 3D WOX provided slicer software. Then a FDM printer (Sindoh, Korea) was employed

to make the prototype. The tube holder was design to have a separable part comprising the lysis tube to perform cell lysis.

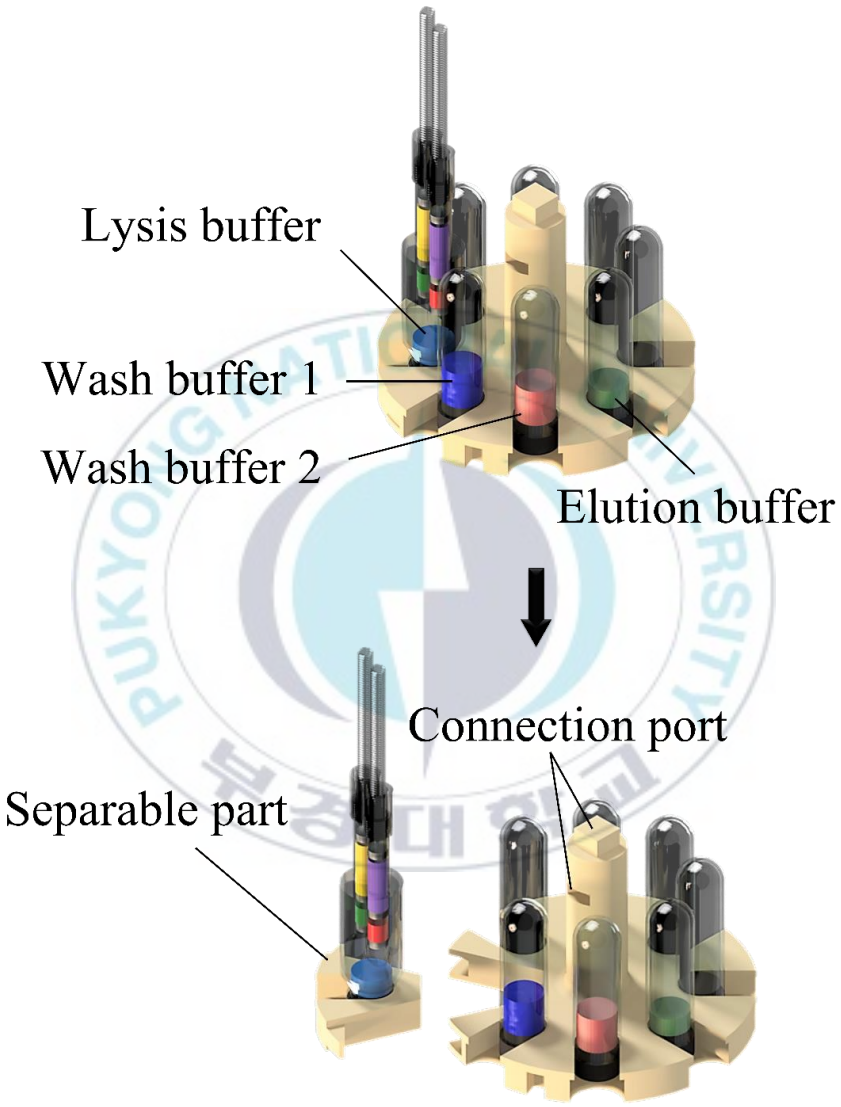

Figure 10. CAD design showing the tube holder consisting of four sets of pre-loaded buffers required for solid-phase DNA extraction. The tube holder was design to have a separable part comprising the lysis tube to perform cell lysis.

The tube holder can be assembled with the extraction machine through the custom-made connector. As visualizing in Figure 10, the tube holder comprises pairs of tube consisting of buffers required for column-based SPE, namely binding to capture nucleic acid on the silica membrane, washing (twice) to remove the impurities and cell debris, and elution to recover the extracted nucleic acid. Each step utilizes two tubes, one is for storing required buffers and one act like a waste reservoir. All buffers used for the experiments were procured from Thermofisher. The tube's caps was adapted from the vacutainer caps (Greiner, Austria). After filling with buffers and loading to the tube array, each tube was compressed or degassed by commercial medical syringes. Specifically, 3 mL syringes were used for inlet to compress air while -30 mL syringes were used for outlet to generate vacuum pressure.

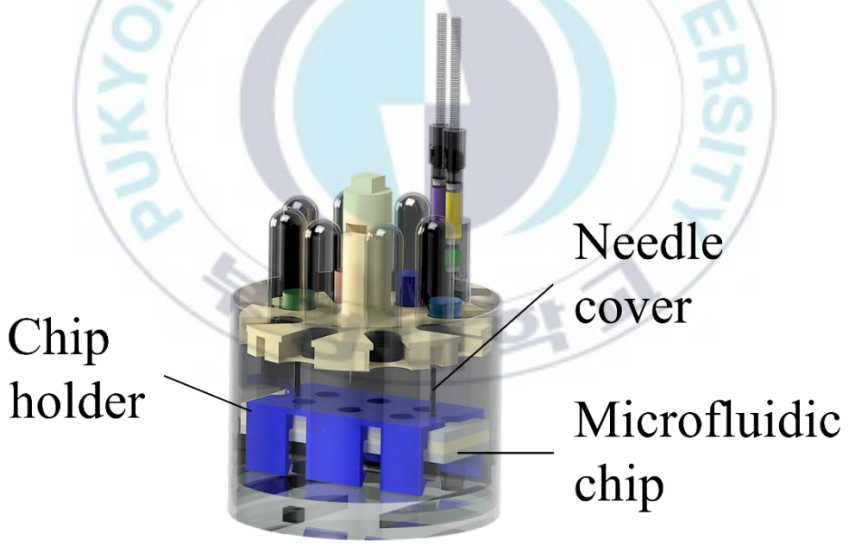

Figure 11. CAD design of the cartridge comprising the chip and the tube holder.

As shown in Figure 11, the cartridge (42 mm in radius, 75 mm in height) comprises the chip and the tube holder. To hold the chip stable in the 3D printed box, a homemade chip's holder utilized. 2 needle's covers were utilized to protect the

needle from the ambient environment. To execute DNA purification, users need to insert the cartridge into the machine, as shown in Figure 14. To switch from the inactive state to active state, the needles need to stab the vacutainer cap. In order to achieve that, 8 holes were made under 8 tubes positions in the tube holder as shown in Figure 12. The tube array has three housing joints which help the tube array fix to the cartridge. At the active state, the buffer will shift from one tube to another through the silica matrix due to pressure force as illustrate in Figure 1.

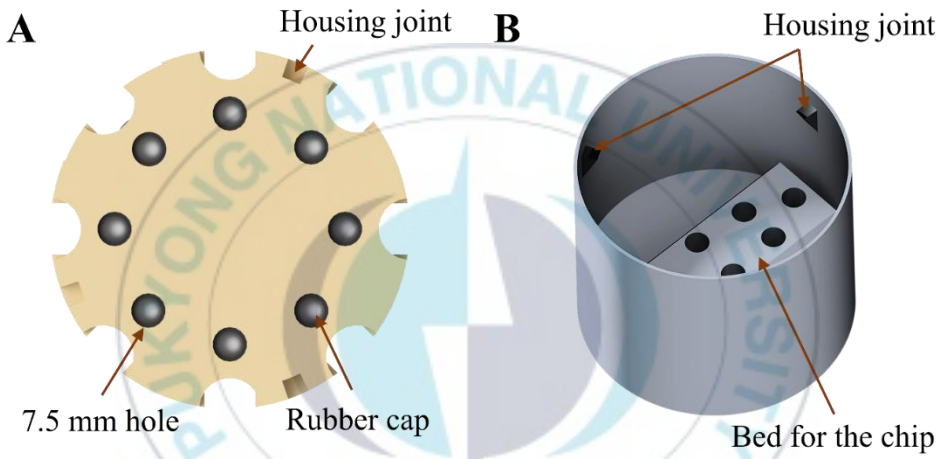

Figure 12. 3D illustration of the (A) bottom of the cartridge and (B) the cartridge without the tube holder and chip. The tube array consists of eight 7.5 mm holes at eight tubes' positions. When the chip's needles stab the vacutainer stoppers, reagents inside one tube will be steered toward another through the silica matrix inside the chip. The tube array has three housing joints which help the tube array fix to the cartridge.

3.6 Evaluation of extracted DNA

To assess the proposed concept, we carried out the extraction with it and measure the concentration of bacterial DNA using Nanodrop (Thermofisher,

America). To confirm the result, we carried out gel electrophoresis using commercial gel electrophoresis machine (Takara, Japan) and commercially available reagents. After letting the DNA move through the gels for approximately 30 min, the agarose gels were placed in a commercial gel imaging system (Davinchi; Korea) to evaluate the result. To run real-time PCR, 2 μL of purified DNA was mixed with 12.5 μL of master mix with intercalating dye, 2 μL of each primer, and the rest is distilled water to make 25 μL final solution. Real-time PCR was conducted by a commercial thermocycler (Thermofisher, America). The running program comprised holding at 95 °C for 5 to 10 minutes and 32 to 40 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds and 72°C for 30 seconds.

CHAPTER 4: RESULT AND DISCUSSION

4.1 Device Operation

Figure 13. Digital image of the 3D printed cartridge.

As shown in Figures 11 and 13, our proposed cartridge design can execute cell lysis and automated DNA extraction. The amount of sample it can process is comparable to conventional method, which is ranging from 25 to 200 μL with the current design and can be expanded easily by increasing the tube and cartridge size. For demonstrating the capability of our device, we did (1) the lysis and (2) cartridge purification of DNA from lab-cultured *Escherichia coli* O157:H7.

Firstly, to begin the test, suspicious sample is loaded into the resin printed tube by a 3 mL syringe. The tube is then detached from the tube holder. Next, users need to twirl the plunger to move the enzyme, namely proteinase K, to the reactor which contains digestion buffer and sample. Then, users have to shake the tube to blend these reagents together and speed up the lysis process. After 10 to 30 seconds, the lysis tube is placed in the oven and baked for 30 minutes up to 4 hours. Then, users need to spin the 2nd plunger to blend the 2nd enzyme, namely RNase A, with the solution in the reactor, shake the tube and incubate it around 2 minutes at ambient environment to eliminate RNA remaining in the mixture. Then, users have to turn both plungers to release the two last reagents to the reactor and mix it well. Finally, the tube should be plugged in the tube holder as shown in Figure 10 and put back to the cartridge as shown in Figure 13.

To perform automatic nucleic acid purification, the cartridge was inserted into the machine, as shown in Figure 14, and the tube array was assembled with the cartridge holder. After pressing the start button, the purification process began with the binding step. The stepper motors moved the tube array downward and two needles of the microfluidic chip were inserted simultaneously into the tubes. As shown in Figure 1, the lysate flowed from the inlet tube to the outlet tube through the microfluidic chip, and the nucleic acid bound to the silica membrane inside the microfluidic chip in the presence of high concentration of chaotropic salts. After transferring all the lysates to the outlet, the inlet and outlet tubes were simultaneously withdrawn from the chip. The tube array was moved upward and rotated 45° to align the first washing set with the needles. It was then moved downward again, and 500 μL of the wash buffer was allowed to flow through the silica membrane. The washing step was performed twice to ensure that all impurities were removed from the silica membrane. After the two washing steps were completed, the tube array was moved up, rotated 45°, and moved down so that the needles punctured the rubber cap. The

elution buffer in the inlet tube flowed through the silica membrane and the nucleic acid was eluted to the outlet tube. Finally, the tube array returned to the initial position after the elution buffer was completely transferred to the outlet tube, and the extraction process was performed. The tube containing the eluate can be removed from the tube array and for immediate eluate retrieval or stored in a 4 °C refrigerator or -20 °C freezer. The eluate can be collected either by inserting a syringe needle through the rubber cap or removing the tube containing the eluate from the cartridge, opening the rubber cap, and collecting the eluate by pipetting. The entire process was controlled by a microcontroller (Arduino Uno and Arduino CNC Shield) located inside the 3D-printed case. The machine was powered by a 12 V DC power source, which can be replaced by a power bank for POC testing.

The total turnaround time for the cartridge nucleic acid extraction process is 36 min, including 33 min for the lysis step (consisting of the time required for releasing the reagents, mixing, and heating for 30 min) and 3 min for the nucleic acid purification step. Meanwhile, conventional nucleic acid extraction method would take approximately 50 min, including 33 min for lysis, and 17 min for purification, which consists of 12 min in total for centrifugation (roughly 3 min for centrifugation repeated four times), and 5 min in total for pipetting and waiting in between each step.

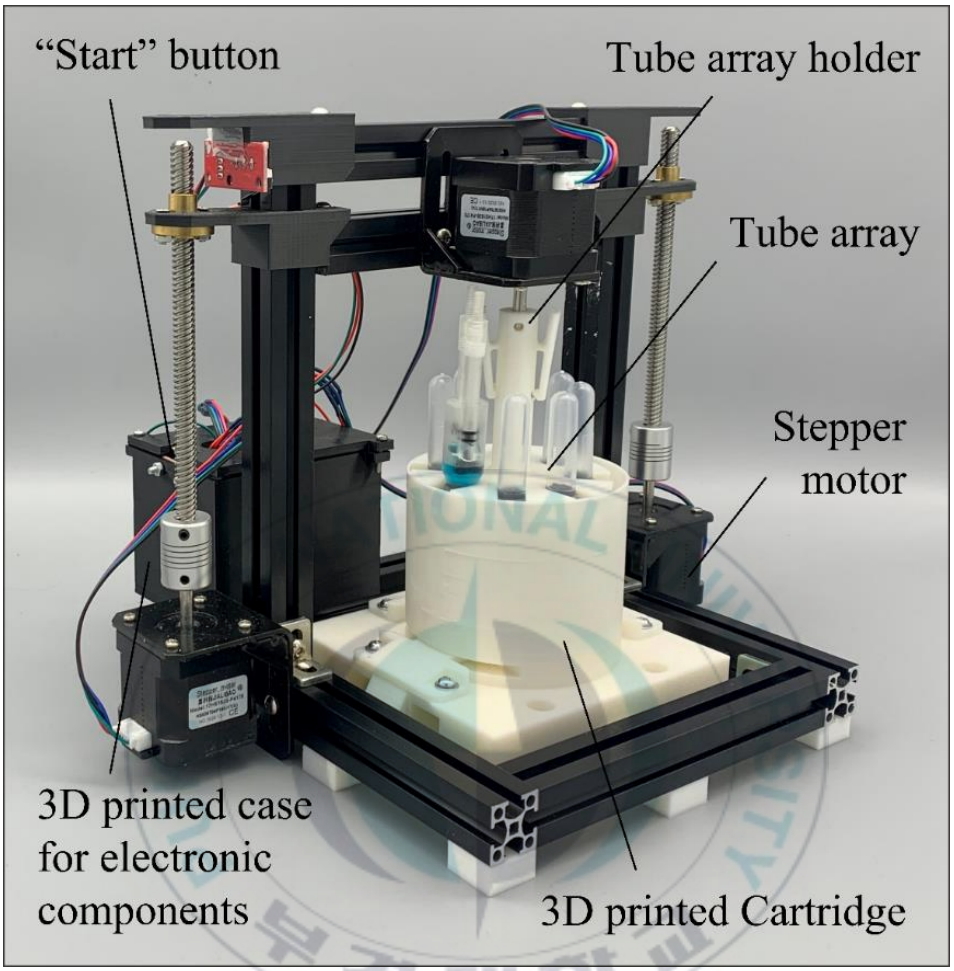

Figure 14. An automated silica-based DNA purification machine utilizing a closed-system pressurized cartridge for bacteria extraction.

4.2 Pressure Characterization

For the appraisal of the pressure and how long each step would take, we did a preliminary study to understand the effect of pressure and the flowrate. We filled the inlet with 0.5 mL ethanol and pressurized the tube with air pressure. A commercial pressure meter was used for monitoring the pressure in the entrance tube. After the needles stabbed the tube, the speed of alcohol flow from one tube to another was

measured by imaging the outlet over time and calculated the change in height of the liquid column. Different values of pressure were tested, for the entrance, 50/100/150/200 kPa cases were tested. For waste reservoir, -80 to -40 kPa was tested.

After analyzing the result using commercial image analysis software Fiji (Image J2), the results were plotted and shown in Figure 15 below. From the chart, we can clearly observe that the higher the pressure is, the shorter time it would take for the alcohol to migrate from the entrance to the exit. As we doing the test, -40 kPa case and 50 kPa case weren't able to deliver the ethanol to another side, and 200 kPa would result in leakage inside the chip frequently which was undesirable. To achieve rapidest machine running time, we decided to compress the entrance tube with 150 kPa while degas the exit tube using -80 kPa. As illustrated in Figure 16, the time each step would take during cartridge purification is measured. Overall, it would take 1.5 min for all the set of tubes to reach equilibrium state after puncturing, and less than 3 min to complete the automated purification when performing with our proposed design.

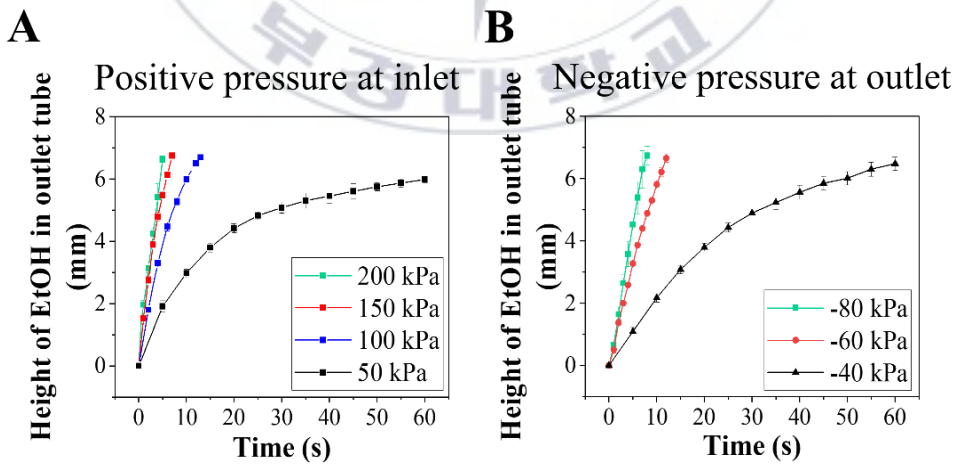

Figure 15. Tube's pressure characterization. Duration needed for 500 μL of 80% alcohol to move from one side to another, in the case of (A) pressurizing inlet, and (B) pressurizing outlet.

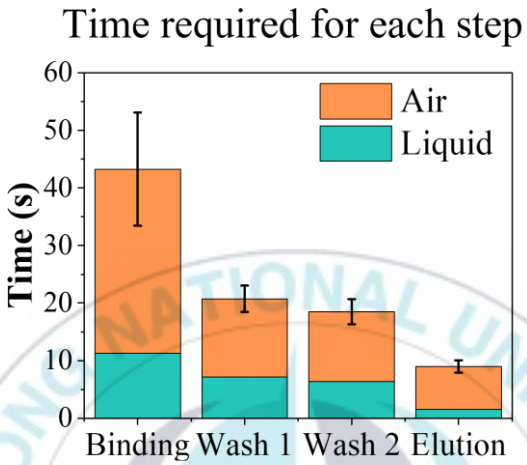

Figure 16. The duration needed for each step of cartridge extraction with optimized amount of pressure. The inlet was pressurized with 150 kPa while the outlet was pressurized with -80 kPa.

4.3 Evaluation of the Resin Printed Tube

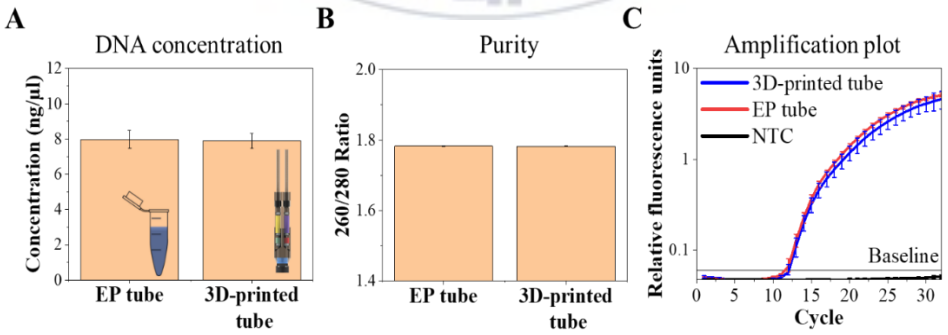

Figure 17. Evaluation of the potential extraction inhibition when utilizing resin printed tube: (A) Concentration of DNA, (B) Measurement of 260/280 ratio, and

(C) real-time PCR plot in comparison with microcentrifuge tube when carrying out the DNA extraction following conventional protocol.

The development of 3D printing technology has enabled fast prototyping with very low cost compared to metal mold. However, DLP printed parts normally inhibit the curing of PDMS because of the chemical remaining on the skin of the printed part [72]. To make sure there is no such inhibition occur during the test, we assessed the resin printed tube carefully by executing nucleic acid extraction and real-time PCR using commercial kit's supplier provided protocol for bacteria. The results are compared against that of using 1.5 mL microcentrifuge tube. From Figure 17, we can observe that the DNA concentration of both cases are around 8 ng/µL while the purity and the real-time PCR results are almost identical. This designates that the 3D printed part with resin doesn't interfere and cause inhibition of the cartridge extraction and real-time PCR when performing the tests.

4.4 Evaluation of Cartridge Extraction

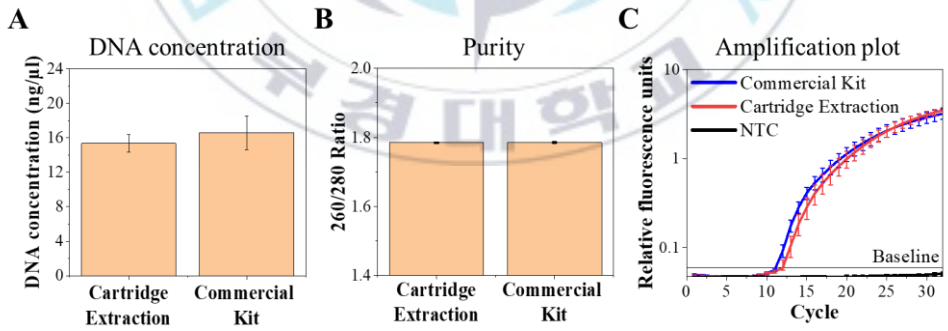

Figure 18. Evaluation of the bacterial DNA achieved from cartridge extraction in comparison to that of conventional method: (A) Concentration of DNA, (B) measurement of 260/280 ratio, and (C) real-time PCR result.

We then evaluated the potential of our design by extracting *E. coli* DNA from lab-cultured samples and compared it with commercial column-based SPE. The

concentration, purity and real-time PCR results were collected and plotted in Figure 18. Figure 18B shows that the purity is identical, which indicating there is no non-trivial interference in the extraction and real-time PCR when isolating DNA utilizing our proposed design. The concentration of DNA and the Ct value of the extraction utilizing our proposed design are a little lower than that of conventional column-based SPE kit. We also did a comparison of extracted DNA concentration following standard extraction protocol versus mingling sample and all lysis buffers together and heating them during cell lysis as illustrated in Figure 19. The result designates that not following suggested protocol will decrease the DNA concentration severely. We suspected chaotropic agents could interrupt with proteinase K and RNase A due to their ability to cause protein denaturation when blending all lysis buffers together [73,74]. Therefore, lysis buffers need to be sectionalized in different chambers as in our lysis tube's design. In short, our design enables extraction process to happen in a closed-system to avoid cross-contamination and endanger users with harmful pathogens while automates the process.

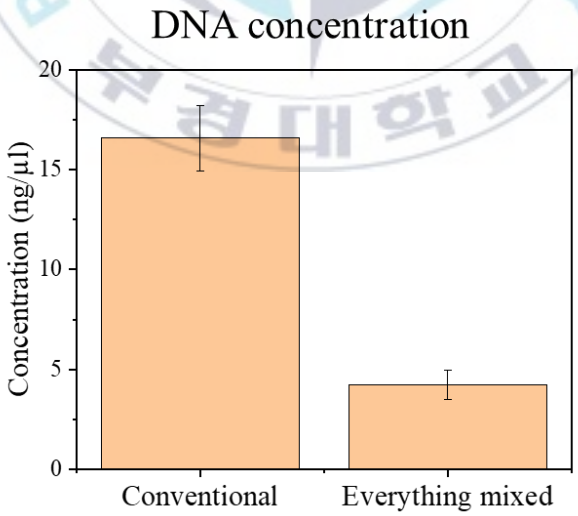

Figure 19. Comparison of extracted DNA concentration following standard extraction protocol versus mingling sample and all lysis buffers together and heating them during cell lysis.

4.5 Assessment of Cartridge Extraction Limit of Detection

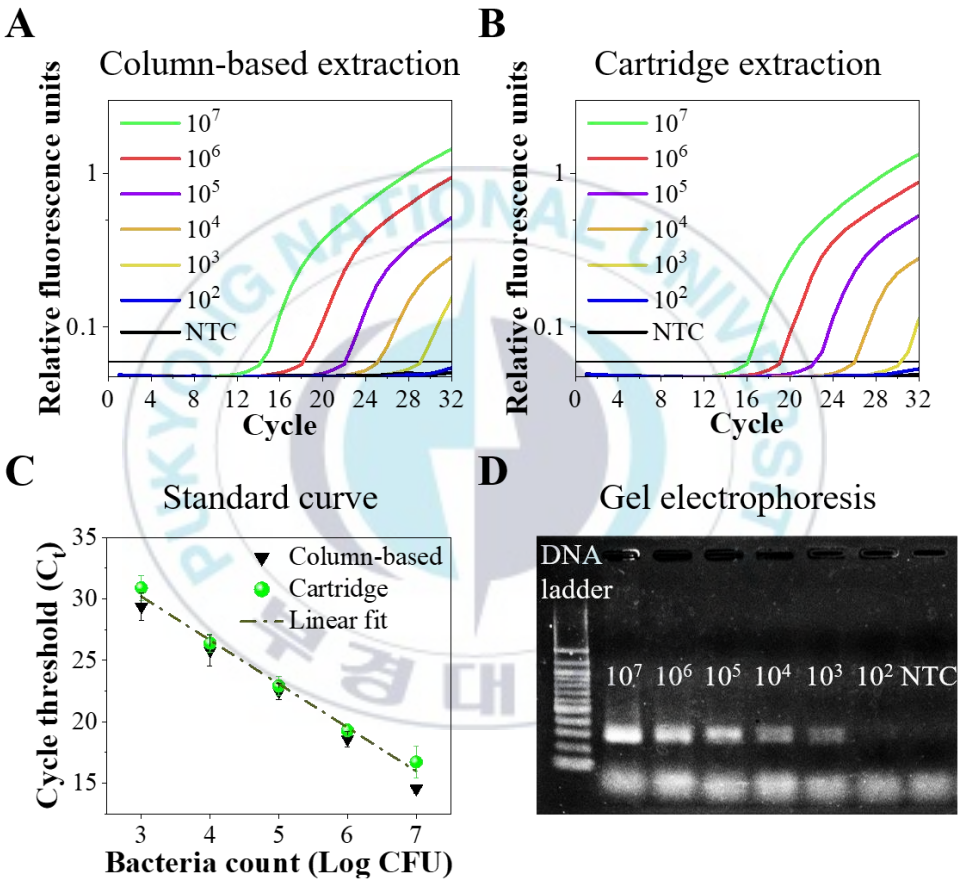

Figure 20. Evaluation of lower detection limit of (A) commercial silica-based SPE kit compared to (B) that of our proposed device when implementing purification and real-time PCR of *E. coli* DNA. (C) Ct values obtained from real-time PCR results were plotted against log base 10 of bacteria concentration to construct the

standard curve. (D) Gel electrophoresis result of real-time PCR results of bacterial samples isolated using our proposed device.

Next, we assessed the extraction LOD of our proposed design when extracting bacterial DNA versus conventional column-based SPE kit. Firstly, we carried out the test to determine the lower limit of detection. As illustrated in Figure 20, our proposed design can extract and successfully amplify as low as 1000 CFU of *E. coli* DNA. This result is comparable to the conventional method and other recent proposed devices as documented in Table 1,2 and 3. Overall, the cycle threshold of cartridge extracted sample is bigger than that of column-based SPE extracted sample. We think the causes of this phenomenon are: (1) the dryness of silica filter in cartridge extraction is not as good as conventional method due to not include a separated drying step, and (2) due to larger amount of elution buffer is used in our proposed design. These drawbacks can be overcome by optimization of the volume of reagents and implement a drying mechanism to the current design. Ct values obtained from real-time PCR results were plotted against log base 10 of bacteria concentration to construct the standard curve as illustrated in Figure 20C. The efficiency of amplification was derived from the following formula [75]:

$$\text{Efficiency} = 10^{-\frac{1}{slope}} - 1$$

Efficiency is 0.91 in comparison with 0.94 of column-based SPE method and shows no significant inhibition. Good amplification efficiency is between 90% and 110% [76], hence this designate our proposed device can provide good quality DNA for downstream applications. Gel electrophoresis result of real-time PCR results of bacterial samples isolated using our proposed device also confirmed there are amplicon at 1000 CFU as shown in Figure 20D.

Then, we tested for the LOD when using a large number of bacteria. Research shows that, the silica-based filter is prone to the clogging of pores and the filter outer layer inactivation by the lysate [77]. Because the supplier of the filter suggests the quantity of bacteria need to be smaller or equal to 2×10^9, we tested the performance of our proposed device when extracting that much of bacteria. As demonstrated in Figure 21, at high concentration the performance of our proposed device is still similar to the column-base SPE kit, including concentration of DNA, 260/280 ratio and real-time PCR result. From Figure 18 and 21, we notice that the cycle threshold is lower when increasing the concentration of bacteria from 2×10^8 to 2×10^9. We suspect that due to the abundance of DNA template, the polymerase might be interfered because of uncompleted denaturation or carry-over of qPCR inhibition components from the extraction [78].

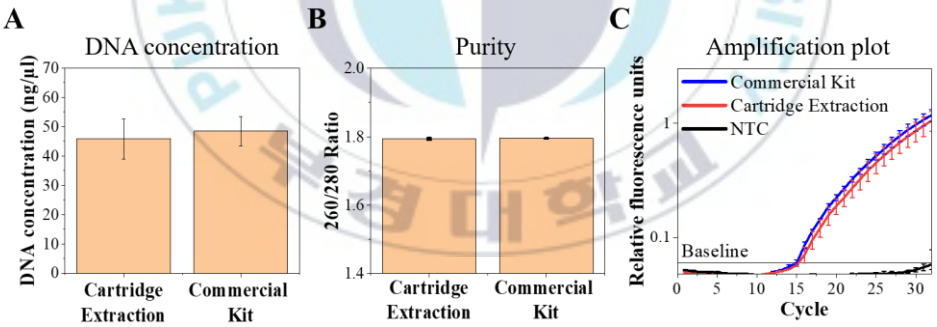

Figure 21. Evaluation of upper detection limit of bacterial DNA extraction utilizing our proposed design compared with that of commercial silica-based SPE kit: (A) concentration of DNA, (B) measurement of 260/280 ratio, and (C) real-time PCR result.

CHAPTER 5: CONCLUSION

In conclusion, we introduced a novel disposable, pressure-driven cartridge with preloaded reagents together with a microfluidic chip that could perform bacterial cell lysis and automate the DNA purification process. While the column-based DNA extraction process might take approximately 1 hour to perform, our proposed cartridge can extract *E. coli* O157:H7 DNA within 36 min, with 33 min to perform cell lysis and the rest for automated nucleic acid purification. By utilizing pressurized air to manipulate the reagents through the microfluidic chip, a closed-system design is achieved, which protects users from exposure to harmful pathogens and eliminates the risk of cross-contamination. Cartridge-extracted DNA shows similar concentration, purity, and amplification results to that of the column-based extraction method. The lower limit of detection of the cartridge extraction is 10^3 CFU *E. coli* O157:H7 which is comparable to that of conventional method. Furthermore, our proposed design only requires two axes movements to automate the DNA extraction process. In addition, the cartridge can be easily modified to extract other targets. Our cartridge is easy to operate and can extract nucleic acid automatically for downstream analysis. a wide range of downstream applications.

Currently, our cartridge only automates the DNA purification process while the lysis step still needs to be done manually. To fully automate the DNA extraction process, two linear actuators can be used to sequentially release the lysis reagents and a custom-made heater can be used to perform the incubation step. Additionally, the motor connecting to the tube array can be programmed to move back and forth continuously to perform the mixing step. A homogenizing tube can be custom-made and integrate into the cartridge to automate RNA extraction. To achieve “sample-in-answer-out”, an amplification module such as qPCR or LAMP and a detection

module such as fluorescent-based detection, colorimetric detection or lateral flow assay can be integrated into the cartridge. Furthermore, other modules can be integrated or built to use together with this device for onsite analysis of environmental samples. For instance, a concentration module can be made to analyze wastewater and aqua-culturing water samples. For plant and soil samples, a homogenizing module can be made to pre-process the sample before nucleic acid extraction. In addition, for urine analysis, the lysis tube and the cartridge size can be modified to process a larger amount of sample. We believe that our proposed cartridge opens up a new horizon for automating the POC nucleic acid testing.

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PUBLICATIONS

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**Oral Presentation*

Patents

1. Joong Ho Shin, Won Han, Thang Le, "Multi-chamber cartridge, nucleic acid extraction module having the same," 10-2022,0058678 (Filling date: 2022.05.13)

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