

Article

3D-Printed Microfluidic Platform Enabling Bacterial Preconcentration and DNA Purification for Molecular Detection of Pathogens in Blood

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Abstract: Molecular detection of pathogens in clinical samples often requires pretreatment techniques, including immunomagnetic separation and magnetic silica bead (MSB)-based DNA purification to obtain the purified DNA of pathogens. These two techniques usually rely on handling small tubes containing a few millilitres of the sample and manual operation, implying that an automated system encompassing both techniques is needed for larger quantities of the samples. Here, we report a 3D-printed microfluidic platform that enables bacterial preconcentration and genomic DNA (gDNA) purification for improving the molecular detection of target pathogens in blood samples. The device consists of two microchannels and one chamber, which can be used to preconcentrate pathogens bound to antibody-conjugated magnetic nanoparticles (Ab-MNPs) and subsequently extract gDNA using magnetic silica beads (MSBs) in a sequential manner. The device was able to preconcentrate very low concentrations of pathogens and extract their genomic DNA in 10 mL of 10% blood within 30 min, and thus allowed polymerase chain reaction (PCR) and quantitative PCR to detect 1 colony forming unit of *Escherichia coli* O157:H7 in 10% blood. The results suggest that the 3D-printed microfluidic platform is highly useful for lowering the limitations on molecular detection in blood by preconcentrating the target pathogen and isolating its DNA in a large volume of the sample.

Keywords: immunomagnetic separation (IMS); bacterial pathogen; 3D printing; preconcentration; DNA purification; molecular diagnostics.

1. Introduction

It is important to accurately detect pathogens in clinical samples at very low concentrations [1,2]. Methods for detecting pathogens in clinical samples, such as blood and saliva, include bacterial culture and polymerase chain reaction (PCR) [3]. However, when detecting pathogens in clinical samples, there are limitations because of the presence of substances that inhibit PCR [3,4]. Thus, such methods of detection still require sample pretreatment to isolate the target microorganisms and purify their nucleic acids [3-5]. Among these pretreatment techniques, immunomagnetic separation (IMS) [5,6] and magnetic silica bead (MSB)-based DNA purification [7-9] are the most popular. However, these two technologies usually rely on handling small tubes containing several millilitres of the sample and manual operation; thus, there is an urgent need for automated systems that can process large volumes of the samples simultaneously because higher concentrations of purified DNA can be obtained by preconcentrating the pathogens and purifying DNA from larger volumes of samples.

In the past few decades, microfluidic devices (μ FDs) have been developed as platforms that can detect pathogens [10-12]. In particular, μ FDs offer several advantages for detection when integrating IMS. For example, μ FDs have a large surface area, thus allowing antibody-conjugated magnetic nanoparticles (Ab-MNPs) and targeted bacterial cells to quickly bind to each other [13]. In addition, the magnetic interaction between the Ab-MNPs and permanent magnets is very strong in the thin

microchannels of the μ FDs, and the bacteria–Ab–MNP complexes can be trapped easily and quickly [14]. Recently, efforts have been made to extract and isolate target DNA using either MSBs or IMS in μ FDs [7–9, 15,16]. However, conventional μ FDs [13–16] are typically not suitable for processing samples larger than 1 mL due to the small dimensions (\sim 1 mm) of their microchannels. In addition, their fabrication requires multiple layers and several bonding steps, making them difficult to be mass-produced. Most recently, we have demonstrated that a 3D-printed μ FD (3Dp μ FD) is an excellent platform for detecting pathogens because of its high speed, integration, and automation [17]. Compared to photolithography and soft lithography, 3D printing has many advantages when printing μ FDs because this technique easily enables printing high aspect ratio structures and does not require complex bonding steps to form a monolithic structure. In recent years, considerable efforts have been devoted to the development of 3D printing microfluidic platforms for separation and detection [17–19]. However, to the best of our knowledge, there have been no reports showing that both IMS and DNA purification functions were integrated into a single device.

In the present study, we report a 3Dp μ FD, which can perform IMS and DNA purification of the target pathogen in samples of volume 10 mL or higher. The performance of the 3Dp μ FD was tested with *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a buffer and spiked blood samples. The performance of the device was verified by standard methods such as colony counting, PCR, and quantitative PCR (qPCR).

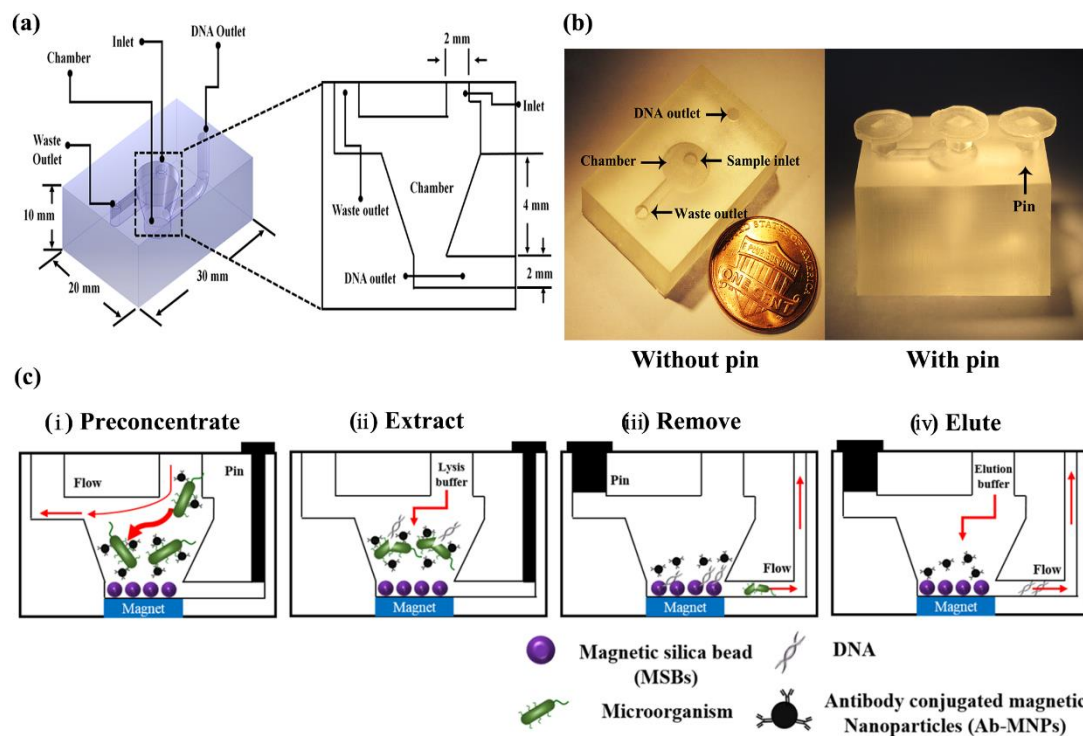


Figure 1. Bacterial preconcentration and genomic DNA (gDNA) purification on the 3Dp μ FD. (a) Design of the 3Dp μ FD. (b) A scale image of the 3Dp μ FD. (c) Schematic of the operational processes: (i) preconcentrating bacteria–Ab–MNP complexes; (ii) extracting gDNA from the complexes with lysis-binding buffer; (iii) removing buffer and bacterial debris by withdrawing flow using a pump; (iv) eluting gDNA from MSBs with elution buffer.

2. Materials and Methods

2.1. 3D printing of μ FD

The μ FD was 3D-printed using a digital light processing (DLP) 3D printer (IM-96) (Carima Co., Seoul, Korea). This 3D-printing technique was based on photopolymerisation by emitting visible light at 405 nm onto a photocurable resin. The 3D sketch of the μ FD was designed using the Student edition of Inventor® Professional (Autodesk Inc., Seoul, Korea). This 3D sketch was cut into 100 μ m thick layers in the z-axis direction using the Carima Slicer software and was separated into 115 layers. Each layer was irradiated for 1.5 s. After the 3D-printing, the μ FDs were washed with 70% ethanol for 5 min to eliminate any uncured resin. Then, the μ FDs were solidified for 10 min using the visible light to improve their mechanical strength. The entire process, including the solidification step, takes 30 min and does not require any additional assembly steps.

Figures 1a and 1b show that the μ FD (width \times length \times height dimensions of 20 \times 30 \times 10 mm³) consists of a cylindrical chamber (diameter: 5 mm, height: 2 mm) connected to two microchannels, a sample inlet (diameter: 2 mm), and DNA and waste outlets (diameter: 2 mm).

2.2. Bacterial culture

The bacterial strains used in this study were *E. coli* O157:H7 (ATCC 43894) and *S. aureus* (ATCC 29213) (Bethesda, MD, USA). The strains were grown overnight in Luria broth (LB) (Becton, Dickinson and Company, NJ, USA) at 200 rpm and 37 °C. The culture was then diluted 100-fold with fresh LB and incubated again at 200 rpm and 37 °C until the optical density of the sample at 600 nm (OD₆₀₀) was 1. Before preconcentration, the samples were serially diluted 10 times with phosphate-buffered saline (PBS) (pH 7.4).

2.3. Synthesis of Ab-MNPs

Amine-modified superparamagnetic nanoparticles (MNPs) of 50 nm diameter were purchased from Chemicell Co. (Berlin, Germany). The MNPs were sonicated for about 40 s to prevent aggregation. A solution of the MNPs (1 mg/mL) in PBS was then made to react with glutaraldehyde (2.5% v/v) in PBS at room temperature (RT) for 1 h using a rotary incubator and then washed with borate buffer (10 mM, pH 7.0) [20]. Aldehyde-functionalised MNPs were then mixed with 50 μ g/mL of affinity purified anti-*E. coli* O157:H7 antibody (SeraCare Life Sciences Inc., Milford, MA, USA) or affinity purified anti-*S. aureus* antibody (SeraCare Life Sciences Inc., Milford, MA, USA) in borate buffer and incubated at RT overnight. Next, Ab-MNPs were washed with 500 μ L of borate buffer and then mixed with 1% bovine serum albumin (BSA) (Thermo Fisher Scientific) in PBS to block unreacted aldehyde groups on the Ab-MNPs at RT for 1 h. To remove the unbound BSA, the Ab-MNPs were washed with 500 μ L of borate buffer again. Then, the Ab-MNPs were treated with 20 mg/mL of sodium cyanoborohydride (Sigma-Aldrich, MO, USA) in borate buffer. Finally, the Ab-MNPs were washed with Tris-HCl buffer (pH 8.0) and stored in PBS at 4 °C until their use.

2.4. Effect of MSB and bacterial concentration on DNA purification

The 3Dp μ FD packed with MSBs (Chemicell co., Berlin, Germany) was prepared by introducing 1 mL of PBS containing different concentrations (10⁹ to 5 \times 10¹⁰ particles/mL) of MSBs at 2 mL/min through the sample inlet while placing a permanent magnet (diameter: 15 mm, height: 1.5 mm, magnetic flux density: 1720 G) underneath the chamber and closing the DNA outlet with a plastic pin. Different volumes (1–100 mL) of PBS containing *E. coli* O157:H7 or *S. aureus* at various concentrations (1–10⁵ colony forming units (CFU)/mL, final concentration) were first mixed with MNPs (10¹³ particles/mL, final concentration) conjugated with antibodies specific to the pathogens, and the mixture was incubated at 37 °C and 200 rpm for 20 min in a beaker. Then, the mixture was injected into the 3Dp μ FD packed with MSBs through the sample inlet using a syringe pump (Harvard Apparatus, Boston, MA, USA) at 2 mL/min, as shown in Fig. 1ci.

To lyse preconcentrated bacterial cells, 100 μ L of Lysis & Binding buffer (Chemicell Co., Berlin, Germany) were loaded into the chamber, as shown in Fig. 1cii. The 3Dp μ FD was agitated by the automated mini-vibration system (DVM-N20 vibration motor) (D&J WITH Co., Ltd., Seoul, Korea) at RT for 5 min and the blocking pins were removed from the 3Dp μ FD before placing on the magnet.

The buffer was removed from the chamber through the waste channel, and then, 500 μL of washing buffer I (Chemicell Co.) and 500 μL of 70% ethanol were injected into the 3Dp μFD sequentially. For the elution step, 50 μL of RNase free water was loaded into the 3Dp μFD without placement on the magnet. After all the inlets and outlets were blocked with the pins again, the device was agitated on a thermo-shaker at 1200 rpm and 65 $^{\circ}\text{C}$ for 15 min. Finally, the DNA released from the MSBs was removed from the device through the DNA outlet.

The number of preconcentrated bacterial cells was confirmed by counting the difference between the number of uncaptured bacterial cells and the total number of bacterial cells using the standard colony counting method [21]. The capturing efficiency for the bacterial cells can be calculated using the following equation [17]:

$$\text{Capturing efficiency (\%)} = (\text{Nt} - \text{Ne})/\text{Nt} \times 100\%, \quad (1)$$

where Nt is the number of bacterial cells in the sample and Ne is the number of uncaptured bacterial cells in the sample.

2.5. Bacterial preconcentration and DNA purification in spiked blood samples

The use of blood was approved by the Institutional Review Board (IRB) of the university (SKKU) and its approval number was SKKU 2017-11-006. Whole blood was purchased from Innovative Research, Inc (MI, USA) and this product was treated with K2 EDTA to prevent blood coagulation. Then, 1 mL of whole blood was mixed with 8.8 mL of PBS, 1 mL of *E. coli* O157:H7 ($10\text{--}10^4$ CFU/mL), and 200 μL of Ab-MNPs. The subsequent procedures were the same as those for the bacterial preconcentration and DNA purification steps.

2.6. Detection of bacteria by PCR and qPCR samples

Purified bacterial genomic DNA (gDNA) was amplified by PCR, and the amplification of target genes was verified by gel electrophoresis. The primers that were designed to amplify the 150-base pairs (bp) of *eae* gene coding intimin adherence protein in *E. coli* O157:H7 consist of a forward primer (GGCGGATTAGACTTCGGCTA) and a reverse primer (CGTTTTGGCACTATTTGCC). The 207-bp of *nuc* gene coding the thermonuclease of *S. aureus* consists of a forward primer (ACACCTGAAACAAAGCATCC) and a reverse primer (TAGCCAAGCCTTGACGAACT). The conventional PCR was performed using a PCR reagent by MJ MINITM thermocycler (Bio-RAD, CA, USA). The PCR products were separated in a 1.5% TAE (Tris base, acetic acid and EDTA) (50 \times) agarose gel at 100 V for 30 min.

The qPCR was performed using LightCycler[®] Nano (Roche, Basel, Switzerland), and its cycle threshold (Ct) value was determined. The same primer sets as those used for the PCR were used here.

3. Results and Discussion

3.1. Optimisation of MSB concentrations for DNA purification using 3Dp μFD

To find the optimal number for MSBs for DNA purification using the 3Dp μFD , it was packed with different particle numbers (1×10^9 to 5×10^{10}) of MSBs before introducing 10 mL of PBS containing 10^3 CFU/mL and Ab-MNPs containing 10^{13} particles/mL. All the bacterial capturing efficiencies were about 90%, which are similar to those mentioned in the previous reports, suggesting that the number of MSBs does not affect the bacterial capturing efficiency on the 3Dp μFD .

Figure 2a shows that gDNA concentrations obtained using the 3Dp μFD s increase as MSB concentrations in the range of 10^9 to 10^{10} particles/mL increase. The maximum gDNA concentration was obtained using the 3Dp μFD packed with 10^{10} particles/mL of MSBs. However, the gDNA concentrations at 5×10^{10} MSBs/mL was lower than that at 10^{10} MSBs/mL. This may be attributed to the

fact that 50 μL of the elution buffer was not sufficient to fully wet the MSBs in the 3Dp μFD . Thus, some DNA might not have been released from some of the MSBs, resulting in lower gDNA concentrations.

When the 3Dp μFD s packed with 10^{10} MSBs/mL was injected along with 10 mL of PBS containing *E. coli* O157:H7 at different concentrations (1– 10^5 CFU/mL) at a rate of 2 mL/min, the gDNA concentration obtained using the 3Dp μFD s increased as the bacterial concentrations increased in the range of 1 to 10^3 CFU/mL, but did not increase any further at concentrations of 10^4 and 10^5 CFU/mL (Figure 2b), thus indicating that the surfaces of the MSBs were saturated with gDNA at such high concentrations. For such high concentrations, the sample solutions should be diluted to obtain accurate measurements.

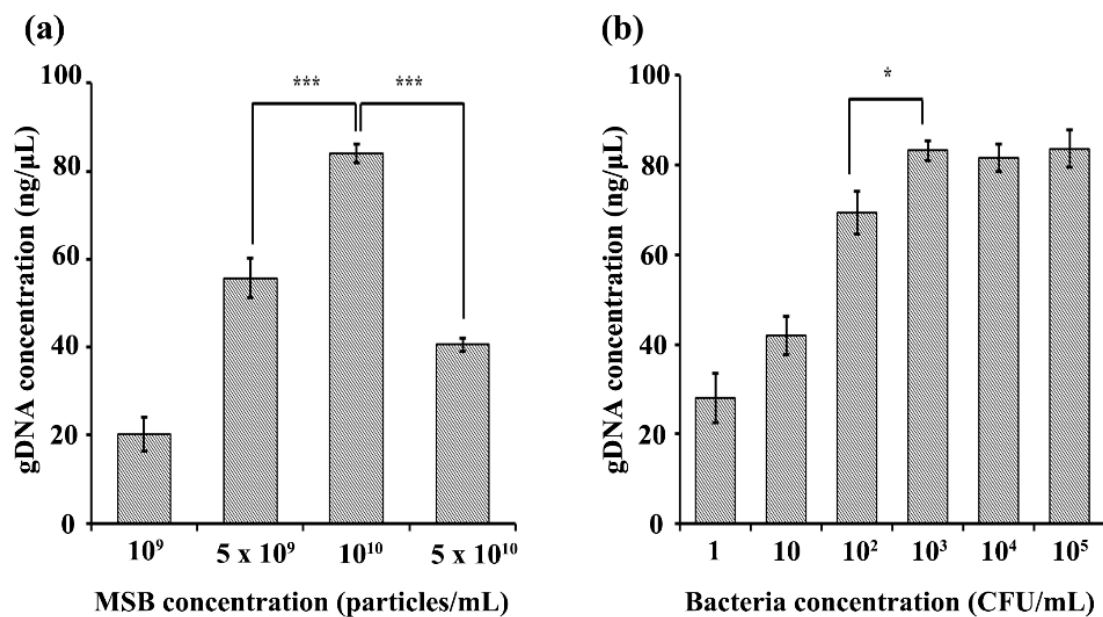


Figure 2. Effect of MSB and bacteria concentrations on DNA purification on the 3Dp μFD : (a) gDNA concentration with different concentrations (10^9 – 5×10^{10} particles/mL) of MSBs for 10^3 CFU/mL of *E. coli* O157:H7; (b) gDNA concentration with different concentrations (1– 10^5 CFU/mL) of *E. coli* O157:H7 using 10^{10} particles/mL of MSBs *: $P < 0.05$, ***: $P < 0.001$. Student *t*-test. Sample number = 3.

3.2. Effect of preconcentration and gDNA purification using 3Dp μFD on molecular amplification of genes in PBS

Once 10 mL of the Gram-negative pathogen *E. coli* O157:H7 at different concentrations (1– 10^3 CFU/mL) was preconcentrated and its gDNA was purified in a sequential manner using the 3Dp μFD , the gDNA was amplified using either PCR or qPCR to verify the yield. The results were compared to those obtained from either untreated samples or samples prepared with only the preconcentration step using 3Dp μFD . In the samples with the preconcentration–purification steps, a concentration as low as 1 CFU/mL was detectable using PCR with gel electrophoresis (Figure 3c), whereas in the untreated samples and samples with only the preconcentration step, concentrations as low as 10^3 CFU/mL and 10 CFU/mL were detectable, respectively (Figures 3a and 3b). A similar trend was observed with qPCR. Considering that Ct values below 35 are reliable, a concentration as low as 1 CFU/mL was detectable in the samples with the preconcentration–purification steps using qPCR because its Ct value was 30.7 (Figure 3f). Concentrations as low as 10^3 CFU/mL and 10 CFU/mL were respectively detectable in the untreated samples and samples with only the preconcentration step

because their respective Ct values were 30.8 and 33.4 (Figures 3d and 3e). This remarkable improvement in the samples with the preconcentration–purification steps in detection using PCR and qPCR can be explained as follows. In the sample with only the preconcentration step, the Ab-MNPs can improve the detection by providing higher numbers of bacterial cells to the PCR and qPCR. However, Ab-MNPs and cell debris can inhibit DNA polymerase, so that the benefit offered by the bacterial preconcentration is decreased due to the modest interference from Ab-MNPs and cell debris in DNA amplification [22]. By including both preconcentration and purification steps in the 3Dp μ FD, only purified gDNA can be provide to the PCR and qPCR. This results in the enhancement in detection using PCR and qPCR.

The performance of the 3Dp μ FDs were further tested by preconcentrating the Gram-positive pathogen *S. aureus* at different concentrations ($1\text{--}10^3$ CFU/mL) and purifying its gDNA using the 3Dp μ FD. Similar to the results (Figure 3) for *E. coli* O157:H7, significant improvements in the detection of *S. aureus* by both molecular diagnostics techniques, PCR-post gel electrophoresis and qPCR, were observed in the samples with the preconcentration–purification steps. The lowest concentration for the detection in the samples with both preconcentration–purification steps using PCR-post gel electrophoresis was 10^2 CFU/mL (Figure 4c), whereas those for the samples with only the preconcentration step was 10^3 CFU/mL (Figure 4b). However, *S. aureus* at all the concentrations ($1\text{--}10^3$ CFU/mL) in the untreated samples was not detectable (Figure 4a). A similar trend was observed using qPCR. The cell walls of Gram-positive bacteria are thicker than those of the Gram-negative ones [7] and is hard to lyse by thermocycling of PCR. As a result, even at 10^3 CFU/mL, the concentration of gDNA released from the lysed cells was not sufficient to be detected using PCR-gel electrophoresis and qPCR. With the preconcentration step, bacterial numbers increased and their gDNA was detectable in the sample containing 10^3 CFU/mL. Further improvement in the detection was possible when the preconcentration and purification steps were included in the 3Dp μ FD because the Lysis & Binding buffer in the DNA purification step induces cell lysis. Together with the results in samples containing *E. coli* O157:H7, these results suggest that gDNA purification is required as an addition in the 3Dp μ FD to improve the detection of bacterial pathogens using molecular diagnostic methods.

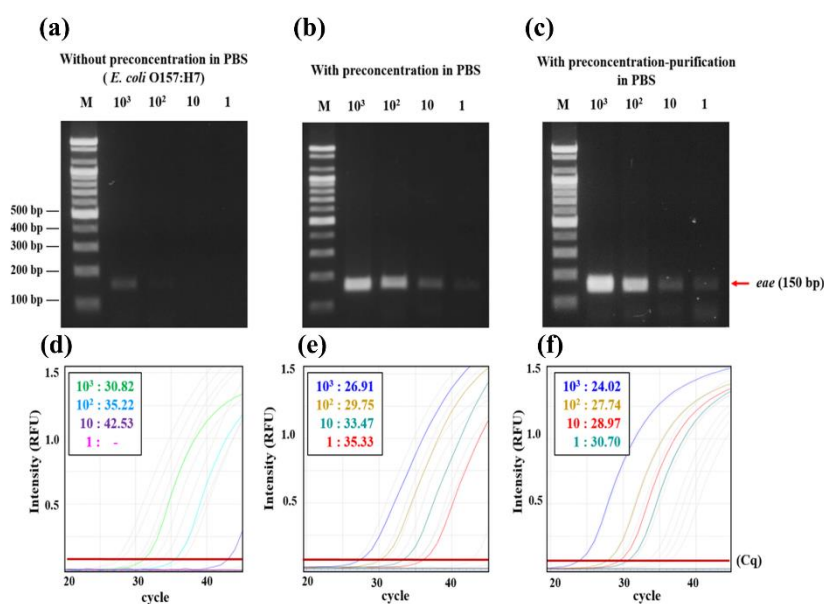


Figure 3. Confirmation of *E. coli* O157:H7 preconcentrating and DNA purifying efficiency using either PCR with gel electrophoresis and qPCR; (a) Gel electrophoresis of PCR products (*eae* gene) from 10 mL PBS containing *E. coli* O157:H7 at different concentration ($1\text{--}10^3$ CFU/mL) before; (b) after preconcentration; (c) after bacterial preconcentration and DNA purification; (d)–(f) qPCR results of (a)–(c), respectively.

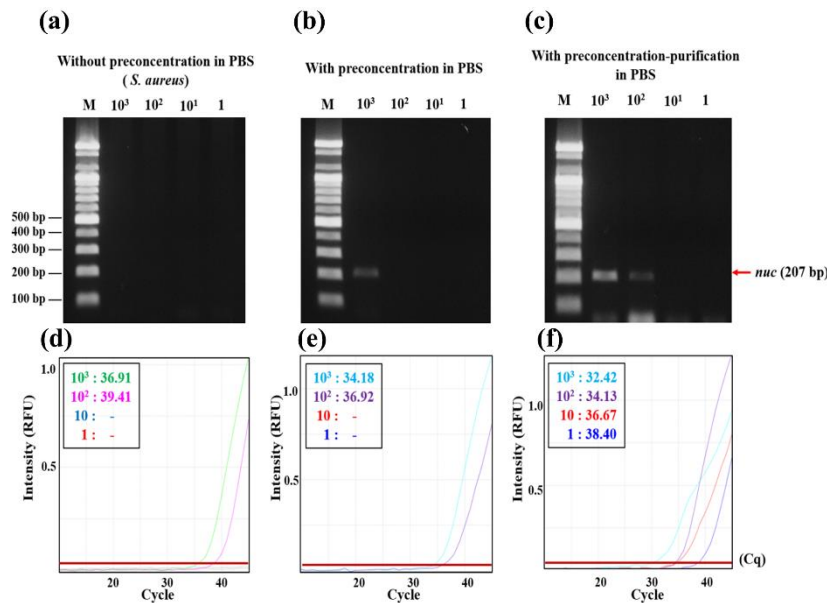


Figure 4. Confirmation of *S. aureus* precentrating and DNA purifying efficiency using PCR with gel electrophoresis and qPCR; (a) Gel electrophoresis of PCR products (*nuc* gene) from 10 mL PBS containing *S. aureus* at different concentration (1–10³ CFU/mL) before; (b) after precentration; (c) after bacterial precentration and DNA purification; (d)–(f) qPCR results of (a)–(c), respectively.

3.6. Spike test in 10% blood

Blood may contain molecular diagnostic inhibitors such as hemin; thus, it is necessary to isolate the DNA from bacteria and diagnostic samples. Figure 5 shows that the performance of the 3D for the sample-preparation from 10 mL of 10% diluted blood with *E. coli* O157:H7 is excellent because *E. coli* O157:H7 can be detected for a concentration of up to 1 CFU/mL with precentration and purification steps, which is 100 and 10 times lower than without sample treatment (Figures 5a and 5b). These results are also confirmed by qPCR through the difference between the C_q values of the samples (Figures 5d–5f). The C_q value of the 1 CFU/mL is 30.6 and that of the 10³ CFU/mL without sample-preparation is 33.8. In general, IgG in the blood interferes with the activation of Taq polymerase as it interacts with single-strand DNA at high temperatures [23]. Further, the blood samples have a lot of molecular diagnostic inhibitors such as hematin and haemoglobin, and they prevent the action of Taq polymerase [24]. For this reason, Figure 5a shows that the limit of detection is 10³ CFU/mL without sample-preparation. Thus, it is very hard to detect pathogens in blood samples using PCR without any sample preparation steps. However, Figure 5c shows that the 3D μ FD can eliminate the molecular diagnostic inhibitors effectively. This is equivalent to a 50 times improvement of sensitivity compared with the microfluidic device for the detection of *E. coli* O157:H7 in 10% blood [25]. These results suggest that 3D μ FD is well suited for improving the level of detection (LOD) in blood by including both bacterial precentration and DNA purification steps

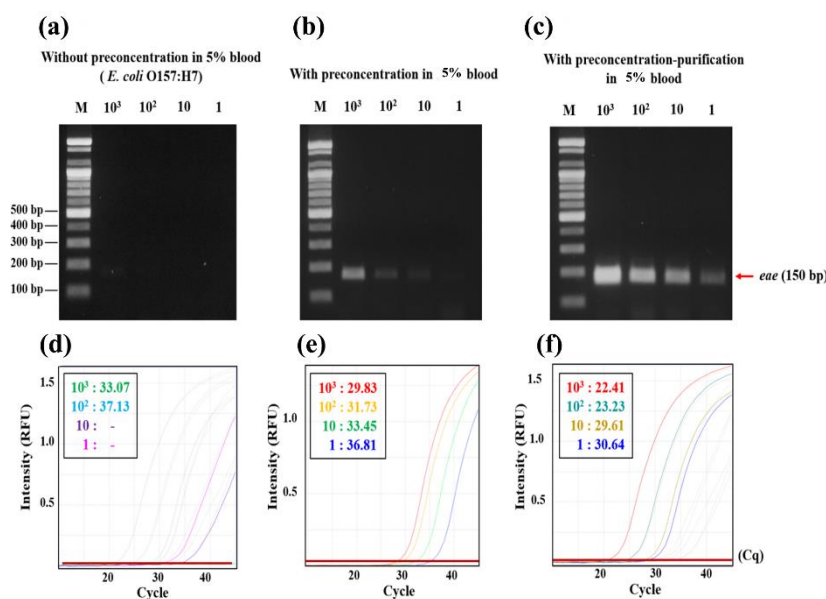


Figure 5. Confirmation of precentrating and DNA purification efficiency of *E. coli* O15:H7 in 10% blood using PCR with gel electrophoresis and qPCR. (a) Gel electrophoresis of PCR products (*eae* gene) from 10% blood in 10 mL PBS containing *E. coli* O157:H7 at different concentration (1–10³ CFU/mL) before; (b) after precentrating; (c) after precentrating and DNA purification; (d)–(f) qPCR results of (a)–(c), respectively.

4. Conclusions

In this study, we report the improvement in detection of molecular diagnostics by successfully integrating both bacterial precentration and DNA purification in the 3DpμFD. The performance of the 3DpμFD shows that it is possible to precentrate very low concentrations of pathogens in 10 mL of 10% blood within 30 min, and 1 CFU/mL of *E. coli* O157:H7 can be detected by PCR with either post-gel electrophoresis or qPCR. These results demonstrate that it is highly useful for improving molecular diagnostic methods in blood samples with addition of the DNA purification step.

There are many commercial kits and systems for the purification of bacterial DNA from blood, such as QIAamp DNA mini kit and ExiPrep bacteria genomic DNA kit. However, these kits are not automated and are difficult for handling large volumes of samples. In addition, they are only suitable for DNA purification and relatively small volumes of samples such as 100 μL. When the 3D-printed microfluidic platform is fully automated, it will be useful for detecting low-levels of pathogens present in clinical samples in a short period of time.

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