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RESEARCH PAPER

Development of an on-site rapid real-time polymerase chain reaction system and the characterization of suitable DNA polymerases for TaqMan probe technology

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Abstract On-site quantitative analyses of microorganisms (including viruses) by the polymerase chain reaction (PCR) system are significantly influencing medical and biological research. We have developed a remarkably rapid and portable real-time PCR system that is based on microfluidic approaches. Real-time PCR using TaqMan probes consists of a complex reaction. Therefore, in a rapid real-time PCR, the optimum DNA polymerase must be estimated by using actual real-time PCR conditions. In this study, we compared the performance of three DNA polymerases in actual PCR conditions using our rapid real-time PCR system. Although KAPA2G Fast HS DNA Polymerase has the highest enzymatic activity among them, SpeedSTAR HS DNA Polymerase exhibited better performance to rapidly increase the fluorescence signal in an actual real-time PCR using TaqMan probes. Furthermore, we achieved rapid detection of *Escherichia coli* in 7 min by using SpeedSTAR HS DNA Polymerase with the same sensitivity as that of a conventional thermal cycler.

Keywords Rapid · PCR · DNA amplification · Polymerase · On-site · Portable

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Introduction

The quantitative detection of specific DNA sequences has been extensively applied in many analyses concerning molecular biology, such as food testing, environmental monitoring [1, 2], the detection of specific pandemic influenza strains [3, 4], and clinical genetic diagnosis [5]. Polymerase chain reaction (PCR) is a molecular biological technique for exponentially increasing the number of copies of a specified DNA fragment present on the initial template DNA by thermal cycling through two or three different temperatures. Furthermore, real-time PCR is an essential method by which the amount of specific DNA present in a sample can be quantified by modifying the PCR method. Real-time PCR employs a fluorescent probe for quantification of the target DNA. Quantification by real-time PCR is based on the calculation of the threshold of fluorescent intensity depending upon the number of DNA fragments amplified in each thermal cycle during PCR. The thermal cycling process in both conventional and real-time PCR systems requires about 1 h for completion, because these PCR instruments use a Peltier heater to perform over 30 thermal cycles, with each cycle requiring about 2–3 min. Therefore, we could not immediately obtain any result about the existence of microorganisms based on the specific DNA sequences.

Over the last two decades, many researchers have tried microfluidic approaches to find faster alternatives for rapid thermal cycling in a PCR [6]. Theoretically, microfluidic PCR techniques should be effective in shortening thermal cycling times because the ramping (heating-and-cooling) rates are independent of the heat capacity of the reactor vessels or the temperature-controlled metal blocks. There are two main approaches for rapid PCR using microfluidic devices. The first approach is the "flow-through PCR" that is composed of a long serpentine microchannel that repeatedly carries the

PCR solution over two or three temperature zones for denaturation, annealing, and extension of DNA [7–16]. In these devices, the PCR solution travels only in one direction along the serpentine microchannel. A serious drawback of the flow-through PCR device is its low sensitivity owing to adsorption of the PCR solution onto the long microchannel. Furthermore, the flow-through PCR chip often lacks versatility, because PCR conditions such as the reaction time and the number of cycles depend on the configuration of the PCR chip. For example, if we need to modify the number of PCR cycles, a new flow-through PCR chip with the modified configuration has to be made. In the second approach, the PCR solution is reciprocated between two or three temperature zones in a short microchannel for thermal cycling. This type of PCR is referred to as "reciprocal-flow PCR" [17–22]. In reciprocal-flow PCR, the adsorption of the PCR solution to the microchannel is negligible owing to the much smaller contact surface area in the microchannel than in that of a flow-through PCR. Furthermore, these reciprocal-flow PCR devices have flexible cycle numbers and reaction times, because the PCR solution is transported back and forth through a single microchannel by programmed flow control.

The first proof of concept of the reciprocal-flow PCR has been reported by Chiou et al. [17]. They performed rapid thermal cycling for 30 cycles in a glass capillary filled with oil by using computer-controlled solenoid valves, and obtained a 500-bp amplicon from genomic λ-DNA in 23 min. However, they used high template concentrations. Real-time amplicon detection based on reciprocal-flow PCR has been demonstrated by Chen et al. [19]. They measured the fluorescent intensity of PCR solutions for each cycle, and obtained an 87-bp amplicon by performing 40 cycles in 43 min. Their PCR achieved sensitivity comparable to that of a commercial thermal cycler; however, their assay was not a rapid PCR. Liu et al. were the first group to establish a highly sensitive and rapid reciprocal-flow PCR [22]. To achieve the high sensitivity of detection, they used a "water in oil" droplet as the reactor of the reciprocal-flow PCR, coated the microchannel with BSA, and optimized the concentration of the DNA polymerase. Although they obtained a 287-bp amplicon by performing 40 cycles in 12 min, detection of the amplicon was performed by electrophoresis after the PCR. Their approach could not be utilized for real-time detection of fluorescence during PCR, and therefore, their device could not be used for quantitative detection.

The extension rate of the DNA polymerase is one of the important factors in rapid PCR, because, in theory, the time taken to change temperatures is negligible when using a microfluidic device. Montgomery et al. reported the activity of DNA polymerase by using a stopped-flow assay where they examined only the extension rate of the DNA polymerase by using an intercalating method [23]. However, in an actual PCR, the melting speed of the DNA duplex in buffer, the period until the primers bind to the target DNA sequence, and the time taken by the DNA polymerase to begin DNA synthesis are important along with the extension rate to achieve rapid PCR. Therefore, the most appropriate DNA polymerase for rapid PCR has to be estimated in actual rapid PCR conditions.

In this study, we developed a sensitive and rapid real-time PCR system based on reciprocal-flow PCR that could be used for on-site detection and compared three DNA polymerases which have different enzymatic activity in an actual rapid real-time PCR. The precise handling of PCR solutions in each temperature zone was achieved by using microblowers instead of a syringe pump for transporting the solution. The evaluation of the optimum DNA polymerase in an actual rapid PCR based on TaqMan probe technology was enabled by this rapid real-time PCR system. Furthermore, in our system, the fastest quantitative cell-direct PCR condition for *Escherichia coli* was examined by using the optimized DNA polymerase. We successfully showed that a rapid and high-sensitivity PCR could be achieved by optimization of the reaction time and the use of an optimum DNA polymerase without coating the microchannel.

Materials and methods

Reagents and chemicals

SpeedSTAR HS DNA Polymerase and Ex Taq Hot Start Version were purchased from TaKaRa Bio Inc. (Japan). KAPA2G Fast HotStart PCR Kit was purchased from KAPA BIOSYSTEMS (USA). TaqMan probe technology was used for fluorescence detection in this study. Forward primer (FP) *uidA*, reverse primer (RP) *uidA*, and probe *uidA* were used in the experiment to optimize the concentration of different polymerases and to quantitate the number of *E. coli* cells. FP 16S ribosomal RNA (rRNA), RP 16S rRNA 1–4, and probe 16S rRNA were used to measure the extension rate of each polymerase. Primer and probe sequences and the expected amplicon sizes are indicated in Table 1. Primers and probes were purchased from Applied Biosystems. The PCR mixture consisted of 0.025–0.2 U μL^{-1} DNA polymerase with its respective buffer at 1× concentration, a 200 μmol L^{-1} dNTP mixture, and primer and probe concentrations as indicated in Table 1.

DNA was extracted from *E. coli* as follows. One hundred microliters of bacterial culture that had been incubated overnight in LB medium was centrifuged at 11,000*g* for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 200 μL of pre-mixed 10 % Chelex solution in a Cycleave PCR Bacteria Screening Kit (TaKaRa BIO Inc., Japan). The suspended bacterial solution was incubated at 99 °C for 5 min and rapidly cooled by placing on ice for

Table 1 Oligonucleotides used as primers and probes

Oligonucleotide	Sequences (5′ → 3′)	Amplicon size (bp)	Final concentration (μmol L^{-1})
FP *uidA*	GTG TGA TAT CTA CCC GCT TCG C		2.7
Probe *uidA*	FAM-TCG GCA TCC GGT CAG TGG CAG T-MGBNFQ		0.2
RP *uidA*	AGA ACG GTT TGT GGT TAA TCA GGA	83	0.9
FP 16S rRNA	GTT TGA TCC TGG CTC A		1.2
Probe 16S rRNA	FAM-CGG GTG AGT AAT GTC TGG-MGBNFQ		0.2
RP 16S rRNA 1	CTT TGG TCT TGC GAC G	188	1.2
RP 16S rRNA 2	GCA TGG CTG CAT CAG	390	1.2
RP 16S rRNA 3	CTG ACT TAA CAA ACC GC	591	1.2
RP 16S rRNA 4	TAC CAG GGT ATC TAA TCC	793	1.2

FAM 6-carboxyfluorescein, fluorescence reporter dye; *MGBNFQ* minor groove binder nonfluorescence quencher

1 min. The solution was again centrifuged at 11,000*g* for 1 min, and the supernatant was recovered as extracted DNA and used as a positive control (PC) for PCR of the *uidA* or 16S rRNA genes. The final concentration of the extracted DNA as PC was 50 μg mL^{-1}. Two nanograms of template DNA as PC was used per reaction. For cell-direct PCR, *E. coli* was cultivated overnight at 37 °C in LB medium. The concentration of the cells was adjusted to 1×10^5 cells μL^{-1} and diluted to the desired concentration with nuclease-free water (Ambion, USA).

Fabrication of the reciprocal-flow PCR chip

A reciprocal-flow PCR chip made of cyclo-olefin polymer (COP) was purchased from j-TAS inc., Japan. The whole length, width, and depth of the microchannel were 176 mm, 700 μm, and 700 μm, respectively. Four microchannels were integrated in the reciprocal-flow PCR chip. Detailed design of the reciprocal-flow PCR chip is available in the Electronic Supplementary Material (ESM), Fig. S1. The reciprocal-flow chip was transparent and had high thermal tolerance. An image of the reciprocal-flow PCR chip is provided in Fig. 1a.

Optimization of DNA polymerase concentration

The reciprocal-flow PCR chip was in tight contact with two heating metal blocks (Fig. 1b). The left block had a higher temperature (95 °C) for denaturation, and the right block had a lower temperature (50 °C) for combined annealing and extension. Thermal cycling for the PCR was performed by shuttling the PCR solution between the two heating metal blocks (Fig. 2). The fluorescence intensity emitted by the TaqMan probe was measured by a fluorescence detector (FLE-510, NSG GROUP, Japan) as the PCR solution repeatedly passed over the fluorescence detection point. The excitation and emission wavelengths were 470 and 530 nm, respectively. First, the concentration of the DNA polymerase was optimized on the chip using SpeedSTAR HS DNA Polymerase, KAPA2G Fast HS DNA Polymerase, and Ex Taq HS DNA Polymerase. For the real-time PCR, 12 μL of the PCR solution containing extracted DNA was incubated at 95 °C for 10 s (as a hot start step) in order to activate the DNA polymerase, followed by 45 cycles of 95 °C (denaturation step) and 50 °C (combined annealing and extension step). For optimization of the concentration for SpeedSTAR HS DNA Polymerase and KAPA2G Fast HS DNA Polymerase, the denaturation time and the time for annealing and extension were 5 and 10 s, respectively. For Ex Taq HS DNA Polymerase, the denaturation time and the time for annealing and extension were 5 and 15 s, respectively.

Efficiency of each DNA polymerase for rapid real-time PCR

To develop a rapid PCR based on TaqMan probe technology, the conditions of real-time PCR using SpeedSTAR HS DNA Polymerase, KAPA2G Fast HS DNA Polymerase, and Ex Taq HS DNA Polymerase were examined on the reciprocal-flow PCR chip. The temperatures of the heating blocks for denaturation and for annealing and extension were 95 and 50 °C, respectively. Twelve microliters of the PCR solution containing the extracted DNA was incubated at 95 °C for 10 s as a hot start step. Next, the shortest cycling times required for each step for different amplicon sizes were examined by performing a real-time PCR on the chip.

Quantification of the number of *E. coli*

The cultivated *E. coli* was quantified by real-time PCR on the reciprocal-flow PCR chip. In this experiment, the *uidA* primers and probe were used to achieve rapid quantification of *E. coli*. The melting temperatures (Tm) of the *uidA* primers and probe were higher than those of the 16S rRNA primers

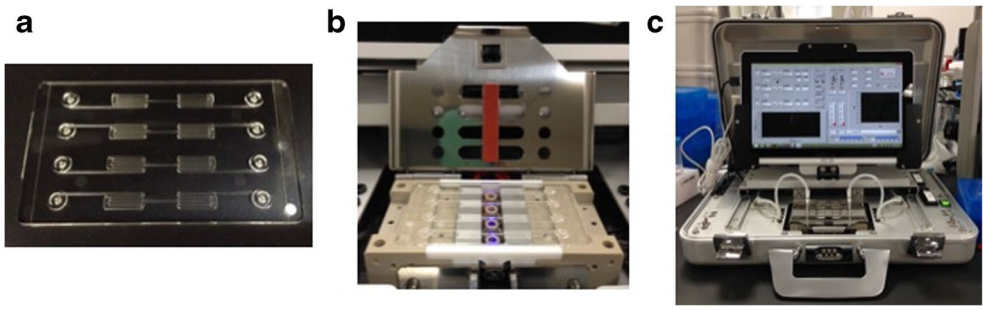

Fig. 1 Portable reciprocal-flow PCR system and chip. **a** Reciprocal-flow PCR chip. **b** The reciprocal-flow PCR chip was fixed onto two metal heater blocks. **c** Portable reciprocal-flow PCR system

and probe. Therefore, the temperatures of the heating blocks for denaturation and for annealing and extension were 95 and 60 °C, respectively. Next, we examined the shortest denaturation and combined extension and annealing times by performing real-time PCR on the chip. In this experiment, the quantification of the number of *E. coli* was performed by using *uidA* primers and probe under optimized rapid PCR conditions.

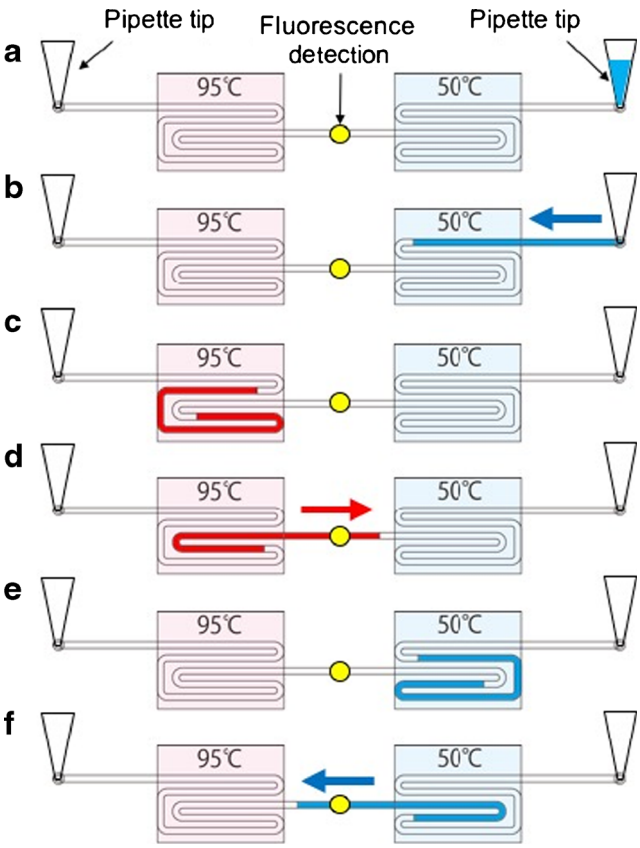

Fig. 2 Rapid PCR on the reciprocal-flow PCR system. **a** Twelve microliters of PCR solution was pipetted; then, the pipette tip was inserted into the right inlet of the reciprocal-flow PCR chip. **b** The PCR solution was introduced into the reciprocal-flow PCR chip. **c** The PCR solution was incubated on the left side heater for denaturation. **d** The PCR solution was moved to the right side heater through the fluorescence detection point. **e** The PCR solution was incubated on the right side heater for annealing and extension. **f** The PCR solution was moved to the left side heater through the fluorescence detection point

Results and discussion

Portable reciprocal-flow PCR system

In this study, we developed a portable reciprocal-flow PCR system that can be transported in an attaché case to realize the goal of on-site detection (Fig. 1). This portable PCR system was composed of a tablet PC for system control, two heating aluminum blocks for thermal cycling, two microblowers (Murata Manufacturing, Japan) for shuttling of the PCR solution, and a fluorescence detector for measuring the fluorescence intensity of the cycling solution during each PCR cycle. The diagram of our system is shown in ESM Fig. S2. The system was powered by a battery (XP18000A, Energizer, USA) for on-site detection. The microblowers were under the top board and were connected to a pipette chip via a silicon tube. The fluorescence detector was also under the top board. The fluorescence signal was detected by the probe arranged between the two heaters under the reciprocal-flow PCR chip. The PCR temperature was measured by thermocouples attached to each heater and regulated by the LabVIEW program. In this portable PCR system, the reciprocating flow of the PCR solution between two heating blocks was achieved by switching the microblowers connected to each end of the microchannel. In this system, 12 μL of PCR solution was pipetted and the pipette tip was inserted into the right inlet of the reciprocal-flow PCR chip. Next, the pipette tip was connected to the microblower via a silicon tube. Then, once the program of the reciprocal-flow PCR system started (Fig. 2), the PCR solution was introduced into the left side heater, passing through the fluorescence detection point, by using the right side microblower. If the fluorescence of the PCR solution decreased after passing through the fluorescence detection point followed by the microblower, the flow was stopped immediately. After the hot start step, the PCR solution was channeled onto the right side heater by the left side microblower. After annealing and extension, the PCR solution was channeled onto the left side heater again for denaturation. As the PCR solution flowed over the center of the microchannel during each cycle, the fluorescent intensity was measured by the fluorescence detector. In this reciprocal-flow PCR system, smooth shuttling of the PCR

solution was achieved by using microblowers instead of conventional micro pumping systems such as syringe pumps or peristaltic pumps. These pumps force the solution through a microchannel based on the compressed and/or depressed pressure between the pump and solution. When the conventional micro pumping systems were used to control the flow of the solution in the reciprocal-flow PCR system, the air between the micro pumping system and PCR solution acted as an air spring. Because of this, the movement of the PCR solution lagged behind the movement of the micro pumping systems. Therefore, the precise and rapid handling of the PCR solution in each temperature zone was difficult. As opposed to this, when the microblower was used to control the flow of the solution in the reciprocal-flow PCR system, the air pressure in the microchannel changed to the atmospheric pressure the instant the microblower stopped. Therefore, the precise and rapid handling of the PCR solution on each temperature zone was achieved. Additionally, we could configure the number of thermal cycles and the reaction time for each temperature step in the reciprocal-flow PCR, which cannot be done in a continuous-flow PCR. The control of this system was programmed using the LabVIEW software. Moreover, in our reciprocal-flow PCR, the microchannel did not need to be filled with oil and the PCR solution was injected as a short segment to prevent bubble formation in the microchannel. The air bubbles that were generated in the segmented PCR solution in the denaturation zone were dislodged easily and promptly from the solution into the air spaces in the microchannel. Furthermore, the ratio of the segmented PCR solution after the completion of reaction to that before initiation was 97.7 ± 0.953 % ($n = 24$). Therefore, the evaporation of the PCR solution was not an issue in our system.

Optimization of the rapid reciprocal-flow PCR

The conditions for the rapid real-time PCR were optimized by using the reciprocal-flow PCR system developed in this study. The optimum concentrations of SpeedSTAR HS DNA Polymerase, KAPA2G Fast HS DNA Polymerase, and Ex Taq HS DNA Polymerase were standardized by using the primers and probe for the *uidA* gene and DNA extracted from *E. coli*. Figure 3 indicates the amplification curve obtained by using different concentrations of SpeedSTAR HS DNA Polymerase. The increase in fluorescence intensity was low when we used 0.025 U μL^{-1} of the DNA polymerase. The fluorescence intensity increased gradually as we increased the amount of polymerase added to 0.1 U μL^{-1} of the polymerase. The number of PCR cycles required to detect an increase in the fluorescence intensity when 0.2 U μL^{-1} of the polymerase was added was almost the same as that observed upon addition of 0.1 U μL^{-1} of the polymerase. Therefore, the optimum concentration of the SpeedSTAR HS DNA Polymerase required for the reciprocal-flow PCR was ascertained to be

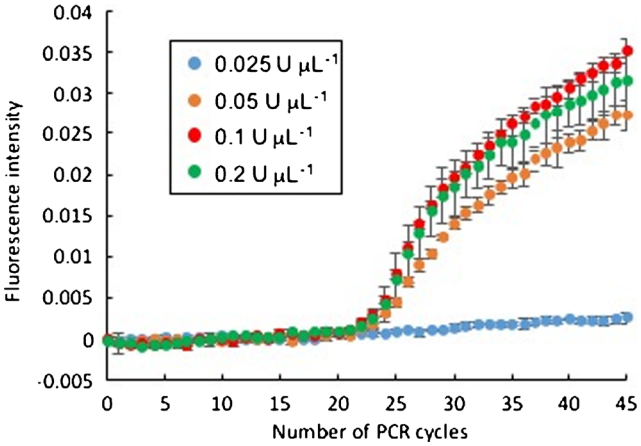

Fig. 3 The amplification curve for the concentration of SpeedSTAR HS DNA Polymerase. *Error bars* indicate standard deviation (SD) ($n = 3$)

0.1 U μL^{-1}. Additionally, the optimum concentrations of KAPA2G Fast HS DNA Polymerase and Ex Taq HS DNA Polymerase were standardized at 0.1 U μL^{-1} for the reciprocal-flow PCR (ESM Fig. S3).

To develop the fastest PCR, we needed to first estimate the shortest possible denaturation time required in an actual PCR. The denaturation time constitutes the time required for the thermal change and melting of the DNA duplex. At first, the shortest possible denaturation times for each DNA polymerase were examined by using the primers and probe for the 16S rRNA gene of *E. coli* (Table 1). The FP 16S rRNA and probe 16S rRNA were the same for detection of different amplicon sizes. RP 16S rRNA 1–4 were used to amplify 188, 390, 591, and 793 bp of the DNA sequence, respectively. To explain the evaluation process of the shortest possible denaturation times, the results of the real-time PCR for detection of the 188-bp amplicon by using SpeedSTAR HS DNA Polymerase are indicated in Fig. 4a. A denaturation time of at least 3 s was required to detect a discernible increase in fluorescence intensity. While the increase in the fluorescence intensity was delayed at a denaturation time of 2 s, it could not be observed even after 45 PCR cycles when the denaturation time was 1 s. These results indicated that the shortest denaturation time was 3 s for an amplicon size of 188 bp by using SpeedSTAR HS DNA Polymerase on the reciprocal-flow PCR chip. Furthermore, the fastest denaturation times for the 188-bp amplicon by using KAPA2G Fast HS DNA Polymerase and Ex Taq HS DNA Polymerase were also examined. As shown in ESM Fig. S4, the shortest denaturation time by using both KAPA2G Fast HS DNA Polymerase and Ex Taq HS DNA Polymerase was also 3 s.

Similarly, to develop the fastest possible PCR, we needed to ascertain the shortest possible time for the combined annealing and extension step in an actual PCR. The time required for the combined annealing and extension step consists of the time required for the thermal change, the binding of primers to the target DNA, extension of the primer by the

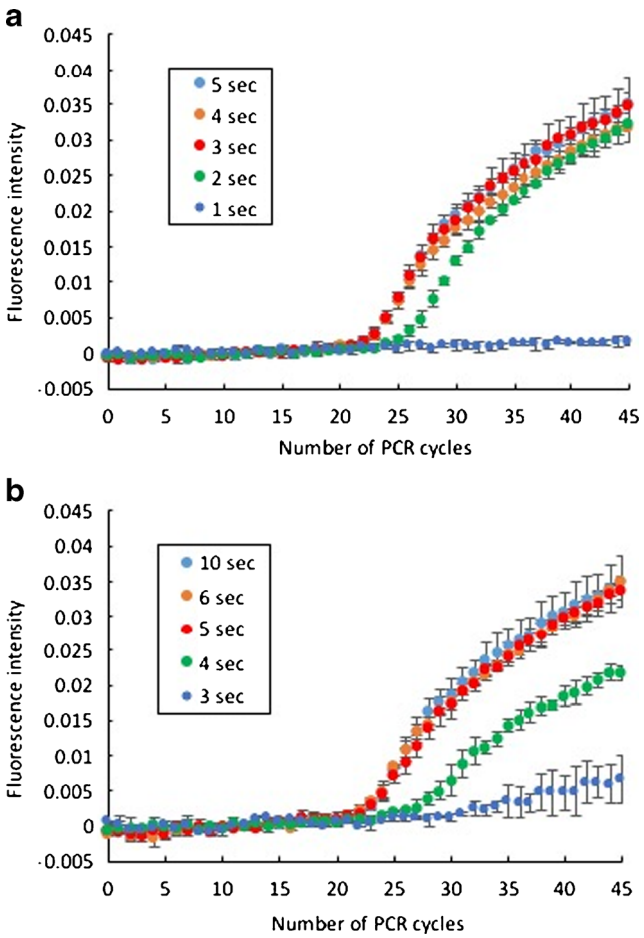

Fig. 4 The examination of the shortest denaturation and combined annealing and extension times for a 188-bp amplicon by using SpeedSTAR HS DNA Polymerase. **a** The shortest denaturation time. **b** The shortest time for annealing and extension. *Error bars* indicate SD ($n = 3$)

DNA polymerase (which depends on its extension rate), and the decomposition reaction of the probe. The shortest times required for the combined annealing and extension for each DNA polymerase were examined by using the primers and probe set shown in Table 1. To explain the evaluation process of the shortest time for the combined annealing and extension, the results of real-time PCR for the 188-bp amplicon using SpeedSTAR HS DNA Polymerase are indicated in Fig. 4b. Discernible increase in the fluorescence intensity was confirmed with a combined annealing and extension time of at least 5 s. While the increase in the fluorescence intensity was delayed at 4 s, it was almost undetectable at 3 s. These results indicated that the shortest time for the combined annealing and extension step was 5 s for a 188-bp amplicon by using SpeedSTAR HS DNA Polymerase. Furthermore, the fastest times for the combined annealing and extension step for a 188-bp amplicon when using KAPA2G Fast HS DNA Polymerase and Ex Taq HS DNA Polymerase were also examined. As shown in ESM Fig. S5, the shortest times for the combined annealing and extension step when using KAPA2G Fast HS DNA Polymerase and Ex Taq HS DNA Polymerase were 7 and 13 s, respectively.

Furthermore, the shortest times for denaturation and the combined annealing and extension step for amplicon sizes of 390, 591, and 793 bp were examined by using each DNA polymerase. The shortest denaturation time for each amplicon size is indicated in Fig. 5a. The *X*- and *Y*-axes indicated the size of the amplicon and the shortest denaturation time, respectively. In case of a temperature increase, the rate was at least 18 °C s^{-1} between 95 and 50 °C because the *Y*-intercept was 2.5. In this experiment, the denaturation time was almost never related to the amplicon size, because the melting of the DNA duplex would be completed until the temperature arrived at 95 °C. The shortest time for the combined annealing and extension step for each amplicon size is indicated in Fig. 5b. The *X*- and *Y*-axes indicated the size of the amplicon and the shortest time for combined annealing and extension, respectively. The inverse of the slope obtained would be regarded as the extension rate, including the decomposition reaction of the probe for each polymerase in actual real-time

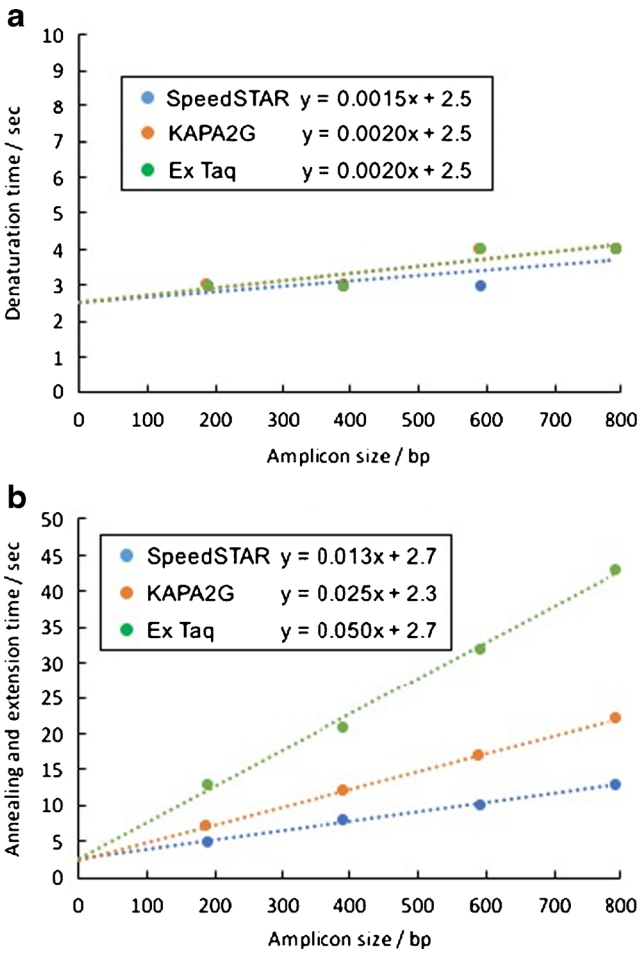

Fig. 5 The examination of the shortest denaturation and combined annealing and extension times for amplicon sizes of 188, 390, 591, and 793 bp by using each DNA polymerase. **a** The shortest denaturation time. **b** The shortest time for annealing and extension

PCR conditions. The extension rates (containing the decomposition reaction of the probe) at 50 °C of SpeedSTAR HS DNA Polymerase, KAPA2G Fast HS DNA Polymerase, and Ex Taq HS DNA Polymerase were 77, 40, and 20 bp s^{-1}, respectively. Although KAPA2G Fast HS DNA Polymerase has the highest enzymatic activity among the three DNA polymerases [23], SpeedSTAR HS DNA Polymerase had the fastest extension rate in actual real-time PCR. In this study, evaluation of the extension rate for each DNA polymerase under real-time PCR conditions by using TaqMan probes was successful in our reciprocal-flow PCR system. Furthermore, the *Y*-intercepts of all slopes indicated the period required for the thermal change and annealing of the primers and the probe to the target DNA sequence. These periods for SpeedSTAR HS DNA Polymerase, KAPA2G Fast HS DNA Polymerase, and Ex Taq HS DNA Polymerase were 2.7, 2.3, 2.7 s, respectively. In this experiment, the rate of temperature decrease and increase would be at least 17 °C s^{-1}. Therefore, the period until the primers bound to the target DNA sequence and the DNA polymerase began to synthesize DNA would be negligibly small for the time of temperature change. Among the three polymerases tested in this study, SpeedSTAR HS DNA Polymerase was the most useful in achieving the fastest real-time PCR employing TaqMan probe technology by using the developed rapid reciprocal-flow PCR system.

Rapid quantitative PCR by the reciprocal-flow PCR system

The rapid quantitative PCR for the *uidA* gene of *E. coli* was examined by using SpeedSTAR HS DNA Polymerase on the reciprocal-flow PCR system. In this study, the shortest times for denaturation and the combined annealing and extension step were examined to realize the rapid quantitative PCR. The temperature of the heating block for the combined annealing and extension was 60 °C because the *uidA* primers and probe set was used in this experiment. As shown in ESM Fig. S6a, the increased fluorescence intensity was normally confirmed when decreasing the denaturation time from 4 to 2 s. However, an increase in the fluorescence intensity could not be confirmed with a denaturation time of 1 s. Therefore, the denaturation time was optimized at 2 s in this system. Furthermore, the fastest time for the combined annealing and extension was examined when the denaturation time was 2 s. As shown in ESM Fig. S6b, in the case of 6, 5, and 4 s for the combined annealing and extension, the fluorescence intensity increased after 26 cycles, although the final fluorescence intensity was slightly lower when the time for the annealing and extension was 4 s. The increase in the fluorescence was delayed when the time for annealing and extension was set at 3 s. Furthermore, sufficient fluorescence could not be detected even at the 45th cycle when the combined annealing and extension step was set at 2 or 1 s. Therefore, the fastest time for annealing and extension was ascertained to be 4 s in this system. In this condition, we estimated the detection sensitivity of a quantitative PCR for purified DNA from *E. coli*, at concentrations of 5×10^4 to 5 copies per reaction. As shown in ESM Fig. S7a, the obvious increase in fluorescence was detected at concentrations between 5×10^4 and 5×10^1 copies per reaction ($n = 3$). In the case of 5 copies per reaction, no increase in fluorescence was detected in one sample of the 3 samples. This would be due to the absorption to the tube or a pipette tip to mix DNA template and PCR reagent. The cycle threshold (Ct) values for each concentration of purified DNA were estimated when a fluorescence intensity of 0.005 could be measured. As shown in ESM Fig. S7b, a high correlativity ($R^2 = 0.997$) was confirmed between the Ct value and the concentrations of purified DNA (5×10^4 to 5×10^1 copies per reaction).

A direct PCR of cells (without an intermediate DNA extraction step) is very important to identify the specific pathogenic bacterium, virus, or parasite causing food contamination. To verify the efficacy of our reciprocal-flow PCR system for screening of food contamination, we estimated the detection sensitivity of a quantitative PCR for *E. coli*, at concentrations of 5×10^4 to 5 cells per reaction by using the optimized conditions for denaturation and combined annealing and extension times. As shown in Fig. 6a, the obvious increase in fluorescence was detected at concentrations between 5×10^4 and 5×10^1 *E. coli* cells per reaction. The cycle threshold (Ct) values for each concentration of *E. coli* were estimated when a fluorescence intensity of 0.005 could be measured. As shown in Fig. 6b, a high correlativity ($R^2 = 0.996$) was confirmed between the Ct value and the concentrations of *E. coli* (5×10^4 to 5×10^1 cells per reaction). The amplification efficiency, calculated from the Ct values, was 84.1 % in our real-time reciprocal-flow PCR system. Furthermore, a real-time PCR involving the same concentrations of *E. coli* was performed by conventional thermal cycler (TP960, TAKARA BIO Inc., Japan). Using this method, a limit of detection of 1×10^2 cells per reaction (25 μL) was achieved. Thus, the limit of detection (LOD) of our reciprocal-flow PCR system was the same as that of the conventional thermal cycler for real-time PCR. Therefore, the quantitative PCR for an 83-bp amplicon of the *uidA* gene of *E. coli* by using the reciprocal-flow PCR system was realized in 7 min for 50 cycles.

For medical applications, not only *E. coli* but also various pathogens, such as viruses and parasites, must be detected. In our system, diverse pathogens can be detected by changing the primers and probe. Furthermore, a sensitivity surpassing 1000 cfu mL^{-1} of blood would be necessary for medical diagnosis. Therefore, the combination with a simplified kit for condensation and purification of DNA is crucial for the on-site assay of real samples at locations lacking adequate auxiliary service options. Recently, we are studying a simple condensation and purification technology that can be used on-site.

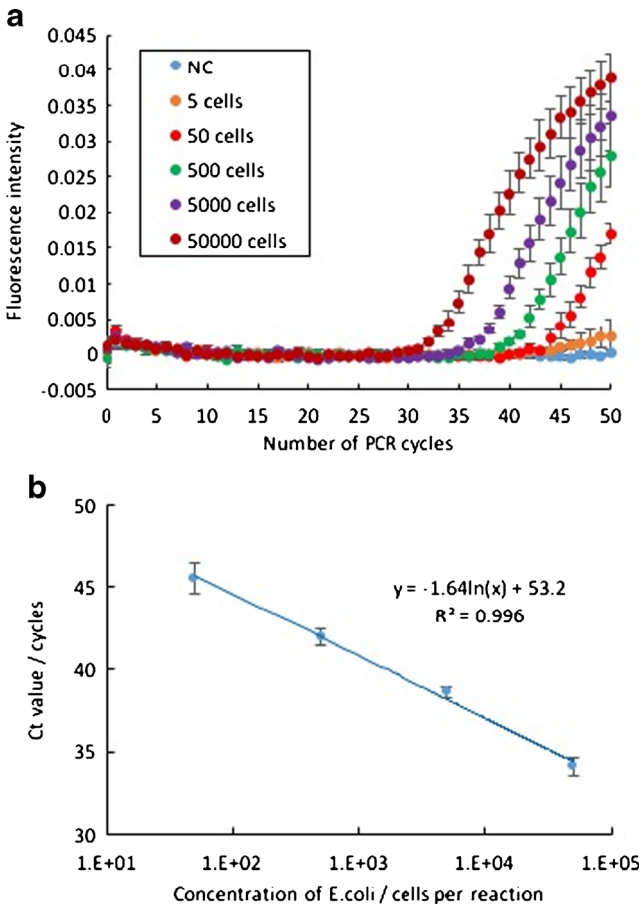

Fig. 6 The rapid quantitative PCR for *uidA* gene of *E. coli* by using SpeedSTAR HS DNA Polymerase. **a** The amplification curve for the concentration of *E. coli*. **b** Correlation between the concentration of *E. coli* and the Ct. *Error bars* indicate SD ($n = 3$)

While assuming that the virus detected in the blood as a real sample (e.g., Ebola virus), we challenged the rapid detection of RNA from the sample spiking synthetic RNA into blood by our system. The rapid and direct detection of RNA in the blood was enabled by using the Ampdirect Plus (Shimadzu, Japan) which is helpful for inactivation of the PCR inhibitors contained in human blood; however, this study using different reagents was not the subject of this paper.

Some of the fast PCR methods have been demonstrated before our work. Neuzil et al. had reported rapid real-time PCR for 40 cycles in less than 6 min [24]. In their method, a real-time PCR (sample with a volume of 100 nL in 1.1 μL of mineral oil) was performed by using infrared radiation as the heat source, at a wavelength specific only for the PCR solution. Although the speed of their assay is almost the same as that of ours, their system is not easily adapted to routine laboratory use or on-site detection. This is owing to the challenges involved in pipetting and loading samples on their device. Furthermore, they had not reported the LOD of their technique. Wheeler et al. had reported a rapid PCR for 30 cycles in 3 min, by convective heat transfer of a thermal fluid through porous media, combined with an integrated electrical heater [25]. Although the speed of PCR was fast, they confirmed the rapid PCR only 3.4×10^4 copies per reaction, and the LOD of their system was not reported. Furthermore, their system is not easily adapted to on-site detection because a further electrophoresis procedure was necessary to detect their PCR product. Our rapid real-time PCR system is more practical than these systems and enables the use of on-site detection. Furthermore, our system could detect template DNA of lower concentration compared to these systems in rapid real-time PCR. The speed of PCR would be limited by the extension rate of DNA polymerase.

On the other hand, as a simple method for the purpose of point-of-care (POC) diagnosis, some of the isothermal amplification methods for the detection of the bacteria have been demonstrated [26–30]. Lee et al. had reported an integrated direct loop-mediated isothermal amplification (Direct LAMP) micro device incorporated with an immunochromatographic strip (ICS) to identify bacteria contaminated in real samples [29]. They realized the detection of *Staphylococcus aureus* and *E. coli* O157:H7 in human whole blood and milk without a sample preparation at a single-cell level within 1 h. Although the sensitivity was very high, the rapid detection was not realized by their method. Kalsi et al. had reported the excellent work for rapid and sensitive detection of extended-spectrum b-lactamases (ESBL) in *E. coli* on a programmable digital microfluidic platform [30]. In this paper, they described the application of active matrix electrowetting-on-dielectric (AM-EWOD) technology for a rapid and sensitive assay for the quantitative detection of ESBL *E. coli* using an isothermal RPA reaction. They realized the highly sensitive detection at fewer than 10 copies (estimated fewer than 222 copies μL^{-1} as a sample) within 15 min. Although the sensitivity of our assay was similar to that of theirs, the detection time of our assay was half that of theirs. The isothermal DNA amplification methods are very useful because they need no temperature changing; however, our rapid PCR is superior to these methods in the point of the detection speed.

Conclusions

A portable reciprocal-flow PCR system was developed to realize on-site quantitative analysis. The precise and rapid handling of the PCR solution at each reaction temperature was achieved by using microblowers in our reciprocal-flow PCR system. Furthermore, the real-time PCR employed fluorescence detection (which was moved on the center of the microchannel) in the PCR solution. The fastest PCR was achieved by using SpeedSTAR HS DNA Polymerase, and its extension rate containing the decomposition reaction of the probe was 77 bp s^{-1} in this study. Furthermore, the fastest quantitative PCR conditions were achieved by using

SpeedSTAR HS DNA Polymerase. By rapid quantitative analysis for *E. coli* in our reciprocal-flow PCR system, we realized detection in 7 min. Moreover, the LOD of our system was the same as that of the conventional thermal cycler.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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