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Development of Portable Multi-fluorescence Detection System Using Indium Tin Oxide Heater for Loop-mediated Isothermal Amplification

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We developed a portable multi-fluorescence detection system for loop-mediated isothermal amplification (LAMP) using an indium tin oxide (ITO) transparent glass heater by improving a previously developed device. By changing the optical design and the material of the heating part, the quantitative performance and the stability of temperature control were improved. We demonstrated the basic performance of the developed device for genetic tests using LAMP by conducting discrimination tests for human genome DNA. We also showed that quantitative analysis of human genomic DNA is possible at initial DNA concentrations of 2.11×10^2–2.11×10^4 ng/μL using the developed device. This genetic testing device is smaller and lighter ($60 \times 90 \times 70$ mm^3, 178 g) than conventional testing devices, giving it considerable potential to facilitate rapid and efficient on-site genetic testing.

1. Introduction

In recent years, the demand for small and inexpensive genetic testing systems has increased.[1–3] Genetic testing systems are used in a wide variety of fields, such as food analysis,[4] environmental analysis,[5] and infectious disease diagnosis.[6] However, many devices are large and expensive, making it difficult to adapt them to on-site testing[1–3] and point-of-care testing.[7] The polymerase chain reaction (PCR),[8] which is used in genetic testing, is the most popular method of gene amplification with high sensitivity. Gene amplification by PCR requires multistep temperature control by cycling for overheating and cooling. Therefore, PCR often requires a long inspection time and complicated device configuration.[9–11]

Loop-mediated isothermal amplification (LAMP),[12] one of the gene amplification methods, also has high amplification efficiency. Genetic testing based on LAMP, which requires

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isothermal control and a short gene amplification time, is suitable for portable genetic testing devices for in situ diagnosis. Therefore, several compact and inexpensive genetic testing systems using LAMP have been developed.[13] Many detection methods have been used for LAMP. Visual detection by the human eye is simple and easy, but it is necessary to confirm the presence or absence of gene amplification after a certain reaction time.[14–16] Fluorescence detection is commonly used for genetic testing based on LAMP.[17,18] In this method, changes in gene amplification during the LAMP reaction are measured as changes in fluorescence intensity. Since the initial stage of amplification can be observed as a change in fluorescence, the inspection time can be shortened. In addition, observation of the behavior of the LAMP reaction by fluorescence detection is also effective in detecting abnormal reactions due to contamination.

When developing a small fluorescence detection device for LAMP, the design of the detection unit is important. Genetic testing using LAMP requires isothermal heating of the sample and fluorescence measurement at the same time. Heaters generally used for LAMP devices are ceramic heaters, Peltier elements, and so forth,[19–21] but most of them are opaque materials, limiting the design of the detection unit. In terms of the optical arrangement, most light sources and fluorescence detectors are arranged in a straight line via light-shielding filters and samples. Since the heater is opaque, when designing a detector unit for multi-measurement, it is necessary to use a detector unit configuration in which the samples are arranged one-dimensionally on a plane. This has often limited the number of samples that can be measured simultaneously and made devices large.[22]

An indium tin oxide (ITO) glass heater[23] is a material in which ITO is deposited on a glass surface, and it has high light transmittance in the visible light region. ITO glass heaters are used widely for cell analysis in biological research,[24] defrosting windows in airplanes,[25] and other fields. We previously developed a portable fluorescent microplate reader for the LAMP method using ITO glass heaters.[26] The detection part of the microplate reader was designed with nine light sources, an ITO glass heater, two optical filters, nine photodiodes, and other components arranged in a straight line. The transparent ITO glass heater was designed with a linear optical arrangement in the vertical direction, making it possible to configure a detector that arranges samples in two dimensions on a flat surface. Using this microplate reader, we succeeded in genetic testing to identify rice varieties using the LAMP method.

In this study, we changed the optical arrangement in the previous study[26] and developed a new portable LAMP genetic testing system using an ITO transparent glass heater. The optical arrangement of the excitation light source and detection unit, vertically aligned with an ITO transparent glass heater, achieves fluorescence detection performance equivalent to that of conventional microplate readers without a long-pass filter. Furthermore, by using commercial microtubes and an aluminum block as sample holders, temperature uniformity and stability were improved compared with those of the previous microplate reader. The possibility of quantitative analysis was also newly demonstrated from the calibration curves of the initial sample DNA concentrations and the logarithm of reaction times measured by the developed device. The performance of this system was verified by LAMP quantitative analysis of human genomic DNA after evaluating the fluorescence detection performance and heating performance.

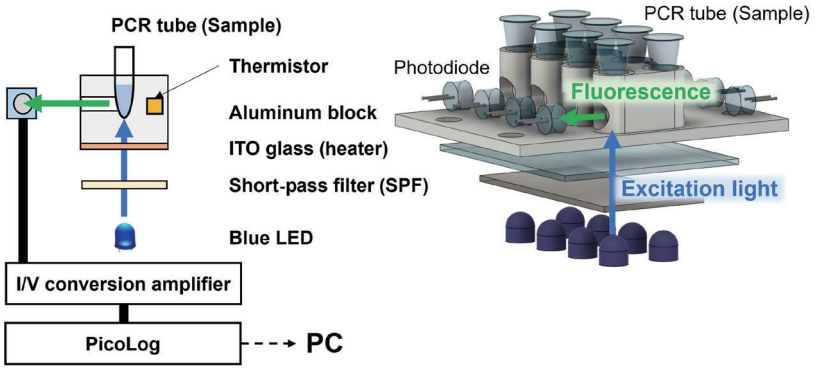
2. Materials and Methods

2.1 Reagents and materials

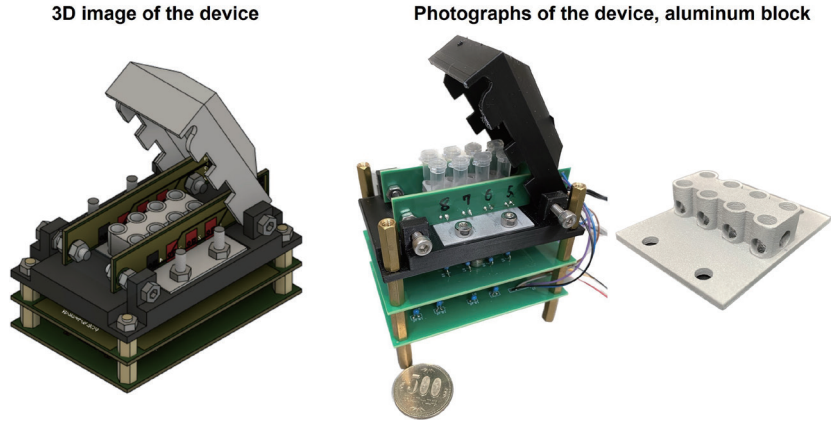
In this study, all chemical reagents and solutions were of analytical grade. Sterile water was purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). Calcein was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). The target DNA in this study, human genomic DNA (female), was the product of Cosmo Bio Co., Ltd. (Tokyo, Japan). Standard oligos including FIP, BIP, F3, B3, LOOPF, and LOOPB were purchased from Eurofins Genomics Co., Ltd. (Tokyo, Japan). Chlorine bleach was the product of Kao Co., Ltd. (Tokyo, Japan). ITO transparent glass (100 × 100 × 1.1 mm^3, No. 1001) was purchased from Geomatec Co., Ltd. (Tokyo, Japan). Ag/AgCl paste was the product of Gwent Group Co., Ltd. (Gwent, UK).

2.2 Development of portable multi-fluorescence detection system for LAMP

The design and a photograph of the portable multi-fluorescence detection system for LAMP in this work are shown in Fig. 1. The fluorescence detection part was composed of eight high-brightness blue LEDs (OSUB5161P, OptoSupply Ltd., N.T., Hong Kong), a short-pass filter (SPF)



(a)



(b)

Fig. 1. (Color online) Portable multi-fluorescence detection system developed in this work. (a) Optical design of the developed system. (b) Three-dimensional data of the developed system.

(SV0490, Asahi Spectra Co., Ltd., Tokyo, Japan), an aluminum block, eight microtubes (1-1599-01, AS ONE Corporation, Osaka, Japan), and eight photodiodes. The SPF was cut to an appropriate size by KADOMI Optical Industry Co. (Tokyo, Japan). The aluminum block was designed using Fusion 360 (Autodesk, California, US) and processed by DMM.com Ltd. (Tokyo, Japan). In this system, the eight blue LEDs were used as excitation light sources, and the eight photodiodes were used as detection parts. To irradiate the excitation lights in the appropriate wavelength range for the samples in the microtubes, the SPF was placed between the microtubes and the blue LEDs. The current of the LEDs was set to 30 mA. The fluorescence of calcein emitted from the sample solution in the microtubes was converted into a photocurrent according to its strength by a photodiode (S13948-01SB, Hamamatsu Photonics, Shizuoka, Japan). The photocurrent was converted to a voltage value by a current/voltage (I/V) conversion amplifier (conversion efficiency 10^6 V/μA), and the voltage value was recorded using a PicoLog ADC-24 data logger (PP309, Pico Technology Ltd., Cambridgeshire, UK). In this study, if the LAMP reagent was positive and the target gene, human genomic DNA, was amplified, the fluorescence intensity of calcein increased during the LAMP reaction.

To perform the LAMP reaction for all reagents in the microtubes, the aluminum block must be heated to around 65 ℃. As a heat source, an ITO glass heater was used. The ITO glass heater consisted of ITO glass and Ag/AgCl paste, and it was placed under the aluminum block. The temperature of the aluminum block heated by the ITO glass heater was monitored by a thermistor (JS8746A-0.15, Amphenol Corporation, Connecticut, US). The temperature of the aluminum block was maintained stably during the LAMP reaction by a proportional–integral–derivative control system using a microcontroller (Arduino Nano 3.0, Gravitech LLC, Nevada, US) based on the temperature monitored by the thermistor. The size and weight of the fluorescence detection system were 60 (L) × 70 (W) × 90 mm^3 (H) and 178 g, respectively.

2.3 LAMP reaction

Genetic testing using LAMP was performed for human genome DNA as the measurement target in this study. Primer mixes (×10) that specifically reacted with human genomic DNA (female) were prepared using sterile water at the concentrations shown in Table 1. In this study, primers targeting human breast cancer 1 (BCRCA1) were used. The template was human genomic DNA. The base sequences were as follows.

A 25 μL sample solution was prepared by mixing 12.5 μL of LAMP Master Mix, 2.5 μL of primer mix (×10), 1 μL of fluorescence detection reagent, 4 μL of sterile water, and 5 μL of human genomic DNA solution (sterile water for NC). In tests on negative control, the same amount of sterile water was added in place of human genomic DNA. After adding the sample to the microtube, 25 μL of mineral oil was added to prevent evaporation of the sample reagent. The microtubes were set into the aluminum block. The LAMP reaction was carried out at 65 ℃ for 40 min. Sample discrimination containing positive and negative controls was performed by comparing the changes in fluorescence emitted from the samples during LAMP.

Table 1
LAMP primer mix.

PRIMER	10 X CONCENTRATION (STOCK) (μmol/L)	1 X CONCENTRATION (FINAL) (μmol/L)
FIP	16	1.6
BIP	16	1.6
F3	2	0.2
B3	2	0.2
LOOP F	4	0.4
LOOP B	4	0.4

F3: TCCTTGAACTTTGGTCTCC
B3: CAGTTCATAAAGGAATTGATAGC
FIP: ATCCCCAGTCTGTGAAATTGGGCAAAATGCTGGGATTATAGATGT
BIP: GCAGCAGAAAGATTATTAACTTGGGCAGTTGGTAAGTAAATGGAAGA
Loop F: AGAACCAGAGGCCAGGCGAG
Loop B: AGGCAGATAGGCTTAGACTCAA

3. Results and Discussion

3.1. Optical design of fluorescence detection system

In this study, the target measurement of fluorescence reagents for LAMP was calcein. The optical design of this system was conducted to detect the fluorescence of calcein, which increases with the progress of the gene amplification reaction based on LAMP, with high sensitivity. Unlike the previously developed microplate reader, this system has an optical arrangement in which the excitation light source and the detector are at a right angle. In this right-angle arrangement, there is no concern about an increase in the background signal from the excitation light source during fluorescence measurements. Therefore, this system does not require the use of a long-pass filter, simplifying the system and reducing its cost. The high-brightness blue LEDs, SPF, and ITO transparent glass used are the same as those in the previously developed microplate reader.

3.2 Evaluation of quantitative performance

To evaluate the fluorescence detection performance of the developed system, the fluorescence intensity of calcein solutions with several concentrations was measured at each detection part. A 50 μL calcein solution diluted with water was added to microtubes. The microtubes were set in each holder of the aluminum block, and the fluorescence intensities were measured using the developed system. The obtained calibration curves for calcein, which were prepared by plotting the fluorescence intensity versus the concentration of calcein at each detection part, are shown in Fig. 2. The fluorescence intensity increased with increasing concentration of the calcein solution and became almost constant in the range of 125–250 μmol/L or more at all measurement parts. For calcein concentrations of 15.6 μmol/L or less, calibration curves with high linearity and a correlation coefficient of 0.9968–0.9996 were obtained at all detection parts. We defined the detection limit (LOD) as three standard deviations, for which the LOD of calcein for this

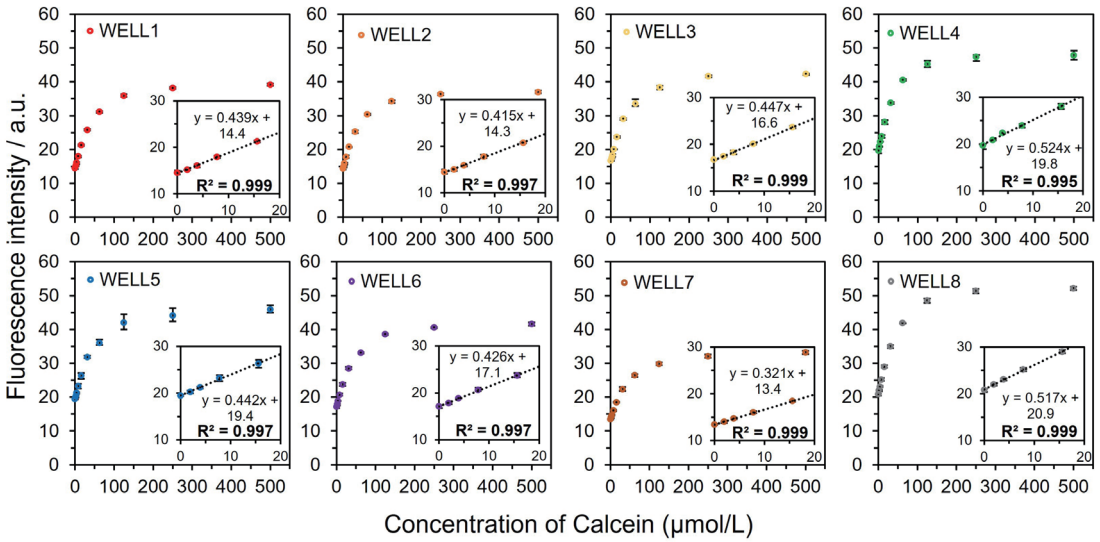
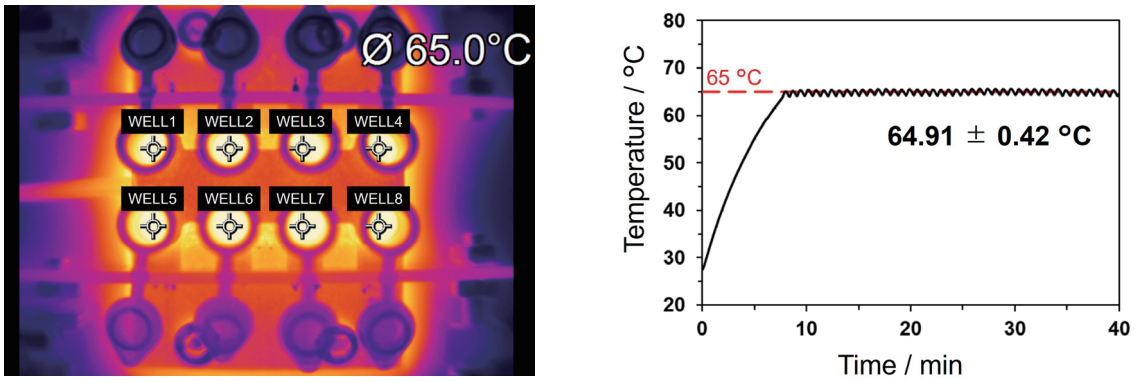


Fig. 2. (Color online) Calibration curves for calcein.

system was estimated to be 0.57–2.34 μmol/L. This was slightly higher than that for the device developed in previous research[26] because the newly developed device does not have a long-pass filter and cannot sufficiently reduce the background noise caused by excitation lights. The relative standard deviation was calculated to be less than 0.25–2.87% at each detection part. These results indicate that this system has the same sensitivity as the previously developed microplate reader and can be used for quantitatively measuring the fluorescence intensity of the calcein solution.

3.3 Heating performance of the ITO glass heater

For the LAMP reaction to proceed in each microtube, the solution in each microtube must be uniformly and stably heated by an ITO glass heater. Thus, the aluminum block was heated to 65 °C using the temperature control unit and the ITO glass heater in the developed system, and the temperature of the water in each microtube was measured with a thermal camera (PI400i, Optris GmbH, Berlin, Germany) to evaluate the temperature uniformity and temperature maintenance performance. The results obtained from the thermogram (Fig. 3) indicated that the temperature of the solution in all microtubes reached 65 °C within 8.6 min from the start of heating and that stable temperature control was achieved for over 30 min thereafter. The 30-min average temperature of the solution in each microtube after reaching 65 °C was recorded as 64.76 ± 0.41 °C–65.21 ± 0.43 °C. These results indicate that the temperature control performance is more stable and uniform than that of the conventional microplate reader[26] and another LAMP analyzer,[27] and it was confirmed that the system developed in this study has sufficient performance to proceed with the LAMP reaction.



(a) (b)

Fig. 3. (Color online) Temperature control performance of the heating unit. (a) Thermal image of the developed device heated by the ITO glass heater. (b) Time course of the temperature in the microtubes. The line shows the average temperature measured in the eight microtubes. The heating was carried out for 40 min.

3.4 Discrimination LAMP test for positive and negative controls of human genome DNA

To confirm that the LAMP reaction occurred appropriately for human genomic DNA using the LAMP reagent containing the prepared primers and master mix, the LAMP reaction was performed at 65 °C for 30 min using a thermal cycler (TCLT-9610, Blue-Ray Biotech Co. Ltd., Zhongshan Dist, Taipei), and the fluorescence of the LAMP reagent before and after the reaction was visually confirmed using a UV illuminator (SLUV-4, AS ONE). If the sample was positive, fluorescence was confirmed in the LAMP reagent after the LAMP reaction, and if it was negative, there was no difference in the light emission of the reagent before and after the LAMP reaction. Our results confirmed that the discrimination of positive and negative controls of human genomic DNA is possible using the LAMP reagent prepared in this study.

Next, to demonstrate the feasibility of the LAMP reaction using the developed system, a LAMP reaction test using positive and negative controls of human genomic DNA was performed at each detection site, and changes in fluorescence intensity during the LAMP reaction were measured in real time. Figure 4 shows the change in fluorescence intensity measured during the LAMP reaction at each detection site. At all detection sites, there was a significant difference in fluorescence intensity between the positive and negative samples during the 40 min reaction. In addition, the starting time of the increase in the fluorescence intensity of the positive control at each detection site was almost the same at 15–20 min, indicating that the heating temperature was appropriately maintained without variations in the heating temperature depending on the detection site. These results show that up to eight samples can be simultaneously measured in real time during the LAMP reaction.

3.5 Quantitative analysis of human genomic DNA

The developed system has sufficient fluorescence detection performance to measure the fluorescence intensity during the LAMP reaction and sufficient heating performance to maintain

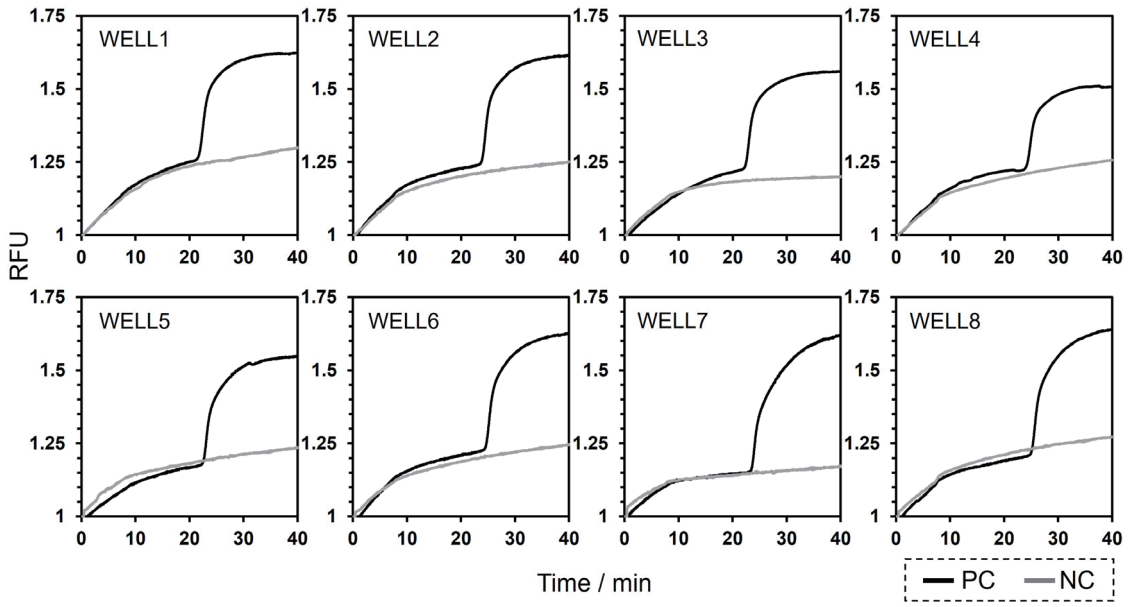


Fig. 4. Time courses of the fluorescence intensity of the solution containing a positive or negative control in the eight microtubes during the LAMP reaction. RFU on the vertical axis stands for relative fluorescence units and is based on the average of the voltage values during the first 10 s of the experiment.

uniform and accurate temperatures at eight measurement sites, making quantitative analysis of the LAMP reaction possible. To demonstrate this possibility, the LAMP reaction was performed with the developed system using positive controls at several concentrations of human genomic DNA, and the fluorescence intensity was measured in real time. Figure 5 shows the measurement results at each of the eight detection sites. In the real-time fluorescence measurement at each detection site, the starting time of the increase in fluorescence intensity became longer as the initial concentration of human genomic DNA decreased. Figure 6 shows the calibration curves for human genomic DNA, which were prepared by plotting the threshold time versus the concentration of human genomic DNA. The threshold time was defined as the time required to reach the RFU (relative fluorescent unit) value, which was the average RFU value during the first 15–20 min of the experiment plus 20 times the standard deviation for the same period when measuring the highest DNA concentration of 2.11×10^4 ng/μL. High linearity was obtained in the calibration curve for each detection site, and it was demonstrated that quantitative analysis of human genomic DNA is possible in the initial DNA concentration range of 2.11×10^2–2.11×10^4 ng/μL using this system. These coefficients of determination are equivalent to or higher than those of other fluorescent LAMP devices.[27,28] However, the LOD of human genomic DNA has not been investigated in detail and is an issue to be considered in the future.

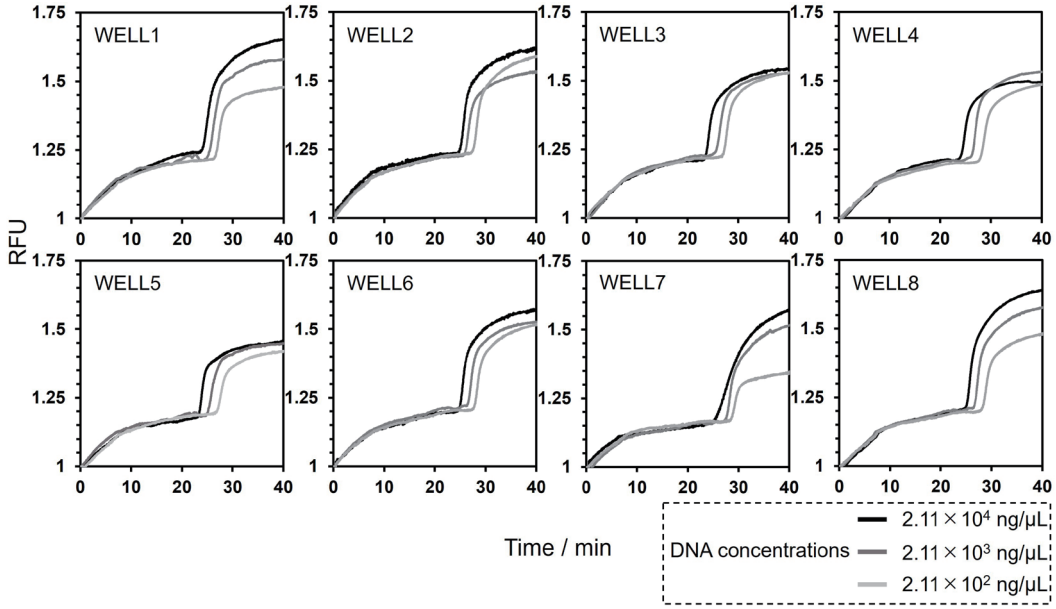


Fig. 5. Time courses of the fluorescence intensity of the solution containing different concentrations of human genomic DNA in the eight microtubes during the LAMP reaction. The RFU on the vertical axis is the same as that in Fig. 4.

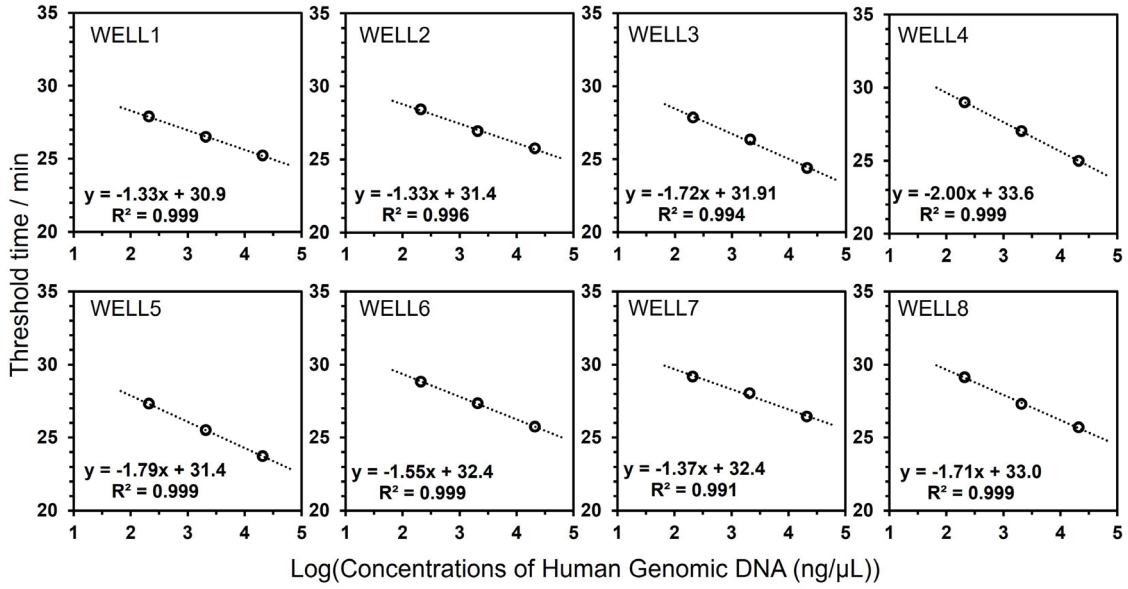


Fig. 6. Calibration curves for human genomic DNA.

4. Conclusion

In this study, we developed a multi-fluorescence detection system for LAMP based on the heating principle of an ITO glass heater proposed previously.[26] This system did not require a long-pass filter and achieved the same size and sensitivity of fluorescence detection as the

previously developed microplate reader. In addition, the heating unit using ITO glass heaters and aluminum blocks in the system improved the accuracy and stability of temperature control for LAMP. The reproducibility of the test using this system was demonstrated by a gene amplification test of human genomic DNA based on LAMP. Furthermore, the results of real-time fluorescence measurement for positive controls of human genome DNA with different concentrations indicated that this system can be applied to the quantitative analysis of human genomic DNA based on LAMP. Although this system is compact and lightweight, it can perform simultaneous genetic testing of up to eight samples. Thus, it is considered to be an extremely useful analyzer for the rapid and simultaneous genetic testing of multiple samples in the field.

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