Lab on a Chip

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COMMUNICATION

Ultrafast Rotary PCR system for multiple influenza viral RNA detection†

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We presented a novel platform for an ultrafast PCR system, called the Rotary PCR Genetic Analyzer, which incorporates a thermal block and resistive temperature detector (RTD) for thermal cycling control, a disposable PCR microchip, and a stepper motor. The influenza viral RNAs from H3N2, H5N1, and H1N1 were simultaneously identified with high sensitivity and speed.

PCR based genetic analysis has been considered as a gold standard method due to the high accuracy and sensitivity, and a variety of on-chip microPCR (μPCR) has been proposed to take full advantages of low reagent consumption, rapid gene amplification by the low thermal mass, and high portability.[1–6] To date, the developed μPCR systems are categorized into two types.[7] One is a stationary PCR in which the target gene is amplified in the microchamber with a micropatterned heater,[8] and the other is a flow-through PCR in which the PCR occurs when the samples pass the three distinct temperature zones.[9,10] The former shows an accurate temperature control but needs a complicated fabrication process to pattern the heater and RTD electrode on a chip. The latter demonstrated higher speed for PCR, but it requires an external syringe pump control and lacks the capability of the multiple genetic analysis.

Herein, we proposed a novel Rotary PCR Genetic Analyzer to perform the ultrafast and multiple RT-PCR reaction by combining the characteristics of the stationary and flow-through PCR. As shown in Fig. 1, the PCR microchip (blue color) is sequentially rotated on the three thermal blocks, which have different temperatures for gene amplification: (i) the block at 94 °C for denaturation, (ii) the block at 58 °C for annealing, and (iii) the block at 72 °C for extension.[11] Firstly, the microchip was placed on the denature thermal block, and then moved to the annealing and extension thermal blocks by a motor to complete one cycle of PCR. The precise position of the microchip and the residence time on each block were automatically controlled by an in-house LabVIEW program (National Instruments, Austin, TX, USA). Because of the rapid transition of the PCR sample between the adjacent blocks similar to the flow-through concept and the fast temperature control for the PCR reactor due to the low thermal mass like the stationary PCR system, we can perform the ultrafast PCR without need for the external pumping instrumentation and complicated fabrication process.

Fig. 2 shows the digital images of the Rotary PCR Genetic Analyzer which consists of three parts: a disposable PCR microchip, thermal blocks and RTD for temperature control, and a stepper motor for precise rotation of the microchip. The PCR chip is made of the glass and PDMS hybrid, and has a 5 mm diameter punctuated hole at the end to be fixed in the central shaft of the stepper motor with direct contact on the heat block as shown in Fig. 2a. Three thermal blocks are embedded in the custom-made Rotary stage and a stepper motor is installed underneath which controls the rotation of the microchip with high accuracy and speed.

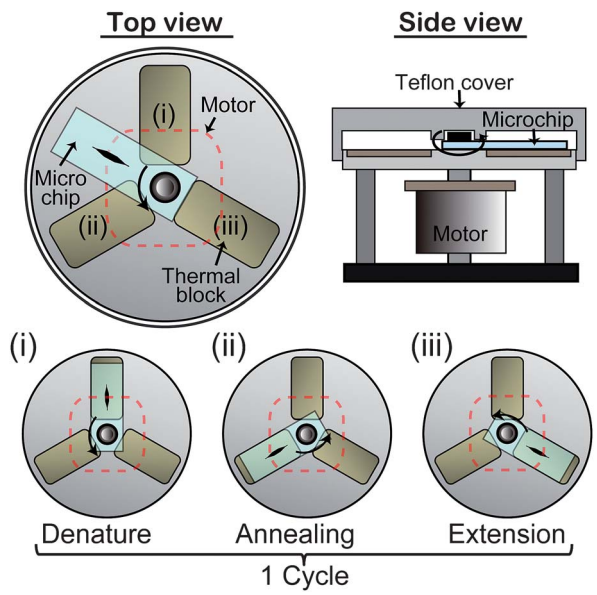

Fig. 1 Schematic illustration of a Rotary PCR Genetic Analyzer which consists of a microchip, three heat blocks, and a stepper motor. Top view (left) and side view (right). Rotary PCR is performed by rotating a PCR chip to the (i) denature (94 °C), (ii) annealing (58 °C), and (iii) extension (72 °C) heat blocks subsequently (bottom panel).

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† Electronic supplementary information (ESI) available: Rotary microchip fabrication, design of the electrical circuits, RT-PCR cocktail preparation, RT-PCR operation in a Rotary system, Fig. S1 and S2, and Tables S1–S3. See DOI: 10.1039/c2lc21269b

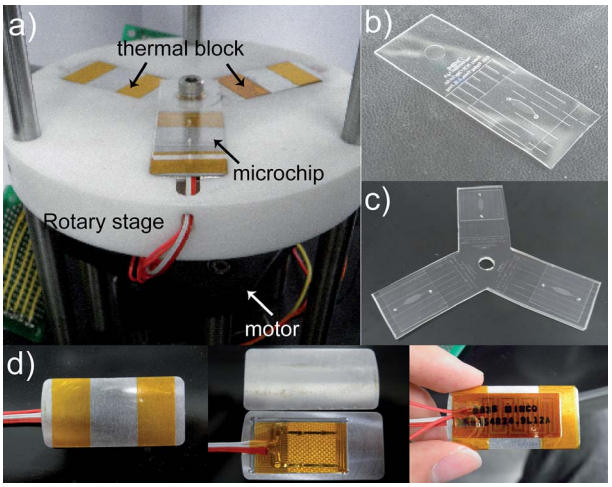

Fig. 2 (a) Digital image of the integrated Rotary PCR system, (b) a disposable PCR microchip, (c) a triple Rotary PCR microchip, and (d) a thermal block; a top view (left), a RTD (middle), a film heater (right).

A disposable PCR microchip was composed of a PDMS layer and a glass cover slip. The PCR chamber and inlet/outlet microchannels were fabricated in the PDMS by using a conventional soft lithography procedure (Fig. 2b). The size of the microchip was 18 × 50 mm, and the volume of the PCR chamber was 1 μL. The thickness of the PDMS layer was 1 mm and the 200 μm thick glass cover slip was attached at the bottom for efficient heat transfer during thermal cycling (Fig. S1†). For multiple genetic analysis, a triple microchip was designed as in Fig. 2c that enables the three PCR reactions separately and simultaneously. Since a number of nanolitre PCR chambers can be fabricated in each PCR chip, a high-throughput PCR reaction can be realized in the future. In this study, we made one PCR chamber on each chip, and targeted three specific gene expressions of influenza viral RNAs by performing a monoplex or duplex RT-PCR reaction. Thermal blocks were made of duralumin, and a film heater and a RTD were incorporated (Fig. 2d). A RTD platinum film (RdF Corporation, NH, USA) was sandwiched between 1.5 mm thick thermal block plates and the film type heater (MINCO™, MN, USA) was attached on the bottom of the thermal block. The integrated thermal blocks were installed onto a recessed area on the top of the Rotary stage. Due to the insulation and heat resistance properties, Teflon was selected as a material for the Rotary stage. To measure the exact temperature during thermal cycling, the design of the electrical circuits for driving the RTD and heater was set up by modifying the previous reports (see ESI†).[1,2] After installation of the PCR microchip, an upper Teflon cover which has a block-shaped hollow was moved downward to tightly seal the Rotary stage, preventing the loss of heat and maintaining the uniform temperature distribution on each heat block (Fig. S2†).

For accurate temperature control, the calibration of the thermal blocks was conducted, and the calibration curve of RTD for each of the three thermal blocks shows linear correlation between the temperature and the measured voltage (Fig. S3a†). The set temperature of denaturation, annealing, and extension on the block was stably maintained for a long time (Fig. S3b†). Thermal cycling profile in the microchip shows the temperature of a PCR chamber reached a desired temperature with a residence time of 60 s on each block (Fig. S3c†).

A stepper motor (NEMA 23, National Instruments) under the Rotary stage can adjust the rotating speed up to 2000 rpm. The designated rotational angle and the residence time were precisely controlled by the in-house LabVIEW software through a DAQ board and a driver interface. The transition time of the microchip from one block to another is 50 ms which reduces the ramping rate drastically compared with that of the stationary PCR system, enabling the ultrafast PCR.

To demonstrate the rapid gene amplification in the Rotary PCR Genetic Analyzer system, we performed a reverse transcriptase PCR (RT-PCR) by using influenza A subtype viral RNAs as templates (three viral strains of influenza A H3N2, H5N1, and H1N1). Firstly, the H3 hemagglutinin (HA) gene of H3N2 subtype virus was targeted and amplified with variation of the thermal cycling time. RT-PCR cocktail prepared from Onestep RT-PCR kit (Qiagen, Germany) with specific primer sets (Table S1†) was injected into the 1 μL PCR chamber whose volume could be handled with ease for downstream detection, and sealed by loading silicone oil in the inlet and outlet holes. RT-PCR was performed according to the designated scheme as shown in Table S2†: 15 min at 50 °C for reverse transcription, 5 min at 95 °C for initial activation, followed by 34 cycles of 60 s at 94 °C, 60 s at 58 °C and 60 s at 72 °C, and finally 7 min at 72 °C for final extension. Then, the PCR product was collected from the microchip and analyzed by micro-capillary electrophoresis (μCE) for identification of target genes. The μCE operation and the laser-induced fluorescence detection on a chip were performed following the previously published literature.[12–14]

The resultant electropherogram displays the major peak for the H3 gene (150 bp) at the elution time of 275 s in Fig. 3a. The PCR step took 102 min when the time for denaturation (D), annealing (A), and extension (E) was fixed at 60 s, which is the recommended protocol offered by the Onestep RT-PCR kit in the conventional thermal cycler. Encouraged by the successful demonstration of the proof-of-concept for the Rotary PCR system, we furthermore decreased the thermal cycling time to 68 (D/A/E = 30/60/30 s), 51 (D/A/E = 30/30/30 s), 34 (D/A/E = 15/30/15 s), and 25.5 min (D/A/E = 15/15/15 s). Since the rapid thermocycling process requires a fast heat transfer between the PCR chamber and the heat block, we tuned the temperature of the thermal blocks to reach the desired temperature in a short time (Table S3†). A rough estimation indicates that the temperature of the denature and extension blocks should be increased by 0.267 °C while that of the annealing block should be decreased by 0.2 °C for each second reduction. For example, in the case of 25.5 min

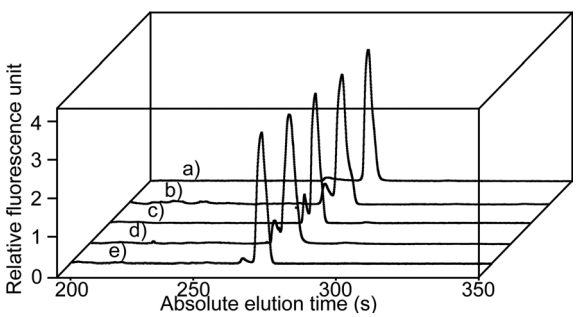

Fig. 3 Electropherogram for H3 gene amplicon separation on a μCE chip with variation of thermocycling time in the Rotary Genetic Analyzer system. Time for denaturation/annealing/extension steps was (a) 60/60/60, (b) 30/60/30, (c) 30/30/30, (d) 15/30/15, and (e) 15/15/15 s.

(D/A/E = 15/15/15 s) PCR reaction, the temperature of the denature block was set to 96 °C and that of the annealing block was fixed at 47 °C. Fig. 3b–e show the fluorescent peaks of the H3 gene amplicon produced with different thermal cycling conditions. Note that even 25.5 min PCR (Fig. 3e) resulted in the equivalent peak height compared with those generated with the prolonged PCR conditions. These results demonstrate that the Rotary Genetic Analyzer can perform RT-PCR to identify the gene expression from the viral RNA sample with high speed and specificity by tuning the temperature of heat blocks.

Under the 25.5 min thermocycling condition, the limit of detection (LOD) test was conducted with serially diluted influenza A H3N2 viral RNA templates (12 pg, 1.2 fg, 120 fg, and 12 ag). Fig. 4 displays the gradually increased peak intensity with proportion to the amount of RNA template, and the target peak produced from 12 ag template which was equivalent to ~2 copy number was clearly and reproducibly detected with a signal-to-noise ratio of more than 3. Thus, the influenza A H3N2 virus could be identified on the proposed Rotary PCR platform with the low LOD and the wide dynamic range of RNA templates.

We redesigned the RT-PCR chip from singlet to triplet for performing gene expression of multiple influenza A viral RNAs simultaneously (Fig. 2c). Influenza A H3N2, H5N1, and H1N1 viral RNAs and the corresponding primer sets for targeting HA genes (H3, H5, and H1) were separately injected into each PCR chamber. In the case of H3N2 subtype, we put an additional primer set for targeting M genes besides the H3 gene to demonstrate the capability of multiplex Rotary RT-PCR. Fig. 5a represents two dominant peaks: one is for the H3 gene (150 bp) and the other is for the M gene (160 bp), verifying the success of influenza A virus subtype H3N2 as well as the multiplexity of Rotary RT-PCR. The second PCR chamber and the third one also produced the target H1 gene (102 bp) and H5 gene (172 bp) at the same time with high reproducibility (Fig. 5b and c). Thus, all three RNAs were amplified in 25 min and the resultant amplicons were separated on the μCE chip in 5 min, thereby subtyping the multiple influenza virus in total 30 min.

In conclusion, we have demonstrated highly sensitive, rapid, and specific viral identification on the Rotary RT-PCR Genetic Analyzer which consists of a disposable microchip, three thermal blocks, and a stepper motor. This novel platform simplifies the RT-PCR chip design without the need for any complicated fabrication process and eliminates the conventional time-consuming ramping step during thermal cycling. The fine tuning of the thermal block temperature and the rapid rotation of the microchip between the thermal blocks enables the target gene amplification in 25.5 min. We could extend the number of PCR chips so that multiple samples could be simultaneously analyzed by monoplex as well as multiplex genotyping. Since the sample pretreatment systems such as blood extraction and cell lysis based on the centrifugal force have been reported,[15,16] the RNA extraction from the cell lysates can be incorporated to the Rotary PCR system for constructing a fully integrated genetic analyzer with sample-in-answer-out capability in the near future.

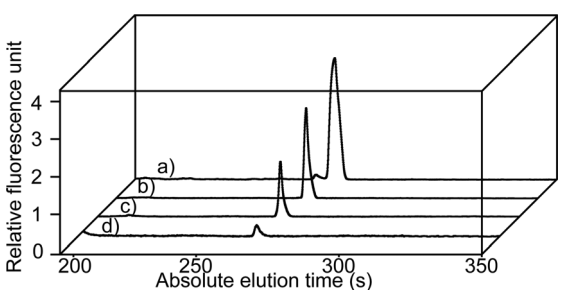

Fig. 4 LOD test for H3 gene expression from influenza A H3N2 virus by using the serially diluted RNA templates on the Rotary PCR system. The amount of RNA templates was (a) 12 pg, (b) 120 fg (c) 1.2 fg, and (d) 12 ag.

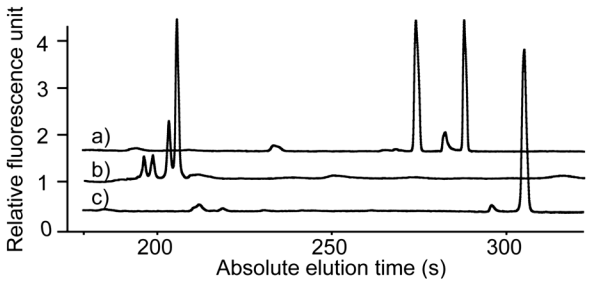

Fig. 5 Simultaneous multiple viral gene expression on the triplex Rotary RT-PCR system. (a) H3 gene (150 bp) and M gene (160 bp) from influenza A H3N2 virus; (b) H1 gene (102 bp) from H1N1 virus, and (c) H5 gene (172 bp) from H5N1 subtype.

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