Rapid detection of viral RNA by a pocket-size real-time PCR system

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We present an economical, battery-powered real-time polymerase chain reaction (RT-PCR) system suitable for field and point-of-care applications; it has a built-in thermal management, a fluorescence-based detection system, and a single chip controller with a graphic touch-screen display.

Containment of infectious diseases is the first line of defense, therefore early diagnosis of infected individuals is crucial. Different strains of influenza or severe acute respiratory syndrome (SARS) repeatedly threaten human populations. These lethal diseases are caused by viruses which can be identified on the basis of their RNA. Hence, fast and reliable tools for RNA detection are required. Such tools have to be economically affordable for point-of-care applications (POC) as the outbreak of the pathologies is likely to occur in rural areas of developing countries. There is also a growing need to detect infected individuals at the countries' check points to prevent a potential spreading of the disease. A suitable method for RNA detection is the reverse transcription polymerase chain reaction (RT-PCR).[1] It is a highly reliable and sensitive technique based on amplification of complementary DNA (cDNA) after it was reverse-transcripted from original viral RNA. Once the process of RT-PCR is completed, the amplified cDNA is detected by post-processing such as electrophoresis or hybridization. A real-time (quantitative) variant of PCR[2] makes it possible to eliminate the post-detection step. Consequently, reverse transcription (RT) followed by a real-time PCR makes the diagnosis faster and simpler, as the PCR product is detected during the reaction. Another benefit of this process simplification is the elimination of sample transfer from the PCR system to the post-processing unit.

A real-time PCR device usually consists of a heater/sensor with a thermoregulator, a fluorescence detection system, a controller and a graphic display. Most commercially available real-time PCR instruments are bulky, heavy and their power consumption is in the order of hundreds of watts. A typical RT-PCR process with 40 PCR cycles takes an hour or more. The systems are also rather expensive for POC applications.

Requirements for POC systems can be met by minimizing the PCR system's thermal mass and by simplifying its fluorescent system. The conventional heating block can be replaced with micro-machined silicon. The pioneering work in this field was presented in 1993.[3] Thus, the system consumed significantly less energy and also allowed fast, time-effective operations. Despite more than 20 years of micro PCR development[4,5,6] and a high number of papers published, only a very few of the various systems[7] were demonstrated to be able to perform RT-PCR. It is possibly due to the high level of degradation of RNA and its sensitivity to contamination.

Conventionally, a metal halide light source or blue laser is used to excite fluorescent light. It can be replaced by a light emitting diode (LED). Further, the detection of the fluorescent light, most commonly performed by a photo multiplier tube (PMT) or a cooled charge-coupled device (CCD) camera, can be accomplished using a photodiode. However, the sensitivity of the photodiode is much lower than that of the PMT, therefore a photodiode-based system requires optimization of all components (*e.g.* the optics and electronics) for signal processing.

As a solution to these problems, a micro-PCR device equipped with a simple fluorescent detection unit could become the system of choice to perform diagnostic assessments for POC applications,[4,8,9] where the reaction chamber is typically made of micro-machined silicon or batch fabrication. On the other side, the PCR chamber has to be disposable to avoid possible sample-to-sample contamination, and the silicon technology for fabrication of a disposable chip is not a cost-effective option.

We have decided to develop a low-cost and easily portable real-time PCR system with a capacity to detect viral RNA. We have chosen to integrate previously reported virtual reaction

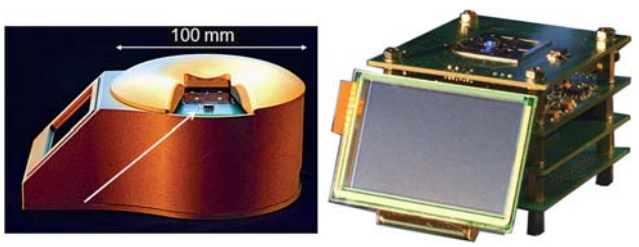

Fig. 1 (left) Photograph of a portable real-time RT-PCR system in an aluminium housing. The PCR is performed in a virtual reaction chamber. The PCR mixture, encapsulated in a droplet of oil (shown by the arrow), is placed on top of a thin film PCR chip for thermocycling. (right) Photograph of a stripped system showing all printed circuit boards with a touch screen display is below. The optical fluorescence system (see Fig. 2) is located underneath the PCR chip. The power consumption of PCR system is only 3W which allows battery operation.

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chamber-based (VRC)[10] PCR device with a miniaturized fluorescent detection unit.[11] In this system, PCR is conducted on a standard disposable microscope glass cover-slip placed on the top of the heater, making the system cheap and affordable. Because the analyzed sample gets into contact only with the disposable glass, the sample-to-sample cross contamination is prevented.

The complete real-time RT-PCR system that consists of 4 blocks, each of them with its own printed circuit board (PCB) (Fig. 1).

The top PCB contains a directly soldered micro-machined silicon chip integrated with a thin film heater, a resistance temperature detector (RTD) and an optical fluorescence unit attached below the PCB (Fig. 2). The optical unit includes an LED as the source of light, an excitation filter, a dichroic mirror, an emission filter, a photodiode detector, and a pre-amplifier. The system was described in detail earlier.[10] The optical housing is aligned with the silicon chip in a way that allowed the light to be focused through a circular hole in the silicon at the VRC.

The second PCB contains a thermal management and a lock-in amplifier for processing of the optical signal. The PCR heater is powered by a pulse width-modulated (PWM) signal using the proportional integration derivative (PID) method. The RTD is connected to an AC-biased Wheatstone bridge. The bridge output signal is amplified 500-fold by a differential amplifier. Afterwards, the resulting signal is processed by a demodulator AD630 (Analog Devices, Inc.) followed by a low pass filter. This set-up provides an overall system temperature sensitivity of 30 mV/°C. The RTD was immersed into the Fluorinert™ 77 temperature-stabilized bath and calibrated using the TSiC™ temperature sensor (IST-AG, Switzerland) with 0.05 °C precision.

Optical signal processing was based on a lock-in amplifier which allows a high amplification factor while preserving sufficient signal-to-noise ratio. The lock-in amplifier technique also allows the system to operate in ambient light, therefore no light shield is required. The LED placed in the housing is powered by current pulses with 1 kHz frequency, 150 mA amplitude, and 10% duty cycle. The fluorescent light is detected by a BPW 21 photodiode (Centronics, Inc.). The photocurrent is converted into voltage by a transimpedance amplifier. This amplifier is placed inside the optical housing (Fig. 2) to enhance the signal-to-noise ratio. At the second PCB, the output voltage corresponding to the photocurrent is fed *via* a high pass filter, and is further amplified and demodulated by AD630 with a subsequent low-pass filter.

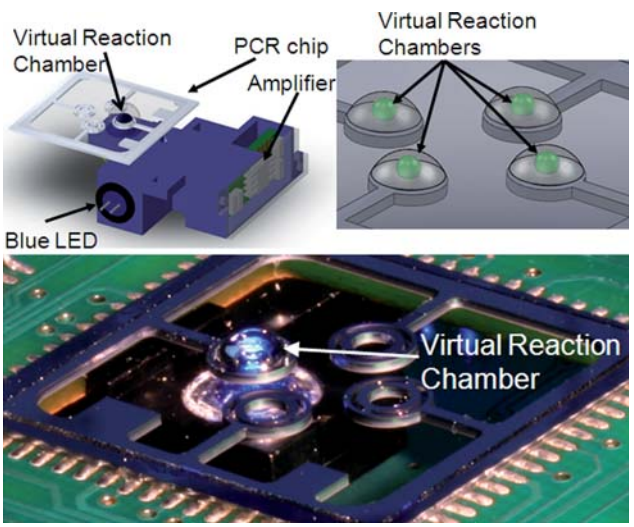

Fig. 2 (Top left) A drawing of the PCR chip with the optical housing underneath. The PCR heater is in a donut shape enabling the light to interact with the PCR sample placed above the heater. (Top right) A drawing of four VRCs at the glass on the top of silicon heaters. (Bottom) Photograph of a system with one optical unit with LED placed underneath emitting blue light into the PCR mixture.

The third PCB houses the microcontroller MC56F8013 (Freescale, Inc.), which controls all PCBs, a touch screen display, processes the data and can be connected to a PC. Alternatively, the system could be externally controlled by a LabView program running on a PC.

All electronics employed in the four PCBs is powered from the fourth PCB, which contains voltage generators comprising a DC adapter or an Li-Ion battery with the total system power consumption of only 3 W.

Performance of the system was verified by performing RT-PCR to detect an *in vitro* transcribed RNA segment of the H5N1 virus. PCR primers for H5N1 were designed by the Genome Institute of Singapore (GIS). The sequence of the forward primer is: 5′-TGCATACAAAA TTGTCAAGAAAGG-3′; the reverse primer: 5′-GGGTG TATATTGTGGAATGGCAT-3′. The single step RT-PCR was set up using the LightCycler® RNA Master SYBR Green I one-step RT-PCR kit from Roche, Inc. (cat. No. 3064760001).

The reaction mixture was prepared by adding 1.3 μl of 50 mM $Mn(OAc)_2$, 0.6 μl of each forward and reverse primers with a final concentration of 0.2 μM, and 7.5 μl of the LightCycler® RNA Master SYBR Green I. An RNA template of 2×10^6 copies in 10 μl was added to the reaction mixture to the total volume of 20 μl immediately before the onset of the reaction. The final template concentration was 10^5 copies per μl.

One μl of the prepared RT-PCR mixture was transferred to a microscope cover slip placed on top of the PCR thermocycler and was covered with 3 μl of mineral oil to prevent possible evaporation.

The reverse transcription was performed at 61 °C for 2.5 min and followed by a 'hot start' at 95 °C for 20 s. A total of 50 PCR cycles were carried out according to the following thermal protocol: denaturation for 4 s at 95 °C; annealing for 20 s at 50 °C and extension for 10 s at 72 °C. The total time required for a single cycle was 34 s. The 'raw' fluorescent signal from the optical unit was recorded during the whole process (Fig. 3). This procedure allowed us to monitor each step of the RT-PCR.

Total time required for the 50-cycle RT-PCR and a melting curve analysis (MCA) was approximately 35 min. The extracted value of critical threshold was 20, which is comparable with the value 20.6 acquired using a commercial thermocycler (Roche LightCycler 1.5). The measured melting temperature T_m of the PCR product was practically identical to that detected by the LightCycler.

The system is currently being designed so that it can be used immediately after conventional extraction of viral RNA (*e.g.* using the Qiagen kit) and the PCR master mix preparation. Our

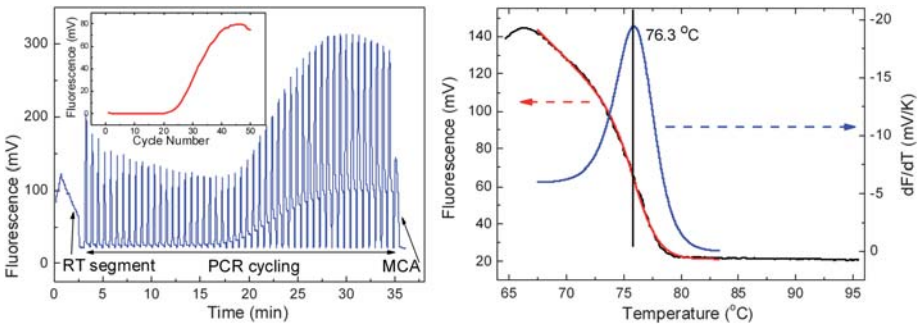

Fig. 3 (left) Fluorescence profile of the RT-PCR, followed by a melting curve analysis (MCA). The average amplitude of the fluorescent signal of the last 2s of the extension segment at 72 °C was recorded as a function of the cycle number, normalized against the background, and plotted into a graph (see inset). (right) Melting curve (black) extracted from PCR data using a ramping rate of $1°Cs^{-1}$. The experimental data were fitted by a sigmoidal function (red). Measured melting temperature of real-time RT-PCR product was 76.3 °C.

future plan is to incorporate this step into the system to develop a truly portable "sample-to-answer" solution.

The system presented here is one of very few micro-PCR instruments reported thus far, capable of performing single-step RT-PCR. Its feasibility to perform real-time PCR was demonstrated by detecting the RNA of HPAI (H5N1) virus in 35 min. Operation of this RT-PCR system is low-cost and user-friendly, as the reaction is conducted on a standard, disposable microscope cover-slip. The chip and sample-to-sample cross-contamination are eliminated by the spacial and functional configuration of the system. Optical detection unit based on lock-in amplification enables the system to be operated without any light protection. At present, the detection device is ready-to-use and is suitable for monitoring avian influenza outbreaks, especially in rural parts of developing countries. In addition, by using different PCR primers, the system can be easily adapted for detection of other infectious diseases, including viral pathologies.

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