Microfluidic devices for gene preparation and detection

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Gene Preparation and Detection**

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**Nanyang Technological University
Singapore**

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**Microfluidic Devices
for
Gene Preparation and Detection**

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Abstract

Lab-on-a-chip systems can perform the various stages of biological and chemical processes in a conventional laboratory, providing great promise in point-of-care diagnostics. This thesis research was focused on the development of methods and devices for the automatic bio-sample preparation and detection in lab-on-a-chip systems. Firstly, a novel sample preparation system was developed in Chapter 2. This system has been subsequently integrated into an all-in-one device developed in Chapter 3 for rapid influenza diagnosis. Lastly, a novel polymerase chain reaction (PCR) array was developed for faster and more accurate PCR mixture delivery and sealing in Chapter 4. We received the Silver Award at the Asian Innovation Awards 2011 for the technology developed in Chapter 3.

Chapter 2 reported the development of a low-cost, disposable DNA/RNA preparation cartridge, which was capable of automatic handling of flexible fluidic sample volumes ranging from 10 μ L to 1 mL. This completely sealed plastic cartridge contained all the necessary reagents for cell lysis, DNA/RNA extraction, impurity washes, DNA/RNA elution and waste processing. The entire sample preparation process was conducted automatically within 6 minutes on a desktop unit using a pneumatic fluid manipulation approach. Transportation of reagents was achieved with a combination of push and pull forces (via compressed air and vacuum, respectively) through the pneumatic inlets at the bottom of the cartridge. The reported automatic lab cartridge successfully extracted and purified the cancer-specific gene from 10 copies of MCF-7 breast cancer cells. The on-cartridge DNA recovery efficiency was ~ 54–63%, which was

comparable to or even better than that obtained by conventional manual approach using silica spin column.

In Chapter 3, an all-in-one system was developed for rapid influenza diagnosis based on WHO-recommended protocols by integrating the automatic DNA/RNA extraction cartridge developed in Chapter 2 with thermal cycler and 1-color, 3-chamber pseudo-multiplexing Real-time Reverse Transcription Polymerase Chain Reaction (RRT-PCR). All necessary chemicals (including the processing of waste generated) were self-contained and completely sealed within a polymer cartridge, thereby minimizing the possibility of human contamination and accidental viral exposure. The system was designed in such a way that the operator only needed to load a patient's nasopharyngeal swab sample with lysis buffer into the cartridge. It allowed for the automatic sample preparation and molecular diagnosis within 2.5 hours. The cartridge design could precisely dispense aliquots for multi-chambered PCR detection. The performance of the all-in-one system was comparable to the commercial real-time PCR with manual RNA extraction and purification. In addition, the multi-chambered capability and sensitivity of the all-in-one system for influenza diagnosis was validated with clinical seasonal H1N1 influenza samples and control experiments based on WHO-recommended protocols and reagents.

In Chapter 4, a new PCR fluidic design for faster and more accurate PCR mixture delivery and sealing was developed. The PCR chip was arranged with a circular pattern, and was connected to a main fluidic channel via an isolation channel. Simulation and experimental results showed that uniform illumination of all wells could be obtained by adopting this configuration to achieve a more accurate detection of fluorescence signals.

With the application of vacuum, the air within the reaction wells could be removed. The filling of PCR mixture within each well could be completed within 8 ms, much faster than other approaches. Liquid wax was then introduced to the fluidic channel to seal the individual reaction chambers. This new PCR fluidic design allowed for the rapid filling of the chambers with PCR mixture at 8 ms per reaction well. The volume of each PCR well could be precisely controlled from 2.99 μL to 3.09 μL . The performance of the chip was successfully tested for (i) NASBA isothermal PCR for the RNA samples from HPIV virus, and (ii) HDA isothermal PCR for the target DNA from *E. coli*.

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List of Abbreviations and Symbols

LOC	Lab-on-a-chip
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
μ TAS	Micro-total analysis system
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RRT-PCR	Reverse transcription real-time polymerase chain reaction
POC	Point of care
PZT	Piezoelectric lead zirconate titanate ceramic material
MEMS	Micro-electro-mechanical system
PDMS	Polydimethylsiloxane
PMMA	Polymethylmethacrylate
PTFE	Polytetrafluoroethylene
PC	Polycarbonate
SU-8	Epoxy-based photoresist
V_{p-p}	Peak-to-peak voltage
DC	Direct current
AC	Alternative current
EOF	Electroosmotic flow
EOP	Electroosmotic pump
EDTA	Ethylenediaminetetraacetic acid
EWOD	Electro-wetting on dielectric
SAW	Surface acoustic wave
NASBA	Nucleic acid sequence-based amplification
LAMP	Loop-mediated amplification
HDA	Helicase-dependent amplification
RCA	Rolling circle amplification
SDA	Strand displacement amplification

Chapter 1

Introduction

1.1 Motivation

Molecular diagnosis offers many advantages over traditional microbiological detection. It offers high specificity and sensitivity for rapid disease detection, on-site environmental monitoring, and food and water-borne pathogens detection [1]. Figure 1.1 shows the work flow for molecular diagnosis, which includes raw sample concentration, removal of debris and other pre-treatments. Once the target cells or the specimens are obtained, the sample is subjected to a lysis process so that the target bio-molecules (e.g., DNA, RNA or proteins) can be released into the lysis buffer solution [2]. These target bio-molecules need to be extracted and purified before detection. Nucleic acids also need to be amplified before detection. To date, most systems for nucleic acid analysis have used the polymerase chain reaction (PCR) for amplification [3-5]. For target molecule detection, techniques such as gel electrophoresis [6], real-time PCR [7] and electrochemical detection [8] have been employed.

Unfortunately, conventional lab-based nucleic acid tests are usually complex and time-consuming. Expensive instrumentation and dedicated laboratory space are required for sample preparation, as well as for amplification and detection of the nucleic acid target. Reagents required for these tests are also expensive, and must be stored at low temperatures. These characteristics have limited the use of certain assays for point-of-care (POC) testing, especially in resource-poor settings.

Figure 1.1: Workflow for molecular diagnosis from raw sample. After concentration/pre-treatment, the patient sample is lysed so that the target DNA/RNA or protein can be extracted and purified. Unlike protein, the eluted DNA/RNA has to be amplified by PCR before detection.

A micro-total analysis system (μ TAS) is an integrated micro-electromechanical system (MEMS) that provides the various steps needed for chemical analysis of a sample (e.g. sampling, sample transport, filtration, dilution, chemical reactions, separation and detection) [1-4, 16]. It enables integration of complex functions, automating repetitive laboratory tasks within a miniaturized device. For this reason, μ TAS is ideal for the rapid detection of DNA, RNA or proteins on-site.

The μ TAS includes two main processes: 1) preparation of DNA/RNA from a raw sample, and 2) amplification/detection of target DNA/RNA. Different specimen types and target cells can be used as raw patient samples for μ TAS. Liquid samples include

whole blood, serum, urine, saliva and cell culture. Solid samples include tissues and stool. DNA, RNA and proteins extracted from these raw patient samples can be used as target molecules [9]. However, prior to the detection step, preparation of biosamples for DNA or RNA analysis may be rather tedious and time-consuming [9-12]. When target molecules are abundant, such as in cell culture, the sample preparation of DNA or RNA targets is relatively simple. If target molecules or cells are rare, for instance circulating tumor cells or circulating fetal cells from whole blood, the sample preparation becomes very complicated [13, 14]. Sample preparation includes many manual steps such as centrifugation, separation, filtration, purification, heating and cooling of reagents, mixing of reagents, etc. [15]. Even for raw samples with an abundance of target molecules, conventional sample preparation often involve intensive manual operation of different instruments.

On the other hand, as the most commonly used method for DNA/RNA amplification, PCR is usually conducted in tubes or well-plates. To perform PCR, operators need to manually load and mix different PCR reagents in the tubes or wells, and then add the DNA/RNA template from the sample preparation. After sufficient target molecules are obtained from PCR amplification, detection is achieved by identifying the existence and quantity of certain nucleic acids. The detection method usually involves several manual steps. For example, gel electrophoresis involves gel preparation and electric field application.

As discussed above, both the conventional sample preparation and PCR amplification/detection are labor-extensive and prone to human error. To provide a fully automatic solution, these two processes can be integrated in a μ TAS, as shown in Figure

1.2. The microfluidic operations include liquid fluid transfer, metering, mixing, thermal cycling, and detection. The integration of various components such as pumps, valves, mixers, separation units, reactors and detectors within a single microfluidic chip is required.

Figure 1.2: Basic microfluidic operations for a lab-on-a-chip (LOC) system.

Due to the advancement in microfluidic systems, μ TAS devices can now perform molecular diagnostics in a clinical laboratory or point-of-care setting [16, 17]. Compared to conventional devices, microfluidic-based instrumentation can realize molecular diagnostic tests in a compact, disposable and automated device with the following advantages: (i) Reduced volume requirements for sample and reagents. (ii) Potential for integrating various functional components into the device with a small footprint. (iii) Multiplexing so that several analyses can be completed simultaneously. (iv) Avoiding cross-contamination and human errors. (v) Unsupervised automation.

The goal of this thesis is to develop a robust, cost-effective and user-friendly μ TAS. For this purpose, the current methods used for fluidic transfer in a LOC system are first reviewed to obtain suitable microfluidic solutions for developing automatic

sample preparation unit. Next, the methods and platforms for amplification/detection of DNA/RNA are reviewed to help select the appropriate approach for our system. Finally, the integrated LOC system is reviewed and compared in an effort to develop a complete and practical device.

1.2 Background

In a microfluidic system, fluid is propelled through microchannels of sub-millimeter cross section. The first microfluidic system was realized on a silicon wafer in 1969 [18] using silicon-pyrex technology. By the end of 1980's, the first micro-valves and micro-pumps based on silicon micro-machining were reported [19-21]. Silicon-based systems were developed to enable the precise control of decreasing fluid volumes and the miniaturization of fluid handling systems. Following such pioneering work, microfluidic components for fluid transport [22-26], fluid metering [27], fluid mixing [28-33], valving, and concentration or separation of molecules [11, 34-37] within miniaturized quantities of fluids were developed in the next two decades.

In LOC applications, microfluidic elements have to be able to perform the basic fluidic unit operations required for molecular diagnostic applications, such as fluid transport, DNA/RNA extraction, fluid metering, fluid mixing, valving and detection.

1.2.1 Fluid transfer in a LOC system

Fluid transfer components are one of the most important aspects of microfluidic devices for the applications of LOC and μ TAS. They are used to drive the microfluid in the LOC [38]. Although externally installed pumps, such as lab-based syringe pumps or

peristaltic pumps are widely utilized for obtaining precise control of reagents [20], due to the size and high cost of the pump, this option is generally limited in portable and/or disposable microfluidic LOC systems. An integrated micro-pump is able to provide pressure to drive liquid fluids within a LOC. Many fluidic transfer methods have been developed and demonstrated by using different actuation mechanisms and fabrication technologies.

1.2.1.1 Reciprocating micro-pump

An integrated micro-pump is one option for transferring reagents within a LOC system. One type of commonly used micro-pumps is called “reciprocating micro-pumps,” which use oscillatory or rotational movements to displace fluid. Other types of micro-pumps include micro-diaphragm pumps and peristaltic micro-pumps [39]. Figure 1.3(a) shows a pump chamber formed by a flexible diaphragm on one side. Oscillatory movement of the diaphragm generates a two-phase pump cycle with periodic volume changes. During the “supply phase”, the underpressure in the pump chamber will suck fluid through the inlet into the pump chamber. During the subsequent “pump phase”, overpressure in the pump chamber will transfer liquid into the outlet. The reagent is delivered in a series of discrete fluid volumes, depending upon the actuator stroke volume, ΔV .

Of the previously developed reciprocating micro-pumps, valveless micro-pumps are of interest since they avoid the use of internal moving parts [39]. Dau and colleagues have developed and characterized a planar fluidic device consisting of a piezoelectric actuated valveless micro-pump and a p-type silicon hotwire that acts as a flow sensor [40, 41] (Figure 1.3(b)). The device was fabricated by standard MEMS processes. The flow

rate and backpressure of the pump were 2.9 mL/min and 0.3 kPa, respectively, when using a 50 V_{p-p} sinusoidal driving voltage at the resonant frequency of 7.9 kHz. A flow rate of 0.05 mL/min was obtained at a low driving voltage of 10 V_{p-p}.

Some mechanical micro-pump designs may contain multiple pump chambers sequentially arranged in series or in parallel. This type of micro-pump design is called peristaltic (see Figure 1.3(c)). Peristaltic movement of diaphragms in the sequentially arranged pump chambers transfers fluid from the inlet to the outlet.

Most peristaltic micro-pumps are fabricated from polydimethylsiloxane (PDMS) with an external pneumatic source for actuation [20, 39]. Chiu et al. developed a peristaltic micro-pump that is actuated and controlled via the periodic generation of electrolytic bubbles [42] (Figure 1.3(d)). The indirect air bubble pushes the solution from inlet to outlet. With an applied voltage of 2.5 V, the maximum pump rates for DI water and whole blood were 121 nL/min and 88 nL/min, respectively. Du and colleagues developed a peristaltic micro-pump driven by two magnetically attracted steel balls with a rotating motor [43]. A flow rate of 5 mL/min with a high backpressure of up to 10 kPa was achieved at a motor speed of 500 rpm.

Figure 1.3: Common reciprocating micro-pump. (a) Principle of membrane micro-pump with 2 check valves. (b) A valveless micro-pump actuated by piezoelectric lead zirconate titanate ceramic material (PZT) with a hotwire for self-measurement of flow rate [41]. (c) Principle of peristaltic micro-pump with three sections of actuating diaphragms. A stroke volume ΔV of reagent will be pumped out. (d) A peristaltic micro-pump actuated and controlled via the periodic generation of electrolytic bubbles [42]. The indirect air bubble pushes the solution from inlet to outlet.

Various micro-actuation techniques have been used for the design of micro-pumps. These micro-actuators make use of piezoelectric [44, 45], thermopneumatic [46, 47], electrostatic [48], electromagnetic [49, 50], and shape memory [51] principles, among others.

1.2.1.2 Electroosmotic pump

The principle of an electroosmotic pump (EOP) is based on the movement of an electrolyte solution relative to a stationary charged surface due to an externally applied electric field [52]. As shown in Figure 1.4, a device driven by electrokinetic forces includes a microfluidic channel and a pair of electrodes or an electrode array, fabricated as MEMS. The platform has a high surface-to-volume ratio.

Electroosmotic pumps possess several advantageous features for LOC applications. They have no moving parts so they can be fabricated using standard microfabrication technologies, and can be readily integrated with LOC devices [53, 54]. They are able to generate both constant and pulse-free flows, the magnitude and direction of which can be easily controlled.

Electroosmosis requires charged solid surfaces to generate electroosmotic flow (EOF). Most surfaces spontaneously acquire a finite charge density when in contact with an aqueous solution. Both direct current (DC) voltage [55, 56] and alternating current (AC) voltage [57, 58] can be used for driving an EOF pump. DC EOPs are commonly operated under high electric voltages. Potential issues associated with these pumps include bubble formation and solution pH variation due to electrolysis. AC EOPs have been developed to address these issues since the application of an AC voltage does not result in strong electrolysis. Both DC and AC EOPs are able to generate a constant flow. AC EOPs use symmetrical electrode arrays to produce flows, and this local flow circulation makes for excellent micro-mixers in the flow channel.

Electrokinetically driven pumps can use a very wide range of actuating voltages (1.2 V to 10 kV) [52]. Depending on the design of the pump, the flow rate achieved ranges from 0.27 $\mu\text{L}/\text{min}$ to 7 mL/min [52]. The highest reported backpressure of EOP is 33 MPa [59].

Figure 1.4: Structure of electric kinetic pumps. (a) The electric double layer and principle of electroosmotic flow in a DC EOP pump. The mobile ions in the diffuse counter-ion layer of the electric double layer are driven by an externally applied electrical field. These moving ions drag bulk liquid through viscous force interactions. The net flow is the superposition electroosmotic flow (EOF) and pressure forces. (b) AC EOP using an asymmetric electrode array. The asymmetry leads to fluid rolls of different sizes over each electrode, resulting in a net fluid flow.

1.2.1.3 Electrowetting actuation

Electrowetting-on-dielectric (EWOD) is based on a liquid droplet placed between two electrodes covered with an insulating layer [39]. As shown in Figure 1.5, the contact angle of the droplet changes when a voltage is applied to the two electrodes. This technology is a well-known method for altering the wettability of surfaces by changing the electrical potential. A thin film insulating layer is used to separate the conductive liquids from metallic electrodes, which can hinder electrolysis. Typically, this insulating

layer is a hydrophobic layer formed by liquid Teflon to enhance the droplet movement [60, 61].

In recent development, several individual addressable electrodes have been used to control droplet path and are located on the bottom of the device. They are typically arranged in linear or two-dimensional arrays. The droplet is enclosed between the two electrode plates in a sufficient volume to cover parts of two addressable electrodes at all times. When a voltage is applied to one of the control electrodes in contact with the droplet, the contact angle at this part of the droplet is reduced, initiating a movement of the droplet along the paths given by the activated electrode.

By configuring the electrode array and applied voltage, this EWOD-driven droplet-based platform can be easily programmed. This allows an operator to perform different assays by running different programs on the same piece of hardware. Both organic solvents, ionic liquids, aqueous solutions and surfactants, as well as biological fluids, such as whole blood, serum, plasma, urine, saliva, sweat and tears have been successfully transported on this EWOD droplet-based platform.

A droplet with a volume as small as 20 nL can be metered with a standard deviation of 2% using EWOD technology [62]. Mixing of the droplet can be performed by merging two droplets into a single one, followed by oscillating the voltage between two electrodes. An increasing oscillating frequency leads to reduce mixing time. The mixing process time can be further reduced by oscillating the droplet over a long linear electrode array. Mixing of 1.3 μ L of fluid could be done within 3 to 4.6 seconds.

Due to voltage losses across the dielectric layer, typically a voltage of ~ 60 V would be required to drive a EWOD device. The lowest recorded voltage to date was 25

V [63-65]. For an initial droplet volume of 0.3 mL and a power of 12 nW, a constant flow rate of 0.02 mL/s was demonstrated [66].

Figure 1.5: Principle and structure of EWOD device. A droplet is enclosed between a common electrode E0 and addressable electrode array E1–E3. The contact angle of the droplet is reduced when voltage is applied to the electrode. The droplet is moved from electrode E1 to E3 when a voltage is applied to E1 follow E2, and then E3.

1.2.1.4 Surface acoustic wave actuation

Surface acoustic wave (SAW) driven fluidic transfer is based on surface waves, which are mechanical waves with amplitudes of only a few nanometers [67, 68]. As shown in Figure 1.6, the surface acoustic waves are generated by a piezoelectric transducer chip fabricated by placing interdigitated electrodes on top of a piezoelectric layer [69, 70]. Liquid droplets situated on the hydrophobic surface of the chip can be moved by SAWs if the acoustic pressure exerted on the liquid droplet is high enough.

SAWs operate at much higher frequencies (10–500 MHz) than conventional ultrasound devices (20 kHz–3 MHz). They involve transverse-axial polarized elliptical electro-acoustic waves traveling across the atomizer's substrate surface. Although the

amplitude of a SAW is very small, the acceleration of the surface is incredibly high (10^7 m/s²) because of the high operating frequency [25, 26].

SAWs offer a powerful method for driving a host of extremely fast microfluidic actuation and micro/bioparticle manipulation schemes. Similar to EWOD, SAWs transfer droplets on a planar surface or through a microchannel. However, SAWs are able to transfer droplets at much higher speeds. Yeo et al. demonstrated droplet transfer at velocities of 1–10 cm/s, which are typically one to two orders of magnitude faster than those afforded by other microfluidic technologies.

Metering of fluid can be accomplished by moving fluid over a small hydrophilic ‘metering spot’ via SAWs. This is done over a hydrophobic/hydrophilic chessboard zone, and a small metered liquid volume is left behind. Since the transport of a droplet depends non-linearly on the droplet size, the initial droplet of a larger volume is constantly moved forward, while small, picoliter-sized droplets remain on the hydrophilic parts of the wetting pattern.

Mixing strategies can be induced in a microchannel through which fluid is pumped via SAWs by changing the SAW frequency to rapidly switch from uniform through-flow to a chaotic oscillatory flow.

SAWs can also be used for microcentrifugation for particle concentration or separation within a microdroplet. Qi et al. reported the separation of liquid, protein molecules from paper [71]. Guttenberg [72] used SAWs for transfer of reagents and hybridization for PCR on a planar chip.

Figure 1.6: Structure of SAW device. (a) Schematic depiction of a liquid droplet placed on the substrate in the propagation pathway of the SAW. (b) The detail of the interdigitated transducers patterned onto the piezoelectric substrate. SAWs are generated from the piezoelectric substrate. Due to the different sound velocities in the substrate and in the liquid phases, the SAW energy leaks into the droplet and forces the droplet in the SAW propagation direction [68].

1.2.1.5 Centrifugal force actuation

A centrifugal force driven device uses centrifugal force to process samples and reagents in a disposable plastic rotor, as shown in Figure 1.7 [73-75]. The current generation of centrifuge devices is made from microfabrication and microfluidic technologies. Therefore, up to one hundred processes can be done in parallel on a disk. This enables a high throughput of tests by highly parallel and automated liquid handling. The assay volumes can also be reduced to less than 1 μL [38].

The liquid transfer, metering, mixing and switching can be achieved by using a centrifugal device controlled by the rotation speed of a disk from one single rotary

engine. Since blood plasma is less dense than the white and red blood cells, extraction of plasma from a blood sample can be finished within 20 seconds by this approach.

Most of the centrifugal devices are fabricated with plastic using injection molding or hot embossing [76]. This makes them cost-effective, disposable and easy to exchange. However, since most of the logic functions and their critical rotating frequencies are permanently fabricated on the disk, the centrifugal platform lacks flexibility as compared to other platforms.

Figure 1.7: Scheme of a segment of microfluidic elements for PCR disk with 14 reaction wells. Processes include reagent transfer, mixing, and metering can be rapidly achieved. The primers are preloaded [73, 74].

1.2.1.6 External pressure actuation

Using off-chip pressure for pushing a reagent loaded pouch or porous PDMS sponge is a simple way to deliver fluids within a LOC device. Dafeng et al. developed an on-chip pouch pump for fluid delivery within a LOC device [77]. In their design, each pouch consists of a cavity milled on a plastic substrate with a rounded end mill and

covered with a thin shell dome. Cha and Kim proposed a novel portable and disposable pressure pump using a porous PDMS sponge [78]. The PDMS sponge with interconnected micropores was fabricated by a sugar leaching technique based on capillary suction of pre-cured PDMS into lumps of sugar. A plunger was used to deform the porous PDMS to drive a fluid sample in this external pressure actuation.

Figure 1.8: Schematic of pressure-driven pumps. Reagent is pre-loaded onto (a) a cavity [77] or (b) a porous PDMS [78]. A plunger is used to deform the pouch membrane or the porous PDMS for fluidic delivery.

1.2.1.7 Fluidic guiding for LOC system

Passive microvalves (without actuation) and active microvalves (with actuation) are used in many microfluidic systems for guiding flow direction and sealing reagents [21]. Since most passive microvalves are check valves that allow fluid flow only in one direction, they are not as diverse in their use as active valves that can open and close the fluid passage.

Thermopneumatic, bimetallic, shape memory, electrostatic, piezoelectric and electromagnetic actuators are most commonly used actuators in conventional active microvalves. A flexible diaphragm is coupled with an actuator to regulate fluid flow

within a channel. Other interesting active microvalves include a thermal responsive hydrogel valve [79] and a pneumatically actuated PDMS valve. Low cost, single-use paraffin valves have also been developed [80, 81].

Figure 1.9: Schematics of an “open-close-open” microvalve using paraffin [81]. (a) The valve is open when the heater is turned off. (b) The valve closes after the heating zone is heated up and then cooled down. (c) The valve opens after the heating zone is heated up again.

The advantages of the microvalve are that they are cost-effective and easy to integrate with microfluidic systems. However, they have poor reliability since the displacement of the diaphragms is typically limited to tens of microns or less. Miniature turning valves have been developed for polymeric LOC and commercial molecular diagnostic cartridges. Some commercial LOC devices such as GenExper also use turning valves for fluidic guiding [82].

1.2.2 DNA/RNA PCR amplification and detection techniques for LOC system

A key step in nucleic acid detection is nucleic acid amplification [83]. This method greatly increases assay sensitivity by generating a large number of target copies through PCR. This makes it a powerful molecular diagnostic tool for identifying and monitoring infectious diseases and genetic disorders.

The materials used for PCR chips are usually silicon or glass substrates [84]. Although silicon has the advantage of ease of fabrication and good thermal conductivity, its poor optical properties and high material and fabrication costs make it unsuitable for use in disposable applications [7, 85]. In contrast, polymeric materials offer benefits in cost, ease of fabrication, disposability, biocompatibility, and optical transparency [86-88]. They include PDMS, polytetrafluoroethylene (PTFE), polypropylene (PP), polycarbonate (PC), polymethylmethacrylate (PMMA), and SU-8. A number of research groups have reported polymer-based PCR microfluidic chips [89-93].

PCR requires three temperature zones: i) denaturation at 95 °C, ii) annealing at 55 °C and iii) extension at 72 °C [94-97]. The conventional PCR reaction chamber cycles between these three different temperatures with the PCR well and PCR solution kept stationary. However, the temperature changes in the heating process may vary, and are affected by the large thermal mass surrounding the chambers. Thus, the PCR process becomes slow and complex. This defect can be overcome with ‘continuous-flow PCR’, where the PCR mixture flows continuously and repeatedly through three different temperature zones necessary for PCR amplification using external pumps such as a syringe pump or peristaltic pump [98-102]. Thermal convection-driven PCR microfluidics is another flow-through PCR method that uses two or three fixed temperature zones to facilitate convection-driven sample flow without using a pump for the fluidic transfer [103, 104].

Figure 1.10: Principle of continuous flow-through PCR fluidics. (a) PCR solution is continuously and repeatedly flowed through three different temperature zones necessary for PCR amplification using an external pump such as a syringe pump or peristaltic pump [99, 105]. (b) Thermal convection-driven PCR microfluidics uses two or three fixed temperature zones to facilitate the convection-driven sample flow without using a pump for the fluidic transfer [104].

The drawback of PCR lies in its requirement of precision thermal cycling between three temperatures during the target DNA/RNA duplication. Low heating/cooling rates and high power consumption are expected when a high thermal mass PCR chip is used. In contrast, isothermal amplification methods do not require any thermal cycling [1, 106-109]. Isothermal amplification is different from PCR in that the rate of enzyme activity is the limiting factor rather than the rate of thermal cycling. It is therefore unlikely that microfluidic isothermal amplification reactions can be significantly accelerated, and acceleration has been achieved by optimizing heat transfer in conventional microfluidic PCR. As a result, the systems developed to miniaturize isothermal amplification have used microchambers with no fluid motion during the reaction. This gives isothermal amplification systems the advantage of simple design requirements as compared to

conventional PCR. The smaller amount of energy required for the operation of isothermal amplification systems makes it a viable option for battery-operated portable molecular diagnostic systems.

Isothermal amplification methods include nucleic acid sequence-based amplification (NASBA) [110, 111], loop-mediated amplification (LAMP) [112-114], helicase-dependent amplification (HDA) [115-118], rolling circle amplification (RCA) [119] and strand displacement amplification (SDA) [1, 3]. Commercial reagents are available for these isothermal amplification techniques.

Figure 1-11: Working principle of (a) NASBA and (b) HDA [82]. Both DNA and/or RNA amplification processes take place at very low temperatures (41 °C and 65 °C, respectively).

1.2.3 Packaging of LOC system

The ideal clinical diagnostic system should be able to deliver rapid, specific and reproducible results, while minimizing the need for specialized laboratory facilities and trained personnel. An all-in-one (AIO) system that can automate all processes from sample input to output would be preferred. Many microfluidic platforms enable the

miniaturization, integration and automation of biochemical assays. They can be classified into three platforms: LOC [80], lab-on-a-foil [74] and lab-on-a-cartridge [77].

Both lab-on-a-chip and lab-on-a-foil systems are characterized by a large, thin area with a small package volume. They are fabricated using lithography/etching or micromolding. The lab-on-a-cartridge package, on the other hand, has a larger package volume. It can be fabricated by conventional machining techniques, such as numerically controlled machining and plastic injection molding. In all 3 types of structures, reagents are required for the entire diagnostic processes, such as reagents for DNA extraction and purification, and PCR reagents and target DNA/RNA primers for PCR amplification. The recently reported planar structure AIO system normally has few PCR wells (e.g. only 1) [120], while the cartridge type structure has as many as 102 wells [121].

To increase the sensitivity of molecular diagnosis, 100 μL to 1000 μL of raw patient samples, such as whole blood, serum, or plasma is usually employed. Large amounts of reagents, including lysis buffer (up to 1000 μL), and washing buffers (up to 600 μL per wash for 2–3 washes) are also used. To reduce the material and fabrication costs, the AIO systems are fabricated with polymers such as PMMA and PC using numerical controlled machining (CNC) or injection molding.

Figure 1.12: Package of integrated, self-contained, AIO polymeric molecular diagnostic systems. LOC from (a) Motorola [80] and (b) Haim's group [77]. (c) Lab-on-foil for a centrifuge PCR disk from Roland's group [73, 122]. The disk is able to distribute 4 samples for detection of 32 target genes. (d) Lab-on-a-cartridge structure from Idaho Technology, USA [121] contains 12 reservoirs. These designs include on-system sample lysis, nucleic acid isolation, and PCR amplification and detection. All the liquids and dry reagents needed for the various reactions are pre-stored in the system. The liquid reagents are stored in pouches.

1.3 Research objectives

Of the various miniaturized analytical systems, PCR microchip/microdevices are most extensively studied. However, due to the lack of development in the area of sample preparation, few complete LOC systems can handle raw samples. Nucleic acids must first be extracted from cells. Residual materials from the samples can inhibit the PCR process and reduce the efficiency of PCR amplification. Cell lysis and nucleic acid extraction systems are therefore rather complex even without an analysis system.

Hence, the objective of this thesis is to develop microfluidic transfer devices for DNA/RNA preparation and DNA detection so as to achieve AIO molecular diagnosis systems.

1.4 Thesis organization

Chapter 1 presents the research motivation and background. The current techniques for fluidic transfer, actuation and guiding in LOC devices were reviewed. The techniques for DNA/RNA amplification and detection based on the PCR technique were presented. The common platforms for LOC systems were introduced. The problems associated with existing techniques were described, along with the objective and scope of this thesis.

Chapter 2 presents a novel device for automatic sample preparation in a low-cost plastic cartridge, which was capable of handling flexible sample volumes of 10 μL –1 mL. This cartridge contained 8 chambers with pre-loaded reagents for raw sample loading, pathogen lysis, DNA/RNA extraction (using a silica membrane), impurity washes, sample elution, DNA collection, and waste storage. In our approach, reagents were

transported by using a combination of compressed air (push) and vacuum (pull) forces, which were connected to the pneumatic inlets at the bottom of the cartridge. The DNA/RNA purification process was automatically conducted within this completely sealed cartridge using a desktop system via a novel fluid manipulation approach. The cartridge was fabricated from PMMA containing no movable components, which made it suitable for mass production by injection molding.

Chapter 3 presents a fully automated system that integrated the sample preparation unit with a PCR amplification/detection unit for rapid infectious disease diagnosis. This AIO system was comprised of sample preparation unit, miniaturized thermal cycler, a 1-color, 3-chambered fluorescence detector for real-time reverse transcription PCR, and a pneumatic fluidic delivery unit. The disposable, self-contained cartridge held all the reagents for viral RNA purification and reverse transcription PCR detection, which took place within the completely sealed cartridge. This system was successfully employed for seasonal influenza A H1N1 typing and sub-typing, with comparable sensitivity to the experiments conducted using manual RNA extraction and commercial thermal cycler. A minimum detectable virus loading of 100 copies/ μ l was been determined by serial dilution experiments.

Chapter 4 presents a rapid filling PCR array chip with up to 35 reaction wells for extension of the detection capability of our AIO system. The wells on the chip were arranged along a circular loop, each connected to a main fluidic channel via the isolation channel. Simulation and experimental results showed that uniform illumination of all wells could be achieved by adopting this configuration, allowing for more accurate detection of fluorescence signals. The filling of each well with PCR mixture could be

completed within 8 ms, which was much faster than other filling techniques. The filling and sealing processes were experimentally characterized, and the chip performance was tested in processes including (i) NASBA isothermal PCR for the detection of RNA samples of HPIV virus, and (ii) HDA isothermal PCR for the detection of *E. coli* DNA.

Chapter 5 presents a summary of the achievements in this thesis, and the recommendation of future studies.

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Chapter 2

Development of a Self-Contained Sample Preparation Cartridge

2.1 Introduction

Molecular diagnosis based on nucleic acids detection is a key technique for disease detection [1-3]. However, several steps are required for nucleic acids extraction from raw biological samples, impurity washes, and DNA/RNA elution. Careful sample preparation is vital to obtaining reliable diagnosis, especially with low copies of pathogens and cells. The sample preparation processes of DNA/RNA extraction and purification are time-consuming and labor-intensive. Several steps are required, including pathogen lysis, nucleic acid extraction, impurity washes, and DNA/RNA elution [3].

Several lab-on-a-chip (LOC) devices have been reported with an integrated sample preparation feature [4-8]. These devices typically have limited sample volume handling capacities of $\leq 1 \mu\text{l}$ due to the height constraints in the fluid channel or chamber. Such low sample volume poses a challenge on early-stage disease diagnosis with a low viral load (< 50 copies/ml) [3, 9, 10]. In addition, the clinical samples commonly have undesired co-existing human or non-specific bacterial cells [9, 10]. The DNA/RNA of these co-lysed impurities may saturate the loading capacity of the miniaturized DNA capture unit. Thus, in typical clinical laboratory settings, sample preparation is often conducted with a sample volume of 200–500 μl and a high loading of porous silica column or silica-coated magnetic beads [11].

A novel device for automatic sample preparation in a polymer cartridge that is capable of handling flexible sample volumes of 10–1000 μl (Figure 2.1) is developed in this chapter [12, 13]. The low-cost cartridge is made of polymethylmethacrylate (PMMA), and contained no moving components such as pumps and valves.

Figure 2.1: Schematic of the sample preparation system, which consists of a cartridge for DNA/RNA purification, a valve manifold for fluid regulation, and two syringe pumps for fluid pumping. The cartridge contains 8 chambers for raw sample loading (C1), DNA/RNA extraction (X), two impurity washes (C2 and C3), ethanol wash (C4), elution buffer (C5), eluent DNA collection (C7), as well as waste storage (C6). Pumping of reagents is achieved using air pressures and vacuum forces, generated by two pumps in a push-pull set-up and regulated by a valve manifold (P1–P5, V1–V2).

The cartridge consists of 8 chambers for the loading and processing of raw sample. Transportation of reagents is conducted with a combination of push and pull forces using compressed air and vacuum, respectively, which are connected to the pneumatic inlets at the bottom of the cartridge. These pneumatic forces are regulated by two pinch valve manifolds and two pneumatic pumps within the desktop system.

DNA/RNA extraction is based on a solid-phase method using a silica membrane. The on-cartridge fluidic processes include DNA/RNA extraction, impurity washes (2×), ethanol flush, elution with elution buffer, and collection of DNA/RNA eluent. The DNA/RNA purification process is automatically performed within this completely sealed cartridge using a desktop unit. The sample preparation process could be achieved in 6 min. We demonstrate the successful use of the cartridge for DNA extraction with an efficiency of 54–63%, which was comparable to or better than the conventional manual approach using silica spin column. In addition, we illustrate the capability of on-cartridge RNA extraction from as few as 10 MCF-7 breast cancer cells using our system. Successful extraction is confirmed using a Bio-Rad CFX-96 commercial real-time polymerase chain reaction (PCR) system for CK19 cancer-specific gene amplification.

2.2 Materials and methods

2.2.1 Device fabrication

The PMMA sample preparation cartridge (40 mm × 23 mm × 30 mm) was designed with SolidWorks, and fabricated by computer numeric control (CNC) machining (Whits Technologies, Singapore) for prototype. The final device was fabricated by cost-effective injection molding. The surface channels were 1 mm in height and 1 mm in width. The through-cartridge pneumatic and fluidic channels were 1 mm in diameter. The chamber volume (Figure 2.2) was designed to accommodate the required amount of reagents for the commercial DNA/RNA extraction kits, such as QIAamp DNeasy Blood and Tissue Kit and QIAamp Virus RNA Mini Kit (Qiagen, Singapore). Currently, the reagents volumes for sample preparation, PCR and other processes are standardized in the various applications. For example, the sample preparation solution

from Qiagen and other companies is always supplied in the volume of 200 μL . The ability of our device to process such a large volume would help to make it more applicable for clinical use than the LOC devices reported earlier.

Figure 2.2: (a) SolidWorks drawing of the sample preparation cartridge with chambers for DNA/RNA extraction and purification. The reagents for DNA/RNA extraction could be preloaded into the cartridge, and sealed by adhesive films. The (b) top and (c) bottom views of the cartridge specified with pressure inlets (P1–P5) and vacuum inlets (V1–V2). Pumping of reagents was achieved using air pressures and vacuum forces generated by two pumps in a push-pull set-up. The red and blue arrows indicate the flow directions of air (pressure or vacuum) and reagents, respectively.

To remove any oil and contaminant, the cartridge was soaked in 0.2% of detergent (Decon 90, Decon Lab. Ltd. UK) for 12 h, rinsed thoroughly with de-ionized water, and oven-dried (60°C, 6 h). Next, the cartridge was dip-coated with 0.5% (w/w) of DuPont AF 1600 fluoropolymer dissolved in 3M FC-40 Fluorinert (filtered with a 10- μm membrane filter (Vacu-Guard, Whatman, Singapore)), and oven-dried (60°C, overnight). The Teflon coating made the surface of chambers and channels hydrophobic, avoiding

the sticking of reagents such as washing buffers on the surface. The Teflon-coated cartridge was soaked in 3% H_2O_2 (MGC Pure Chemicals) for 12 h, rinsed with 0.1% diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, Singapore) to remove RNases and DNases, and oven-dried (60°C, 6 h). FujiFilm silica membrane was used for DNA/RNA extraction (Quickgene RNA Cultured Kit S, FujiFilm, Singapore). The membrane was cut from the silica column with its support (to a height 4 mm). The membrane was inserted at the bottom of the membrane chamber, as shown in Figure 2.2(c). The top and bottom of the cartridge were then sealed with MicroAMP optical adhesive film (Cat. #4306311, Applied Biosystems, Singapore).

2.2.2 Fluidic pumping and regulation

Most planar microfluidic structures require on-chip valves for directing fluids [14-18]. The necessity of having more components increases the cost per chip. Although surface acoustic wave [19, 20] and magnetic force [21] are able to direct fluids without using valves, the fluids are exposed to air during transportation, which raise the concern for cross contamination. In our system, the fluids were manipulated using a combination of compressed air and vacuum forces. The reagents within our cartridge's chambers were automatically isolated due to gravity. The novelty of our approach was that the cartridge was designed with no moving components for guiding and transferring fluids. The pressure and vacuum forces were generated by two custom-built pumps in the desktop unit (Figure 2.3). The pneumatic forces were directed to the appropriate chambers within the cartridge using the pinch valve manifolds. The pumps and pinch valve manifolds constituted the main external pneumatic system, which controlled the fluidic motion

within the chambers of the cartridge. This structure has greatly simplified the cartridge assembly, and would allow for the mass production of cartridges via injection molding, thereby significantly reducing the cartridge cost. Since the reagents were self-contained within the fluidic chambers and channels throughout the entire operation, the potential run-to-run and hardware cross contaminations were eliminated.

When the cartridge was loaded into the pneumatic interface slot, the stainless steel tubes (Figure 2.3(e)) would puncture the adhesive film covering the inlets of the pneumatic channels. Pressure and vacuum could then be applied to each fluidic chamber through the pressure inlets (P1–P5) and vacuum inlets (V1–V2) of the cartridge (see Figures 2.1 and 2.2). The operation of the sample preparation cartridge is illustrated in Figure 2.1 with the status of each pinch valve indicated. The biological sample was first loaded into the sample chamber (C1) by a needle syringe, and the cartridge was then re-sealed with an adhesive film. After the cartridge was loaded onto the desktop system, pinch valves P1 and V1 were opened, while other valves remained closed. The pressure pump and vacuum pump ran at a flow rate of 0.5 ml/min. As shown in Figure 2.1, the pressure source from the pressure pump passes through the pinch valve P1, through a vertical pneumatic channel (shown in Figure 2.2(a)), to the top of the sample chamber C1. In the meantime, the vacuum source from the vacuum pump (Figure 2.1) passes through the pinch valve V1 and a vertical pneumatic channel, to the top of the waste chamber C6. The pressure source and the vacuum source would not come in contact with the sample solution, thus avoid any contamination.

Figure 2.3: Photographs of the sample preparation system. (a) The sample preparation cartridge (1) and the desktop system (2). (b) Photograph of the desktop unit with a pinch valve manifold (3), two syringe pumps (4 and 5), a syringe pump controller (6), and a power supply (7). (c) The reagent was automatic isolated in vertical and upside down position due to a combination of gravity and air pressure. (d) The cartridge was connected to the external pressure and vacuum forces via the o-ring sealed interface (8). (e) Photograph of the o-ring sealed interface with stainless steel tube with 1 mm protrusion for piercing the sealing film and connecting pressure or vacuum source to the cartridge.

The biological sample containing the target DNAs was then lysed and filtered through the silica membrane. The DNAs were captured by the membrane, while the filtrate waste was directed to the waste chamber. Like the DNA capturing step, the impurities (trapped within the silica membrane) were washed out sequentially by wash buffer 1 (C2), wash buffer 2 (C3), and ethanol flush (C4) at a flow rate of 1 ml/min. Before elution step, by turning on pinch valve P4 and V1, the system was extensively flushed with air (flow rate = 10 ml/min, 2 min) from chamber C4 to chamber C6 to remove any remaining wash buffer in the channels and the silica membrane. The purified DNA was then released from the silica membrane with the elution buffer (C5), and directed to the eluent chamber (C7), with only valves P5 and V2 opened. The entire process took only approximately 6 min. Since all the reagents are preloaded inside the cartridge and the sample preparation processes are conducted within the cartridge automatically, human error and cross-contamination were avoided.

Two in-house fabricated syringe pumps with a volume of 25 ml were used to generate the air pressures and vacuum forces. These syringe pumps were driven by a linear actuator (E43H4N-12, Haydon, USA) and a step motor driver (DCS 4010, Haydon, USA) with a maximum flow rate of 12 ml/min. The air pressures and vacuum forces (connected to inlets P1–P5 and V1–V2, respectively, in Figures 2.2(b) and (c)) were regulated by a pinch-valve manifold (P/N 075P2NC12-23S, Bio-Chem Fluidics, UK). These pneumatic forces were connected to the cartridge via o-rings and pneumatic connectors (M-3AU, SMC, Japan) through protruded stainless steel tubes (of 1 mm in length), which pierced through the bottom sealing film upon cartridge loading. The entire system was controlled by an industrial controller (PMX-4EX-SA, Arcus Inc., USA).

The automatic system was powered by a power source of 12 V and 35 W (S-35-12, MeanWell, Taiwan). The currents required for each pinch valve, linear actuator and controller were 0.24, 0.3 and 0.5 A, respectively. Since only two pumps, two pinch valves and the controller were working at the same time, the average power consumption was about 19 W (12 V, 1.58 A). Hence, the system can be powered by a car battery.

2.2.3 Pressure required for transferring solutions

When a fluid flows through a closed channel such as a pipe or between two flat plates, depending on the velocity of the fluid, laminar flow or turbulent flow may occur. Laminar flow tends to occur at lower velocities, whereby the fluid moves in parallel layers, with no disruption between the layers. In contrast, turbulent flow is a less orderly flow regime characterized by eddies or small packets of fluid particles, which result in lateral mixing.

Reynold's number (Re), a dimensionless number that gives a measure of the ratio of inertial forces to viscous forces, is usually adopted to characterize different flow regimes, such as laminar or turbulent flow. It is normally defined in the following forms:

$$R_e = \frac{D_l \cdot V \cdot r}{m} = \frac{D_H \cdot V}{t}, \quad (2-1)$$

where D_l = the diameter of the channel,

D_H = the hydraulic diameter of the flow channel,

V = the flow velocity,

r = the fluid density,

m = the dynamic viscosity,

t = the kinematic viscosity.

When $Re = 1$ – 100 , the fluid flow is laminar with strong Reynold's dependence. For a fully developed flow in a pipe, experimental observations show that laminar flow occurs when $Re < 2300$ and turbulent flow occurs when $Re > 4000$. According to the classical theory of fluid mechanics, the pressure drop associated with a viscous liquid with a flow rate of Q_v through a long microchannel (length/diameter ratio on the order of 10 – 100) with a constant cross-sectional area can be calculated from the following equations:

$$\Delta P = Q_v \cdot \frac{CmL}{2AD_H^2} \quad (2-2)$$

$$D_H = \frac{4A}{u} \quad (2-3)$$

where ΔP denotes the pressure drop across the microchannel, A and L are the cross-sectional area and length of the microchannel, respectively, D_H is the hydraulic diameter, C is the friction factor, m is the dynamic viscosity of the fluid, and u is the wetted perimeter.

For easy fabrication of the cartridges using conventional fabrication technique, the channels' dimensions used in our design are shown in Table 2.1. The total lengths of the horizontal and vertical channels were 21 mm and 30 mm, respectively. There are two vertical channels, one for upward flow and the other for downward flow.

Table 2.1: Dimensions of the channels in the cartridge.

Name	Cross-Section	Dimension (mm)
Vertical channel	Circular	$\phi 0.5$
Horizontal channel	Rectangle	0.5×0.5
Chamber	Circular	$\phi 5$ to $\phi 7$

In order to calculate the pressure required to transfer solution, the flow path (from C1 to C6) of the cartridge shown in Figure 2.1 was simplified as a vertical channel of 21 mm and a horizontal channel of 60 mm, with a porous silica membrane in between that works as the DNA/RNA extractor (see Figure 2.4). Positive pressure was needed to overcome the collective flow resistance of the three compartments.

Figure 2.4: Simplified flow path model for the sample preparation cartridge. The DNA/RNA extractor is a porous silica membrane.

First, we considered the pressure required to transfer the solution through the vertical and horizontal channels. To prepare 200 μL of biosample, 200 μL of lysis buffer and 200 μL of ETOH were required, giving a solution with a total volume of 600 μL . If the 600 μL of solution was to be transferred within a period of T seconds, the averaged flow rate Q_v was $600/T$ $\mu\text{L/s}$. This was substituted into Equations (2-2) and (2-3) to calculate the corresponding pressure needed to drive the fluid. The results for fluid transfer time durations of 20–80 s are shown in Figure 2.5.

Figure 2.5: Calculated pressure required for transferring 600 μL of solution through the vertical and horizontal channels in 20–80 s.

The DNA/RNA extractor was a porous silica membrane with a thickness of 0.1 mm and a pore size of 90 nm. For flow-through porous medium, the pressure drop can be described by Darcy's law:

$$Q_v = \frac{-kA}{m} \frac{\Delta P}{L}, \quad (2-4)$$

where k is the permeability of the porous medium, and A and L are the cross-sectional area and thickness of the membrane, respectively. Since it would be complicated to determine the membrane permeability, the actual pressure drop across the membrane was measured experimentally instead by using a digital pressure sensor (ZSE10F-M5R, SMC, Singapore). A plot of the actual pressure required for pushing and pulling 600 μL of solution is shown in Figure 2.6.

Figure 2.6: Measured pressure required for transferring 600 μL of solution through the DNA/RNA extractor in 20–80 s.

As shown in Figures 2.5 and 2.6, the pressure required to drive the fluids decreases with the increasing time for liquid transfer. This is not surprising because it is always easier to transfer the same amount of liquid over a longer duration. A comparison of the three compartments showed that a higher pressure would be required for the DNA/RNA extractor and the vertical channel than the horizontal channel. The pressure required for the vertical channel could be reduced by decreasing the height of the cartridge or increasing the diameter of the channel. The pressure required for the DNA/RNA extractor could be lowered by increasing the pore size or the area of the membrane used.

The total pressure required for transferring solutions along the flow path shown in Figure 2.4 through all three compartments was measured using the digital pressure sensor

mentioned above (see Figure 2.7). The calculated pressure (note: the membrane resistance was obtained experimentally) was shown to agree well with the experimental data.

Figure 2.7: Comparison of the calculated and measured pressures required for transferring solutions along the flow path as shown in Figure 2.4.

2.2.4 DNA recovery efficiency

DNA extraction was conducted using the reagents from QIAamp DNeasy Blood and Tissue Kit (Qiagen, Singapore) based on the manufacturer's instructions. Serial dilutions (50–200 ng/ μ l) of mouse liver DNA (200 μ l) were added with 200 μ l of ethanol (96–100%) and 200 μ l of buffer AL in a 1.5-ml tube. The mixture was then transferred to the sample chamber of the cartridge, which was preloaded with the QIAamp's reagents as follows: 500 μ l of wash buffer AW1 in the wash 1 chamber, 500 μ l of wash buffer AW2 in the wash 2 chamber, 500 μ l of ethanol (96–100%) in

the ethanol flush chamber, and 200 μ l of elution buffer in the elution buffer chamber. The top of the cartridge was then sealed with MicroAMP optical adhesive film, and loaded into the desktop unit, which performed the DNA extraction automatically. The total DNA eluted was collected in the product chamber, and measured using the Nano-drop ND-1000 spectrophotometer (Thermo Scientific, Singapore).

Control experiments were performed with the same Qiagen kit according to the manufacturer's protocols. Briefly, the sample mixture as in the cartridge experiment was transferred to the spin column, spun at 8000 rpm for 1 min, washed with 500 μ l of wash buffer AW1 (8000 rpm, 1 min), washed with 500 μ l of wash buffer AW2 (14000 rpm, 3 min), and finally eluted with 200 μ l of AE elution buffer (8000 rpm, 1 min).

2.2.5 RNA extraction from cell and real-time reverse transcription PCR (RRT-PCR)

The RNA extraction of MCF-7 cancer cells was conducted using the reagents from QIAamp Virus RNA Mini Kit (Qiagen, Singapore) based on the manufacturer's instruction. Serial dilutions (10 – 10^4 cells in 70 μ l of phosphate buffered saline (PBS)) of MCF-7 breast cancer cells were added with 280 μ l of cell lysis AVL buffer and 2.8 μ l of carrier RNA (1 μ g/ μ l in AVE buffer) in a 1.5-ml tube. The mixture was incubated at room temperature for 1 min. Next, 280 μ l of ethanol (96–100%) was added to the mixture, which was then transferred to the diethyl pyrocarbonate (DEPC) treated cartridge (sample chamber). The cartridge was preloaded with essentially the same amount of QIAamp's reagents as in the DNA extraction experiment, except that the pre-loaded elution buffer volume was 100 μ l. After automatic RNA extraction, the eluted total RNA was collected in the product chamber for RRT-PCR.

Control experiments were performed with the same Qiagen kit according to the manufacturer's protocol. Briefly, the sample mixture as in the cartridge experiment was transferred to the spin column, spun at 8000 rpm for 1 min, washed with 500 μ l of wash buffer AW1 (8000 rpm, 1 min), washed with 500 μ l of wash buffer AW2 (14000 rpm, 3 min), and eluted with 100 μ l of AVE elution buffer (8000 rpm, 1 min).

Keratin, type I cytoskeletal 19 (CK19) gene from MCF-7 cells was selected for characterization of RNA extraction efficiency. RRT-PCR of CK19 was performed in a Bio-Rad CFX96 system with 20 μ l of reaction mixture, which included 10 μ l of 2 $\times$ RT-PCR Mix (Cat. 4392587, Applied Biosystems, Singapore), 0.5 μ l of 40 $\times$ TaqMan RT Enzyme Mix (Cat. 4392939, Applied Biosystems, Singapore), 1 μ l of 20 $\times$ LC Green Plus (Idaho Technology, USA), 8 μ l of extracted RNA, and 400 nM of CK19 primers (forward primer: ATGAAAGCTGCCTTGGAAGA; reverse primer: TGATTCTGCCGCTCACTATCAG; Sigma Aldrich). The RRT-PCR process included: 48 $^{\circ}$ C (10 min) for the reverse transcription process, followed by 95 $^{\circ}$ C (10 min) and 50 cycles of 95 $^{\circ}$ C (15 s) and 60 $^{\circ}$ C (1 min) for gene amplification and detection.

2.3 Results and discussion

2.3.1 Performance of fluidic transfer

The displacement velocities for the pressure and vacuum pumps were the same. Before the fluidic pumping began, the pressure pump was at its maximum volume position ($\sim$ 25 ml), and the vacuum pump was at its minimum vacuum volume position. Once fluidic transfer was initiated, the respective pinch valves would start their operation. Pressure was applied to the fluidic chamber (e.g. C1 to C2), and vacuum was applied to the waste chamber (C6) or eluent chamber (C7).

The relationship of the pump displacement volume and the fluid volume transported from reagent chambers is illustrated in Figure 2.8. The fluid volumes transported were very similar at a given pump displacement volume for pump displacement velocities of 0.1 ml/min and 0.2 ml/min. Although there was an initial compression volume of $\sim 47.4 \mu\text{l}$ before the fluid would start to move, the volume of fluid transported has a linear relationship with the pump displacement volume. Hence, the fluidic transportation could be easily controlled.

When clinical samples are used, some debris such as blood blocks may be present inside the sample. As such, pre-filtration of the sample using 20–50 μm filter would be required.

Figure 2.8: The fluid volumes transported as a function of the displacement volumes of vacuum and pressure pumps were very similar at pump displacement velocities of ($\diamond$) 0.1 ml/min and ($\square$) 0.2 ml/min. Their averaged linear curve (—) has a slope of 0.907, with $R^2 = 0.998$. Error bar: standard deviation, $n = 4$.

2.3.2 Efficiency of on-cartridge DNA extraction

Nucleic acid assays based on PCR [6, 22, 23] or biosensors [24-26] are relatively robust. However, a variety of contaminants can inhibit amplification and affect gene detection [3, 11]. In order to circumvent this problem, DNA must be extracted and purified from a raw sample through a variety of lysis protocols and purification techniques [4, 27-32]. The most common method is chemical lysis of the raw sample, followed by DNA purification using a solid-phase extraction method.

Rat total DNA was used for the on-cartridge DNA extraction and purification. The sample was first lysed, and the genes were protected in the lysis buffer with high ionic concentration. When transferred to the sample chamber of the cartridge, the lysis buffer containing genes was first passed to the silica membrane chamber. The DNA was adsorbed onto the silica membrane surface, while the unbound impurities passed through the membrane to be stored in the waste chamber. Purification of the bounded DNA was achieved by passing the washing buffer twice and ethanol flush once through the silica membrane. The membrane was then dried by passing air through it. The adsorbed DNA was released into the elution buffer with low ionic concentration using nuclease-free water to dilute the DNA.

To test the efficiency of the on-cartridge DNA extraction, the mouse liver total DNA was selected to mimic the clinical biological sample with co-existing human total DNA and virus DNA. ND-1000 spectrophotometer was used to measure the DNA concentration of the sample loaded in the cartridge and in the elution buffer after the on-cartridge sample preparation. The volume of DNA loaded in the cartridge and the eluent volume were both 200 μ l. Since the measurement limits of the ND-1000

spectrophotometer were 2–1000 ng/ μ l, the input pure sample DNA concentration was selected to be 50–200 ng/ μ l.

The DNA recovery efficiency via the on-cartridge DNA extraction is shown in Figure 2.9. The concentration of recovered DNA has a linear relationship with the concentration of DNA loaded into the cartridge. The recovery efficiency varied from 54% to 63%. Using a manual spin column to process the same DNA with the same Qiagen reagents, an average DNA recovery efficiency of $54 \pm 5\%$ was obtained ($n = 3$, input DNA concentration = 200 ng/ μ l). This demonstrated that our automated cartridge-based process has a comparable or better performance as compared to the conventional spin column process.

When clinical sample is used, very few of the targeted DNA/RNA molecules may be present. As such, a larger raw sample volume may be required.

Figure 2.9: The recovered DNA concentration (●) and DNA recovery efficiency (■) as a function of the input DNA concentration. The volume of DNA sample loaded into the cartridge and the eluent volume were both 200 μ l. The DNA recovery efficiency was ~ 54–63%. Error bar: standard deviation, $n = 3$.

2.3.3 RNA extraction with MCF-7 breast cells

RNA extraction in the sample preparation for RRT-PCR required several steps [29, 33]. Similar to the purification of DNA, the RNA adsorbed onto the silica membrane surface was purified by passing the washing buffer twice and ethanol flush once through the silica membrane. The adsorbed RNA was then released into the elution solution under a low ionic concentration. These manual, labor-intensive processes have been integrated in our on-cartridge RNA extraction. Reagents from Qiagen QIAamp Viral RNA Mini Kit was used to extract $10\text{--}10^4$ MCF-7 breast cancer cells with our cartridge. The Qiagen Kit employed Carrier RNA (2.8 μg) to enhance the RNA extraction [34, 35], which made it difficult to quantify the recovery rate of the target RNA directly by measuring the quantity of eluted RNA using spectrometer, since the excess amount of Carrier RNA would co-elute with the target RNA. We have utilized the RRT-PCR method to tackle this problem.

The RNA from on-cartridge extraction was examined using Taqman One-Step RRT-PCR Kit. The kit combined reverse transcription and cDNA amplification in the same mixture. A commercial thermal cycler (CFX-96, Bio-Rad, Singapore) was employed to amplify the CK19 gene in the cancer cells. The enzyme reverse transcriptase (RT) process was conducted at 48°C for 15 min. The RNA strand was first reverse transcribed into its DNA complement (cDNA). The CK19 gene was amplified under denaturation of the DNA complement at 95°C for 10 s, followed by a combined annealing and extension step at 60°C for 1 min. The total number of amplification thermal cycles was 50; the RRT-PCR curves are shown as red lines in Figure 2.10.

Control experiments were performed with the Qiagen spin column using the same reagents to extract RNA from the same amount of breast cancer cells. Their RRT-PCR curves are shown as black lines in Figure 2.10. To test for any contamination in the RRT-PCR reagents, negative controls with no template RNA and with mouse liver total RNA sample (Cat #7801, Ambion, Invitrogen Singapore) that did not have CK19 gene were run using the same RRT-PCR reagents, and amplified under the same thermal cycling conditions as for the samples. As shown by the blue and pink lines in Figure 2.10, nothing was amplified in these negative controls, indicating that the RRT-PCR amplification was specific.

The cycle threshold (C_T) values for 10 and 10^4 cells were 29.3 and 25.6, respectively, using our cartridge for RNA extraction. The C_T values were 30.3 and 26.6, respectively, when spin column was employed for the RNA extraction. Ideally, for a 10-fold difference in input cell number, the difference in C_T values should be ~ 3.3 . However, we found that the differences in C_T values between 10^4 and 10^3 cells, between 10^3 and 10^2 cells, and between 10^2 and 10 cells were 1.7, 1.7 and 0.3, respectively. When using the spin column, the corresponding differences in C_T values were 0.8, 2.6 and 0.3, respectively. This could be due to two reasons: (i) the input cell number might not be accurate at the low cell concentrations, and (ii) the gene expression of individual breast cancer cells might be different.

Clinical samples are very complex and may contain PCR inhibitors. This may affect the downstream PCR results.

Figure 2.10: The real-time fluorescence curves of total RNA extracted from (*) 10, (×) 10^2 , (■) 10^3 and (◆) 10^4 MCF-7 cells using the cartridge (red lines) and the Qiagen spin column (black lines), reverse transcribed and amplified using Bio-Rad CFX-96. The blue (—) and pink lines (—) were negative controls that have been run using water and rat total RNA with no CK19 gene, respectively.

2.4 Conclusion

In conclusion, we have successfully developed a novel self-contained cartridge and a desktop unit for automated DNA/RNA purification from raw sample within 6 min. The cartridge used a combination of compressed air and vacuum forces for fluidic transfer. The on-cartridge DNA recovery efficiency was 54–63%, which was comparable to or better than the conventional manual approach using silica spin column. In addition, the on-cartridge RNA extraction was successfully demonstrated using 10 – 10^4 MCF-7 breast cancer cells as samples. RRT-PCR of CK19 cancer-specific gene was achieved with as little as 10 MCF-7 cells using lab-based PCR machine. This cartridge system could also be integrated with components such as real-time PCR and DNA microarray to provide a point-of-care device for rapid disease diagnosis.

2.5 References

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Chapter 3

Development of Self-Contained All-in-One Diagnostic Cartridge for Influenza Detection

3.1 Introduction

Nucleic acid-based molecular diagnosis involves three major steps: (i) DNA/RNA sample preparation, (ii) nucleic acid amplification by PCR, and (iii) detection of amplified DNA. The microfluidics-based cartridge system developed in Chapter 2 has effectively met the need for DNA/RNA sample preparation [1]. The goal of this chapter is to develop a DNA/RNA amplification/detection device that can be conveniently integrated with the sample preparation system for molecular diagnosis applications.

With its simplicity and effectiveness, real-time PCR remains the most popular and robust method for pathogen diagnosis [2], although detections with DNA microchips [3-5], a label-free approach [6] and electrophoretic analysis [7] have also been reported. A number of miniaturized disease diagnostic devices have been developed [8-11]. Most of them are focused on either sample preparation [12-16] for the pathogen, DNA/RNA purification or on-chip PCR amplification with built-in microvalves, heaters and sensors [6, 7, 17-22]. Despite impressive advances [5, 19, 23-28], the integration of sample purification and molecular detection remains a major challenge for portable disease diagnostic devices [7, 29]. The lack of multiplexing capability has also limited the applicability of these devices towards detecting viruses, such as influenza and enterovirus including the specific hand, foot and mouth disease-causing enterovirus (EV71) [30, 31], which contain various serotypes with similar patient symptoms. In addition, the typical

open device design for external introduction of reagents and release of processed waste is prone to hardware cross contamination and accidental virus exposure.

Several issues need to be addressed in order to integrate all necessary steps of PCR-based molecular diagnosis onto a chip or cartridge platform in a user-friendly way suitable for non-specialized operators. These include fluid regulation, reagent supply, hardware cross contamination, cost of device fabrication, applicability of device, and the different storage conditions of various reagents for cell and pathogen lysis, RNA/DNA extraction, and enzymes for RNA reverse transcription and DNA amplification. To meet the need for a truly low-cost automated platform, disposable components should be manufactured using an inexpensive fabrication method, with minimal on-chip active components and device assembly steps. In addition, it would be desirable that the cartridge is self-contained and ready-to-use with all the necessary reagents, can process the waste, and is flexible for various disease diagnoses with a plugged-in RT-PCR assay.

To our knowledge, only a few examples of fully integrated microfluidic devices for nucleic acid detection with self-contained reagents have been published to date [5], which, however, require a complicated manufacturing process. Most of the integrated PCR-based microfluidic systems still require a substantial amount of operator handling of the precision syringe pump to supply reagents to the system in order to run the assay [7, 10, 12, 19, 23-28]. Such situations are prone to hardware cross contamination. Moreover, the device and reagent storage issues are often not addressed in the published papers.

In this chapter, we integrated pure DNA/RNA aliquot, thermal cycling and 1-color, 3-chamber pseudo-multiplexing RRT-PCR to the automatic sample preparation cartridge developed in Chapter 2 for an all-in-one system as shown in Figure 3.1 [32]. The performance of the system was then tested for rapid influenza diagnosis with WHO

recommended protocols. The outbreak of infectious influenza A, H1N1-2009, posed a severe threat to global public health. H1N1-2009 was highly contagious. Thirty-nine countries reported 8480 cases of H1N1- 2009 infection and 72 deaths officially to the World Health Organization (WHO) in less than a month after the first reported case surfaced on April 23, 2009 [33]. The conventional method for influenza diagnosis requires several manual processes, including the lysis of virus particles, viral RNA extraction and molecular diagnosis, which are often conducted within the confines of centralized laboratories [2]. The entire protocol takes 5–6 h, and requires skilled operators who are subjected to the risk of accidental virus exposure and disease contraction. Although the fatality rate of H1N1-2009 is lower than initially expected [34], the global fear of its spread demonstrates the need for a fully automated system to rapidly identify infected patients. Such a system should allow for diagnosis in decentralized locations such as airports, train stations and immigration check points to contain the spread of highly contagious diseases, and to alleviate the burden on healthcare personnel in the diagnosis of an overwhelming number of suspect cases.

In our all-in-one system, all necessary chemicals (including the process waste generated) were self-contained and completely sealed within a polymer cartridge, minimizing the possibility of human contamination and accidental viral exposure. The operator would only need to load the patient's nasopharyngeal swab sample with lysis buffer into the cartridge, and the system would automatically perform the entire sample preparation and molecular diagnosis within 2.5 h. The cartridge design provided for precise dispensing of aliquots for multi-chamber PCR detection. The performance of the integrated RNA extraction, custom-built thermal cycler and optical detection system compared favorably with that of manual RNA purification and commercial real-time

thermal cycler. The multi-chamber capability and sensitivity of our all-in-one system has been demonstrated with clinical seasonal influenza H1N1 samples, and compared with control experiments performed using WHO-recommended protocols and reagents.

Figure 3.1: Real-time PCR system with integrated sample preparation and 3-channel fluorescent detection on an all-in-one cartridge. This system automatically performs DNA/RNA extraction from a raw sample, reagents metering and aliquot, and real-time reverse transcription PCR for disease diagnosis.

3.2 Materials and methods

3.2.1 Fluidic pumping and regulation

Similar to our sample preparation cartridge (Section 2.2.1), this all-in-one cartridge is fabricated by computer numeric control (CNC) machining using PMMA. It has a dimension of 33.7 mm × 34.1 mm × 69.1 mm. The removal of oil and contaminants of the cartridge is as detailed in Section 2.2.1.

Two in-house fabricated syringe pumps with a volume of 25 mL were used to generate the pressure and vacuum forces. These syringe pumps were driven by a

linear actuator (E43H4N-12, Haydon) and a step motor driver (DCS 4010, Haydon) with a maximum flow rate of 12 ml/min, controlled by LabView (National Instruments). The pressure and vacuum forces (connected to inlets p1–p6 and v1–v6 respectively, in Figure 3.2(b)) were regulated by separate pinch valve manifold (P/N 075P2NC12-23S, Bio-Chem Fluidics, UK), powered by a 15-V power source (S-35-15, MeanWell). LabView (National Instruments) was used to control these pinch valves. The pneumatic sources were connected to the cartridge via pneumatic connectors (M-3AU, SMC, Japan) and o-rings, which pierced through the bottom sealing film upon cartridge loading.

(a)

(b)

Figure 3.2: (a) SolidWorks drawing of the all-in-one cartridge with chambers for DNA/RNA extraction, reagent aliquot and real-time PCR. The reagents for DNA/RNA extraction could be pre-loaded into the cartridge, and sealed by adhesive films. The PCR mixtures were frozen and stored in the standard 0.2-ml PCR tubes and inserted into the cartridges. (b) The top and bottom views of the all-in-one cartridge with pressure (p1–p6) and vacuum (v1–v6) inlets. Reagents pumping was achieved by using air pressure and vacuum forces generated by two syringes pumps in a push-pull method. The red and blue arrows indicate the flow directions of air (pressure or vacuum) and reagents, respectively.

3.2.2 Sample preparation

The RNA extraction was demonstrated using the reagents from QIAamp Viral RNA Mini Kit (Qiagen, Germany) based on the manufacturer's instructions. Serially diluted ($1-10^5$ folds or 1000–0.01 ng/ μ l) mouse total liver RNA (10 μ l) was added with 280 μ l of AVL buffer, 2.8 μ l of carrier RNA (1 μ g/ μ l in AVE buffer) and 160 μ l of nuclease-free water (AM9938, Applied Biosystems, Singapore) in a 1.5-ml tube. The mixture was incubated at room temperature for 10 min, followed by adding 280 μ l of ethanol (96–100%). The sample mixture was transferred to the diethyl pyrocarbonate (DEPC)-treated cartridge (sample chamber), which was preloaded with QIAamp's reagents, including 500 μ l of prepared wash buffer AW1 (in Wash 1 buffer chamber), 500 μ l of wash buffer AW2 (in Wash 2 buffer chamber), 200 μ l of elution buffer (in Eluent buffer chamber) and 500 μ l (96–100%) of ethanol (in Ethanol flush chamber). The cartridge (top layer) was re-sealed with MicroAMP optical adhesive film, and loaded into the fluidic pumping unit, which automatically performed the DNA/RNA extraction.

Control experiments were performed with QIAamp Mini Spin Column, according to the manufacturer's protocol. Briefly, the prepared sample mixture (same mixture as in the cartridge experiment) was transferred to the spin column and spun at 8000 rpm for 1 min, washed with 500 μ l of wash buffer AW1 (8000 rpm, 1 min) and 500 μ l of wash buffer AW2 (14000 rpm, 3 min), and eluted with 200 μ l of AVE elution buffer (8000 rpm, 1 min). A second control experiment with un-treated mouse liver total RNA was also conducted with adjusted RNA concentration according to the elution buffer volume (200 μ l). Briefly, 10 μ l of serially diluted ($1-10^4$ folds) liver total RNA was added with 190 μ l of Nuclease-free water. Finally, the RNA extraction

efficiency was characterized by reverse transcription PCR (RT-PCR).

3.2.3 Principle of PCR and real-time PCR

PCR is a technique for amplifying DNA molecules. The technique has become the cornerstone of modern molecular biology. During this process, the polymerase enzyme serves as a catalyst to drive the PCR reaction, and the primer synthesizes a complementary sequence of bases to any single-strand of DNA, providing that it has a double-stranded starting point. This primer is very useful because we can choose the gene we want to amplify in a mixed DNA sample by adding small pieces of DNA complimentary to the gene of interest.

Two or three temperature steps are usually used to control the activity of the polymerase and the binding of primers during a PCR process:

Step 1: To start the PCR reaction, the temperature is raised to 95°C. At this temperature, all double stranded DNA is dissociated to single strands.

Step 2: The temperature is decreased to ~ 50°C to allow the primers to bind to the gene of interest. Thus, the polymerase can start copying the DNA strand.

Step 3: The optimal temperature for the polymerase to function is 72°C. Therefore, the temperature is raised to 72°C to allow the enzyme to work with optimal activity. Currently, most PCR reagents available in the market use only Step 1 and Step 2 for the PCR amplification.

After the PCR amplification, millions of copies of the target gene are obtained. Conventionally, these target genes are detected by agarose gel electrophoresis of PCR products. A dye such as GelRedTM can be used to stain the DNA molecules to make it visible. However, agarose gel electrophoresis for target gene detection is usually time-consuming and unable to provide quantitative results.

Real-Time PCR is identical to normal PCR except that the amount of DNA molecules amplified during the reaction is monitored by a fluorescence intensity detector such as a CCD camera or photomultiplier tube detector in “real time”. Real-time PCR is advantageous over normal PCR because the efficiency and rate of the reaction can be observed during the PCR process. Also, there is no need to run the PCR product on a gel after the reaction.

In real-time PCR, the fluorescent marker that binds to the amplified target DNA is used to monitor the PCR process. The fluorescence intensity increases with increasing number of gene copies. The fluorescent marker can be a fluorescent dye or fluorescent probe.

Fluorescent dyes are the simplest and cheapest way to monitor PCR process in real time. These dyes can be bound to double-stranded DNA molecules. During the PCR reaction, when the number of target DNA copies increases, more dyes will be bound to the DNA molecules, increasing the fluorescence intensity. The most commonly used fluorescent dye for real-time PCR is SYBR Green, which is an asymmetrical cyanine dye. The DNA-SYBR Green dye complex absorbs blue light ($\lambda_{\text{max}} = 497 \text{ nm}$) and emits green light ($\lambda_{\text{max}} = 520 \text{ nm}$). The main disadvantage of using a fluorescent dye for real-time PCR detection is the lack of specificity, because the dye will report any DNA that is amplified, rather than the target gene alone. This problem can be solved by using the following fluorescent probe method.

Fluorescent probes are pieces of DNA complimentary to the target gene of interest that are labeled with a fluorescent dye. The most commonly used probe is the Taqman probe. It is labeled with a fluorescent reporter molecule at the 5'-end of the oligonucleotide probe, and a quencher at the 3'-end. Since the quencher quenches the

fluorescence of the reporter under normal circumstances, the fluorescence emission from the probe is low. During the PCR process, the probe binds to the gene of interest and becomes cleaved by the polymerase. When the copy number of target DNA increases, more reporters and quenchers are physically separated, resulting in the increase of fluorescence intensity from the sample.

Molecular beacon is another commonly used probe. It is also a small piece of DNA complimentary to the target gene of interest, with a fluorescent reporter and a quencher molecule labeled on opposite ends of the probe. The probes are designed to fold onto themselves to bring the reporter and the quencher to closer proximity. Hence, under normal condition, fluorescent emission is low. When the probe binds to the target gene, it adopts a linear form, and the reporter and the quencher are separated to release the fluorescence. With an increasing copy number of target DNA molecules, more probes will change to the linear form, and a higher fluorescence intensity will be detected.

3.2.4 Characterization of on-cartridge RNA extraction and real-time PCR

In this project, mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected for the characterization of the in-house developed thermal cycler and real-time PCR detection system. Mouse liver total RNA (1000 ng, Cat. #7810, Ambion, Invitrogen Singapore) was reverse transcribed using Taqman Reverse Transcription kit (Cat. #N8080234, Applied Biosystems, Singapore) performed in a Bio-Rad CFX-96 instrument with 100 μ l of reaction mixture according to the manufacturer's protocol. The randomly reverse transcribed cDNA mixture was serially diluted 1–10⁴ folds with nuclease-free water (Cat. #AM9939, Applied Biosystems, Singapore), and the serially diluted GAPDH cDNA (2 μ l in 20 μ l of

mixture) was amplified using Taqman Fast Universal PCR master mix (Cat. #4352042, Applied Biosystems, Singapore) and Taqman Assays-by-Design containing primers and probe encoding for GAPDH (Cat. # Mm99999915_g1, Applied Biosystems, Singapore) according to the manufacturer's instructions, using the custom-made thermal cycler. Briefly, 20 µl of PCR mixture was covered with 15 µl of liquid wax (Chill-outTM Liquid Wax, Bio-Rad, Singapore), and heated to 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 60 s (combined annealing and extension). Fluorescence arising from the DNA replication was recorded versus cycle number.

Mouse liver total RNA was selected for the characterization of RNA extraction efficiency of the all-in-cartridge system. The reverse transcription PCR (RT-PCR) was performed with Taqman RNA-to-C_T 1-step kit (Cat. # 4392938, Applied Biosystems, Singapore) in a Bio-Rad CFX-96 instrument with 20 µl of reaction mixture, including 0.5 µl of TaqMan RT Enzyme Mix, 10 µl of TaqMan RT-PCR Mix, 1 µl of Taqman Assays-by-Design (Cat. # Mm99999915_g1, Applied Biosystems, Singapore), and 8.5 µl of serial diluted purified or unpurified mouse liver total RNA (7810, Ambion). The real-time RT-PCR (RRT-PCR) was performed with 48°C for 15 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

3.2.5 Seasonal influenza screening and sub-typing

Patients' nasopharyngeal swab samples in viral transport media (UTM-RT 330C-3ml, Copan, USA) were provided by the Molecular Diagnosis Centre of National University Hospital, collected for the 2009-H1N1 screening activity with the Institutional Review Board (IRB) approval. Nasal samples were serially diluted 1–10⁴

folds with viral transport media (AM9939, COPAN, USA), and influenza viral RNA (200 μ l) was extracted with QIAamp Virus RNA Mini Kit (Qiagen, Germany) using either all-in-one cartridge or QIAgen spin column with the protocol described in Section 3.2.2. The RRT-PCR was performed with the influenza A virus matrix gene-specific primers and probe for influenza A typing, and H1-specific primers and probes for seasonal H1N1 sub-typing (Table 3.1). All probes were labeled at the 5' end and 3' end with the 6-carboxyfluorescein (FAM) reporter dye and 6-carboxytetramethylrhodamine (TAMRA) quencher dye, respectively. The RRT-PCR assays were performed using a Qiagen QuantiTect RT Probe Kit (one-step RT-PCR) with 40 μ l of reaction mixture, including 0.4 μ l of QuantiTech RT Mix, 20 μ l of QuantiTect Probe RT-PCR Master Mix, 20 pmol of each primer, 10 pmol of probe, and 10 μ l of extracted RNA mixture. The RRT-PCR was performed with 95°C for 2 min, followed by 50 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 30 s, and the final extension at 72°C for 10 min either in the custom-made thermal cycler (with the all-in-one cartridge) or Bio-Rad CFX96 thermal cycler (control).

For the all-in-one cartridge system detection, 30 μ l of the RRT-PCR mixture without the target RNA was preloaded in standard 0.2-ml tubes and covered with 15 μ l of liquid wax. As illustrated in Fig. 2(a), these preloaded PCR tubes were fixed onto the all-in-one cartridges prior to sample extraction and detection. After sample extraction, extracted viral RNA (10 μ l) was automatically aliquoted into each PCR tube. Thermal cycling and detection were subsequently performed by the system's real-time PCR hardware.

Table 3.1: PCR primer and hydrolysis probe sequence.

Primer/probe	Sequence (5'-3')	Region (bp)
<i>Influenza A</i>		
Forward	AGATGAGTCTTCTAACCGAGGTCG	25-48
Reverse	TGCAAAAACATCTTCAAGTCTCTG	101-124
Probe	(FAM)-TCAGGCCCCCTCAAAGCCGA-(TAMRA)	64-83
<i>Seasonal H1N1</i>		
Forward	AACATGTTACCCAGGGCATTTCGC	247-270
Reverse	GTGGTTGGGCCATGAGCTTTCTTT	338-361
Probe	(FAM)-GAGGAACTGAGGGAGCAATTGAGTTCAG-(TAMRA)	278-305

3.2.6 Real-time PCR thermal cycling and optical signal detection

The real-time PCR was performed by using an in-house fabricated thermal cycler, which included a fan, a thermoelectric (TE) heater/cooler (9501/127/030, FerroTec) and a thermoelectric control kit (FerroTec, USA) consisting of FTA600 H-bridge amplifier, and FTC100 temperature controller (see Figure 3.1). The thermoelectric heater was powered by the FTA600 amplifier, which was controlled by the FTC100 temperature controller. A T-type thermocouple (5TC-TT-T-40-36, OMEGA Engineering, USA) was mounted on the TE heater to measure the temperature, and used as a feedback to the FTC100 temperature controller. The temperature difference between the thermoelectric heater and actual temperature inside the PCR chamber was calibrated by measuring the temperature inside the PCR chamber directly with the same volume of PCR reagents and paraffin oil.

The optical detection system (Figure 3.1) comprises three blue LEDs ($\lambda_p = 470$ nm, $\Delta\lambda = 25$ nm, LLB52050, Dotlight, RS Singapore), a photo-multiplier tube (PMT) (H5784-20, Hamamatsu, Japan), a collimating lens (AC254-040-A1, Thorlabs, USA) and a filter set (ex: BG-12, Edmund; em: HQ535/50m, Chroma, USA), targeted for

FAM and SYBR Green I fluorescent dyes. Fluorescent measurement was performed at the end of each extension cycle (usually at 72 °C) by sequentially lighting each LED for 200 ms, as controlled by a power supply (NI9265, National Instrument, USA). Fluorescence from the excited fluorescent probes in the PCR tubes was collected and collimated to the PMT where the acquired signal was averaged 50 times (within 200 ms) by a data acquisition card with a sample rate of 1 kHz (NI9206, National Instrument, USA). The LEDs were tilted at 45° related to the PCR tubes, so as to minimize the transmitted stray light to the PMT detector.

3.3 Results and discussion

3.3.1 Device operation

Prior to operation, the all-in-one cartridge was pre-loaded with RNA extraction reagents (QIAamp Viral RNA Mini Kit, recommended by WHO for influenza virus RNA extraction [35]) in the respective chambers, and the top and bottom surfaces of the cartridge were sealed with MicroAMP adhesive tape. Pre-loaded PCR tubes were also fixed onto the cartridges (Figure 3.2) as previously described. The operator only needed to introduce the biological sample into the designated sample chamber via a syringe needle. The pierced film was then resealed with an adhesive tape. When the cartridge was loaded into the system, the pneumatic connectors of the system's hardware would pierce the cartridge's bottom film, connecting the pressure and vacuum inlets (Figure 3.2(b)) of the cartridge to the external pneumatic system.

In our approach, the manipulation of fluidics was achieved by using a combination of compressed air (push) and vacuum (pull) forces, generated by two

custom-made syringe pumps, and two pinch-valve manifolds that direct the pneumatic forces to the appropriate chambers within the cartridge. These syringe pumps and pinch-valve manifolds made up the main external pneumatic system that controlled the fluidic motion within the chambers of disposable self-contained cartridge (Figure 3.3.). This highlighted the novelty of our approach as internal pumps or fluidic valves did not need to be incorporated into the cartridges. This significantly reduced the complexity of assembling the cartridges and would drive its manufacturing cost down. The cartridges (Figure 3.2) were designed to be compatible with cost-effective scale-up manufacturing methods such as injection molding.

The cartridges were designed with two separate networks of pneumatic and fluidic channels. During the entire operation, the pre-loaded reagents and biological samples flowing through the fluidic channels was not exposed to the pneumatic channels that were linked to the system's external hardware, eliminating the potential run-to-run cross contamination. Each chamber was designed with one pneumatic inlet channel on its upper surface (Figure 3.2(b)), which either supplies a positive pressure in a form of compressed air from the first syringe pump or a vacuum force generated by the second syringe pump. Separately, each chamber has a fluidic outlet at the bottom (Figure 3.2). These fluidic outlets were connected to the designated chamber's inlet via channels that ran through the cartridge body. These through-cartridge channels connected the bottom network of fluidic outlets to the top inlet channels of the cartridge chambers as illustrated in Figure 3.2.

To move liquid from one chamber to another, a positive pressure was applied to the source chamber, while a vacuum was generated within the target chamber. The resultant pressure gradient caused the reagents to drain from the bottom of source

flow up through the cartridge channels and into the top fluidic inlet of the target chamber. Each chamber's pneumatic channel is independently manipulated by the external pinch valves controlled by the system's LabView software. Pinch valves opened to connect the chambers to the hardware's pneumatic system, while closed pinch valves isolated the chambers from the external pneumatic manipulation. This allowed the fluidic movement to be independently directed to the relevant chambers, isolating the neighboring chambers from any pneumatic interference.

The overall device operation of the all-in-one cartridge is illustrated in Figure 3.3 with the status of each pinch valve indicated. Lysed biological sample was first loaded into the Sample Chamber (C1) by a needle syringe, and resealed with an adhesive tape. The biological sample containing target RNAs was then filtered through the silica membrane, with the RNAs captured by the membrane and remaining filtrate directed to the Waster Chamber (C5). The impurities (trapped within the silica membrane) were washed out sequentially by Wash Buffer 1 (C2), Wash Buffer 2 (C3), and Ethanol Flush (C4) (flow rate: 1 ml/min), and extensively flushed with air (flow rate: 10 ml/min; 2 min) to remove the remaining wash buffer residue. The purified RNA was then released from the silica membrane, directed to the Eluent Chamber (C7) with the Elution Buffer (C5), and dispensed to the three aliquot reservoirs sequentially (flow rate: 0.1 ml/min), while the excess elution mixture was delivered to the Excess Eluent Chamber (C8). Subsequently, the purified RNA remaining within the connection channel was air-flushed to the Excess Eluent Chamber (C8) (flow rate: 5.62 ml/min), while the RNA aliquots were held in position with the help of surface tension of the aliquot reservoirs. The purified RNA was then dispensed as aliquots to the corresponding PCR tubes containing RT-PCR pre-

mixture. The dense RNA sample would drop through the surface layer of liquid wax directly into the RT-PCR mixture. The liquid wax would remain on the surface of the RT-PCR mixture during thermal cycling due to its much lower density and prevent any surface evaporation during the entire real-time PCR detection. Wax has been successfully used as a vapor barrier for PCR reactions. The hardware's fluorescence excitation and detection took place at the bottom of the PCR tube. Thus, the surface meniscus of the liquid wax would not cause any interference to the hardware's acquisition of fluorescence signal.

Figure 3.3: Schematic diagrams of device operations. (a) Biological sample (C1) was loaded into silica membrane chamber (X1), lysed and sequentially washed with Wash 1 buffer (C2), Wash 2 buffer (C3) and ethanol (C4). (b) Purified DNA/RNA eluted with elution buffer (C5) into eluent chamber (C7). (c) Purified DNA/RNA was dispensed as aliquots with fluidic meters integrated with passive valves (M1–M3). (d) Excess DNA/RNA was flushed into the excess eluent chamber (C8). (e) Extracted RNA was dispensed into PCR tubes containing RT-PCR pre-mixture (T1–T3). (f) RT-PCR. Liquid wax remained on the surface of the RT-PCR mixture and prevented evaporation during thermal cycling. The status of each pinch valve was illustrated in the figure (×: close; ↑: open).

3.3.2 Dispensing of reagents aliquots

Disease diagnosis or screening would require multiple PCRs with aliquots of extracted RNA for disease typing and sub-typing, and establishing the positive control (confirming the PCR enzyme mixture) [31, 36]. In our approach, we utilized the concepts of aliquot-metered reservoirs and surface tension valves (Figure 3.5(a)) adopted from lateral microfluidic structures [37, 38] to distribute extracted RNA samples into each PCR vial. Extracted RNA was sequentially filled to the constriction of the passive valve of aliquot reservoir, and the fluid remaining in the connection channel was air-flushed. The volume of the extracted RNA aliquots was effectively defined by the physical dimensions of the aliquot reservoirs. The standard deviation in volume for 16 repeated aliquots per reservoir was $0.9\ \mu\text{l}$ for an average reservoir volume of $\sim 10\ \mu\text{l}$ (measured with water) (Figure 3.5(b)). The variations were associated with the cartridge fabrication (CNC milling) and the fluid shear-off on the bottom of meters during connection channel air flush, which could be minimized by reducing the dimension of connection channel and injection molded cartridges.

Figure 3.4: Design of reagents metering and aliquot dispensing using passive valves. The red and blue arrows indicated the flow directions of air (pressure or vacuum) and reagents, respectively.

(a)

(b)

Figure 3.5: (a) Time sequence photographs of aliquot dispensing of RNA eluent, demonstrated with blue food dye. (1) The eluent passed through the silica membrane, and (2) began to fill up the eluent chamber. (3–5) As the eluent chamber was filled, passive valves were sequentially filled up to the constriction. (6) Excess eluent was directed to the excess eluent chamber. Note that the fluid within each aliquot reservoir was isolated. (7–9) Precise dispensing of eluent aliquots into the respective PCR tubes 1–3. (b) Accuracy of fluid aliquots across the three aliquot reservoirs with a target volume of 10 μl . $\diamond$: average volume of 16 repeated measurements with water. Error bar: 3 standard deviations.

3.3.3 RNA extraction

RNA extraction from biological sample for RT-PCR requires several steps [13, 39]. RNA is first adsorbed onto the silica surface under high ionic strength conditions. The unbound impurities are washed away, and then the adsorbed RNA is released into solution with a higher pH. These manual, tedious and labor-intensive processes were integrated into an on-cartridge RNA extraction process, as demonstrated using Qiagen Viral RNA Mini Kit to extract serially diluted mouse liver total RNA (0.1–1000 ng/ μ l) (Chapter 2). The Qiagen Kit uses Carrier RNA (2.8 μ g) [40, 41] to enhance the RNA extraction, which made it difficult to measure the recovery rate of the target RNA directly by measuring the quantity of eluted RNA since the excess amount of Carrier RNA would co-elute with the target RNA. In addition, the residual lysis and wash buffer from the RNA extraction process could also potentially inhibit the RT-PCR. Therefore, the on-cartridge extraction using the RT-PCR was examined. One-step RRT-PCR (whereby reverse transcription and cDNA amplification were combined in the same mixture) was conducted to amplify the mouse GAPDH gene using a commercial thermal cycler (Bio-Rad CFX-96). Control experiments were performed with either the Qiagen spin column or the original untreated sample. The mouse liver total RNA was chosen to mimic clinical biological sample with co-existing human total RNA and virus RNA. The on-cartridge extraction of liver total RNA gave a linear recovery curve and showed an optimal RT-PCR efficiency, indicating that RNA was quantitatively reisolated and of high purity (Figure 3.6, insert). In comparison with the Qiagen spin column, no significant difference was found with respect to their cycle thresholds (C_T) (Table 3.2) and amplification efficiencies (Figure 3.6 insert; on-cartridge: 87%, spin column: 97%) in the subsequent mouse GAPDH RRT-PCR. The variance was most likely due to the inherently

degenerated efficiency of one-step RT-PCR, as indicated by the low efficiency of the original untreated sample (94%).

Figure 3.6: The real-time fluorescence curves of serial diluted ($1-10^4$ folds or $1000-0.1$ ng/ μ l) total liver RNA: extracted using (—) the all-in-one cartridge and (—) the Qiagen spin column, and reverse transcribed amplified using Bio-Rad CFX-96 ($\square = 1000$ ng/ μ l, $\Delta = 100$ ng/ μ l, $\times = 10$ ng/ μ l, $\diamond = 1$ ng/ μ l; $\bullet = 0.1$ ng/ μ l). The inset showed the C_T values of the fluorescence curves. The solid lines were the linear regression fits for (—) the all-in-one cartridge (slope = -3.68 , $E = 87\%$, $R^2 = 0.990$), (—) the Qiagen spin column (slope = -3.40 , $E = 97\%$, $R^2 = 0.972$), and (—) unpurified sample (slope = -3.48 , $E = 94\%$, $R^2 = 0.994$), where $E = 10^{(-1/\text{slope})} - 1$ was the RT-PCR efficiency. The abnormal fluorescence signals in the initial cycles (≤ 10) were due to trapped bubbles.

Table 3.2 C_T values of real-time RT-PCR of RNA extracted by all-in-one cartridge vs. Qiagen spin column, and the original untreated sample.

Loaded Total Liver RNA (ng/ μ l)	Untreated Liver RNA (C_T)	Qiagen Spin Column (C_T)	All-in-One Cartridge (C_T)
1000	18.4	19.0	19.5
100	20.9	23.2	22.5
10	25.3	27.2	26
1	28.7	29	30.6
0.1	31.9	— [†]	— [†]

[†] RT-PCR failed.

3.3.4 Real-time PCR thermal cycling

A thermoelectric module with heat sinks and fan was utilized for thermal cycling. Figure 3.7(a) illustrates the temperature profiles of the thermal cycler obtained from a feedback temperature sensor. Temperatures at the heater surface and within the PCR chamber were measured and calibrated. The heating and cooling rates estimated from Figure 3.7(a) were 2.5°C/s and 2.2°C/s, respectively, which were comparable with those of commercial thermal cyclers. The overshoot was less than 1°C for each temperature setting, and thermal stability was maintained within $\pm 0.1^\circ\text{C}$. These temperature stability and precision fulfilled the requirement of PCR molecular diagnosis [42].

The all-in-one cartridge contained three 0.2-ml PCR tubes intended for disease typing, sub-typing and positive control, respectively, which were subjected simultaneously to the same PCR cycling conditions. Figure 3.8 shows the on-cartridge real-time fluorescent curves and C_T values of serially diluted ($1-10^4$ folds) mouse GAPDH cDNA. The PCR efficiency covering a dynamic range of 3 orders of magnitude was close to 100% (Figure 3.8), whereby the R^2 correlation coefficient of 0.999 indicated the linearity of the linear regression fit. The PCR efficiency decreased to 81% for 10^4 -fold dilution. We hypothesized that the PCR efficiency in our system was affected by the stray light of LEDs. The fluorescent signals were homogeneous across the three PCR tubes (Figure 3.7(b)).

Figure 3.7: (a) Thermal cycling profiles of the custom-built PCR thermal cycler (solid line: set temperature; dash line: measured temperature). The heating and cooling rates estimated from this figure were 2.5°C/s and 2.2°C/s, respectively. (b) Real-time PCR curves of the 3 PCR tubes ($\circ$: left tube, $\diamond$: central tube, $\square$: right tube), conducted with 10-fold diluted GAPDH cDNA mixture. The normalized fluorescent intensities were highly consistent across the 3 PCR tubes.

Figure 3.8: The real-time fluorescent curves of serial diluted ($1-10^6$ folds) GAPDH cDNA, amplified and measured with the custom-built thermal cycler and detection system ($\times$), MJ Research Opticon ($\diamond$), and Bio-Rad CFX96 (Δ). The solid lines were the linear regression fits obtained for the all-in-one cartridge (blue; slope = -3.89; $E = 81\%$; $R^2 = 0.999$), MJ Research Opticon (red; slope = -3.84; $E = 82\%$; $R^2 = 0.998$) and Bio-Rad CFX96 (black; slope = -3.71; $E = 86\%$; $R^2 = 0.994$), where $E = 10^{(-1/\text{slope})} - 1$ was the RT-PCR efficiency.

3.3.5 Rapid flu diagnosis and sub-typing

Influenza virus typing and sub-typing verification are important RT-PCR experiments needed to identify and diagnose the virus contracted by the patient. In particular, influenza type A with a H1 sub-typing is the required protocol to identify infected H1N1 patients. As previously described, our all-in-one system is able to distribute extracted RNA samples evenly into 3 PCR tubes in a single pass. To demonstrate this important multiplexing capability, influenza A primers were premixed in PCR tube 1, H1 sub-type primers were introduced to PCR tube 2, and a positive control mixture was preloaded in PCR tube 3. The positive control PCR mixture consisted of influenza A primers and Spin Column extracted influenza A RNA, previously verified by Bio-Rad CFX96 system. Its role is to verify the functionality of the real-time PCR's hardware and the presence of possible contaminants in the event of a negative result for type A and H1 sub-typing test. Patients' nasopharyngeal swab samples, infected with influenza A H1N1 (human) were introduced to a preloaded cartridge. The extraction and detection were done entirely within the all-in-one system and completed within 2.5 h. As shown in Figure 3.9, the system identified the sample to be positive for influenza type A ($C_T = 24.23$) with a H1 sub-type ($C_T = 27.45$). The higher C_T value for the H1 sub-typing was due to the different set of primers and probe used for the typing and sub-typing RT-PCR reaction.

Figure 3.9: The real-time RT-PCR fluorescence curves of seasonal influenza (human) H1N1 virus detected by all-in-one cartridge system with sub-typing classifications. (●) type A ($C_T = 24.23$), (■) sub-type H1 ($C_T = 27.45$), and (□) positive control* ($C_T = 24.38$). *Identical influenza H1N1 patient's sample extracted by Qiagen Spin Column protocol and previously verified by Bio-Rad CFX 96 system. C_T values were obtained at a normalized threshold value of 0.2.

3.3.6 Detection limit and benchmarking

To push the detection limit of the all-in-one system, influenza A positive nasal samples were serially diluted $1-10^4$ folds with viral transport media and tested with the system. The performance of the all-in-one system was benchmarked against the conventional Qiagen Spin Column manual RNA extraction method and RRT-PCR detection with the Bio-Rad CFX96 system. As a control to evaluate the system's RNA extraction performance, excess extracted RNA samples from the cartridge's Excess Eluent Chamber were removed and detected with the Bio-Rad CFX96 system. As shown in Table 3.3, the all-in-one system was able to detect influenza A with 10^3 folds of dilution. However, the conventional benchmark Spin Column method and the cartridge-extracted samples detected by Bio-Rad CFX 96 were able to detect as low as 10^4 folds of dilution. The PCR efficiency achieved with the Bio-Rad system over the dilution range was close to 100% (Figure 3.10). In contrast, the amplification efficiency for the all-in-one system dropped to 90% (Figure 3.10). Earlier, the all-in-

one system's real-time PCR hardware benchmark tested against MJ Research Opticon and Bio-Rad CFX96 commercial systems showed no significant difference (Figure 3.7). Thus, the PCR efficiency decrease from 99% to 90% and the reduced sensitivity for the all-in-on system were not likely to be due to the system's extraction or real-time PCR hardware. Instead, this difference could be attributed to the thorough mixing of RT-PCR reagents and RNA sample achieved by manual pipetting methods, versus the automated RNA sample dispensing in the all-in-one system that purely relied on the diffusion of RNA sample to mix with the preloaded reagents in the PCR vials. While diffusion-based mixing was sufficient for the detection of concentrated samples (1 to 10^3 dilutions), it caused the system to fail with the more diluted sample (10^4 dilutions). Further development and improvements such as the incorporation of a magnetic-initiated mixing to the system would address this issue. Nonetheless, the all-in-one system's detection ability after 10^3 folds of dilution demonstrated its potential as a self-contained diagnostic system for influenza. The mean viral load in the nasal samples of patients with seasonal influenza A was previously reported to be 3.28×10^8 copies/ml [35]. Hence, we could estimate that the all-in-one system was able to detect samples with viral loads of $\sim 10^5$ copies/ml, which was well below the reported mean viral load (1.84×10^8 copies/ml) of the 2009 pandemic H1N1 influenza patient samples.

Table 3.3: C_T values of the real-time fluorescent curves of serial diluted ($1-10^4$ folds) influenza A patient samples. The virus RNA was extracted by the Qiagen Spin Column or the all-in-one cartridge system. Bio-Rad CFX96 was utilized for detection in columns 1 and 2. C_T values for column 3 were obtained by utilizing the complete extraction and detection modules of the all-in-one cartridge system.

Influenza A (Dilution folds)	Spin Column Extraction, Bio-Rad CFX96 (C_T)	All-in-One Extraction, Bio-Rad CFX96 (C_T)	All-in-One System for Extraction and Detection (C_T)
1	23.03	24.70	25.74
10^1	27.23	28.57	30.26
10^2	30.14	32.40	33.03
10^3	33.96	35.20	37.68
10^4	36.49	38.24	† ₋

† RT-PCR failed

It was noteworthy that the recovery rate of viral RNA extraction depended on the biological samples, the extraction kits and the processing conditions. The kit insert for the QIAamp kit states that the RNA kit is designed for purification of viral RNA from plasma, serum and cell-free body fluids, and it will co-purify DNA if present in the sample. Thus, the extraction kits need to be carefully chosen to suit the requirement of clinical samples in practical applications. In addition, different storage conditions are required for lysis buffer (prepared fresh), RNA extraction reagents (room temperature), and reverse transcriptase and DNA polymerase (-20°C). Depending on the applications, the lysis buffer may contain DNase, RNase and proteinase K, thus it is recommended that the lysis buffer be freshly prepared. While this work was demonstrated with the virus particle lysis prepared externally in a microfuge tube, the cartridge design was flexible and could be extended to accommodate chambers for integrated virus particle lysis with on-site introduced fresh lysis buffer.

Figure 3.10: The C_T values of the real-time fluorescence curves of serial diluted ($1-10^4$ folds) influenza A patient samples, obtained with (Δ) the Qiagen Spin Column plus Bio-Rad CFX96, ($\diamond$) the on-cartridge RNA extraction plus Bio-Rad CFX96, and ($\times$) the all-in-one system. The solid lines are the linear regression fits obtained for (—) Qiagen Spin Column with Bio-Rad CFX96 (slope = -3.37 , $E = 99\%$, $R^2 = 0.994$), (—) the on-cartridge RNA extraction with Bio-Rad CFX96 (slope = -3.37 , $E = 99\%$, $R^2 = 0.995$), and (—) the all-in-one system (slope = -3.59 , $E = 90\%$, $R^2 = 0.991$), where $E = 10^{(-1/\text{slope})} - 1$ was the RT-PCR efficiency.

This chapter was demonstrated with $200\ \mu\text{l}$ of elution buffer, which would dilute the concentration of the extracted viral RNA. The minimum eluent volume ($100\ \mu\text{l}$) of the current design for consistent RNA elution was limited by the relatively large diameter ($7\ \text{mm}$) of the thin-film Fujifilm silica membrane employed in our on-cartridge RNA extraction. A dead volume of $\sim 5\ \mu\text{l}$ was generated between the silica membrane and the bottom of the membrane chamber in our current membrane assembly. In addition, an uneven reagents drainage was observed across the membrane, causing a ring of fine residues deposited at the edge of the membrane chamber. An extra step of ethanol flush ($500\ \mu\text{l}$) was added before introducing the eluent buffer to completely flush out the wash buffer AW2 of QIAamp kit, which could have potentially inhibited the RRT-PCR. Improvement in reducing the elution buffer volume could be achieved by incorporating a commercial or monolithic silica column with reduced diameter [43, 44].

3.4 Conclusion

We have successfully developed a disposable self-contained all-in-one cartridge-based diagnostic system that integrated the viral RNA extraction and RRT-PCR detection. The viral RNA extraction and detection took place within the sealed chambers of the all-in-one cartridge, minimizing sample contamination and reducing the operator's exposure to the viral samples. The system's overall operation has been simplified, only requiring the operator to load the sample into the cartridge and run the system with a preloaded programme for sample extraction and detection, which closely followed the standardized protocols as recommended by WHO. The all-in-one system we developed was a desktop machine that could be operated in decentralized testing facilities by non-specialists. The system demonstrated its multiplexing capability with influenza type A and H1 sub-typing of real clinical nasal swab samples. Its detection performance was further validated with a direct comparison against conventional spin column extraction protocols and commercial real-time PCR systems. The system was able to detect clinical influenza samples that were diluted by 10^3 folds. In addition, our novel microfluidic manipulation approach that utilized external pneumatic pumps and valves reduced the complexity of the disposable cartridges. The cartridge design was flexible and could be extended to accommodate multiple channels for multiplexing detection, without significant design modifications. This would enable the simultaneous detection of a panel of respiratory virus infections [45, 46]. In short, we presented a practical, low-cost, self-contained and fully integrated diagnostic system that has demonstrated its ability to handle real clinical influenza samples.

3.5 References

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Chapter 4

Rapid-Filling Omega PCR Array Chip

4.1 Introduction

PCR is a well-developed method for nucleic acid amplification and gene detection [1-10]. This process is usually conducted in devices such as PCR tubes and well plates. However, microfluidics chip-based PCR devices have been increasingly examined for various applications, including food safety testing, environmental monitoring and point-of-care clinical diagnostics [1-6]. Compared to the conventional devices, the microfluidic/nanofluidic chip-based PCR systems provide many advantages, such as rapid operation, small sample volume, ease of sample transport to analytical stage, and parallel amplification in multiple wells [3, 11-15]. One such example is the lab-on-cartridge system with 3 PCR reaction wells [16, 17]. To further enhance the detection capability of this system, a PCR chip with many more wells has been developed in this work, in an effort to meet the needs of applications such as cancer and infectious disease diagnosis whereby the targets could be more than 10.

According to the method used for microfluidic transfer, PCR platforms can be classified into three categories: chamber-stationary PCR system [18], continuous-flow PCR system [10, 19] and thermal convection-driven PCR system [20]. Among them, the chamber-stationary PCR system is most suitable for multi-chamber PCR amplification with high-throughput. However, there are quite a number of challenges to overcome to effectively conduct PCR on a single chip. For example, it is not easy to uniformly deliver

a small amount of PCR mixture to individual chambers. On the other hand, the PCR chambers have a very small volume, and hence it would be important to prevent any evaporation of reaction mixture at high temperatures (for example, 95°C). In addition, bubbles may form during the PCR process, which can significantly affect the chip operation and reduce PCR efficiency. The most commonly used method to address these issues is the integration of pumps [21] and valves [22] onto a chip for PCR mixture loading and sealing [20, 21]. However, integration of many components onto a single chip complicates device fabrication and operation. An alternative method is to utilize capillary-driven microfluidics for the loading of sample into the reaction chambers, which can be achieved by using surfactants to minimize the contact angle between the PCR mixture and the PCR chip [8, 23, 24]. However, the surfactants added often have undesirable effects on PCR amplification efficiency. Centrifugal force is another option since it is easy to be applied to disk-type microfluidic device for accurate PCR mixture delivery [25-27]. However, fluidic transfer based on centrifugal force is not flexible, especially when the PCR portion is integrated with other bio-sample preparation components [28, 29].

This paper presented a new PCR fluidic chip for the fast and accurate PCR mixture delivery and sealing. The wells on the chip were arranged along a circular loop, each connected to a main fluidic channel via the isolation channel. This formed the shape of ‘Ω’ (Figure 4.1(a)), and hence the chip was referred to as the ‘Omega PCR array chip’. Simulation and experimental results showed that uniform illumination of all wells could be obtained by adopting this configuration, and therefore more accurate detection of fluorescence signal could be achieved. To operate this chip, vacuum was first applied to

the chip to remove air from the reaction wells. Next, PCR mixture was introduced at the chip inlet to automatically fill the individual wells that were under vacuum, mixing with the PCR probes preloaded in the wells. The mixture remaining inside the main fluidic channel was then removed from the chip. Next, liquid wax was used to fill the emptied channel to seal the individual reaction chambers. By using this approach, the filling of PCR mixture into each well could be completed within 8 ms, much faster than other filling techniques. The wells could be sealed easily and effectively after they were filled. Both the filling and sealing of wells were driven by vacuum, which was easy to operate.

A fluorescence light excitation and signal detection method has been specifically developed for monitoring the amplification progress in the Omega PCR array chip using a CCD camera [30, 31]. We then tested the chip's performance in PCR application in two detection processes: (i) the NASBA isothermal PCR for RNA samples from HPIV, and (ii) the HDA isothermal PCR for the target DNA from *E. coli* bacteria.

4.2 Methodology

4.2.1 Chip design and fabrication

As shown in Figure 4.1, the prototype chip has a sandwich structure. The main body was a polycarbonate (PC) substrate of 1.5 mm in thickness, featured with open wells and channels. Two optical adhesive tapes of 0.1 mm in thickness were used to seal the top and bottom of the substrate, forming an enclosed flow space within the chip. The chip contained 26 wells, uniformly located outside a circular fluidic channel. Each well has a diameter of 2.0 mm and a depth of 1.0 mm, corresponding to a sample volume of ~ 3 μ L. The wells were linked to the fluidic channel via a short channel of 0.5 mm in width and 0.5 mm in depth, as indicated by the ‘isolation channel’ in Figure 4.1. The fluidic channel for delivering PCR mixture to individual wells also has a width and a depth of 0.5 mm and 0.5 mm, respectively. At the ends of the fluidic channel, there were two holes of 1 mm in diameter, which were to be connected with the inlet and outlet tubes.

The chip design was generated using the CAD software SolidworksTM (Dassault Systèmes SolidWorks Corp., France). The polycarbonate substrate was then fabricated using a computer numeric controlled (CNC) machine (WHITS Technologies, Singapore). The fabricated PC substrate was later soaked in 0.2% detergent (Decon 90, Decon Lab Ltd.) solution for 12 h, followed by rinsing thoroughly with de-ionized water so as to remove any oil contamination from the CNC machining process. Next, the chip was soaked in 3% H₂O₂ (MGC Pure Chemicals, Singapore) for 12 h, rinsed with 0.1% diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, Singapore) to remove RNases and DNases, and oven-dried at 60°C for 6 h. Pipette tips (volume: 20 μ L) were cut into segments of 7 mm in length for use as the inlet and outlet tubes; they were glued to the two holes at the ends

of the fluidic channel. Optical adhesive films (MicroAmp™, Applied Biosystems Inc.) were laser-cut into the desired size using a 40-W CO₂ cutting system (Helix 40 Epilog, USA) under 20% laser power, and then applied to the top and bottom surfaces of the polycarbonate substrate.

Figure 4.1: Design of the Omega PCR array chip. (a) 3D model of the PCR chip with circular fluidic channel (top and bottom tapes not shown). (b) Detailed chip configuration showing the fluidic channel and isolation channels. (c) Cross-sectional view of the chip with preloaded primer in the PCR well. The top and bottom of the PCR chip are bonded with pressure-sensitive tapes with optical transparency.

4.2.2 Operation of the microfluidic PCR chip

The vacuum system for loading PCR mixture and liquid wax into the chip consisted of a motorized-driven 10-mL syringe (NE-1000, New Era Pump Systems Inc. USA), and two pinch valves (V1 and V2) for turning on/off the vacuum and the liquid flow (Figure 4.2(a)). A 200- μ L Eppendorf tube was used as the reservoir for holding PCR mixture. It was connected to the inlet of the chip via a silicone tube (O.D. = 2.2 mm and I.D. = 0.5 mm, Cole Parmer, USA). The outlet of the PCR chip was connected to the

syringe via a silicone tube of the same size. Note that the PCR chip was always mounted horizontally during the loading process. The operation of the microfluidic PCR array chip included the flowing steps:

Step 1. Initially, the vacuum syringe was set to its zero volume. The inlet pinch valve V1 was closed while the outlet valve V2 was open. The vacuum syringe moved backwards to generate the vacuum for air removal from the channel and the wells. The target volume of the syringe was set to 2.5, 5, 10 or 15 mL, depending on the vacuum level needed for the subsequent filling of wells. The actual pressure in the wells corresponding to the vacuum volume of syringe pump was calibrated, as shown in Figure 4.2(b).

Step 2. Valve V1 was opened. Due to the vacuum generated in the channel and the wells, the PCR mixture moved into the fluidic channel and filled the wells one by one within 0.5 s. Once the PCR mixture reached the outlet of the chip, valve V2 was closed to isolate the chip from the vacuum in the syringe pump. The filling process continued due to the vacuum remaining in the channel and the wells. After ~ 10 s when all the wells were completely filled, V1 was also closed.

Step 3. The Eppendorf tube with PCR mixture was removed. At the same time, the vacuum in the syringe pump was released. Valves V1 and V2 were then opened. The syringe pump was started and run at a slow speed of 50 $\mu\text{L}/\text{min}$. As a result, the PCR mixture remaining inside the main fluidic channel was gently drawn out of the chip. After that, an Eppendorf tube containing 100 μL of liquid wax (Bio-Rad, Singapore) was attached to the inlet of the chip. With the pumping from the syringe, the silicone oil

gradually filled the fluidic channel, and its immiscibility with water helped to effectively seal all the individual wells in the chip.

To study the details of the PCR mixture filling/sealing process, a high-speed video camera (MotionPro X4, DEL Imaging Systems, USA) was employed to monitor the above process at a recording rate of 600 frames/s. Deionized water was used to simulate the PCR mixture solution, with the addition of ink to improve the image contrast for easier observation. The temporal information could be obtained from the time point when the corresponding frame was obtained.

Figure 4.2: (a) Schematic diagram for the delivery of PCR mixture and liquid wax to the Omega chip. The fluidic delivery includes 3 steps. (i) The motorized syringe pump moves backwards to generate vacuum (i.e. V1 is closed while V2 is open). (ii) The PCR mixture enters the fluidic channel and PCR wells sequentially until all the wells are fully filled (i.e. both V1 and V2 are open). (iii) The remaining PCR mixture in the fluidic channel is gradually pumped out and replaced by the liquid wax that helps to effectively seal all the PCR wells (i.e. both V1 and V2 are open). (b) Pressure in the wells corresponding to different vacuum volumes in the syringe pump.

4.2.3 On-chip NASBA amplification for virus detection

NASBA is a useful isothermal PCR amplification process. It works under a relatively low temperature of 41°C, which makes it very suitable for a low-power-consumption hand-held device. NASBA amplification processes mimic the retroviral RNA replication without the need for thermal cycling. During the amplification, NASBA uses three enzymes, reverse transcriptase, RNase H and T7 DNA-dependent RNA polymerase. NASBA has a high amplification factor. For example, the reaction can produce about one billion complementary RNA molecules in 1.5 h using two primers specific to the target RNA. Real-time NASBA is achieved by using molecular beacons.

On-chip NASBA process has been used for virus detection and bacteria detection, but only one target was tested [31-33]. Herein we applied NASBA amplification to the detection of two types of HPIV on our chip. HPIV is one of the major cause for upper and lower respiratory tract illnesses in pediatric patients, which break out annually in winter. There are four recognized serotypes of HPIV: HPIV1, HPIV2, HPIV3 and HPIV4, but only HPIV1 and HPIV2 are commonly found. In this experiment, we chose HPIV 1 and HPIV 2 for testing purposes.

The required reagents for NASBA were adopted from a NucliSens Basic Kit (bioMérieux, UK). They were prepared according to the protocol recommended by the manufacturer before the experiment was conducted. Briefly, 80 µL of reagent diluent was added to one reagent sphere from the kit to make the ready-to-use reagent solution. The ready-to-use KCl solution was prepared by mixing 14 µL of KCl stock solution in the kit with 16 µL of NASBA water. The enzyme solution was prepared by adding 45 µL of enzyme dilution from the kit to one enzyme sphere. The RNA molecules of HPIV1 (Cat.

VR-94D) and HPIV2 (Cat. VR-92D) were purchased from the American Type Culture Collection (ATCC) (USA).

Since each well has a volume of 3 μL , filling a 28-well PCR chip requires a total sample volume of 84 μL . Therefore, we prepared 120 μL of PCR mixture for the filling and testing, which contained 30 μL of virus RNA template, 39 μL of reagent solution, 15 μL of KCl solution, 30 μL of enzyme solution and 6 μL of water.

Primers and molecular beacon probes were purchased from Eurogentec (Cat. NB-PAI01-48 for HPIV1 detection using Fam-Dabsyl molecular beacon, Cat. NB-PAI02-48 for HPIV1 detection using Rox-Dabsyl molecular beacon). The primers were pre-deposited into individual wells during the chip assembly.

4.2.4 Bacteria culture and plasmid DNA preparation

E. coli (ATCC25922) bacteria used for HDA amplification study were obtained from ATCC (USA). They were cultured in the tryptic soy broth (TSB) buffer (BD, USA) contained in a 1.5-mL Eppendorf tube placed on a thermal mixer (Thermal Mixer Comfort, Eppendorf Co., USA) at 41.5°C.

After overnight culture, the bacteria were harvested by centrifuge. 10 μL of serially diluted bacteria sample was introduced into a C-Chip (DHC-N01, Incyto Co. Korea) and the image of bacteria was captured by the CCD camera of an optical microscope (IX51, Olympus, USA). The number of bacteria was counted from the image, and the corresponding density was calculated. About 10^3 to 10^9 bacteria cells were collected into a 1.5-mL tube for plasmid DNA extraction using the Plasmid Minit Kit (Cat. 12143, Qiagen, Singapore). According to the user manual from the manufacturer,

the extraction included 3 main steps: (i) bacteria cell lysis and lysate collection, (ii) DNA extraction and purification, and (iii) DNA elution. In the bacteria lysis step, the cell suspension was centrifuged at 8000 rpm for 10 min. After the supernatant was removed, 250 μ L of buffer P1 was added to the tube to re-suspend the bacteria. Next, 250 μ L of buffer P2 was added and mixed thoroughly by gently inverting the tube 5 times. After 2 min of incubation, 350 μ L of buffer N3 was added and mixed thoroughly by inverting the tube 10 times. After the lysis steps, the solution was centrifuged at 10000 rpm for 10 min. The supernatant was collected and transferred to a QIAprep spin column for DNA extraction and purification. The DNA extraction was done by spinning the solution at 3000 rpm for 1 min. The DNA molecules were solid-phase extracted onto the spin column, while the flow-through was discarded. The purification process involved adding 0.75 mL of buffer PE to the column, and centrifuging at 3000 rpm for 1 min. In the elution process, the column with purified DNA molecules was placed in a clean 1.5-mL tube. 50 μ L of buffer PE was added to the tube. After 1 min of incubation, the tube was centrifuged at 3000 rpm for 1 min, and the pure plasmid DNA molecules in the eluted solution were collected for downstream PCR amplification.

4.2.5 Real-time helicase-dependent PCR amplification

The IsoAmp tHDA-III kit from New England Biolabs (Beverly, USA) was used for the isothermal PCR amplification detection of *E. coli* plasmid DNAs. The tHDA kit contained the required reagents for the PCR amplification, except the DNA template, forward primer and reverse primer. A tHDA reaction mainly consisted of the following materials: 1 $\times$ annealing buffer, 25 $\times$ enzyme mix, 14 $\times$ dNTP solution, 3–4.5 mM of

MgSO₄, and 20–50 mM of NaCl. In this study, target M13 gene of the *E. coli* DNA was amplified. According the sequences of primer 1233 and 1224 (New England Biolab, USA), the forward and reverse primers were 5'AGCGGATAACAATTTACACAGGA 3' and 5' CGCCAGGGTTTTCCCAGTCACGAC 3', respectively.

The IsoAmp tHDA kit did not come with the reporting dye for real-time PCR. Currently, SYBR Green I, Eva Green and LG Green are the most widely used reporting dyes for real-time PCR. Although both Eva Green and SYBR Green could be used, previous studies have shown that the Eva Green has much lower PCR inhibition than other dyes [14]. Hence, we chose Eva Green as the reporting dye for real-time HDA.

4.2.6 Real-time PCR system

A real-time PCR system for characterizing our PCR chip was constructed and tested. A Peltier thermoelectric device (TEC) was used to generate the required temperature for PCR reaction. Aluminum cooling heat sink was attached to the backside of the TEC. Furthermore, a small cooling fan was attached to the aluminum block to enhance the cooling effect. Plate temperature was measured by a 2.252-k Ω resistive temperature detector (RTD) (Cat. #201347, FerroTec, USA). A digital temperature controller (FTC100) and an H-bridge amplifier (FTA600) from FerroTec were used for the feedback temperature control.

An optical system was developed for the detection of fluorescence of SYBR Green I/Eva Green in the real-time PCR system, as shown in Figure 4.3(a). The light from a solid state switchable light source (Lumencor, Spectra Light Engine, Lumencor Inc., Beaverton, OR) was transmitted via an optical fiber (400 μ m, 0.75 m UV-SR, Ocean

Optics), and filtered by an excitation filter (D480/30X, Chroma Technology Co., USA) to generate the 480-nm blue excitation beam. The light beam was reflected by a mirror, and laterally excited the detection PCR chip that was located 10 cm vertically below it. The light emitted from the samples in the PCR chip was filtered by an emission filter (HQ535/50M, Chroma Technology Co., USA) to generate the fluorescence signal at 520 nm. This signal was focused by a compact lens with a fixed focal length (Cat. 59871, Edmund Optics, USA). The working distance between the lens and the PCR chip ranged from 15 to 25 cm. The focused light was then detected by a cooled CCD camera (Retiga EXi, QImaging, Canada), and the light intensity over the entire chip was obtained by analyzing the images captured at certain time points by the camera.

Both photodiode and CCD camera can be used for the detection of fluorescence signal of PCR [30, 31]. Since photodiode is a point detector, in order to monitor more PCR wells simultaneously, fiber-optic cables and an optical multiplexer have to be used. This would become increasingly complex and expensive as throughput is further increased. CCD camera detects signal over an area instead a point, hence the number of PCR wells monitored is irrelevant. Because of the requirement of some distance between the light source and the PCR wells, and between the PCR wells and the camera, the sensitivity of camera-based detection is generally less than fiber-optic cable-based detection, which suffers little transmission loss over distance. This reduced sensitivity is generally not a problem for PCR product detection. With the availability of more sensitive and cost-effective CCD camera, more and more commercial real-time PCR system would use CCD camera as a detector.

A power meter (Model 841-PE, Newport Corp., CA, USA) was used to measure light intensity (in $\mu\text{W}/\text{cm}^2$) at various lateral displacements from the optical axis. The data (not shown) agreed well with the simulation results based on Gaussian normal distribution (MATLAB, Mathworks Inc., USA) (see Figure 4.3(b)). The distribution of the power of the incident light was axisymmetric around the optical axis, and a sufficiently high intensity could be obtained for displacements ranging from -15 mm to 15 mm, which was comparable to the dimensions of the Omega chip. Given that both the Omega chip and the Gaussian distribution of light intensity were radially symmetrical, we concluded that each well would receive light of the same intensity when the chip and optical detection were positioned concentrically. This was an important feature of our optical detection system, which allowed similar sensitivity to be achieved for each well.

Figure 4.3: Optical detection for Omega PCR array chip. (a) Schematic diagram of the optical detection system. (b) The distribution of light intensity over the PCR chip from simulation. Dark red: brightest, dark blue: dimmest.

4.3 Results and discussion

4.3.1 Rapid filling of PCR wells

Rapid and accurate filling of the PCR wells is important for high-throughput DNA/RNA amplification. Different methods for rapid PCR filling have been developed [34]. For example, Takamura's group and Gong's group developed a PCR array chip that automatically sucked the PCR mixture into the distribution channels and reaction wells by silanizing the surfaces of channels/wells to enhance the capillary force for driving fluid [23]. Unlike the above methods, in our case, vacuum was directly applied to each reaction well via the inlet/outlet of the chip. Because vacuum could be easily applied to microfluidic systems [14], our PCR mixture filling approach was expected to be more effective, which has been confirmed through the following experiments.

The outlet of the chip was connected to a 10-mL syringe that was controlled by a motorized syringe pump. The plunger of the syringe was pushed to the end with zero volume. Prior to the start of sample loading, the inlet and outlet pinch valves were set at the 'close' and 'open' positions, respectively. When the plunger began to be moved backwards, the air originally sealed in the chip channel and wells (ambient pressure = P_0 , volume = V_0) expanded. When the air volume was increased to V , the pressure P inside the channel/wells could be calculated from the ideal gas law:

$$P = P_0 V_0 / (V_0 + V)$$

In this study, the syringe vacuum volume V was usually set at 1–10 mL, which generates a vacuum of -79.9 to -89.9 kPa. The vacuum was maintained for 10 s for pressure stabilization before the inlet pinch valve was opened. After the inlet valve was opened, the PCR mixture in the reservoir, which was subjected to the pressure difference of ($P_0 -$

P), moved along the fluidic channel and entered every single reaction well. Figure 4.4 shows the sample loading snapshots from a video taken with a high-speed camera. It could be seen that the wells were filled sequentially — the ones near the inlet were filled earlier than those near the exit. For a 35-well PCR chip, the filling process was completed within 300 ms.

Figure 4.4: Snapshots of the sample loading process in the PCR chip, as captured by high-speed camera. Air inside the loading channel and reaction wells was sucked out by the external syringe, and then the PCR mixture was introduced to fill the loading channel and reaction wells. The total filling time was less than 300 ms for a vacuum volume of 10 mL. Scale bar: 5 mm.

The dependence of the average filling volume per well on the applied vacuum is shown in Figure 4.5(a). The volume of liquid in each well was calculated by dividing the weight measured from a precision weight balance (AB204-S, Mettler Toledo, USA) with the liquid density. The filling volume was found to be insensitive to the range of vacuum volumes optimized for this application (5–15 mL). For example, the filling volumes corresponding to the vacuum volumes of 5, 10 and 15 mL were 3.02, 3.11 and 3.06 μL , respectively. These values were very close to the theoretical liquid volume in a fully filled well of 3.14 μL calculated from the well dimensions (diameter = 2.0 mm and depth = 1.0 mm), indicating that the wells were fully or almost fully filled in the sample loading process. Figure 4.5(b) shows the volume filled in different wells along the fluidic channel. The first well has a slightly higher filled volume of 3.09 μL , while the last well has a filled volume of 2.99 μL . Their difference in filled volume was 3.3%, indicating a good uniformity in the filling of different wells. The filled volume only decreased very slightly for the wells along the flow channel. This minor difference could be due to a gradually reduced vacuum level during the filling process, i.e. the downstream wells might have been slightly less filled under a slightly lower vacuum level.

Figure 4.5: (a) The average filling volume per well on the Omega chip under a vacuum volume of 5–15 mL. (b) Liquid volume filled in different wells along the fluidic channel at a vacuum volume of 10 mL.

4.3.2 Optimization of the applied vacuum level

One parameter important for vacuum filling was the vacuum level applied to drive the fluid. Inadequate vacuum would slow down the filling process. It could further result in the significant trapping of air in the reaction wells, which might lead to the failure of the PCR process due to expansion of the trapped air at high temperatures causing problems in amplification and detection. On the other hand, if the vacuum level was too high, bubbles might form as a result of water evaporation at low pressures, which would negatively affect the filling process by blocking the fluidic and isolation channels. To obtain the optimal vacuum level, vacuum volumes of 1–22 mL were tested for sample loading, and the corresponding filling quality was examined and compared. As shown in Figure 4.6(a), several PCR chambers were not filled fully for vacuum volumes of < 5 mL. On the other hand, if the vacuum volume applied was higher than 22 mL, bubbles would

form easily inside the fluidic channel and the wells (Figure 4.6(b)); in this case, some of the PCR mixture solution might evaporate. Moreover, the high filling velocity would also generate flow turbulence, and as a result, air might be trapped as bubbles within the filling liquid. A suitable vacuum volume would be in the range of 5–15 mL.

Figure 4.6: PCR chamber filling performance as a function of the vacuum level. (a) Several wells (as indicated by the arrow) were only partially filled with PCR mixture when an insufficient vacuum volume of less than 5 mL was used. (b) Bubbles formed in the channel and wells (indicated by arrows) when a vacuum volume of 22 mL was used. Scale bar: 5mm.

4.3.3 Sealing of the reaction wells

Preventing loss of PCR mixture is a challenge for microfluidics-based PCR systems. The three main causes for sample loss are as follow. (i) The air trapped in reaction wells during sample loading expands under high temperatures, and pushes the sample solution out of the wells. Several methods have been developed to avoid the trapping of air, including improvement of the design of PCR well shape and modifying the well surface to render it highly hydrophilic. (ii) The gas originally dissolved in the PCR mixture is released under high temperatures due to its reduced solubility; this can

also push the sample solution out of the wells. De-gasification of the PCR mixture before loading can help to minimize this effect. (iii) The PCR mixture will evaporate at high temperatures, leading to the loss of sample volume; this is especially significant after multiple thermal cycles. In order to resolve this problem, individual valves are used to seal the reaction chambers after filling them [35]. An alternative approach is to add a layer of silicone oil or liquid wax as a liquid-vapor barrier to prevent evaporation. Both silicone oil and liquid wax have boiling points well above water [36], hence, they would remain as stable liquids at the highest operating temperature of the PCR process, and prevent the transmission of water vapor.

In our approach, liquid wax was used for sealing and isolating each PCR well. Figure 4.7 shows the steps taken to seal the PCR wells. After the PCR mixture was loaded into the wells, the outlet pinch valve was closed and the vacuum in the syringe pump was released to atmosphere. Next, the pinch valve V2 was opened again, and the syringe pump slowly pumped away the PCR mixture remaining in the loading channel at a flow rate of 50 $\mu\text{L}/\text{min}$ (Figures 4.7(a)–(c)). After the PCR mixture was fully removed, liquid wax was introduced from the inlet and filled the void space in the loading channel. The PCR mixture was sealed inside the reaction wells by the liquid wax, unable to evaporate and escape from the Omega chip (Figures 4.7(d)–(f)). Both the inlet and outlet valves were then closed, and the entire chip was subjected to PCR thermal cycling.

Figure 4.7: Process for removing the excess PCR mixture from the loading channel, and sealing the PCR wells with liquid wax. (a–c) After loading the PCR mixture (highlighted by blue ink for better visualization), the excess PCR mixture remaining in the loading channel was removed by the syringe pump at a flow rate of 50 $\mu\text{L}/\text{min}$. The arrows in red show the interfaces between the PCR mixture and air in the loading channel. (d–f) Liquid wax (highlighted by pink ink for better visualization) was loaded into the fluidic channel to seal and isolate the PCR mixture in each of the wells. The arrows in red show the position of the liquid wax in the loading channel. Scale bar: 5 mm.

4.3.4 On-chip detection of HPIV RNA by NASBA

Rapid detection of various bacteria is critical for food safety testing and clinical diagnosis. Conventional PCR or reverse transcription PCR (RT-PCR) is commonly used for bacteria detection. However, it suffers from one important drawback, i.e. it requires rapid and precise thermal cycling for the denaturation, extension and annealing of target DNA molecules during the reaction processes. In contrast, isothermal PCR does not

require thermal cycling, and usually works at relatively low temperatures (e.g. 41°C) [36, 37]. As it does not involve any thermal cycling processes, isothermal PCR system allows for a simplified design and much lower energy consumption. Previous studies have shown that isothermal PCR is most suitable for portable, battery-operated PCR detection systems. There are about 8 types of isothermal methods [37], including the most commonly used NASBA, loop-mediated isothermal amplification (LAMP), HDA, rolling circle amplification (RCA) and strand displacement amplification (SDA) [33, 38-41].

The NASBA assay involves a continuous, isothermal process without the need for thermal cycling. The sensitivity of the NASBA assay was tested using the original virus stock solution, and dilutions by 10 to 10000 folds. The diluted template was mixed with other components to prepare the 120- μ L PCR mixture. To prepare the negative control, water free of DNase and RNase was used instead of the diluted virus sample.

The PCR mixture was introduced to the wells of the Omega chip using vacuum, and then sealed with liquid wax, as previously described. Next, the entire chip was mounted onto the TEC heater, and PCR amplification was run for 1.5 h at 41°C. The image of the chip was obtained with the CCD camera every 5 min.

Figure 4.8 shows the amplification curves of the original stock RNA sample and its 10 $\times$ to 10000 $\times$ dilutions in real-time detection on the Omega chip. After $\sim$ 20 min of reaction, the fluorescence intensity began to increase almost linearly, except for the negative control. The time-to-positive for the positive amplifications varied from 20 to 30 min. There was a clear relationship between the rate of fluorescence intensity increase and the input template RNA concentration. As expected, a higher RNA concentration led to a faster increase in signal intensity.

Figure 4.8: Sensitivity of HPIV real-time detection on Omega chip with the original stock RNA sample of HPIV2 viruses, and its 10 $\times$ to 10000 $\times$ serial dilutions. The red line is the negative control (water).

Figure 4.9 shows the image of the simultaneous end-point detection for HPIV1 and HPIV2 viruses on the Omega PCR array chip. A color Grasshopper2 CCD-based RGB camera (Point Grey Research Inc., Richmond, BC, Canada) with a 25-mm focus lens was used for this study. A full quad-band filter set (Semrock Inc., Rochester, NY, USA) was used as the excitation light filter. The wells were preloaded with either Fam-Dabsyl or Rox-Dabsyl molecular beacon. The former displayed a blue color for positive identification of HPIV1, while the latter displayed a red color for HPIV 2. A mixture of HPIV1 and HPIV2 samples in 1:1 volume ratio was used as the RNA template in this study. A strong blue fluorescence was observed from the wells deposited with Fam-Dabsyl, and a red fluorescence was seen in the wells with Rox-Dabsyl. The wells without any primer did not show any fluorescence signal, confirming that there was no cross-talk between the different wells. This study demonstrated that the Omega PCR array chip could be employed for the simultaneous detection of multiple targets.

Figure 4.9: Simultaneous end-point detection for HPIV1 and HPIV2 viruses on the Omega PCR array chip. Fam-Dabsyl molecular beacon (blue) was used for HPIV1 detection, and Rox-Dabsyl molecular beacon (red) was employed for HPIV2 detection. The wells without fluorescence signal were not pre-deposited with any probes (×). Scale bar: 5 mm.

4.3.5 On-chip detection of *E. coli* DNA by HDA

HDA was based on the natural mechanism of DNA replication fork. It can work at a single temperature of 65°C. The IsoAmp tHDA kit can amplify a short DNA sequence of 70 to 130 bp. Successful tHDA amplifications have been achieved on products as short as 85 bp, and as long as 129 bp.

Although the liquid wax sealing of reaction wells discussed in Section 4.3.3 helped to prevent gas from escaping from the chip, it did not eliminate water evaporation inside the wells at high temperatures. To solve this problem, the PCR mixture was pre-mixed with a certain amount of glycerol to reduce the vapor pressure. According to Raoult's law, the vapor pressure of a water/glycerol mixture P can be approximated as,

$$P_{(T)} = P_{W(T)}x_W + P_{G(T)}x_G ,$$

where $P_{W(T)}$ and $P_{G(T)}$ are the vapor pressures of water and glycerol at temperature T , respectively, and x_W and x_G are the mole fractions of water and glycerol, respectively. The molecular weights of water and glycerol are 18 and 92, respectively; the weight percentages of water and glycerol used in experiments can be converted to mole fractions with these molecular weights. Since glycerol has a much lower vapor pressure than water at the same temperature, the above equation shows that the addition of glycerol may decrease the vapor pressure of a PCR mixture. At the boiling temperature, the vapor pressure of the mixture is equal to the ambient pressure of 1 atm. The boiling temperatures calculated for different glycerol concentrations are shown in Figure 4.10.

Figure 4.10: Calculated boiling temperatures of glycerol-water mixtures of different glycerol concentrations.

Takamura's group examined the addition of 5% to 20% (w/w) glycerol [14], and found that the fluorescence intensity and the PCR C_T value were not significantly

affected. In our experiments, 15% (w/w) of glycerol was added to the PCR mixture. At this concentration, the mixture's boiling temperature was $\sim 103\text{ }^{\circ}\text{C}$.

In our study, the primer pair specific for the target region of the bacteria DNA was preloaded into the wells. Plasmid DNA extracted from about 10^3 , 10^5 , 10^7 and 10^9 bacteria cells was introduced to 4 different chips, and subjected to isothermal PCR amplification. The fluorescence signal from individual wells was captured every 2 min. The PCR amplification became very obvious after ~ 27 min (Figure 4.11). The time required was not significantly different for extractions from different bacteria concentrations, indicating that HDA might not be suitable for the quantitative detection of *E. coli*.

Figure 4.11: Isothermal HDA curves of DNA extraction from 10^3 – 10^9 bacteria cells on the Omega PCR array chip.

Uniformity of fluorescence signal was important in the quantitative detection of genes. In order to check the detection uniformity in the Omega PCR array chip, we preloaded the same amount of primers, and vacuum-filled the same PCR mixture

(including the DNA sample extracted from 10^9 bacteria cells) into each well. After 40 min of HDA at 65°C , the end-point fluorescence signals captured is shown in Figure 4.12(a). Well-by-well signal intensity was quantitatively measured by ImageJ software (NIH, USA) (see Figure 4.12(b)). The signal intensity reading for the 28 wells ranged from 1208 to 1533. The average signal intensity for the 28 wells was 1454, and the standard deviation was ± 93 , which was $\sim 6.3\%$ of the average signal intensity, indicating a fairly uniform distribution in fluorescence signals amongst the different wells.

Figure 4.12: Signal uniformity measurement for isothermal real-time HDA of bacteria DNA on the Omega chip. (a) Fluorescence image of Omega chip after 40 min of PCR amplification. (b) Fluorescence signal intensity measured over the 28 PCR wells. The average signal intensity of the 28 wells was 1454, and the standard deviation was ± 93 . Scale bar: 5 mm.

4.4 Conclusions

This paper described the development of Omega PCR array chip with multiple wells for the detection of multiple targets. The well arrangement in a circular shape facilitated the uniform distribution of light intensity to all the wells from a point light

source. Rapid filling of the wells with PCR mixture could be achieved by applying vacuum to the chip as the driving force for liquid flow. A single well could be filled within 8 ms, and uniform filling volume for different wells could be attained. The optimum vacuum volume ranged from 5 to 15 mL. The performance of the chip has been successfully demonstrated in processes including NASBA isothermal PCR for RNA samples from HPIV virus, and HDA isothermal PCR for target DNA from *E. coli*.

4.5 References

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Chapter 5

Conclusions and Future Work

5.1 Conclusions

This thesis focused on the research and development of microfluidic systems for automatic molecular diagnosis, including a cost-effective, reliable and easy-to-use lab cartridge for automatic DNA/RNA extraction and purification, an all-in-one molecular diagnosis system, and an advanced PCR array chip.

First, a self-contained cartridge has been developed for automatic sample extraction and purification. This plastic sealed cartridge contained all the necessary reagents for pathogen and cell lysis, DNA/RNA extraction, impurity washes, DNA/RNA elution and waste processing. The entire sample preparation processes could be automatically completed within the cartridge on a desktop unit using a pneumatic fluid manipulation approach. Reagents transportation was realized with a combination of push and pull forces with compressed air and vacuum, respectively. This automatic sample preparation cartridge has been found to have on-cartridge DNA recovery efficiency of 54–63%, which was comparable to or even higher than the conventional manual approach using silica spin column. The capability of the on-cartridge RNA sample preparation has been successfully demonstrated by extracting and purifying RNA from 10 copies of MCF-7 breast cancer cells, followed by the verification of the CK19 cancer-specific gene in a lab-based PCR machine.

Subsequently, an all-in-one system has been developed for rapid influenza diagnosis by integrating the automatic DNA/RNA sample preparation cartridge developed in Chapter 2 with a thermal cycler and a 1-color, 3-chamber pseudo-multiplexing RRT-PCR. All necessary chemicals (including the process waste generated) were self-contained and completely sealed within a polymer cartridge, minimizing the potential human contamination and accidental viral exposure. The operation only involved loading the patient's nasopharyngeal swab sample with lysis buffer into the cartridge, and the system could automatically perform the entire sample preparation and molecular diagnosis within 2.5 h. The cartridge design allowed for precise dispensing of aliquots for multi-chamber PCR detection. This all-in-one system was capable of detecting influenza type A and H1 sub-typing in real clinical nasal swab samples with dilution factor of 10^3 folds. The results were comparable with the commercial real-time PCR systems using conventional spin column extraction protocols. Moreover, the cartridge design in the all-in-one system was flexible and could be easily extended to accommodate multiple channels for multiplexing detection.

Finally, to further improve the PCR detection capability of our lab-on-cartridge system developed in Chapter 3, a new PCR chip has been developed for rapid PCR mixture delivery and sealing. The PCR chip was arranged in a circular manner and connected to a main fluidic channel via an isolation channel. Simulation and experimental results showed that uniform illumination of all wells could be obtained by adopting this configuration and therefore more accurate detection of fluorescence signal would be achieved. The fluidic delivery was realized by applying a vacuum source, which was compatible with the fluidic delivery method in our sample preparation

cartridge. This new PCR fluidic design allowed for rapid filling of PCR mixture at a rate as fast as 8 ms per reaction well with uniform filling volume of 2.99 μL to 3.09 μL . The performance of the chip has been successfully tested in (i) NASBA isothermal PCR for the RNA samples from HPIV virus, and (ii) HDA isothermal PCR for the target DNA from *E. coli*.

In summary, this thesis has achieved the objective of developing microfluidic transfer devices for DNA/RNA preparation and DNA sample detection for all-in-one molecular diagnostics systems.

5.2 Limitation of current work

Although we have successfully demonstrated our sample preparation cartridge (Chapter 2) and lab-on-cartridge device (Chapter 3) for molecular diagnostic, the configurations of these systems have not been optimized in terms of easy fabrication and cost reduction. The company that has licensed our technologies has been working on the mass production of these cartridges.

The top and bottom layers of our rapid filling Omega PCR chip involved optical pressure-sensitive tapes. Although the tape worked well for the isothermal PCR, which has a maximum temperature of 65 °C, it was found not to be bonded well onto the surface of the chip in normal PCR runs, whereby the highest temperature was 95 °C. Better bonding methods and materials would be needed. One solution would be to use a thin sheet of poly(methyl methacrylate) (PMMA) or polycarbonate (PC) instead of the optical tape, and to employ ultrasound welding for bonding with the chip. Both PMMA and PC are transparent and compatible with PCR [1, 2].

5.3 Recommendations for future work

Future work will focus on integrating the rapid-filling omega PCR chip with the sample preparation chip to develop a fully integrated molecular diagnostic system with multiple target detection capability. Figure 5.1 shows one of the methods for the integration.

Figure 5.1: Method for integrating sample preparation cartridge and rapid-filling PCR chip as an all-in-one integrated molecular diagnostic system. The sample preparation cartridge and the PCR chip are de-attachable. All reagents will be preloaded into the system.

The sample preparation cartridge and the PCR chip are de-attachable. Reagents can be preloaded into the sample preparation cartridge, based on the types of patient

samples used. Primers for different diseases can be preloaded into and freeze-dried in each well in the PCR chip.

The integrated system should be robust enough for fast screening and diagnosis of infectious diseases, cancers and bacteria. For example, six PCR reaction wells may be required for dengue fever diagnosis [3, 4]. Clinical samples will be used for testing the device performance.

5.4 References

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List of Publications, Patents and Awards

A. Journal and conference publications:

- 1) Jeremy Loh, Guolin Xu, James Hsieh, Rosa Qi, Daniel Lee, Chunyan Zhang and Jackie Y. Ying, "Making molecular diagnosis and bio-sample preparation a breeze through LabVIEW-inspired system automation," National Instruments ASEAN Virtual Instrumentation Applications Contest (2006) 1-8.
- 2) Guolin Xu, Daniel Lee, Jeremy Loh, Chunyan Zhang, James Tseng-Ming Hsieh, Namyong Kim and Jackie Y. Ying, "Rapid avian virus sample preparation with an automatic self-contained polymeric cartridge," MEMS Technology and Devices, 2007, 360-364, Pan Stanford Publishing. (Part of Chapter 2)
- 3) Guolin Xu, Tseng-Ming Hsieh, Daniel Y. S. Lee, Emril Mohamed Ali, Hong Xie, Xing Lun Looi, Evelyn S.-C. Koay, Mo-Huang Li and Jackie Y. Ying, "A self-contained all-in-one cartridge for sample preparation and real-time PCR in rapid influenza diagnosis," Lab Chip, 2010, 10, 3103-3111. (Part of Chapter 3)
- 4) Guolin Xu, Daniel Yoke San Lee, Hong Xie, Deon Chiew, Tseng-Ming Hsieh, Emril Mohamed Ali, Xing Lun Looi, Mo-Huang Li and Jackie Y. Ying, "A self-contained polymeric cartridge for automated biological sample preparation," Biomicrofluidics 2011, 5, 034107. (Part of Chapter 2)

B. Patent applications:

- 1) Jackie Y. Ying, Guolin Xu and Jeremy Loh, "Fluid processing and transfer using inter-connected multi-chamber device," US Patent Application No. 20110030809, 2011. (Part of Chapter 2)
- 2) Mo-Huang Li, Jackie Y. Ying, Guolin Xu, Daniel Yoke San Lee, Emril Mohamed Ali and Tseng-Ming Hsieh, "Method and device for the accurate dispensing of reagent aliquots," PCT Patent Application No. WO2011139234, May 2011. (Part of Chapter 3)

C. Awards:

- 1) Asian Innovation Silver Award, The Wall Street Journal Asia, November 2011.
- 2) Best Innovation Award in Biomedical and Pharmaceutical Applications, 2009 ASEAN Virtual Instrumentation Applications Contest, October 2009.