Microfluidics nucleic acid testing for point-of-care liver assessment

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NANYANG
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MICROFLUIDIC NUCLEIC ACID TESTING
FOR
POINT-OF-CARE LIVER ASSESSMENT

LIM GEOK SOON
SCHOOL OF ELECTRICAL AND ELECTRONIC ENGINEERING
2016

**MICROFLUIDIC NUCLEIC ACID TESTING
FOR
POINT-OF-CARE LIVER ASSESSMENT**

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School of Electrical and Electronic Engineering

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TABLE OF CONTENTS

TABLE OF CONTENTS.....	i
ABSTRACT.....	iv
AUTHOR’S PUBLICATIONS.....	vi
LIST OF FIGURES.....	vii
LIST OF TABLES.....	xi
LIST OF ABBREVIATIONS.....	xiii
Chapter 1 Introduction.....	1
1.1 Background and Motivation.....	1
1.2 Objectives.....	7
1.3 Contributions.....	9
1.4 Organization.....	12
Chapter 2 Literature Review.....	15
2.1 Gold Standards for Liver Assessment.....	16
2.1.1 Histopathology.....	16
2.1.2 Liver Function Tests.....	19
2.1.3 Gene Expression Profiling.....	25
2.2 PCR Microfluidics.....	29
2.2.1 Genetic Materials Used for Nucleic Acid Amplification.....	31
2.2.2 Materials Used for Biochip Fabrication.....	33
2.2.3 Microfluidic Biochip Layout and Design.....	38
2.2.4 Heating/Thermal-Cycling Systems.....	43
2.2.5 Lab-On-A-Chip Nucleic Acid Detection Systems.....	46
2.2.6 Applications of Reported LOC Platforms.....	50
2.3 Preliminary Selection of Liver-Critical Genes of Interest.....	53

2.4	Summary of Literature Review.....	55
Chapter 3	First Integrated LOC System Embodying Stationary PCR Approach and Real-Time Detection.....	57
3.1	Introduction.....	57
3.2	Materials and Methods.....	60
3.2.1	Biological Samples.....	60
3.2.2	Sample Preparation and mRNA Extraction Chip Design and Fabrication.....	61
3.2.3	Multiplex RT-PCR Chip Design and Fabrication.....	64
3.2.4	Liver Tissue Sample Preparation and mRNA Extraction on Microfluidic Chip.....	65
3.2.5	Preloading of PCR Primers on Microfluidic Chip.....	68
3.2.6	Multiplex RT-PCR on Microfluidic Chip.....	68
3.2.7	Fluorescent Detection of Amplified cDNA.....	73
3.3	Results and Discussions.....	76
3.3.1	Microfluidic Sample Preparation and mRNA Extraction.....	76
3.3.2	Reconstitution of Dried Preloaded Primers.....	73
3.3.3	Microfluidic Multiplex RT-PCR.....	78
3.3.4	Fluorescent Detection and Gene Expression Analysis.....	85
3.4	Preliminary Selection of Liver-Critical Genes of Interests.....	90
3.5	Conclusions.....	94
Chapter 4	Second Integrated LOC System Embodying Continuous-Flow PCR Approach and End-Point Detection.....	95
4.1	Introduction.....	95
4.2	Materials and Methods.....	99
4.2.1	Microfluidic Chip Design.....	99
4.2.2	Microfluidic Chip Fabrication.....	102
4.2.3	Microfluidic RT-qPCR Setup.....	102

4.2.4	Surface Treatment.....	105
4.2.5	Sample Preparation and Assay Loading.....	106
4.2.6	Segmented RT-qPCR.....	108
4.3	Results and Discussions.....	112
4.3.1	Reconstitution of Preloaded Primers.....	112
4.3.2	Segmented RT-PCR.....	116
4.3.3	Fully-Quantitative Detection with an Increased Assay Flow Rate for Rapid Gene Expression Analysis.....	121
4.4	Conclusions.....	125
Chapter 5	Conclusions and Future Work.....	128
5.1	Conclusions.....	128
5.2	Recommendations for Future Work.....	132
5.2.1	“Lab-in-a-Needle” Concept Device.....	132
5.2.2	Verification of Critical Target Gene List.....	135
5.2.3	Printed Electronics in Microfluidics.....	136
	Bibliography.....	138

ABSTRACT

Liver assessment is currently one of the most important medical diagnostics. The inherent complexities of current gold standards of liver assessment such as histopathology and Liver Function Tests are generally incompatible with point-of-care testing (POCT). Hence, there is a real need for a truly practical Lab-on-a-Chip (LOC) system POCT liver assessment. The embodiment of such a system should feature *rapid, full-automation, ease-of-use*, and *sample-in-answer-out* diagnostic capabilities. To date, reported microfluidic devices for POCT liver assessment remain largely rudimentary as they embody semi-integrated, semi-quantitative and single gene detection capabilities.

In this thesis, we propose the use of microfluidics Nucleic Acid Testing (NAT) to demonstrate the feasibility for a practical and clinically pertinent POCT liver assessment device. In our research, we propose and realize two different integrated LOC systems for multiplex gene expression studies in POCT hepatotoxicity assessment. For our first integrated LOC system, we propose and describe an LOC system (stationary PCR approach) which encompasses the entire NAT process for *sample-in-answer-out* capabilities. Our first integrated LOC system embodies the integration and *semi-automation* of a tissue sample preparation module, a polymeric chip-compatible thermal cycler for multiplex RT-PCR, and a real-time fully-quantitative fluorescent detection methodology. To evaluate the feasibility of our first integrated LOC system in POCT hepatotoxicity assessment, we select and validate two different liver-critical genes of interest (GOIs) – namely, alanine transaminase and aspartate transaminase, based on critical liver protein biomarkers used in Liver Function Tests. To validate our integrated LOC system, we perform a preclinical drug toxicity animal study via the administration of cyclophosphamide, and subsequent microfluidic gene expression analysis depicts up-regulations in the GOIs' expression levels – consistent with results obtained from conventional methodology.

For our second integrated LOC system, we propose and describe an LOC system (continuous-flow PCR approach) which features a small equipment footprint and embodies an easy-to-assemble fluorescent detection module. With a contact-based isothermal heating setup, a polymeric biochip embodying a segmented flow approach, and a fully-quantitative coordinate-based detection methodology, we describe a method for multiplex gene expression analysis on a continuous-flow PCR platform. We demonstrate the *rapidness* of our second LOC system using the same drug-treated mice tissue samples, and illustrate significant reductions in RT-qPCR completion, wherein the entire process can be completed in <20mins – 3.9-fold faster than a conventional thermal cycler and ~3.15-fold faster than our first integrated LOC system embodying the stationary PCR approach.

In short, both of our integrated LOC systems are designed to be somewhat close to a practical POCT liver assessment device, and feature *semi-automation*, relative *ease-of-use*, and *sample-in-answer-out* capabilities for multiplex gene expression analysis. Collectively, the contributions from both of our integrated LOC systems are pertinent towards the realization of a truly practical POCT liver assessment device.

AUTHOR'S PUBLICATIONS

Provisional Patent

- [1] J. S. Chang, G. S. Lim, L. Zhang, Z. P. Wang, R. Wu, K. Cui, and S. Wong, “A Lab-in-a-Needle Device for Point-of-Care Testing, and a Method of Using Microfluidic Nucleic Acid Testing for Tissue Assessment”, filed on March 13, 2015 and accorded US provisional application number 62/133,014.

Technical Disclosure

- [2] J. S. Chang, G. S. Lim, L. Zhang, Z. P. Wang, R. Wu, K. Cui, and S. Wong, “A Novel, Rapid, Low Cost Lab-in-a-Needle System for On-The-Spot Drug Toxicity Classification”, filed with Nanyang Technological University on September 29, 2014, NTU Ref: TD/270/14.

Journal Papers

- [3] G. S. Lim, J. S. Chang, L. Zhang, R. Wu, Z. P. Wang, K. Cui, and S. Wong, “A Lab-on-a-Chip System Integrating Tissue Sample Preparation and Multiplex RT-qPCR for Gene Expression Analysis on Liver Function Test Biomarkers for Point-of-Care Hepatotoxicity Assessment”, *Lab Chip*, 15, 4032-4043, doi:10.1039/c5lc00798d (2015).
- [4] G. S. Lim, J. S. Chang, R. Wu, Z. P. Wang, K. Cui, and S. Wong, “A Segmented Multiplex Continuous-Flow Polymeric RT-PCR Microfluidic System with a Coordinate-based Quantitative Detection for Point-of-Care Gene Expression Analysis”, submitted for publication in *Biomedical Microdevices* on Aug 25, 2015, manuscript currently revision.

Conference Papers

- [5] G. S. Lim, R. Wu, and Z. P. Wang, “A Rapid, Low-cost Lab-on-a-Chip System for On-the-Spot Liver Toxicity Assessment ”, *Lab-on-a-Chip World Congress*, Sep 25-26, 2012, San Diego, USA.
- [6] G. S. Lim, R. Wu, and Z. P. Wang, “Segmented Continuous-Flow RT-PCR with Coordinate-based Quantitative Detection”, *Lab-on-a-Chip Asia*, May 19-20, 2015, Singapore.
- [7] G. S. Lim, R. Wu, and Z. P. Wang, “Microfluidic RT-qPCR for Point-of-Care Liver Toxicity Assessment”, *Advances in qPCR and dPCR*, May 21-22, 2015, Singapore.

LIST OF FIGURES

Figure 1-1	Simplified process chain for Nucleic Acid Testing.....	5
Figure 2-1	Procedural flow chart for histopathological tissue preparation.....	17
Figure 2-2	Consolidated flow model indicating the flow of information and artifacts between all users involved in an Anatomic Pathological assessment.....	19
Figure 2-3	a) Key liver biomarkers involved in liver disease classification; b) Diagnostic flowchart for Liver Function Tests.....	22
Figure 2-4	Complete flowchart of DNA microarray analysis (Step 1) for gene expression profiling, followed by confirmation procedures (Step 2) to affirm the validity of results. The RT-qPCR confirmation step is a feasible approach for microfluidic NAT.....	27
Figure 2-5	a) Quartz-based Affymetrix® GeneChip used in DNA microarray analysis; b) Affymetrix® fluidics station used for washing, staining and amplification of the hybridized cRNA.....	28
Figure 2-6	Progression timeline of genetic materials used in microfluidic PCR.....	32
Figure 2-7	Some designs of stationary, chamber-based PCR microfluidic chips.....	39
Figure 2-8	Some designs of stationary, multi-chamber-based PCR microfluidic chips.....	40
Figure 2-9	Some designs of continuous-flow PCR microfluidic chips.....	42
Figure 2-10	Typical setup of capillary electrophoresis.....	47
Figure 2-11	Components involved in a real-time detection module integrated with a DNA amplification system.....	48
Figure 2-12	Schematic diagram of the semi-quantitative microfluidic borosilicate glass device featuring integrated RNA extraction and RT-PCR for single gene analysis.....	53
Figure 3-1	Sample preparation microfluidic chip design: a) Schematic layout of the microfluidic chip indicating the various features; b) Model of the microfluidic chip in an exploded view illustrating the different PMMA layers, tissue microhomogenizer attachment for tissue lysis, mechanical screws/nuts and silicon membranes for valve control, and attached NanoPort™ ferrules for fluid input and actuation.....	63
Figure 3-2	RT-PCR microfluidic chip design: a) Schematic layout of the microfluidic chip indicating the various features; b) Model of the microfluidic chip in an exploded view illustrating the different PMMA layers, aluminium heat block, and attached NanoPort™ ferrule.....	65

Figure 3-3	Schematic depicting the tissue sample preparation setup featuring a syringe pump, PMMA microfluidic chip with preloaded microbeads and buffers in reagent reservoir chambers, external Eppendorf tubes for collection, and an external air compressor to provide a chamber mixing function.....	67
Figure 3-4	Schematic depicting the multiplex microfluidic RT-qPCR setup featuring a syringe pump, PMMA microfluidic chip on an aluminium heater connected to an Arduino microcontroller, and 3MP CMOS camera module with 550nm longpass filter and 470nm blue excitation LEDs connected to a computer.....	71
Figure 3-5	Illustration of RT-PCR microfluidic chip preparation with colored dye and brown adhesive tape: a) Loading of RT-PCR assay into single loading inlet to be sequentially distributed into preloaded reaction chambers; b) Sealing of vent outlets and loading of immiscible mineral oil to separate individual reaction chambers to prevent cross-contamination.....	72
Figure 3-6	RT-PCR and fluorescent detection setup: Photograph of entire microfluidic setup with a chip-compatible thermal cycler and attached 3MP CMOS camera module with 550nm longpass filter and 470nm blue excitation LEDs.....	75
Figure 3-7	Comparison of A260/280 ratios as a measure of mRNA purity between microfluidic and manual extraction methods from five random mouse tissue samples.....	78
Figure 3-8	Reconstitution of dried preloaded primers with varying final concentrations in RT-PCR reagent assay for ALT target gene followed by on-chip thermal cycling: a) Gel electrophoresis image showing varying final-primer concentrations (0.1 μ M, 0.25 μ M, 0.5 μ M, 0.75 μ M and 1.0 μ M) against a 100-bp DNA ladder; b) Signal intensity plot (r.f.u) against final-primer concentration using data derived from gel electrophoresis electropherogram plots. Experiments were performed in triplicates, with error bars shown accordingly.....	80
Figure 3-9	Agarose gel electrophoresis image taken under trans-UV illustrating the specificity of multiplex RT-PCR targeting both ALT and AST genes of interests, and β -actin housekeeping gene from 3x-dosaged mice liver tissue samples. The 12 reaction chambers on our microfluidic chip allowed four replicate groups, each with three target fragments.....	82
Figure 3-10	Comparison of the differences in AST, ALT and β -actin cDNA concentrations after varying levels (1x and 3x) of drug treatment (cyclophosphamide, 200mg/kg body wt.). Experiments were performed in triplicates, with results normalized to the untreated control.....	83

Figure 3-11	Quantification of real-time fluorescence emission for a) AST, b) ALT and c) β -actin at 3x drug dosage using ImageJ, with images captured at the annealing/extension phase of each PCR cycle.....	89
Figure 3-12	Procedure for microarray data analysis from GEO.....	91
Figure 4-1	Continuous-flow RT-PCR microfluidic chip design: a) Schematic layout of microfluidic chip with different regions indicated; b) Model of microfluidic chip in exploded view illustrating the PCB copper trace heater, with attached NanoPort™ ferrules and tubings.....	101
Figure 4-2	Schematic setup of our continuous-flow RT-PCR system.....	104
Figure 4-3	Circuit diagram for temperature control.....	104
Figure 4-4	Our fluorescence detection module with camera unit and mounted blue transilluminator.....	105
Figure 4-5	Illustration of flow sequence in the microchannel of our segmented continuous-flow RT-PCR chip. A single RT-PCR assay is used to reconstitute the dried preloaded primers in the GOI primer chambers before entering into the central microchannel. Assay droplets with a volume of ~50nL were separated with immiscible PCR-grade mineral oil to prevent cross-contamination.....	109
Figure 4-6	Illustration of coordinate system used by our end-point quantitative detection methodology to determine C_t values of GOIs. In this system, the loops of the annealing/extension region would determine the integer C_t value while the position of the targeted droplet along the length, $L=50\text{mm}$, of the microchannel would allow the C_t value to be resolved to 1 decimal place (i.e. C_t value is a function of loop number and length, L of microchannel).....	111
Figure 4-7	a) Agarose gel electrophoresis image illustrating the effect of GOI primer chamber design (where Design 1 is a 2.5 loop serpentine microchannel; Design 2 is a rectangular chamber; and Design 3 is a single loop serpentine microchannel) on primers reconstitution and cDNA amplification efficiency, and tested using ALT as the gene of interest on the untreated control at a flow rate of $0.5\mu\text{L}/\text{min}$; b) Comparison of the effect of surface area-to-volume ratio on primer reconstitution and cDNA amplification efficiency.....	114
Figure 4-8	a) Agarose gel electrophoresis image taken under trans-UV illustrating the functionality of duplex RT-PCR targeting both ALT and AST genes of interests from untreated mice liver tissue samples, with the signal band intensities indicating the amount of cDNA amplicons synthesized at different flow rates; b) Average amplification efficiencies of continuous-flow RT-PCR at $0.5\text{--}3.0\mu\text{L}/\text{min}$ flow rates. Experiments were performed in triplicates, with results normalized to the positive control.....	118

Figure 4-9	a) Average PCR completion times for the conventional thermal cycler and different flow rates of our continuous-flow RT-PCR setup; b) Plot of average amplification efficiencies (%) of both ALT and AST against average rate of RT-PCR reaction (min^{-1}). Experiments were performed in triplicates, with error bars shown accordingly.....	120
Figure 4-10	A sample fluorescence image captured by the fluorescence detection module integrated with the microfluidic RT-PCR setup illustrating the fluorescence intensity gradient along the annealing/extension region of the microfluidic chip. The non-fluorescent segments of the continuous-flow assay belonged to the mineral oil layer separating both ALT and AST RT-PCR assays.....	123
Figure 5-1	An example of use of “Lab-in-a-Needle” device with liver biopsy for liver assessment.....	134

LIST OF TABLES

Table 2-1:	Comparison of gold standards for liver assessment and microfluidic NAT...	24
Table 2-2:	Types of liver toxicity analytical methods.....	26
Table 2-3:	Material properties of common polymeric substrates.....	37
Table 2-4:	Two different categories of thermal-cycling systems and their embodiments.....	43
Table 2-5:	Tabulation of different microfluidic heating systems and their required amplification times.....	46
Table 2-6:	Comparison of different reported LOC devices and their applications.....	51
Table 3-1:	RT-PCR targets and primers sequences (5'-3').....	73
Table 3-2:	Comparison of average cycle threshold (C_t) values of conventional qPCR machine (CFX96 Touch™) with our microfluidic setup.....	88
Table 3-3:	Compilation of the top 25 genes with highest change factor after rat hepatotoxicity drug treatment.....	93
Table 4-1:	Different assay flow rates through our continuous-flow PCR microfluidic chip, the time taken per PCR cycle, and the total time taken to complete 35 cycles of RT-PCR.....	121
Table 4-2:	Evaluation of C_t values of the different GOIs from untreated and 3x dosage mice liver tissue samples by observing the x-y positions on the annealing/ extension region of the continuous-flow microfluidic chip where the fluorescence intensities are 20% higher than that of an NTC assay.....	123
Table 4-3:	Comparison of C_t values of conventional qPCR machine (CFX96 Touch™) with our continuous-flow RT-PCR microfluidic setup.....	125

LIST OF ABBREVIATIONS

POCT	Point-of-Care Testing
LOC	Lab-on-a-Chip
NAT	Nucleic Acid Testing
RNA	Ribonucleic Acid
mRNA	Messenger RNA
GOI	Gene of Interest
PCR	Polymerase-Chain Reaction
DNA	Deoxyribonucleic Acid
RT-PCR	Reverse-Transcription PCR
ALT	Alanine Transaminase
AST	Aspartate Transaminase
ALB	Albumin
ALP	Alkaline Phosphatase
TBIL	Total Bilirubin
DBIL	Direct Bilirubin
GGT	Gamma Glutamyl Transpeptidase
cDNA	Complementary DNA
cRNA	Complementary RNA
RT-qPCR	Quantitative RT-PCR
MEMS	Micro-Electromechanical Systems
PDMS	Polydimethylsiloxane
PC	Polycarbonate
PMMA	Polymethylmethacrylate

COC	Cyclic Olefin Copolymer
CE	Capillary Electrophoresis
UV	Ultraviolet
bp	Base Pairs
TE	Tris-EDTA
TAE	Tris-Acetate-EDTA
C _t	Cycle Threshold
NTC	No Template Control
PCB	Printed Circuit Board
SAV	Surface Area-to-Volume Ratio

CHAPTER 1 Introduction

This introductory chapter describes the background/motivation, objectives, contributions and organization of the thesis.

1.1 Background and Motivation

The “third revolution of medicine” largely embodies multidisciplinary fields involving the convergence of life sciences, physical sciences, mathematics and engineering. The outcome of this third revolution includes the innovation of revolutionary medical discoveries, procedures, devices, etc. One such possible practical and clinically pertinent medical device is a point-of-care testing (POCT) diagnostic device for liver assessment. Such a device requires a technological ‘pull’ provided by the engineering field and a clinical ‘push’ from the domain of medical and life sciences. POCT is defined by the Centers for Disease Control and Prevention (CDC, USA) as “tests designed to be used at or near the site where the patient is located, that do not require permanent dedicated space, and that are performed outside the physical facilities of clinical laboratories” [1].

The Medical Device Manufacturers Association (MDMA, USA) that the biomedical device industry would grow rapidly, with a worldwide market of US\$164B in 2010 to an estimated gargantuan US\$228B by 2015 [2]. This rapid growth in the biomedical device industry mirrors the increasing demand for medical diagnostics, where the overall global market for the latter was valued at US\$45.6B in 2012, and expected to

grow at approximately 7% to an estimated US\$64.6B in 2017 [2]. Of particular interest, the microfluidics (or Lab-on-a-Chip (LOC)) device market for POCT applications forms a critical subset of this biomedical device industry, wherein the pharmaceutical, biomedical science and *in-vitro* diagnostics domains are its key driving factors.

Liver assessment is one of the main domains in medical diagnostics, largely due to the pertinence of liver health in society. The American Liver Foundation [3] reports that one in ten Americans suffers from liver disease, and liver disease is ranked as one of the top ten causes of death in USA. In some countries, such as those in Southeast Asia, liver cancer is even more prevalent. For example, liver cancer ranks as the fourth most common type of cancer amongst Singaporean men [4]. Additionally, liver toxicity (or hepatotoxicity) is critical during drug administration for therapeutic intervention (e.g. during chemotherapy). In view of these concerns, medical diagnosis for liver assessment is imperative, particularly for liver health and hepatotoxicity.

Liver assessment generally falls under two main categories [5] – liver health (including liver disease, liver damage and liver cancer) and hepatotoxicity. Liver disease and hepatotoxicity diagnostics traditionally involve two commonly used analytical methods – histopathology (such as microscopy and tissue study) and blood parameter analysis (otherwise known as Liver Function Tests). Both methods are generally accepted as traditional gold standards by the medical community for liver assessment. Nevertheless, despite their acceptance, it is well known that they suffer from several shortcomings, particularly their inherent associated complexities which are largely incompatible with POCT. For example, the requirements of traditional

liver assessment often involve trained personnel, multistep procedures, and complex logistics, including requiring multiple tools and equipment, and significant amount of processing/administrative time [5-7]. These shortcomings largely conflict with the basic requirements of POCT.

To resolve the aforementioned intrinsic issues of traditional liver assessment, LOC techniques may be employed to realize system miniaturization and procedural simplification. This would alleviate the aforesaid issues, including reduced consumption of reagents, integration of several modules, the possibility of system automation and shorter process completion time [8]; thereby congruous to POCT. With POCT, liver assessment tests could be brought “conveniently and immediately to the patient” [9], enabling these on-site liver assessment tests to be compatible with emerging trends in medical diagnostics such as theranostics, genomic medicine, companion diagnostics and personalized healthcare [10].

At this juncture, microfluidic devices reported in literature for liver assessment (such as hepatotoxicity) are largely rudimentary. Particularly, they often require extensive off-chip sample preparation procedures (such as liver tissue lysis and nucleic acid extraction), lack multiplex gene expression analysis capabilities, lack on-chip fully-quantitative detection functions [11], etc. Although these microfluidic devices serve as an important step towards a POCT for liver assessment, their applications hitherto to a practical POCT are limited. In our opinion, a truly practical LOC system for POCT liver assessment necessitates the embodiment of rapid, fully-automated, ease-of-use, and sample-in-answer-out diagnostic capabilities for multiplex gene expression analysis. To the best of our knowledge, no such device has been reported to date.

Interestingly, the current art of microfluidics in liver assessment (particularly hepatotoxicity studies) embodies *in-vitro* liver drug metabolism and toxicity studies via cell/tissue culture maintained in biomimetic microenvironments [12-14], and *in-situ* quantification of cellular fluorescence intensities to observe enzymatic reactions or expressions in toxicity-induced hepatocytes [15]. This present-day approach to microfluidic hepatotoxicity assessment is largely an adaptation of the aforementioned traditional histopathology transposed onto LOC platforms. This is, hence, generally incompatible with POCT diagnostics, particularly “on-the-spot” patient care because it requires time-consuming cell or tissue culture growth and the subsequent analysis.

At this juncture, there are several reported microfluidic nucleic acid testing (NAT) work [8, 16-18], comprising a biomolecular diagnostic approach targeting different applications such as pathogen/disease detection and identification of environmental pollutants. Although these microfluidic NAT work have reportedly demonstrated their pertinence to POCT, their practical applications in companion diagnostics and personalized healthcare are however limited. This is largely due to differences in the nature of their applications (e.g. in forensic DNA studies [19, 20] vis-à-vis the diagnosis of a patient’s health) or the lack of a specific target application [21]. Interestingly, despite the advancement of microfluidic devices in NAT work, reported POCT *ex-vivo* liver assessment NAT-based devices in literature remain exiguous, largely due to the following challenges:

- (i) Sample preparation in terms of tissue handling and extraction of ribonucleic acid (RNA) in a microfluidic device;
- (ii) Multiplex gene expression analysis; and

- (iii) Selection of target messenger RNAs (mRNAs) that are indicative of liver damage [8, 22].

The need to accommodate the aforementioned challenges would possibly provide for the realization of a fully-integrated LOC microfluidic device with the following functions – simple and rapid tissue sample preparation and RNA extraction, multiplex nucleic acid amplification, and real-time fluorescence detection (see Figure 1-1). This envisioned LOC device would be capable of performing the entire NAT process depicted in Figure 1-1, and would feature fully-quantitative detection capabilities to analyze a select group of liver-critical mRNAs with significant (and detectable) responses to liver damage.

Figure 1-1 Simplified process chain for Nucleic Acid Testing

In short, there is a real need to explore the possible integration of NAT into an LOC platform, specifically for liver assessment in order to demonstrate the potential for a POCT NAT-based liver diagnostic device. The liver assessment arising from the extent of liver damage (e.g. from liver disease or hepatotoxicity) could be diagnosed using gene expression studies (a companion bio-diagnostic technique to NAT [23]) on liver critical genes of interest (GOIs). The cellular response of these GOIs may be observed in terms of the changes in their gene expression levels to an external event or

stimuli, e.g. liver damage arising from alcoholic cirrhosis or drug administration. Such a microfluidic NAT diagnostic device for liver assessment, presently unavailable, would effectively be a viable and highly advantageous alternative to the current gold standards of liver assessment, particularly in terms of a practical POCT.

From a practical POCT application perspective, the device *should* feature the following:

- (i) Sufficiently rapid processing capability to complete the entire NAT process in a short interval, e.g. within 30 minutes, thereby largely allowing for “on-the-spot” liver assessment;
- (ii) Full-automation of the sample preparation, nucleic acid amplification and nucleic acid detection functions to simplify the diagnostic process, and for ease-of-use (user-friendliness);
- (iii) Sample-in-answer-out capabilities with integrated sample preparation and real-time gene expression analytical functions, and a simple operating interface to minimize procedural error and without requiring the device operator to be trained in molecular biology; and
- (iv) Low cost, thereby an attractive alternative to current gold standards of liver assessment, while adhering to established safety protocols.

From a biomedical device design perspective, such a device would be congruous with the aforementioned “third revolution of medicine” for the realization of advanced, practical and useful medical diagnostic devices. The multidisciplinary effort would be a symbiotic collaboration between multiple parties of diverse disciplines – convergence of life sciences, physical sciences, mathematics and engineering.

1.2 Objectives

In view of the real need for the aforesaid POCT NAT-based liver diagnostic device, the *Grand Objectives* of the overall research program is the design and realization of a practical and clinically pertinent POCT liver assessment device featuring of *rapid, fully-automated, ease-of-use, and sample-in-answer-out* diagnostic capabilities for multiplex gene expression analysis on a select group of liver-critical GOIs. This *Grand Objectives* would further include the realization of said POCT liver assessment in a highly compact form – possibly even as a “Lab-in-a-Needle” system as described in our technical disclosure (NTU Ref: TD/270/14) and provisional patent (US Application No.: 62/133,014). This research program is a collaboration between Nanyang Technological University (NTU), Singapore Institute of Manufacturing Technology (SIMTech) and Houston Methodist Research Institute (HMRI), affiliated with Weill Cornell Medical College (Cornell University, USA).

The *Specific Objectives* of this research program pertain to the second and third chains of Figure 1-1 (Nucleic Acid Amplification and Nucleic Acid Detection) for the design and realization of said practical and clinically pertinent POCT liver assessment device. The objectives for these two chains and their combination thereof are now stipulated in turn.

For the nucleic acid amplification process (second chain of NAT in Figure 1-1) of the proposed LOC device, the *Specific Objectives* are to:

- (A-i) Establish a control experimental protocol where the results thereof serve as a benchmark for the outcome in polymerase-chain reaction (PCR) microfluidics;
- (A-ii) Select and validate critical GOIs that serve as precursors to critical Liver Function Tests' protein biomarkers;
- (A-iii) Design an appropriate polymer biochip capable of multiplex on-chip nucleic acid amplification, including ease-of-use attributes;
- (A-iv) Design a thermal cycler system compatible with said polymer biochip, featuring capabilities including rapid temperature ramping/cooling rates to allow for high-speed deoxyribonucleic acid (DNA) amplification, and stable and accurate temperature control; and
- (A-v) Simplify the liver diagnostic gene list obtained from gene expression profiling datasets to allow selection of up to 12 liver-critical GOIs for liver assessment on a microfluidic chip.

For the nucleic acid detection process (third chain of NAT in Figure 1-1) of the proposed diagnostic device, the *Specific Objectives* are to:

- (B-i) Investigate, select and design a fluorescence detection scheme compatible with our thermal cycler system (*Specific Objective* (A-iv) above) and the multiplexing requirement for DNA amplification (*Specific Objective* (A-iii) above); and
- (B-ii) Establish a fully-quantitative detection methodology to analyze changes in gene expression levels of selected GOIs (*Specific Objective* (A-ii) above).

For the embodiment of an integrated POCT NAT-based prototype device (largely comprising of the combination of the second and third chain of NAT in Figure 1-1), the *Specific Objectives* are to:

- (C-i) Integrate said two separate modular components of nucleic amplification and detection to form a functional single-unit prototype; and
- (C-ii) Validate said integrated prototype using drug-treated mouse liver samples to ascertain the viability of using microfluidic NAT for hepatotoxicity assessment.

1.3 Contributions

In the perspective of the *Grand Objectives* delineated in the preceding section and pertaining to the second and third chains of NAT (and their integration thereof), we made a number of contributions towards the realization of a practical and clinically pertinent POCT liver assessment device. In our contributions, congruous to our *Specific Objectives*, we designed and validated two different integrated LOC systems in multiplex gene expression studies for POCT hepatotoxicity assessment. Our contributions for each of the two NAT-based LOC systems are now succinctly delineated in turn.

In our first integrated LOC system where we adopted a stationary PCR approach (wherein the PCR reaction assay remained static within a fixed chamber), our first contribution is the design of a contact-based thermal cycling system capable of multiplex reverse-transcription PCR (RT-PCR) using polymeric biochips (*Specific*

Objectives (A-iii) and (A-iv)). Our second contribution is the integration of a tissue sample preparation module (contributed by a colleague in this research program) and a fully-quantitative real-time detection module (*Specific Objectives* (B-i) and (B-ii)) with the aforementioned thermal cycling system. This enabled us to realize an integrated POCT NAT-based microfluidic device (*Specific Objective* (C-i)) embodying *semi-automation* and *sample-in-answer-out* capabilities.

Our third contribution pertains to the benchmarking of the output of our first integrated LOC system (*Specific Objective* (A-i)) where we ascertained the gold standard methodology for performing NAT at HMRI (USA), and established control experimental protocols in SIMTech (Singapore). In our fourth contribution, we evaluated the feasibility of our first integrated LOC system in POCT hepatotoxicity assessment, by selecting two different liver-critical GOIs (i.e. alanine transaminase (ALT) and aspartate transaminase (AST)) based on critical liver protein biomarkers [24-27] used in Liver Function Tests (*Specific Objective* (A-ii)), and performed multiplex gene expression analysis on the two GOIs after a preclinical drug toxicity animal study (*Specific Objective* (C-ii)).

In our second integrated LOC system where we adopted a continuous-flow PCR approach (wherein the PCR reaction assay would be actuated through different isothermal temperature zones), our fifth contribution is the design and realization of a contact-based isothermal heating setup, as well as a polymeric biochip capable of multiplex RT-PCR via a segmented continuous-flow approach (*Specific Objectives* (A-iii) and (A-iv)). Using the fluorescent detection module from our first integrated LOC system, in our sixth contribution, we established a fully-quantitative coordinate-based

end-point detection methodology for multiplex gene expression studies (*Specific Objectives* (B-i), (B-ii) and (C-i)).

In short, collectively from our six contributions pertaining to our two integrated LOC systems and with the aforementioned drug toxicity study, we demonstrated the efficacy of our integrated LOC systems featuring *semi-automation*, relative *ease-of-use* and *sample-in-answer-out* capabilities for rapid POCT hepatotoxicity assessment. Overall, this is somewhat close to a practical and clinically pertinent POCT liver assessment device with *rapid, fully-automated, ease-of-use*, and *sample-in-answer-out* diagnostic capabilities for multiplex gene expression analysis.

For completeness, further to our aforesaid contributions, our contributions include the initiation of a preliminary selection process by performing a statistical analysis on a database repository of microarray gene expression profiling experiments from comprehensive animal studies [23, 28-31]. This preliminary selection process is imperative for the identification and selection of the critical GOIs (*Specific Objectives* (A-v)) to be used in using microfluidics NAT for liver assessment. Overall, this contribution is critical towards the realization of comprehensive NAT diagnosis for a practical and clinically pertinent liver assessment.

1.4 Organization

This thesis is organized as follows:

Chapter 1 presented an introductory background and motivation to this research program, together with the objectives and contributions of this thesis.

A comprehensive literature review is presented in Chapter 2, which includes background information on current gold standards for liver assessment, and a literature survey on PCR microfluidics research. This review serves as a preamble to Chapters 3 and 4, and largely delineates reported work on NAT microfluidic devices. To identify crucial design factors for the realization of a practical POCT device, pertinent aspects of reported NAT microfluidic devices are succinctly outlined – including the different types of sample used; materials used in biochip fabrication; design components of microfluidic chips, thermal cycling and detection systems; and the applications of current LOC platforms; etc. It is interesting to note that a fully-integrated LOC platform with sample-in-answer-out capabilities for POCT liver assessment remains unreported. We also describe in this chapter a process for a preliminary selection process to identify other liver-critical GOIs (apart from the aforementioned AST and ALT) – crucial towards a clinically pertinent liver assessment.

In Chapter 3, we describe, for the first time, an integrated LOC system that is somewhat close to a practical POCT hepatotoxicity assessment device – it embodies both tissue sample preparation and multiplex real-time RT-PCR. It features semi-automation, relatively easy-to-use, and sample-in-answer-out capabilities for multiplex gene expression analysis. Our tissue sample preparation module, contributed by a

colleague in this research program, incorporates both a microhomogenizer and surface-treated paramagnetic microbeads for high purity mRNA extracts. We also describe a primer preloading surface treatment procedure and single-loading inlet on our multiplex real-time RT-PCR prototype which allows us to simplify off-chip handling procedures for ease-of-use. To demonstrate the efficacy of our LOC system for POCT hepatotoxicity assessment, we perform a preclinical animal study with the administration of cyclophosphamide, followed by a gene expression analysis on two critical Liver Function Tests' protein biomarkers (i.e. AST and ALT). In short, in this chapter, we illustrate the feasibility of multiplex gene expression analysis in an integrated LOC system as a viable POCT means for hepatotoxicity assessment. Additionally, we perform a statistical analysis on a database repository of microarray gene expression profiling experiments. This process allows us to identify and rank a group of critical GOIs related to hepatotoxicity in extensive drug studies.

In Chapter 4, we describe a rapid segmented continuous-flow RT-PCR microfluidic system featuring a polycarbonate microfluidic chip-based setup with small equipment footprint and an easy-to-assemble fluorescence detection module. We also describe, for the first time, a primer preloading procedure in a continuous-flow PCR system to simplify off-chip handling procedures and to allow for ease-of-integration with potential upstream modules. We demonstrate our multiplex microfluidic system by the duplex amplification of said two GOIs (i.e. AST and ALT), and demonstrate the use of an adopted coordinate-based quantitative detection methodology. We validate our microfluidic system in a POCT application by performing a gene expression analysis for said GOIs on the same drug-treated mice tissue samples. In short, in this chapter, we demonstrate the efficacy of our multiplex continuous-flow RT-PCR

microfluidic system with small equipment footprint, and its applicability in rapid POCT hepatotoxicity assessment.

Chapter 5 concludes this thesis and describes potential directions for further research on Microfluidic NAT for POCT Liver Assessment. We first describe a conceptual device, termed as “Lab-in-a-Needle”. We propose the embodiment of such a device and illustrate an example of use in POCT liver biopsy assessment. We also describe in this chapter the need to validate the preliminary group of liver-critical GOIs selected and ranked in Chapter 3 using extensive animal studies for hepatotoxicity and liver disease classification. Finally, we outline the viability of integrating printed electronics with microfluidics for the realization of practical POCT devices.

CHAPTER 2 Literature Review

In this literature review, we will first provide a brief introduction to traditional gold standards for liver assessment – histopathology and Liver Function Tests. A more recent gold standard for comprehensive liver assessment known as gene expression profiling will thereafter be described to illustrate the pertinence of PCR (and NAT) in liver assessment.

Secondly, we will provide a comprehensive literature review on PCR microfluidics (pertinent for POCT) from its conception to the current state-of-the-art. In the design phase of an LOC device for specific applications, a researcher faces a plethora of design and material choices and these would affect the performance, amplification/detection efficiencies, sample-type compatibilities and other tradeoffs. This literature review serves to identify reported crucial focus areas for our realization of an integrated LOC system using microfluidics technology for POCT liver assessment. We will also explore reported microfluidics devices for liver assessment to delineate their limitations for a practical POCT.

Thirdly, we describe in this chapter our process for a preliminary selection process for liver-critical GOIs (apart from the aforementioned AST and ALT), where a statistical analysis has to be performed on a database repository of microarray gene expression profiling experiments. Such a selection process is pertinent towards a comprehensive liver assessment, and towards the aforesaid microfluidic device for practical and clinically pertinent liver assessment.

The information delineated in this chapter is in part extracted from our Qualifying Examination Report submitted to Nanyang Technological University in 2011.

2.1 Gold Standards for Liver Assessment

In this section, we will provide background information and methodologies of the gold standards of liver assessment, namely the following:

- (i) Histopathology;
- (ii) Liver Function Tests; and
- (iii) Gene expression profiling.

The first two approaches are traditional gold standards for liver assessment, while the third approach is increasingly accepted as a gold standard. We will also delineate the inherent associated complexities of these traditional gold standards that render them largely incompatible with POCT, and outline the general methodology of gene expression profiling to illustrate the pertinence of NAT in liver assessment.

2.1.1 Histopathology

Histopathology for liver assessment is largely a microscopy examination based on liver biopsies, and is the first traditional gold standard for liver assessment. It is a qualitative methodology wherein the extent of liver damage is based on the “determination of the degree of fibrosis and architectural change” [32] of the extracted

liver tissue. Clinical manifestations of liver failure or liver disease is often indicated by “architectural changes of the liver parenchyma” [32] due to liver damage caused by advanced stages of liver fibrosis. It is hence not unexpected that the histological diagnosis of the degree of liver fibrosis is largely accepted as the gold standard for liver assessment in histopathology. However, the usage of histopathology in liver assessment has several critical limitations in the perspective of POCT, and these will now be discussed.

In the histopathology process, the process involves several labor-intensive and time-consuming preparation steps. Same-day results are generally not possible due to current technological limits (due to multiple processing steps, complex equipment, etc.) and most histopathological tissue processing are usually done overnight [6]. Figure 2-1 below depicts a typical histopathological process flow embodying several critical steps, each with their own necessary tools or equipment. In Figure 2-1, it can be easily appreciated that this process is largely inefficient, with multiple waiting times between each step required for sample preparation.

Figure 2-1 Procedural flow chart for histopathological tissue preparation [6]

Notwithstanding the complexity of the preparation stage, the diagnostic process which commences with the “initiation by the clinician with the removal of a tissue specimen from the patient and terminates with the receipt of a final pathology report at the clinician’s office” [7], is equally convoluted and involves multiple personnel (see Figure 2-2). Furthermore, the histopathological scoring of the degree of liver damage is largely based on qualitative assessment of architectural change and hence is prone to “considerable intra-observer and inter-observer errors” [32]. Put simply, although histopathology is a well-established diagnostic methodology for liver assessment, the aforementioned limitations make this diagnostic method inherently incompatible with POCT.

Figure 2-2 Consolidated flow model indicating the flow of information and artifacts between all users involved in an Anatomic Pathological assessment [7]

2.1.2 Liver Function Tests

Liver Function Tests is the second traditional gold standard for liver assessment. It is embodied by a series of different biochemical tests covering a broad spectrum of liver

biomarkers. Besides the aforementioned AST and ALT, other liver biomarkers include albumin (ALB), alkaline phosphatase (ALP), total bilirubin (TBIL), direct bilirubin (DBIL), gamma glutamyl transpeptidase (GGT), etc. [5] Each of these liver biomarkers can either be manually processed with individual colorimetric activity assay kits (with an accompanying fluorometer) to ascertain its relative concentration in the patient's blood sample, or obtained using multi-analyte assays with fully-automated biochemistry analyzers (such as Roche[®], Beckman Coulter[®] and Abbott[®]).

The general principle of Liver Function Tests is that the presence of foreign synthetic chemicals or invasion of pathogens in the liver results in liver damage. The varying degree of liver damage would subsequently cause atypical changes in the concentrations of aforementioned liver protein biomarkers (which are detectable in blood serum). These said abnormal changes, which are the underlying detection principle behind Liver Function Tests, are generally used in a clinical setting as a non-invasive preliminary screening test to provide a reasonable estimate of liver damage [5].

There are several key liver biomarkers (typically 7 to 8 different target protein biomarkers) that are tested for during a Liver Function Test. With regard to hepatotoxicity, two of the more critical liver protein biomarkers are ALT and AST; these are the two biomarkers used in our integrated LOC systems for POCT hepatotoxicity assessment (see Chapters 3 and 4 later). The elevations of both ALT and AST are often strongly suggestive of hepatocellular injury or liver damage (see Figure 2-3b where an abnormal increase in AST/ALT ratio of $\geq 4\text{-}5\text{x}$ during drug therapy is often indicative of hepatotoxicity) [33]. Similarly, for liver disease

classification, the different combinations in the concentrations of the various key liver biomarkers often provide clinicians a preliminary (or screening) insight to the type of liver disease a patient is suffering from [5] (see Figure 2-3a).

The main disadvantages for Liver Function Tests are that the manual process is largely labor-intensive and requires trained technicians to process the assays; while the biochemical auto-analyzers are generally bulky (requires a significant equipment footprint) and somewhat expensive (typically costing tens of thousands of dollars). Consequently, Liver Function Tests typically require a few days to a week (logistically slow) for the final blood analysis test report to be delivered to the clinician's office [5].

Besides the aforementioned incompatibilities of Liver Function Tests with POCT, they also suffer from several fundamental shortcomings [5]. Although they are appropriately suitable as a form of initial evaluation of liver health, these tests are largely perceived to be unreliable in terms of sensitivity and specificity, and could suffer from fluctuations in test results depending on the period whereby the blood is drawn from the patient. Additionally, because the test sample used is blood drawn from the patient, Liver Function Tests are not liver-specific (e.g. AST can originate from elsewhere, e.g. damaged muscle tissue) and could result in nonspecific test outcomes.

Put simply, it is hence of worthwhile interest to use biopsied liver tissue samples (vis-à-vis blood samples in Liver Function Tests) to avoid nonspecific test results, and to adopt the established methodology of Liver Function Tests to NAT for liver assessment. This is the premise of the research reported in this thesis.

a

Summary of Liver Function Test Abnormalities				
Disease	ALT	AST	GGT	ALP
Viral hepatitis	+++	+++	++	N/+
Drug induced hepatitis	++	++	++	N/+
Chronic active hepatitis	++	++	++	++
Infectious mono-nucleosis hepatitis	++	++	++	N
Primary biliary cirrhosis	++	++	+++	++
Alcoholic cirrhosis	N	++	+++	N/+
Intrahepatic cholestasis	++	++	+++	++
Extrahepatic cholestasis	++	++	+++	+++
Hepatoma	N/+	++	++	++

b

Figure 2-3 a) Key liver biomarkers involved in liver disease classification; b) Diagnostic flowchart for Liver Function Tests [5]

For the sake of completeness, Table 2-1 summarizes the current limitations of histopathology and Liver Function Tests in the perspective of POCT for liver assessment. Additionally, the grand objectives aforementioned in Section 1.2 are also indicated in the table to illustrate the advantages of microfluidic NAT in liver assessment for POCT.

Table 2-1: Comparison of gold standards for liver assessment and microfluidic NAT

	Present-day Gold Standards [5]		Our Grand Objectives
	Histopathology	Liver Function Tests	Microfluidic NAT
Sample Preparation Process	Complex, Multistep	Simple, Multistep	Simple, Single-step
Sample Preparation Time	Extremely Long	Long (Manual) Short (Automated)	Short
Equipment Required	Multiple	Multiple	Stand-Alone
Types of Equipment	Expensive, Bulky	Expensive, Bulky	Low Cost, Small footprint
Trained Technician Required?	Yes	Yes	No
Separate Facility Required?	Yes (Pathology Laboratory)	Yes (Diagnostic Laboratory)	No
Type of Diagnosis	Qualitative	Quantitative	Quantitative
Diagnostic Process	Complex	Simple	Simple
Diagnostic Time	Extremely Long	Immediate	Immediate
Deliverance of Test Results	1-2 weeks	3-4 days	On-the-Spot

2.1.3 Gene Expression Profiling

Histopathology and Liver Function Tests are the two traditional gold standards for liver assessment. Of late, gene expression profiling is increasingly accepted as a gold standard. Gene expression profiling is a high-throughput biomolecular technique involving DNA microarray technology. It is often used to determine the physiological state and cellular response of an organism to particular drug treatments, hence critical in liver toxicity assessments. This diagnostic method is considered to be semi-quantitative, and is typically used to identify crucial GOIs (precursors to the aforesaid protein biomarkers) [23].

Historically, gene expression profiling was first conceived in 1982 [34], and evolved from a DNA analytical method known as Southern Blotting. With rapid advancements in DNA microarray technologies, it has significantly improved its throughput, from the first reported simultaneous analysis of 378 differential gene sequences to the present ability to process tens of thousands of genes sequences simultaneously [23]. The simultaneous measurement of the genotypical (genetic expression) activity of tens of thousands of differential genes sequences allows researchers to construct an overall view of the phenotypical cellular function (physical expression). This is critical for investigating cellular response in toxicology studies.

Being a more recently developed toxicogenomics approach (as opposed to traditional histopathology and Liver Function Tests, see Table 2-2), microarray gene expression profiling allows for much shorter term drug dosing periods (e.g. 24 hours) in animal toxicity studies, compared with a minimum of two to four weeks dosing periods for

the traditional methods. Consequently, this allows for identification of adverse events in the liver before they histopathologically manifest themselves – this ‘early-stage’ hepatotoxicity assessment is hence highly advantageous and desirable in view of therapeutic treatment, if any.

Table 2-2: Types of liver toxicity analytical methods

Gold Standards for Liver Assessment	Characteristics
Histopathology	<ul style="list-style-type: none"> • Traditional liver assessment methods • Requires at least 2-4 weeks dosing periods in an animal studies
Liver Function Tests	
Gene Expression Profiling	<ul style="list-style-type: none"> • More recently developed toxicogenomics approach • Allows for short term (24hrs) dosing periods • Early gene expression changes observed at 24hrs are useful in predicting pathological changes after 2 weeks • Good estimate of ‘early-stage’ hepatotoxicity

Step 1 of Figure 2-4 illustrates a flowchart of the entire microarray gene expression profiling process. Step 2 depicts the commonly used confirmation techniques to verify the statistical data obtained from gene expression profiling; the RT-qPCR (i.e. quantitative RT-PCR) approach in Step 2 mirrors the NAT process delineated in Figure 1-1 which we integrated onto a microfluidics platform (see Chapters 3 and 4 later).

Figure 2-4 Complete flowchart of DNA microarray analysis (Step 1) for gene expression profiling, followed by confirmation procedures (Step 2) to affirm the validity of results. The RT-qPCR confirmation step is a feasible approach for microfluidic NAT

The entire microarray gene expression profiling process (Step 1) will now be delineated. The initial step involves the isolation and purification of total RNA extracted from the cells after cell lysis (using commercial lysis and extraction kits). The purified RNA is thereafter quantified and assessed to ensure suitable RNA integrity for ensuing downstream applications. Following that, the RNA is converted or synthesized into complementary DNA (cDNA) and subsequently complementary RNA (cRNA). This is a relatively crucial step as RNA is highly fragile and prone to degradation. For this reason, some research groups [31] outsource this step to specialized laboratories or institutes (such as the Roswell Park Cancer Institute, USA) to ensure high quality RNA would be available for hybridization onto Affymetrix® GeneChips (see Figure 2-5a).

The next step involves transferring the hybridized cRNA in the GeneChip into a compatible fluidics station wherein the sample would be washed to remove unwanted reagents, stained with fluorescent dyes and amplified to improve signal emission. The system then performs a scan to ascertain the amount of RNA bound to particular regions of the DNA microarray via fluorescence emissions. The data is subsequently collected and analyzed using specific bioinformatics tools.

As DNA microarray gene expression profiling is a diagnostic tool to identify important regulatory genes and is considered semi-quantitative [23]. In Step 2 (Figure 2-4), RT-qPCR is typically performed to confirm the up- or down-regulations of the previously identified genes. Finally, traditional liver toxicity studies such as histopathological examination or Liver Function Tests are, on occasion, carried out to further validate the results.

Figure 2-5 a) Quartz-based Affymetrix[®] GeneChip used in DNA microarray analysis; b) Affymetrix[®] fluidics station used for washing, staining and amplification of the hybridized cRNA [23]

It has been reported [35], although somewhat contentious, that after the DNA microarray process wherein thousands of genes have been analyzed, it is necessary “to

rank these genes according to their degree of participation in a physiological process”. However, this process is largely logistically complicated, expensive [23] and is time consuming.

Of specific interest, RT-qPCR (‘Step 2’) is now largely considered as the gold standard for qualitative and quantitative gene expression analysis. RT-qPCR is largely suitable for routine use, requires significantly reduced sample input, and is comparatively simple to be adapted onto an LOC platform. With the advantages of microfluidics technology, such as full-automation (from sample preparation to reagent treatment to analysis), reduced cost in terms of reagents used and equipment involved, diagnostic completion time, small equipment footprint, etc., RT-qPCR is a feasible approach for POCT.

2.2 PCR Microfluidics

As aforementioned in Section 2.1.3, PCR (and NAT) is pertinent for liver assessment. We will now review PCR microfluidics in view of POCT compatibility.

PCR microfluidics is a relatively mature field of study, with a development history of 20 years. The first PCR microfluidic device was reportedly developed in 1993 by Northrup *et al.* [36], embodying a stationary, chamber-based PCR silicon chip, built using the technologies derived from the semiconductor industry. The primary advantage it offered was the significant reduction of time needed for PCR completion. In 1998, Kopp *et al.* [37] developed the first continuous-flow PCR chip. Using a 20-

cycle PCR amplification of a specific DNA fragment from *Neisseria gonorrhoeae* on a flow-through glass microchip, Kopp *et al.* managed to significantly further reduce the time needed for PCR completion [37].

PCR microfluidics technology has since continuously progressed, both for stationary-chamber and continuous-flow PCR. At this juncture, it is well recognized that solely reducing the time needed for PCR completion is insufficient. For example, for microfluidic PCR to match the capabilities of current conventional benchtop PCR technologies, there is an increasing need for the former to have an output efficiency that is comparable to the latter. By developing a fully integrated system, the entire process (from sample preparation to PCR to detection) could be automated to provide yet another significant advantage; for POCT, the ability to perform sample-in-answer-out diagnosis is advantageous.

In the perspective of our research, particularly the application and identification of current state-of-the-art LOC technologies to an original field of study in liver assessment, we will now review the literature in the following six aspects of PCR microfluidics:

- (i) Genetic materials for nucleic acid amplification;
- (ii) Materials used for biochip fabrication;
- (iii) Microfluidic biochip layout and design;
- (iv) Heating/Thermal-cycling systems;
- (v) Lab-on-a-Chip nucleic acid detection systems; and
- (vi) Applications of reported LOC platforms.

2.2.1 Genetic Materials For Nucleic Acid Amplification

In early microfluidic PCR research, a large number of research groups used synthetic DNA templates known as λ -DNA [38] for proof-of-principle as well as basic characterization of their PCR biochips. These λ -DNA samples often have a pre-determined nucleic acid sequence and fragment size, making them easy and convenient for subsequent detection and identification. Subsequently, the advancement of microfluidic PCR research resulted in the use of DNA obtained and purified from actual cells. For example, Xiang *et al.* [39] performed their experiments on *Escherichia coli* cells and were able to amplify a specific DNA fragment from the cells cultured using specific complementary oligonucleotide primers for the identification of that particular fragment.

To explore the application aspect of microfluidic PCR, diagnosis or detection of diseases and pathogens were reportedly pertinently demonstrated. For example, Hagen *et al.* [16] used influenza viral RNA as their starting material, while Cady *et al.* [40] tested on cultured *Salmonella typhimurium* bacteria cells, and used specific oligonucleotide primers to identify a particular *S. typhimurium* DNA fragment. Lee *et al.* [41] obtained RNA from human embryonic kidney cell cultures infected with the human immunodeficiency virus (HIV). In the field of cancer diagnostics, Backhouse *et al.* [42] successfully demonstrated DNA amplification and detection of multiple myeloma from individually isolated, cancerous white blood cells.

There are several advantages of using pure sample material based on actual viral RNA/DNA, individual cancer cells or laboratory grown pathogen-infected cell

cultures, such as the assurance of the purity of the sample genetic material, and sufficient quantity of the base materials to ensure a high signal detection output. If raw or crude samples such as tissues or blood were used instead, there would be an additional need to homogenize the tissues, or isolate the specific type of cells required in the blood. Moreover, it would be necessary to purify the sample in order to improve the efficiency of the PCR process and to prevent unwanted entities from interfering with the DNA amplification. In the case of Zhang *et al.* [21], crude human blood was used as the sample material, and after a DNA purification process, a particular fragment of human genomic DNA was selected and amplified via oligonucleotide primers. Similarly, Qiu *et al.* [43] used raw saliva samples containing *B. cereus* bacteria cells as their sample material, and had to include an additional sample purification module into their microfluidic device. To illustrate the progression in the samples-of-choice embodied by the difficulties in sample preparation, Figure 2-6 shows the rough outline of the timeline of genetic materials used in microfluidic PCR research.

Figure 2-6 Progression timeline of genetic materials used in microfluidic PCR

As aforementioned in the introduction in Chapter 1, the usage of crude tissue samples and ensuing RNA extraction remains exiguous in literature due to the difficulties in terms of tissue handling and extraction of RNA in a microfluidic device [8, 11]. In the perspective of this research program and our grand objectives, we will delineate in Chapter 3 our proposal to use crude biopsied liver tissue samples in our microfluidic PCR application, and the ensuing RNA extraction (i.e. third chain of the progression timeline in Figure 2-7). On the basis of our review, we remark that that PCR microfluidics for POCT liver assessment is unreported in literature (save microfluidic hepatotoxicity assessment by Shaw *et al.* [11]); and the idea of using purified liver tissue lysates as our sample material is interesting and has the potential of achieving the aforesaid objectives delineated earlier in our *Grand* and *Specific Objectives*.

2.2.2 Materials Used For Biochip Fabrication

From the mid-1990s to the early-2000s, PCR microfluidic chips were primarily made of silicon [36, 44, 45]. The primary reason for this was that mature semiconductor technologies such as photolithography and silicon wafer etching techniques could easily be transferred and adapted to micro-electromechanical systems (MEMS) research. This facilitated PCR microfluidic chips with relatively accurate and precise dimensioning of their microchannels and chamber designs. Additional components such as micro-heaters and temperature sensors could likewise be built and integrated onto the silicon wafer. Further, silicon has excellent thermal conductivity, which is advantageous for fast temperature ramping, a necessity for quicker PCR completion [8].

However, it soon became apparent that new materials were required to replace silicon. This was because silicon has an inhibitory effect on the PCR process and could severely decrease the DNA amplification efficiency. Ke *et al.* [46] attempted to circumvent this limitation by growing a thin layer of silicon dioxide via thermal oxidation process on the inner surface of the fluidic chamber (to ensure its compatibility with the PCR reaction). Nevertheless, silicon remains incompatible with real-time PCR, in part because of its opacity. This is because real-time PCR requires the transmission of excitation light through the substrate material to irradiate the PCR assay.

Glass as a substrate subsequently became a popular alternative choice of material to silicon [8, 47, 48]. Besides the ease of fabrication (similar to that of silicon substrate), glass has two notable advantages over silicon, namely excellent optical transparency and biocompatibility. These advantages render glass appropriate as a material for PCR as well as real-time detection, without the need for additional substrate treatment or modifications. Nevertheless, as the fabrication cost of both silicon and glass substrates is relatively high, they are not ideal as disposable biochips [8].

Since the early-2000s, there has been an increasing interest in the use of polymers for the fabrication of PCR biochips. This is because of a number of potentially superior attributes to silicon, and they include the following. First, their fabrication is easier, leading to lower cost and hence the ensuing disposability. Second, the fabrication of polymer chips is faster (shorting the process from design to fabrication to testing), allowing experiments to be conducted more expeditiously. Third, the physical and chemical properties of polymers can also be easily fine-tuned to fit specific

applications. This is by means of either altering and optimizing the type, composition and dispersion of different component polymers and their stabilizers, or mixing in modifier additives [8]. For example, the polymeric materials could be made biocompatible to preserve the integrity of the biomolecules; or hydrophobic to prevent adsorption of the DNA to the surface walls of the PCR biochip, as well as to prevent water stiction [49]. Lastly, the optical properties of polymeric materials could also be adjusted. This is particularly applicable where optical transparency is an important consideration, for example in fluorescence PCR detection [8], as in the case of our experiments (see Chapters 3 and 4 later).

Of all the various polymeric materials, the common ones include polydimethylsiloxane (PDMS) [21, 38, 39, 50], polycarbonate (PC) [51-53], polymethylmethacrylate (PMMA) [20, 54, 55], SU-8 [56, 57] and cyclic olefin copolymer (COC) [41, 58]. Their popularities are largely due to their compatibility to PCR, including a glass transition temperature greater than 95°C to ensure thermal stability during the denaturation temperature phase, good chemical resistance to cleaning solvents and buffers required for PCR, excellent dimensional stability, as well as high optical transparency [8]. Some of the more pertinent material properties of several key polymers are summarized by Becker *et al.* [59] in the following table.

In efforts to exploit the beneficial properties of the individual substrates, ‘hybrid’ chips such as silicon-glass [60, 61], silicon-polymer [57] or glass-polymer [62] have been reported. In view of the large plethora of material choices for biochip fabrication, it is imperative that the choice of material used for microfluidic biochips be made with a number of considerations. With respect to our research program, we have primarily

worked with PMMA and PC for their attractive properties in terms of chemical resistance, glass transition temperatures as well as optical properties, and also for the ease of chip design, fabrication and bonding in SIMTech's facilities. These aspects were considered in our biochips' designs and fabrication in Chapters 3 and 4.

Table 2-3: Material properties of common polymeric substrates [59]

Polymeric Substrate	Polymer Type	Glass Transition Temperature, T_g (°C)	Useful Temperature Range (°C)	Thermal Conductivity (W/mK)	Visible Light Transmissivity (%)	Chemical Resistance			Native Surface Charge	Fabrication
						Acid	Alkalis	Organic Solvent		
PMMA	Thermoplastic	106	70-100	0.186	92	Good	Excellent	Poor	Yes	Injection molding, hot embossing, laser ablation, micro-milling
PC	Thermoplastic	150	130-140	0.21	89	Good	Poor	Poor	Yes	Injection molding, hot embossing, micro-milling
COC	Thermoplastic	90-136	73-80	0.16	92-94	Good	Good	Poor-Fair	No	Injection molding, hot embossing, micro-milling
Polyimide	Thermoplastic	285	73-240	0.2	87	Fair-Good	Fair-Good	Fair	No	Injection molding, hot embossing, laser ablation, micro-milling
PS	Thermoplastic	100	40-70	0.18	90	Good	Good	Poor	Yes	Hot embossing, micro-milling
PDMS	Elastomer	120	40-110	0.17-0.9	91	Fair-Good	Poor-Fair	Poor	Yes	Soft lithography

2.2.3 Microfluidic Biochip Layout And Design

There are two general types of microfluidic PCR systems – stationary, chamber-based PCR; and continuous-flow PCR. In the former (first developed by Northrup *et al.* [36], and improved upon by Daniel *et al.* [63]), the PCR assay remains static within a fixed chamber while the thermal heating system cycles through the three specific PCR-required temperatures, heating and cooling both the PCR assay as well as the device. On the other hand, the latter (first developed by Kopp *et al.* [37]) does not require a thermal cycling system. The design of the PCR chip would, as expected, be significantly different, with microchannels etched into the substrate chip. The ensuing PCR assay would subsequently flow or move through three fixed temperature zones, and the PCR assay would be heated or cooled as it passes from one zone to another.

The primary advantages of the stationary, chamber-based PCR are its simplicity of the microfluidic chip design, as well as the ensuing mitigation of complications of thermal cross-talk between the reaction chambers [8]. They are also readily configured to accommodate the number of amplification cycles, simply by increasing the number of thermal cycles and ensuing time needed. Examples of stationary, chamber-based PCR [41, 44] are depicted in Figure 2-7 below. The common feature in both example designs is the presence of a single fixed-volume PCR reaction chamber, in which thermal cycling occurs.

Figure 2-7 Some designs of stationary, chamber-based PCR microfluidic chips [41, 44]

The limitation of the single-chambered, stationary PCR is that it is largely incompatible with high-throughput or multiplex PCR, wherein multiple samples have to be amplified simultaneously for increased efficiency. Not unexpectedly, multi-chambered, stationary PCR was subsequently reported. In these designs, it is imperative to ensure that there is thermal uniformity to maintain the repeatability and reliability of multiple simultaneous PCR reactions amongst the chambers. This is typically achieved by means of careful thermal design and the chamber layout. Figure 2-8 depicts a couple of reported multiplex stationary PCR devices [21, 39]. A similarity in these designs is the multiple fixed-volume PCR reactions chambers, thereby permitting parallelization (i.e. simultaneous) of several PCR reactions.

Figure 2-8 Some designs of stationary, multi-chamber-based PCR microfluidic chips [21, 39]

Continuous-flow PCR has gained increasing popularity in recent years, in part due to the inherent limitations of stationary, chamber-based PCR. Furthermore, because of the intrinsic limitation of thermal cycling of the latter wherein time is required for temperature ramping to switch between the three different temperatures, stationary, chamber-based PCR is generally slower than continuous-flow PCR.

The advantages of continuous-flow PCR include faster heat transfer due to the fact that only the PCR assay is required to be heated and not the entire device, and shorter thermal cycling time intervals due to the elimination of temperature ramping. This not only reduces the total time needed for PCR completion, but complex or highly

efficient thermal heating systems are also not required. This is because temperature feedback control to ensure accurate thermal cycling is unnecessary [22].

The design of a continuous-flow PCR is more complicated than that of stationary, chamber-based PCR. This is largely for two reasons. First, the configuration and dimensions of the microchannels that pass through the three different temperature zones. Second, the sample flow rate has to be optimized and calibrated to obtain the required efficiency in terms of output and time taken for completion. Due to the need for flow and depending on the length of the microchannels, the PCR assay volumes in continuous-flow PCR are usually greater than that in stationary, chamber-based PCR. In applications where a small volume is critical, continuous-flow PCR would be disadvantageous. Furthermore, unlike the stationary, chamber-based PCR, continuous-flow PCR has an inherent fixed number of amplification cycles, due to the predetermined layout of the microchannels. Finally, there is a need for proper thermal insulation between the three temperature zones to reduce thermal cross-talk.

Two examples [40, 64] of common continuous-flow PCR designs are depicted in Figure 2-9.

Figure 2-9 Some designs of continuous-flow PCR microfluidic chips [40, 64]

It is imperative to consider both the advantages and disadvantages of the two different types of microfluidic PCR biochip designs. With regard to our aforesaid requirement for multiplex amplification of several different GOIs simultaneously, it is prudent to consider using stationary, chamber PCR as our initial starting point for proof-of-principle for microfluidic NAT (see Chapter 3). However, in terms of improving the speed of completion of the entire NAT process, we also developed and realized a continuous-flow PCR LOC system (see Chapter 4). In short, we exploit the idiosyncratic advantages of the two types of microfluidic PCR biochip designs according to our specific needs.

2.2.4 Heating/Thermal-Cycling Systems

The thermal heating system in microfluidic PCR is one of the more critical components in a microfluidic PCR device. There are two important considerations. First, it is desirable that the rate of temperature ramping of the thermal heating system be sufficiently high to ensure that the PCR completion time is kept short. Secondly, the thermal heating system must be able to maintain a stable temperature because the activity of Taq Polymerase, a key component of the PCR assay, is extremely temperature-sensitive [8].

There are two general categories of heating systems – contact and non-contact thermal heating, and they are depicted in Table 2-4 below. The former utilizes the resistive heating of thin metal films or metal/ceramic blocks which are in direct contact with either the PCR assay or with the microfluidic chip. In the latter however, the heating element does not come into direct contact with microfluidic chip.

Table 2-4: Two different categories of thermal-cycling systems and their embodiments

Categories of Thermal Cycling Systems	Embodiments
Contact Heating	<ul style="list-style-type: none">• Thin Film Heating• Metal Block Heating• Ceramic Block Heating• Continuous-Flow Water-Mediated Heating
Non-Contact Heating	<ul style="list-style-type: none">• Rapid Hot Air Heating• Infra-Red Radiation• Laser-Mediated Heating• Inductive Heating• Inductive Heating• Microwave Irradiation

For thin film contact thermal heating, the thin metal films are often fabricated together with the microfluidic chip via thin film deposition such as sputtering, electroplating, chemical vapor deposition or electron beam evaporation. In these heating systems, the most commonly used materials are platinum [47, 65, 66] and polysilicon [36, 67]. Platinum is a metal that has several desirable properties, including being easily micro-machined using thin film deposition, excellent resistive heating, high melting point, as well as good chemical and oxidation resistance. Polysilicon is a polymer that is stable at high temperatures and has variable resistive heating properties depending on the doping [8].

For metal or ceramic block contact thermal heating [37, 40, 45], the heating element is typically simple by using metal blocks or thermoelectric ceramic blocks, without the need for micromachining. However, the primary disadvantage of heating blocks is their bigger thermal mass and larger specific heat capacities. This results in lower rates of temperature ramping and hence the ensuing long PCR completion time. However, precise temperature control (critical for high efficiency PCR) is comparatively easier to establish in heating blocks [8]. The implementation of a metal heating block in our first integrated LOC system (Chapter 3) allowed us to quickly establish a proof-of-concept device for POCT hepatotoxicity assessment.

For non-contact thermal heating systems, the main issue of a thermal mass hindering a rapid temperature ramping rate is eliminated [8]. There are several reported types of non-contact thermal heating systems. The more common and developed systems will now be described in turn.

The rapid hot air temperature cycling [41, 68] method accomplishes quick cycling and switching between different air streams of appropriate temperature. Because air has a lower heat capacity, the temperature ramping rate is rapid. The infra-red radiation for thermal heating is reported by several groups [41, 69, 70], and is arguably considered to be one of the more effective means of non-contact thermal heating, with extremely rapid rates of temperature ramping. Laser-based heating systems involve the photo-thermal heating effect from a diode laser. Being a point source, the main advantage of this method is that it allows for the fastest heating/cooling rates, while maintaining very precise localized heating. Nevertheless, temperature control with this thermal system is difficult [71, 72]. Lastly, a system based on inductive thermal heating reported by Pal *et al.* [73] allows for easier fabrication techniques, while maintaining a fast temperature ramping rate. This system has a small power consumption requirement, hence making it attractive as a portable thermal heating system operated by batteries.

Based on our literature review, Table 2-5 summarizes the different types of microfluidic heating systems, and their corresponding required amplification time. In view of the myriad combinations of different possible designs and intentions of the reported work in this Table 2-5, this table is largely a general indication of these heating systems. For example, the biochemical aspect of PCR is an important consideration, which strongly depends on the type of sample or length of target DNA to be amplified [74].

Table 2-5: Tabulation of different microfluidic heating systems and their required amplification times

Research Group	PCR System	Substrate Material Used	Heating System	PCR Reaction Volume (μL)	Number of Amplification Cycles	Amplification Time Required (min)
Daniel <i>et al.</i> (1998) [75]	Stationary Chamber	Silicon	Platinum Resistor	1.5	30	< 12
Ke <i>et al.</i> (2007) [46]	Stationary Chamber	Silicon-Glass	Platinum Resistor	3.0	30	28
Huang <i>et al.</i> (2006) [50]	Stationary Chamber	PDMS-Glass	Platinum Resistor	0.5	20	< 22
Lagelly <i>et al.</i> (2004) [65]	Stationary Chamber	Glass	Titanium/Platinum Resistor	0.2	30-35	< 30
Xiang <i>et al.</i> (2005) [39]	Stationary Chamber	PDMS-Glass	Thin Film Heater	0.9-7.0	35	< 81
Qiu <i>et al.</i> (2011) [43]	Stationary Chamber	PC	Thermoelectric Module Heater	25	25	30
Easley <i>et al.</i> (2004) [76]	Stationary Chamber	Glass	Infrared Radiation	0.55	30	11
Giordano <i>et al.</i> (2001) [70]	Stationary Chamber	Polyimide	Infrared Radiation	1.7	15	4
Hühmer <i>et al.</i> (2000) [77]	Stationary Chamber	Glass-Polyimide	Infrared Radiation	0.16	10	< 12
Kim <i>et al.</i> (2006) [38]	Continuous-Flow	PDMS-Glass	Thermoelectric Module Heater	50	30	4 – 28
Sayers <i>et al.</i> (2006) [78]	Continuous-Flow	FEP-Teflon	Thin Film Heater	0.25	50	< 84
Han <i>et al.</i> (2011) [79]	Continuous-Flow	PC	Polyimide Heater	25	35	< 23

2.2.5 Lab-On-A-Chip Nucleic Acid Detection Systems

The majority of research groups employ the well-established method of laser-induced fluorescence detection [8] for their LOC nucleic acid detection systems. A minority, nevertheless, employ the electrochemical detection approach [80, 81] which correlates the changes of the impedance of the reaction solution to the increase in amplified

products. The reason for the wider adoption of the former is because this detection setup is largely more sensitive, and is easier to implement and integrate with the upstream nucleic acid amplification module [8].

There are two general types of detection methods – end-point detection and real-time detection. For end-point detection, one of the more common methods in a microfluidic platform is based on the conventional gel electrophoresis method (used for validation of our microfluidic results in Chapters 3 and 4), and is known as capillary electrophoresis (CE). The concept behind CE is similar to that of gel electrophoresis [47, 82], where the amplified nucleic acid products are first loaded into an electrophoretic microchannel (or capillary) containing a CE sieving medium. Thereafter an external electric field is applied across both ends of the microchannel to separate the nucleic acid fragments by size. Finally, detection or identification of the fragments separation is carried out using a single intercalating dye, a light excitation source, and a CCD/CMOS camera or sensor to capture the fluorescence emission. An example is depicted in Figure 2-10 [50].

Figure 2-10 Typical setup of capillary electrophoresis [50]

One of the first CE component to be integrated with a nucleic acid analytical device was reported by Wooley *et al.* [60] in 1996. They reported rapid CE separation of the amplified DNA fragments (on glass substrates) in less than two minutes – establishing the feasibility of attaining fast DNA sample analyses in integrated microfluidic devices. Subsequently, integrated PCR-CE microfluidic systems were developed using a wide variety of substrates, such as polymeric materials, monolithic glass, and hybrid materials (e.g. silicon-glass, PDMS-glass) [8].

The primary shortcoming of CE is its semi-quantitative detection methodology (similar to gel electrophoresis, see Chapter 3) which requires conversion of the qualitative separated fragment emission intensities to useful semi-quantitative data for gene expression studies [11, 83]. To circumvent this shortcoming, several research groups employ fully-quantitative real-time detection methods, for example that which is depicted in Figure 2-11 [84]. Real-time detection involves the continuous monitoring of the fluorescence emission from the reaction solutions. It also involves the detection of the increase in fluorescence intensities from the fluorophoretic intercalating dyes during amplification.

Figure 2-11 Components in a real-time detection module integrated with a DNA amplification system

[84]

Two notable real-time PCR detection systems were reported by Cady *et al.* [40] and Xiang *et al.* [39]. In the former, Cady *et al.* [40] reported a continuous-flow PCR chip, whereby the fluorescence emission from the SYBR[®] Green I dye was detected during the 72°C extension step of every PCR cycle. In the latter, Xiang *et al.* [39] reported a stationary, chamber-based PCR chip, where the fluorescence emission from the SYBR[®] Green I dye was captured during the 55°C annealing phase of the PCR process using a fluorescence microscope.

In terms of multiplexing capabilities, real-time PCR detection suffers from a somewhat severe drawback. Typically, for simultaneous detection of multiple DNA sequences, TaqMan probes can be used for the selection and amplification of specific fragments [85]. This embodiment in a PCR system is known as a homogenous multiplex PCR system (where multiple gene sequences are being identified in a single chamber), and is limited by a relatively low multiplexing grade [85]. Because there is no fragment separation, there is a consequent restriction on the number of fluorophoretic intercalating dyes that can be spectrally differentiated. Hitherto, if the number of fluorophores used is greater than three, there would be significant spectral overlap, rendering it too unwieldy for signal processing and analysis [47].

To address the aforementioned problem of low multiplexing grade, separate individual homogenous PCR chambers can be employed. This spatial separation is desirable because it avoids any spectral cross-talk [85], a somewhat popular concept explored by several groups, including Zhang *et al.* [21], Ramalingam *et al.* [84], etc.

An alternative to the homogeneous multiplex PCR system is the heterogeneous multiplex detection system. In one study, Steigert *et al.* [86] demonstrated an increase in the multiplexing grade of different DNA fragments, without the requirement for spatial separation or sample partitioning for parallel DNA amplification and detection. With this approach, up to a hundred different color-coded beads coated with specific binding fluoroprobes can be optically distinguished, thereby allowing for a greater number of different nucleic acid fragments to be identified and quantified by fluorescence.

On the basis of our literature review, our detection setup for our integrated LOC systems delineated in Chapters 3 and 4 later embodies the assembly of excitation light sources, a CMOS camera, and fluorophoretic intercalating dyes. We employ a fully-quantitative real-time detection methodology with separate homogenous PCR chambers in our first integrated LOC system (Chapter 3); and a coordinate-based fully-quantitative end-point detection methodology in our second integrated LOC system (Chapter 4).

2.2.6 Applications of Reported LOC Platforms

The applications of integrated LOC platforms or devices (embodying sample preparation, nucleic acid amplification and nucleic acid detection) reported in literature are now delineated. The most common type of application of these LOC platforms largely involves the identification or detection of certain bacteria or virus strains by targeting specific GOIs intrinsic to these bacteria/viruses. This is largely

due to the ease of the sample preparation step for bacteria/viruses, hence minimizing the need for complex microfluidic designs or procedures.

In the field of microfluidic POCT diagnostics, LOC devices have also been reported for use in other types of applications (besides bacterial or virus analysis), including analysis of diseases, pathogens and environmental pollutants. LOC devices have also been reported for use in forensics DNA analysis, helping to speed up and simplify the diagnostic process. Table 2-6 below tabulates the representative reported LOC devices with their targeted applications.

Table 2-6: Comparison of different reported LOC devices and their applications

Reported LOC Devices	Application	Comment
Our proposed device [87, 88]	Liver disease classification/Hepatotoxicity	Emerging diagnostic application for liver assessment
Lounsbury <i>et al.</i> [20]	Clinical and forensic DNA analysis	Other types of applications such as bacterial/virus, disease and environmental pollutants analysis
Pernagallo <i>et al.</i> [17]	Nucleic acid analysis	
Qiu <i>et al.</i> [43]	Nucleic acid analysis	
Ramalingam <i>et al.</i> [84]	Detection of waterborne pathogens	
Schumacher <i>et al.</i> [89]	Detection of human pathogens	Not reported for liver assessment
VanHeirstraeten <i>et al.</i> [90]	Detection of lower respiratory tract infections	
Zhang <i>et al.</i> [21]	Gene analysis	

The rapid development of reported microfluidic devices in PCR/NAT work has opened up new possibilities in liver assessment (e.g. hepatotoxicity studies). Nonetheless, at this juncture, there have been little POCT *ex-vivo* liver diagnostic

NAT-based devices reported in literature [87], largely due to the challenges in handling RNA in a microfluidic device as well as the complexity in the stringent selection criteria of liver-related GOIs that are indicative of liver damage due to liver disease or hepatotoxicity.

Based on our review, other than our work [87] detailed in Chapter 3, there is arguably only one other reported *ex-vivo* POCT NAT-based liver assessment device. Shaw *et al.* [11] developed a semi-quantitative microfluidic borosilicate glass chip (see Figure 2-12) featuring integrated RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR) capable of interrogating up-regulations of CYP1A2 (derived from cytochrome P450) gene expression levels during hepatotoxicity. This particular microfluidic device, however, embodied only a single chambered glass chip capable of a single gene expression analysis per experimental run, and a semi-quantitative methodology which required off-chip end-point analysis via capillary gel electrophoresis. Further, it required extensive off-chip sample preparation procedures (such as a liver tissue lysis), lacked multiplex gene expression analysis capability, and did not have an on-chip fully-quantitative (or real-time) detection methodology. Put simply, although this microfluidic device is an important step towards a POCT for hepatotoxicity assessment, its application to a practical POCT is somewhat limited.

Figure 2-12 Schematic diagram of the semi-quantitative microfluidic borosilicate glass device featuring integrated RNA extraction and RT-PCR for single gene analysis [11]

In summary, to the best of our knowledge, reported work on microfluidic devices for POCT liver assessment remains exiguous and largely rudimentary, with limited applications for practical POCT. As aforementioned in our *Grand Objectives* in Chapter 1, our proposed LOC device will contentiously be the first-ever integrated and fully-quantitative LOC device featuring rapid, fully-automated, ease-of-use, and sample-in-answer-out diagnostic capabilities for multiplex gene expression analysis in POCT liver assessment.

2.3 Preliminary Selection of Liver-Critical Genes of Interest

Congruous to our *Specific Objectives* in delineated in Chapter 1, it is necessary to determine a comprehensive gene list for liver assessment. In our preliminary selection process for critical GOIs in liver assessment, we have to perform a statistical analysis

on a database repository of microarray gene expression profiling experiments [23, 28-31]. Specifically, two published microarray datasets were obtained from Gene Expression Omnibus (National Center of Biotechnology Information, USA) database repository which contains a large collection of high throughput gene expression data. Both of these selected datasets were generated based on the Affymetrix[®] Rat Genome U34 Array.

The first microarray dataset (GEO accession reference number: GSE17388) was based on a reported analysis [30] of gene expression changes performed using the Affymetrix[®] chip and the aforementioned U34 array. Said reported work was strongly correlated to hepatotoxicity in the presence of 24-h (short duration) and 336-h (long duration) drug toxicity study using two different drug compounds (i.e. R1 and R2) at different doses. In view of the incomplete nature of the first microarray dataset, we incorporated only the data for R1 treatment in our analysis; see Table 3.3 later.

The second microarray dataset (GEO accession reference number: GSE5789) was based on two studies by Ovando *et al.* [28, 31]. The experimental methodology of this second microarray dataset was similar to that of the first, and involved the use of the Affymetrix[®] Rat Genome U34 Array. This second dataset investigated the administration of five different drug compounds (TCDD, PeCDF, PCB126, Binary, PCB153) at different dosages for a 13-week (i.e. extremely long or subchronic duration) period.

The analysis of the microarray datasets is succinctly described as follows: the original microarray image cell files were analyzed using the GeneChip Operating Software

(Affymetrix[®] Inc., USA). Probe-level microarray data were subsequently background subtracted and normalized using the GC-Robust Multiarray Analysis (gcRMA) method [28]. Finally, statistical analysis of this processed microarray data was performed using the Gene Set Enrichment Analysis (GSEA) platform to consolidate the intensity data per probe to the intensity for each data. This GSEA computation method would allow us to determine whether an a priori (i.e. earlier) defined set of genes shows statistically significant and concordant differences in gene expression between two biological states (e.g. treated and control samples). Collectively, the analysis of both microarray datasets would allow us to identify critical GOIs related to hepatotoxicity in extensive drug studies.

On the basis of the aforesaid two datasets, we perform statistical analysis to ascertain the preliminary list of liver-critical GOIs. This will be described in Chapter 3 later.

2.4 Summary of Literature Review

In this chapter, we have succinctly reviewed the gold standards for liver assessment, namely histopathology, Liver Function Tests and gene expression profiling, and delineated their limitations to POCT liver assessment. Further, we have illustrated the advantages of using NAT (specifically RT-qPCR) for liver assessment, in terms of potential early-stage liver damage detection as well as ease of adaptation onto a microfluidic platform.

We have presented a comprehensive and critical literature review on current state-of-the-art PCR microfluidics research – the basis for our two integrated LOC systems

embodying stationary PCR (see Chapter 3) and continuous-flow PCR (see Chapter 4) with fully-quantitative detection. Of particular interest, we have illustrated the largely rudimentary state of reported microfluidic devices for POCT liver assessment, and identified the real need for a practical POCT liver assessment device.

We have also described our process for a preliminary selection process for liver-critical GOIs where a statistical analysis has to be performed on a database repository of microarray gene expression profiling experiments. The combination of a group of relevant and liver-critical GOIs is crucial towards a clinically pertinent liver assessment.

Chapter 3 First Integrated LOC System Embodying Stationary PCR Approach and Real-Time Detection

3.1 Introduction

The work delineated in this chapter is largely extracted from our paper accepted for publication in *Lab on a Chip - Miniaturisation for Chemistry and Biology* [87].

The work delineated in this chapter largely serves as a proof-of-concept for our *Grand Objectives* delineated in Chapter 1 where we propose, design and realize an integrated microfluidic NAT system that is arguably the closest device to a practical POCT hepatotoxicity assessment device. Our integrated LOC system is based on the stationary PCR approach, and is largely compatible with the three chains of NAT (see Figure 1.1 earlier). Our integrated LOC system embodies both tissue sample preparation and multiplex real-time RT-PCR. Compared to the state-of-the-art, our microfluidic system features semi-automation, relative ease-of-use, and sample-in-answer-out capabilities for multiplex gene expression analysis (vis-à-vis the semi-quantitative, single-gene POCT microfluidic device reported by Shaw *et al.* [11]).

As aforementioned in our contributions delineated in Chapter 1, our tissue sample preparation module incorporates both a microhomogenizer and surface-treated paramagnetic microbeads [91] to perform on-chip tissue lysis and mRNA purification, allowing us to obtain high purity mRNA extracts – considerably better than manual extraction means; as delineated in Chapter 1, this tissue preparation aspect is the work of a colleague in our research group. A primer preloading surface treatment procedure

and single-loading assay inlet on our multiplex real-time RT-PCR module simplifies off-chip handling procedures, such as pre-mixing of different RT-PCR assays for GOIs, for ease-of-use. Compared to previously reported designs in the literature for microfluidic POCT hepatotoxicity assessment, our proposed device is advantageous in terms of being able to directly interrogate a crude liver tissue sample, and investigate multiple gene targets in a single experimental run. Further, with an integrated fully-quantitative methodology, we are able to reduce post-amplification procedural steps and diagnostic time, thereby allowing for sample-in-answer-out diagnostic capability.

With regard to targeted mRNA sequences used to investigate hepatotoxicity, genes related to cytochrome P450 enzyme (and its isoforms), such as the aforementioned CYP1A2 gene, are currently widely used in hepatotoxicity studies due to their role in drug metabolism [11, 29, 83, 92]. For the sake of a more comprehensive hepatotoxicity assessment, we describe in this chapter our investigation into the feasibility of mRNA genes derived from Liver Function Tests' protein biomarkers in hepatotoxicity assessment. As delineated in our literature review in Chapter 2, two critical liver protein biomarkers [24-27] that we adopt herein are AST [25] and ALT [24], where their elevations are often strongly suggestive of hepatocellular injury or liver damage. Additionally, by using said GOIs, our microfluidic NAT device may be applied to other applications, for example liver disease classification [5], wherein different liver diseases often have different AST/ALT ratios. To the best of our knowledge, the approach embodying the application of microfluidic NAT based on the protein biomarkers used in Liver Function Tests is presently unreported and largely unexplored. Further, congruous to our *Specific Objectives* in Chapter 1 to ascertain other liver-critical GOIs for comprehensive liver assessment, we describe in this

chapter our process for a preliminary selection process for liver-critical GOIs where we perform a statistical analysis on the aforementioned database repository of microarray gene expression profiling experiments (see Section 2.3 earlier).

Collectively, we describe in this chapter our proposed microfluidic NAT system, designed for a practical POCT hepatotoxicity assessment, embodying three functions: tissue sample preparation, multiplex RT-PCR and real-time fluorescent detection for gene expression analysis. To establish its feasibility for an *ex-vivo* hepatotoxicity assessment, laboratory mice were subjected to a short-term drug toxicity study, wherein cyclophosphamide, an alkylating agent often used in the cancer therapy and treatment of autoimmune disorders [93], was administered in varying dosages. In our experiments, we were able to obtain high purity mRNA extracts, and illustrate that the final cDNA concentrations of AST and ALT increased by 58.7% and 33.3% respectively after 3x drug dosage treatment. Our results demonstrated the validity of our selected mRNA gene targets in hepatotoxicity assessment. Moreover, our integrated microfluidic NAT system illustrated normalized fold changes of 1.62 and 1.31 for AST and ALT respectively during gene expression analysis. The results from our LOC system and from conventional methodology were largely consistent, thereby demonstrating the viability of using microfluidic NAT in POCT hepatotoxicity assessment.

3.2 Materials and Methods

In this section, we will first delineate the protocols involved in our preclinical drug toxicity study to investigate the effects of hepatotoxicity on our GOIs. Secondly, we will discuss the design and assembly of our semi-integrated microfluidic chips for sample preparation and mRNA extraction, and for multiplex RT-PCR. Thirdly, we will delineate the primer preloading procedure carried out to prepare the RT-PCR microfluidic chip, followed by describing the microfluidic sample preparation and multiplex RT-PCR protocols. Lastly, we will discuss the fluorescence detection of amplified cDNA, including the duplex amplification of the two GOIs and their ensuing gene expression analysis.

3.2.1 Biological Samples

A short-term (5 days) preclinical drug toxicity study was performed on common black laboratory mice (Strain name: C57BL/6J; Age: 5-7 weeks). All experiments were performed in accordance with the National Institutes of Health Guide for the care and use of laboratory animals (NIH Publications No. 80-23, revised 1996) and conducted after approval by the Institutional Animal Care and Use Committee of Houston Methodist Research Institute (HMRI).

A total of eight mice were divided into two equal groups, with one mouse in each group designated as the control and the remaining three subjected to drug treatment. The drug used was cyclophosphamide (200mg/kg body wt.) [94], and the control used

was saline with concentration of 0.9% w/v NaCl. The drug was administered through intraperitoneal route, with the first group having a single dose administered on the 1st day only; and the second group having 3 doses administered on the 1st, 3rd and 5th days. The animals were subsequently euthanized following standard carbon dioxide anaesthesia procedure on the 5th day after final drug administration. The mouse liver tissues were thereafter silated, and cut into equal small pieces (~20-30mg) and stored in RNAlater RNA Stabilization Reagent (Qiagen[®], USA) at 4°C to prevent RNA degradation.

3.2.2 Sample Preparation and mRNA Extraction Chip Design and Fabrication

Our proposed PMMA microfluidic chip for tissue sample preparation and mRNA extraction is depicted in Figure 3-1a. This microfluidic chip (and the subsequent RT-PCR microfluidic chip) was designed using Solidworks[™] software and comprised five PMMA layers (Figure 3-1b) patterned using CO₂ laser ablation with a 35W CO₂ laser (VersaLASER VLS2.30, Universal[®] Laser Systems, USA). Each PMMA layer was subsequently cleaned in a bath sonicator with 10% v/v isopropanol for 15mins and dried in a vacuum oven at 80°C overnight. All five layers were clamped between two pieces of 5mm thick borosilicate glass pieces and thermally bonded in an oven at 128°C under a 1atm pressure for 2hrs, followed by natural cooling to room temperature.

The proposed PMMA chip comprised four main components (see Figure 3-1a) which were all largely patterned in the 3rd PMMA layer, including a tissue lysis chamber,

five reagent reservoirs, a mixing chamber, and seven mechanical valves. The tissue lysis chamber had a volume of $\sim 250\mu\text{L}$, and operated in conjunction with a tissue microhomogenizer attachment (ClaremontBio[®], USA) which shredded and homogenized liver tissue samples placed within the chamber. Furthermore, an array of micro-pillars with dimensions $500\mu\text{m} \times 500\mu\text{m}$ helped to remove any unwanted tissue debris from homogenized tissue lysate.

Our chip design included four reagent reservoirs with volumes of $450\mu\text{L}$ to preload and store buffers, and another $150\mu\text{L}$ reagent reservoir for the paramagnetic microbeads from the Dynabeads[®] mRNA DIRECT[™] Purification Kit (Ambion[®], USA). During the mRNA extraction process, these buffers and microbeads were actuated from their respective reservoirs using external syringes.

For fluidic manipulation and control, mechanical valves were incorporated into our microfluidic chip. The mechanical force exerted by the external screws (see Figure 3-1b) allowed the flexible 0.25mm thick silicon membrane (BISCO[®] Silicones, Rogers Corporation, USA, catalogue number HT-6240) to be directly deformed, thereby blocking microchannels and preventing solutions from passing through [95].

Lastly, the mixing chamber had a volume of $150\mu\text{L}$, and had the critical function of mixing the tissue lysate with the microbeads and relevant buffers for mRNA purification and extraction. This mixing function was accomplished via an adopted aeroelasticity-based fluid agitation technique [96, 97] which used the spontaneous vibration of a silicone membrane (or diaphragm) induced by an external air flow.

Figure 3-1 Sample preparation microfluidic chip design: a) Schematic layout of the microfluidic chip indicating the various features; b) Model of the microfluidic chip in an exploded view illustrating the different PMMA layers, tissue microhomogenizer attachment for tissue lysis, mechanical screws/nuts and silicon membranes for valve control, and attached NanoPort™ ferrules for fluid input and actuation

3.2.3 Multiplex RT-PCR Chip Design and Fabrication

The proposed PMMA chip consisted of 12 reaction chambers connected to a central microchannel for multiplex RT-PCR reactions. Structurally, the RT-PCR reaction chambers were cut into the 1mm thick middle layer, and their top and bottom surfaces were enclosed respectively by 0.5mm thick PMMA top and bottom layers. This construction ensured minimization of the surface roughness within the reaction chambers which would otherwise result in unwanted bubble formation during the PCR thermal cycling process [98].

Our chip design comprised a single assay loading inlet with attached NanoPort™ PEEK ferrule connectors (Upchurch Scientific®, USA) and 1/32" OD tubing. The RT-PCR assay derived from the upstream sample preparation microfluidic chip was actuated through this inlet, and was equally distributed into the 12 reaction chambers. Each reaction chamber had a volume of ~10µL, with microchannels of 5mm by 0.2mm to allow the RT-PCR assay to be loaded. To minimize the undesirable effect of evaporation and the formation of bubbles, transparent adhesive PCR film seal (ThermoScientific®, Singapore) was used to seal the vent outlets during the RT-PCR process.

Figure 3-2 RT-PCR microfluidic chip design: a) Schematic layout of the microfluidic chip indicating the various features; and b) Model of the microfluidic chip in an exploded view illustrating the different PMMA layers, aluminium heat block, and attached NanoPort™ ferrule

3.2.4 Liver Tissue Sample Preparation and mRNA Extraction on Microfluidic Chip

For this procedure, all instruments, the microfluidic chips and equipment used were first sterilized with the UltraClean® Lab Cleaner (Mo-Bio Laboratories®, USA) and the experiment performed in a biosafety level 2 cabinet to minimize the presence of

RNases. To prepare the microfluidic chip for sample preparation, 150 μ L of Dynabeads[®] microbeads solution stored in the reagent reservoir was actuated into the mixing chamber using a 250 μ L Hamilton[®] glass syringe (Sigma-Aldrich[®], Singapore, catalogue number 24538-U), and a programmable syringe pump (New Era Pump Systems[®] Inc., USA, model number NE-1002x) at a flow rate of 100 μ L/min (see Figure 3-3, Dynabeads[®] microbeads). 450 μ L Lysis/Binding buffer stored in another reagent reservoir on the chip was actuated into the mixing chamber to pre-condition the microbeads for subsequent mRNA binding. The mixing function was provided by the aeroelastic vibrational action of a thin-film silica diaphragm caused by the external air compressor [96, 97]. Once the paramagnetic microbeads were sufficiently pre-conditioned, the excess Lysis/Binding buffer was actuated into the Waste Collection Eppendorf tube whilst the microbeads were held within the mixing chamber with external neodymium magnets.

A liver tissue sample, weighing approximately 20-30mg, was loaded directly into the tissue lysis chamber. The tissue sample was secured within the chamber when the tissue microhomogenizer attachment was fitted into the opening of the lysis chamber. Subsequently, 250 μ L Lysis/Binding buffer was loaded into another 250 μ L glass syringe (see Figure 3-3, Sample Inlet), and actuated into the tissue lysis chamber at a flow rate of 200 μ L/min. For tissue lysis, the microhomogenizer attachment was activated for ~1min to allow the liver tissue to be shredded and homogenized. After the completion of tissue lysis, the unwanted tissue debris and supernatant were removed by the micro-pillars as the homogenized tissue lysate was actuated into the mixing chamber, where the lysate was mixed with the microbeads for ~8mins to allow for mRNA binding.

The subsequent mRNA extraction and purification steps were performed using 450 μ L Washing buffers A/B as per a modified version of the manufacturer's suggested protocol, with a final extraction volume of 30 μ L (using the Elution buffer). The purified mRNA from each mouse liver tissue sample was collected in an Eppendorf tube preloaded with an RT-PCR master mix. To validate the efficacy of our sample preparation microfluidic chip, purified mRNA solutions extracted using our microfluidic chip was tested for their purity using the NanodropTM 2000 (ThermoScientific[®], Singapore), to ensure an A260/280 ratio of ~2.0-2.2 before proceeding to the subsequent downstream applications of RT-PCR and nucleic acid detection.

Figure 3-3 Schematic depicting the tissue sample preparation setup featuring a syringe pump, PMMA microfluidic chip with preloaded microbeads and buffers in reagent reservoir chambers, external Eppendorf tubes for collection, and an external air compressor to provide a chamber mixing function

3.2.5 Preloading of PCR Primers on Microfluidic Chip

Both forward and reverse primers of our two GOIs (AST and ALT) and our housekeeping gene β -actin were preloaded through the vent outlets/primer loading inlets (Figure 3-1a) after the PMMA microfluidic chip had been thermally bonded and sterilized under ultraviolet (UV) radiation for 15mins. 50 μ M of each primer (forward and reverse) was diluted in nuclease-free water to make up a final-volume of 10 μ L and final-primer concentration of 0.5 μ M. The preloading process was performed using a micropipette, and the 12 chambers were separated into three groups to be treated with the AST, ALT and β -actin primer pairs respectively. Subsequently, the preloaded chip was dried in a convection oven at 65°C for 15mins to allow the water in the primer solution to evaporate, leaving the dried primers deposited as powder form in the individual reaction chambers. This surface treatment process would allow simultaneous amplification of multiple GOIs with a single RT-PCR assay.

3.2.6 Multiplex RT-PCR on Microfluidic Chip

Once the purified mRNA had been extracted from the liver tissue samples, 30 μ L of the purified mRNA extract (i.e. elution buffer with the microbeads) was collected in an Eppendorf tube containing the following RT-PCR composition: 75 μ L 2x SYBR Green RT-PCR reaction mix (BioRad[®], Singapore), 3.0 μ L iScript One-Step RT-PCR reverse transcriptase (BioRad[®], Singapore) and 42 μ L nuclease-free water. This 150 μ L final RT-PCR assay was subsequently transferred from the Eppendorf tube into a 250 μ L

Hamilton[®] glass syringe connected to the RT-PCR microfluidic chip via a 1/32" OD tubing.

To demonstrate the functionality of our multiplex RT-PCR microfluidic chip, the purified mRNA extracted from the mouse liver tissue samples were subjected to thermal cycling on our chip-compatible aluminium block thermal cycler. Our chip-compatible thermal cycler was based on the open-source OpenPCR[®] (Chai Biotechnologies, USA) platform, using a custom-designed aluminium metal block for contact-based thermal cycling and an Arduino microcontroller for precise temperature control (see Figure 3-4).

For multiplex gene expression analysis, two different GOIs (i.e. AST and ALT) were amplified concurrently in each experimental run. The subsequent final cDNA concentrations of said GOIs were compared between the animals with and without drug treatment for gene expression analysis. Intron spanning primers were used for our two GOIs to ensure specific amplification of the transcribed mRNA and not the genomic DNA (see Table 3-1). Our AST and ALT primers were designed using the PrimerQuest software (Integrated DNA Technologies[®], Singapore) and synthesized *de novo* from the same company. For our housekeeping gene, β -actin was selected and used in our qPCR experiments for normalization of data. β -actin is a common housekeeping gene that is widely used as a positive control in qPCR of mouse tissues due to its constitutive expression [99, 100]. The β -actin primer assay was obtained from Qiagen[®], Singapore (Catalog No. PPM02945B), with a band size of 154-bp.

To prepare the microfluidic chip for multiplex RT-PCR, the RT-PCR assay was actuated into the 12 reaction chambers using a programmable syringe pump at a flow rate of 50 μ L/min. Once all 12 reaction chambers were sequentially filled with the RT-PCR assay, their vent outlets were sealed with the aforementioned transparent adhesive PCR film seal. Subsequently, 30 μ L of an immiscible PCR-grade mineral oil (Sigma-Aldrich[®], Singapore) was actuated using the syringe pump to remove the excess RT-PCR assay remaining in the central microchannel (into the waste reservoir). The separation of the RT-PCR assays in individual reaction chambers by the immiscible mineral oil prevents any cross-contamination during the thermal cycling process. The sequence of events illustrating the microfluidic chip preparation process is depicted in Figure 3-5.

To provide a benchmark reference to our chip, the same RT-PCR assays (with addition of forward/reverse primers at 0.5 μ M concentration) were amplified separately in Eppendorf tubes on a conventional CFX96 Touch[™] Real-Time PCR Detection System (BioRad[®], USA).

The thermal cycling process was as follows: 70°C for 2mins to allow dehybridization of the captured mRNA from the microbeads, followed by 50°C for 10mins for reverse transcription, subsequently with an initial denaturation step of 95°C for 5mins and finally 35 cycles of PCR, comprising two stages: denaturation at 95°C for 10s and annealing/extension at 55°C for 30s.

Figure 3-4 Schematic depicting the multiplex microfluidic RT-qPCR setup featuring a syringe pump, PMMA microfluidic chip on an aluminium heater connected to an Arduino microcontroller, and 3MP CMOS camera module with 550nm longpass filter and 470nm blue excitation LEDs connected to a computer

Figure 3-5 Illustration of RT-PCR microfluidic chip preparation with colored dye and brown adhesive tape: a) Loading of RT-PCR assay into single loading inlet to be sequentially distributed into preloaded reaction chambers; b) Sealing of vent outlets and loading of immiscible mineral oil to separate individual reaction chambers to prevent cross-contamination

Table 3-1: RT-PCR targets and primers sequences (5'-3')

Sequence name	Nucleic acid sequence	Number of base pairs
<i>AST</i> (Target)	GCT GAC TTC TTA GGG CGA TGG TAC AAT GGT ACA GAT AAC AAG AAC ACA CCA ATC TAC GTA TCA TCA CCA ACC TGG GAG AAC CAT AAT GCT GTG TTT TCT GCC GCC GGT TTT AAG GAC ATT CGG CCC TAT TGC TAC TGG GAT GCG GAG AAG AGA GGA CTG GAC CTC CAG GGT TTC CTG AAT GA	182
<i>AST</i> Forward Primer <i>AST</i> Reverse Primer	GCT GAC TTC TTA GGG CGA TG TCA TTC AGG AAA CCC TGG AG	
<i>ALT</i> (Target)	CTG CAG ACC CGA ACA ACA TAT TTC TGT CCA CAG GGG CCA GCG ATG CCA TCG TGA CCA TGC TCA AGC TGC TGG TAG CCG GCG AGG GCC GTG CGC GAA CCG GTG TAC TCA TTC CCA TTC CTC AGT ACC CAC TGT ACT CAG CTG CGC TGG CTG AGC TGG ACG CCG TGC AAG TGG ACT ACT ACC TGA ACG AAG AGC GCG CCT GGG CTC TTG ACA TCG CTG AGC TG	221
<i>ALT</i> Forward Primer <i>ALT</i> Reverse Primer	CTG CAG ACC CGA ACA ACA TA CAG CTC AGC GAT GTC AAG AG	

3.2.7 Fluorescent Detection of Amplified cDNA

To enable simultaneous fluorescent detection of amplified cDNA for a fully-quantitative methodology, we developed an image capturing system embodying a camera module with blue excitation LEDs to be mounted on our chip-compatible thermal cycler (Figure 3-6). A 3MP CMOS camera with mounted 550nm longpass optical filter (Island Optical Systems[®], Singapore) was fitted onto a black opaque acrylic frame with a 470nm blue LED module (IORodeo[®], USA) to provide an excitation source for the SYBR Green 1 fluorescent dye present in the RT-PCR assay. The blue LED module was affixed at an angle of ~45° to the plane of the microfluidic

chip to minimize excitation light interference on the captured light pathway of the camera unit.

Fluorescence images were captured during the annealing/extension phase (55°C) of each RT-PCR cycle [39, 101, 102] and the mean intensity from each reaction chamber was measured subsequently after RT-PCR completion using ImageJ image processing and analysis software (National Institutes of Health, USA). A standardized rectangular region (of fixed area) was used to select the fluorescence emission intensities from each reaction chamber, followed by using the measurement analysis tool to obtain the mean fluorescence intensities from the selected region of each chamber. The fluorescence intensity results were then plotted into real-time PCR amplification curves to allow us to perform gene expression analysis on said GOIs.

To evaluate the specificity of our multiplex RT-PCR process, the amplified PCR products were also visualized and analyzed off-chip using conventional gel electrophoresis with a 3.0% agarose gel (Tris-acetate-EDTA or TAE) prestained with ethidium bromide (BioRad[®], Singapore). The gels were typically run for 45mins at a constant voltage of 100V in 1X TAE buffer.

Figure 3-6 RT-PCR and fluorescent detection setup: Photograph of entire microfluidic setup with a chip-compatible thermal cycler and attached 3MP CMOS camera module with 550nm longpass filter and 470nm blue excitation LEDs

3.3 Results and Discussions

In this section, we discuss the results obtained from the experiments delineated in the previous section. Firstly, we will discuss the feasibility of microfluidic sample preparation and mRNA extraction in terms of the purity of the mRNA solution. Secondly, we will discuss the viability of dried preloaded primers in PMMA chips in RT-PCR after reconstitution in reagent assay, as well as determining the optimum final-primer concentration to be preloaded. Thirdly, we will analyze the PCR amplification specificity and efficiency of our on-chip RT-PCR system by performing off-chip gel electrophoresis on amplified PCR products extracted from the chip after RT-PCR completion. Finally, we will examine the feasibility and functionality of our image capturing system integrated with our microfluidic RT-PCR system for real-time fluorescent detection to establish a proof-of-principle for a POCT NAT-based hepatotoxicity assessment device.

3.3.1 Microfluidic Sample Preparation and mRNA Extraction

The Dynabeads[®] microbeads used in our microfluidic mRNA extraction protocols were coated with oligo (dT)₂₅ residues to allow for base binding with the polyA tails at the 3' end of most mRNA polynucleotides, hence resulting in an highly specific isolation protocol. Rather than the capture of total RNA [11, 90], the specific capture of mRNA molecules would ensure a more relevant nucleic acid sample material for ensuing gene expression analysis.

To validate the feasibility of microfluidic sample preparation and mRNA extraction, we compared the purity of the mRNA extracts obtained from our microfluidic chips as well as from the conventional manual process of using the purification kit. The purity of an RNA sample can be quantified by measuring its absorbance in a spectrophotometer (e.g. Nanodrop[™] 2000) at different wavelengths of light (i.e. 260nm and 280nm) [103]. Specifically, the ratio of absorbance of an RNA aliquot at 260nm and 280nm is used to assess its purity where an A260/280 ratio of ~2.14 is largely considered to be a pure RNA sample dissolved in Tris-EDTA (TE) buffer at pH 8 (which is the base component of the elution buffer) [103]. Lower values are often indicative of the presence of contaminants, such as proteins and phenols, which are strongly absorbent near or at the 280nm wavelength.

Figure 3-7 depicts the comparison of the purity of mRNA extracts between our microfluidic device and manual extraction method from 5 random mouse tissue samples. We observed that the best-fit horizontal mean from microfluidic mRNA extraction and from manual extraction mRNA extraction had an A260/280 ratio of ~2.17 and ~2.05 respectively. This indicated that the purity of our microfluidic mRNA extracts was considerably better than the manual mRNA extracts. This noticeable improvement in mRNA purity could be attributed to the sterile enclosed environment provided by on-chip preparation and extraction procedures. The increased quality of the purified mRNA would subsequently ensure more specific and more efficient cDNA amplification in the downstream RT-PCR process. In addition, our microfluidic mRNA extraction protocols also reduced the number of handling steps compared to the manual extraction process, and avoided unnecessary exposure to the external contaminants.

Figure 3-7 Comparison of A260/280 ratios as a measure of mRNA purity between microfluidic and manual extraction methods from five random mouse tissue samples

3.3.2 Reconstitution of Dried Preloaded Primers

To perform on-chip RT-PCR, the RT-PCR assay (comprising purified mRNA hybridized to the microbeads and RT-PCR master mix) was loaded into our RT-PCR microfluidic chip and distributed to the 12 reaction chambers to mix with the different sets of dried primers during reconstitution. During the reconstitution process, the chip was left at room temperature for 10mins to allow the dried primers to passively fully dissolve in the reagent assay. Active mixing was not performed to avoid any bubble generation which would otherwise result in uneven heat distribution within the reaction assay during thermal cycling.

During our experiments, there were concerns regarding the viability of the primers maintaining their nucleotide structure and functional integrity when exposed to high temperatures during the drying process. If the primer concentrations had decreased due to high heat degradation, the efficiency of PCR would drop significantly [104]. To verify that the primers maintain their intended functionalities after being subjected to high temperatures, different concentrations of preloaded primers were tested using the ALT target gene and corresponding primers.

Figure 3-8 depicts the differences in PCR amplification efficiencies after on-chip thermal cycling of varying final concentrations of the re-suspended dried primers. From Figure 3-8, we observed that high temperature treatment on the primers during the drying process did not have any adverse effect on the functional integrity of the primers. In Figure 3-8b, we also observed that the signal intensity of the amplified DNA product after gel electrophoresis saturated at a final-primer concentration of 0.5 μ M and higher, indicating that we were able to preload an optimum final-primer concentration of 0.5 μ M in the chip without any loss in primers functionality after the drying process.

The process of preloading the primers in dried form was critical towards the design of a microfluidic chip. In our microfluidic chip design, we were able to incorporate a single assay loading inlet to distribute the RT-PCR assays into different reaction chambers preloaded with different primer sets. This surface treatment procedure removed the need to pre-mix different primer sets for multiple RT-PCR assays, thereby significantly simplifying off-chip handling procedures (particularly for multiplex gene expression analysis).

Figure 3-8 Reconstitution of dried preloaded primers with varying final concentrations in RT-PCR reagent assay for ALT target gene followed by on-chip thermal cycling; a) Gel electrophoresis image showing varying final-primer concentrations (0.1 μ M, 0.25 μ M, 0.5 μ M, 0.75 μ M and 1.0 μ M) against a 100-bp DNA ladder; b) Signal intensity plot (r.f.u) against final-primer concentration using data derived from gel electrophoresis electropherogram plots. Experiments were performed in triplicates, with error bars shown accordingly

3.3.3 Microfluidic Multiplex RT-PCR

Multiple RT-PCR was verified on our PMMA microfluidic chip embodying 12 reaction chambers, preloaded with 0.5 μ M final-primer concentrations. Each microfluidic multiplex RT-PCR run embodied a purified mRNA extract from a single liver tissue sample targeting our two GOIs (i.e. AST and ALT) and our housekeeping gene (i.e. β -actin).

To ascertain the specificity of our multiplex RT-PCR reactions, the RT-PCR amplicons were extracted from the chip and analyzed off-chip using conventional gel electrophoresis (see Figure 3-9). We observed three clear bands on the agarose gel electrophoresis image for each replicate group (for a total of 12) in Figure 3-9, wherein the bands at 221-bp, 182-bp and 154-bp belonged to the ALT, AST and β -actin target fragments respectively. This clear band separation with minimal band smearing indicated that our multiplex RT-PCR reaction was highly specific, and verified the usage of mineral oil as a separation medium to eliminate cross-contamination between reaction chambers.

Figure 3-9 Agarose gel electrophoresis image taken under trans-UV illustrating the specificity of multiplex RT-PCR targeting both ALT and AST genes of interests, and β -actin housekeeping gene from 3x-dosaged mice liver tissue samples. The 12 reaction chambers on our microfluidic chip allowed four replicate groups, each with three target fragments

In addition, the average signal intensities obtained from the gel electrophoresis image could be converted into an electropherogram and used for qualitative analysis of end-point RT-PCR [11, 83]. From the electropherograms, we were able to determine the differences in final cDNA concentrations from liver tissue samples with/without drug treatment, wherein the strength of signal intensities are proportional to the quantity of amplified gene product present [104]. This qualitative analytic technique could be used to estimate gene expression levels of our GOIs, and investigate any changes due to cyclophosphamide administration [11]. In Figure 3-10, the signal intensities for the RT-PCR amplicons from the treated tissue samples (both 1x dosage and 3x dosage) were normalized to those from untreated control tissue sample. This allowed us to compare and average the data obtained from three biological replicates (i.e. each run encompassing one out of the three drug-treated mice from each treatment group).

In view of the statistical significance for the drug-treated groups vis-à-vis the untreated controls, we performed a 2 way ANOVA statistical analysis. Our statistical analysis comprised the data obtained from three biological replicates and three technical replicates (from each treatment group), and depicted that the variations of our two GOIs were statistically different (i.e. p-value <0.0001) between the drug-treated groups and untreated controls.

Differences in Final cDNA Concentrations

Figure 3-10 Comparison of the differences in AST, ALT and β -actin cDNA concentrations after varying levels (1x and 3x) of drug treatment (cyclophosphamide, 200mg/kg body wt.). Experiments were performed in triplicates, with results normalized to the untreated control

From Figure 3-10, we observed that the differences in final cDNA concentrations at 1x drug dosage largely had no significant difference when compared to the untreated control samples. We speculated that this lack of increase in gene expressions levels at

1x drug dosage could be attributed to low (or delayed) drug metabolism of the administered drug in the mice [105].

There were, however, significant increases in final cDNA concentrations for both AST and ALT at 3x drug dosage of cyclophosphamide (200mg/kg body wt.), with 58.7% and 33.3% increases for AST and ALT respectively. These results support our hypothesis of using a biomolecular diagnostic approach (or NAT) as a viable alternative to conventional Liver Function Tests. These elevations in gene expression levels were generally consistent with data from genetic testing using laboratory mice, wherein significant elevations (in particular ALT) were observed after excessive alcohol consumption [26] or in mouse fatty liver tissues [27], and could be used as evidence of hepatocellular damage. Additionally, we observed that β -actin mRNA expression levels remained relatively unchanged after a 1x and 3x drug dosage. These results illustrated the constitutive expression behavior of β -actin and helped to validate it as an appropriate choice of housekeeping gene and positive control for our qPCR experiments.

In a separate microfluidic experiment where DNA amplification was performed without the reverse transcription step, genomic contamination was observed to be kept to a minimum. Gel electrophoresis results showed no signal bands for both GOIs as well as the housekeeping gene, indicating the absence of an amplified PCR product. From this result, we were able to unambiguously demonstrate the validity of our intron-spanning primers used for AST, ALT and β -actin mRNA targets.

In short, the increase in genes expression levels for our two GOIs at 3x drug dosage validated the potential for using mRNA target genes based on Liver Function Tests protein biomarkers, and demonstrated the viability of a POCT NAT device for hepatotoxicity assessment.

3.3.4 Fluorescent Detection and Gene Expression Analysis

To evaluate the viability of our image capturing system in determining the differences in fluorescence emissions between RT-PCR assays targeting different GOIs, we performed real-time fluorescence emission image captures embodying the 12 reaction chambers. Fluorescence images were captured at each PCR cycle during the annealing/extension phase (55°C) [39, 101] for a total of 35 cycles.

The intensities in fluorescence emissions derived from captured images were plotted in Figure 3-11 to obtain a real-time fluorescence emission curve for both GOIs (i.e. AST and ALT) and the housekeeping gene (i.e. β -actin). With three biological replicates in each treatment group, we were able to account for variation between samples for more precise gene expression measurements [106]. From the real-time fluorescence amplification plots in Figure 3-11, we were able to estimate the initial mRNA concentrations of our samples used via their respective average C_t values; C_t (or cycle threshold) values are often used in quantitative PCR as an indicator of starting/initial concentration of the nucleic acid template [39, 84, 107].

To determine the number of cycles required for the fluorescence signal to exceed that of the background level [108], the user-defined C_t threshold value in Figure 3-11 was selected based on No Template Control (NTC) fluorescence intensities. Our C_t threshold was defined by post-experimental results analysis wherein the number of cycles required for the fluorescence intensities to cross a pre-determined fluorescence threshold (i.e. 20% higher than those of the NTCs) were consistent with the results obtained from a conventional real-time thermal cycler. The C_t values of all six samples (AST-control, AST-3x dosage, ALT-control, ALT-3x dosage, β -actin-control, and β -actin-3x dosage) were correspondingly interpolated and tabulated in Table 3-2, and compared to determine the relative changes in the mRNA initial concentrations. Table 3-2 shows that the average C_t value of AST on our microfluidic chip decreased from 20.5 to 19.9 after a 3x drug dosage, while the average C_t value of ALT decreased from 28.8 to 28.5. Conversely, the average C_t value of β -actin remained relatively constant – once again illustrating the constitutive expression behavior of our housekeeping gene.

The presence of the housekeeping gene, β -actin, allowed us to normalize our qPCR experimental data, to minimize possible variations from the experimental setup such as sample preparation and handling, reverse transcription efficiency between sample-sample/run-run repetitions, and DNA amplification efficiencies [108, 109]. To obtain a normalized target gene expression level, we used the Livak method [108] to first determine the difference in C_t values between a target gene and the housekeeping gene for a particular sample, before obtaining the $\Delta\Delta C_t$ values (indicative of normalized fold change) [108]. From our microfluidic experimental data, we demonstrated that the normalized fold changes (or up-regulation) for AST and ALT were 1.62 and 1.31

respectively. These values largely indicated that the 3x drug dosaged samples had a higher concentration of initial nucleic acid (or target mRNA in our case) concentration when compared to an untreated control. In the perspective of the differences of up-regulations between AST and ALT, we observed that ALT had a relatively lower increase in gene expression levels compared to AST. We speculate that this might be due to the reduced (or delayed) response of ALT to cyclophosphamide administration (vis-à-vis AST). Nonetheless, our statistical analysis indicated that AST and ALT collectively illustrated significant differences between the 3x drug dosaged samples and untreated controls.

These results were consistent with the estimates of gene expression changes (i.e. 58.7% and 33.3% increases for AST and ALT respectively) from our previous qualitative analytic approach (derived from final cDNA concentrations). This consistency of up-regulated gene expression changes from both our qualitative analytic approach and fully-quantitative fluorescent detection methodology herein demonstrated the efficacy of the latter, and its integration with our microfluidic RT-PCR system. More importantly, we validated the AST and ALT GOIs derived from protein biomarkers used in Liver Function Tests, which were illustrated to have been up-regulated after 3x cyclophosphamide treatment.

Collectively, we have designed and demonstrated an integrated LOC system embodying tissue sample preparation, multiplex RT-PCR and real-time fluorescent detection for POCT hepatotoxicity assessment – hence the viability of POCT for hepatotoxicity assessment.

Table 3-2: Comparison of average cycle threshold (C_t) values of conventional qPCR machine (CFX96 Touch™) with our microfluidic setup

No.	mRNA Target	CFX96 Touch™	Microfluidic Chip
1	AST – Control	20.07±0.02	20.5±0.2
2	AST – 3x Dosage	19.34±0.05	19.9±0.2
3	ALT – Control	27.28±0.10	28.8±0.2
4	ALT – 3x Dosage	26.75±0.07	28.5±0.3
5	β-Actin – Control	22.93±0.11	23.0±0.2
6	β-Actin – 3x Dosage	23.02±0.08	23.1±0.2

Figure 3-11 Quantification of real-time fluorescence emission for a) AST, b) ALT and c) β -actin at 3x drug dosage using ImageJ, with images captured at the annealing/extension phase of each PCR cycle

3.4 Preliminary Selection of Liver-Critical Genes of Interest

As aforementioned in our literature review (see Section 2.3), it is imperative to investigate other liver-critical GOIs related to hepatotoxicity and liver diseases, and to ascertain an encompassing gene list for comprehensive liver assessment. In our preliminary selection process for said critical GOIs in liver assessment, we performed a statistical analysis on a database repository (i.e. Gene Expression Omnibus) of microarray gene expression profiling experiments. Figure 3-12 below depicts our approach for organizing the statistical microarray data from GEO. Put simply, we adopted the data analysis approach by Roth *et al.* [30] to determine the differences in gene expression levels – termed as change factors. From the raw microarray cell intensity data, the probe-level intensity values of different samples were first compressed to obtain their respective mean log2 values [110]. Subsequently, the change factors of different GOIs were determined as the “ratio of signals between two experimental conditions (treated and control)” [30], and calculated as the $[(\text{Mean of treated samples} / \text{Mean of control samples}) - 1]$.

The original cell files were downloaded and the background correction, normalization and pm correction are applied using the R package of 'limma' under default parameters.

After obtaining the above probe intensity data, the tool of 'CollapseDataset' in the GSEA platform are used to consolidate the intensity data per probe to the intensity for each data.

The Change Factor indicating differences in gene expression is calculated as:

$$\text{Change Factor} = (\text{Mean of treated samples} / \text{Mean of control samples}) - 1$$

e. g.
 A Change Factor of 0.5 indicates that the normalized mean of treated samples is higher than that of control samples by 50%;
 A Change Factor of -0.5 indicates the normalized mean of treated samples is lower than that of control by 50%.

And the top 25 genes are selected as the result.

Columns 3)-7) are generated using the same scheme.

Figure 3-12 Procedure for microarray data analysis from GEO

We subsequently ranked the top 25 genes according to the largest change factor in response to R1 drug treatment over a 24-h dosage period and tabulated them in Table 3-3. The values tabulated in Table 3-3 can be interpreted as follows. A positive change factor would indicate an up-regulation of the gene, while a negative change factor would indicate a down-regulation. The greater the value of the change factor (either positive or negative) of a particular gene, the more highly involved the gene was in the response of the rat to the hepatotoxicity drug treatment studies.

In Table 3-3 below, we color-coded the tabulated data to indicate the changes in gene expression activities – wherein a darker color indicates greater change factor. Specifically, blue refers to a positive fold-change, wherein the particular gene experienced an up-regulation (or increase in activity). Conversely, red indicates a negative change factor, wherein the gene experienced a down-regulation (or decrease in activity). A large number of the top 25 genes ranked in Table 3-3 are related to major drug metabolism and transportation pathways, or are transcriptomes of drug-metabolizing enzymes [111]. These genes play an important role in the conjugation, biotransformation, and subsequent elimination of potentially toxic xenobiotics, drugs and other endogenous compounds. The combination of an encompassing gene list and a POCT liver assessment device would offer the possibility of longitudinal monitoring of gene expression changes of liver-critical genes. This envisioned POCT platform would hence be able to provide both an early indicator of liver damage as well as early response of drug efficacy in chemotherapy – serving as a potential molecular stratifier for personalized medicine and companion healthcare.

It is imperative to note that our preliminary statistical analysis would need to be supported by in-depth validation of the combination of the selected critical genes (see Future Work in Chapter 5). The gene list we compiled in Table 3-3 using the published datasets from Gene Expression Omnibus largely serves as a guide on the selection criteria. Extensive drug toxicity studies on mouse/rat subjects would have to be carried out for said validation of a select group of critical GOIs.

Table 3-3: Compilation of the top 25 genes with highest change factor after rat hepatotoxicity drug treatment

Gene Name	24h	336h	TCDD	PeCDF	PCB126	Binary	PCB153
HMGCR	0.639392	0.418377	0.075496	0.169527	0.16539	0.304791	0.072521
UGT1A2	0.597631	0.75365	0.004355	-0.02388	-0.0332	0.054181	-0.05392
ACAT2	0.51479	0.527717	0.038834	0.004488	-0.03214	0.016629	0.040942
HSD17B7	0.485223	0.276891	0.041601	-0.12899	-0.20605	-0.07413	0.051226
CYP3A3 /// CYP3A1	0.439307	0.645778	0.009509	0.145011	0.152889	0.093578	0.104337
SQLE	0.438903	0.412838	0.012563	-0.028	-0.055	-0.05708	0.013883
IDI1	0.420713	0.392266	-0.00893	-0.01009	-0.03416	0.16085	0.017543
PSAT1	0.405249	0.255835	0.114829	0.224258	0.212259	0.264903	0.042255
UGT1A1	0.379162	0.220365	0.009957	-0.02219	-0.02781	0.144161	0.079122
LSS	0.357694	0.197262	0.049054	-0.00369	-0.01542	0.060572	0.06088
CYP51A1	0.357663	0.302709	0.047803	0.136265	0.111698	0.270863	0.035527
HMGCS1	0.338444	0.151405	0.0354	0.02556	0.011767	-0.00748	0.042814
SC4MOL	0.330305	0.303309	0.055204	0.011264	-0.02628	0.053015	0.067266
RGD1359202 /// LOC29	0.325073	0.476248	0.056533	-0.06678	-0.02095	0.237179	-0.016
MVD	0.314592	0.157854	0.019345	-0.03512	-0.02642	-0.02368	0.040531
FDFT1	0.288257	0.322913	-0.00472	-0.04737	-0.10784	0.018952	0.069587
ME1	0.279605	0.388577	0.225654	0.59388	0.453453	0.541062	0.148135
CES2	0.27432	0.702867	-0.05965	0.181713	0.173709	0.363135	0.330448
GSR	0.273046	0.207834	0.084352	-0.01702	-0.05306	0.101594	0.108193
GPX2	0.254818	0.615201	-0.03508	0.09502	0.099204	0.120569	-0.009
SLCO1A4	0.22436	0.261247	-0.12813	-0.04399	-0.05613	-0.10837	0.029626
NOL3	0.208257	0.434901	-0.00884	-0.03907	-0.01558	-0.00426	0.008046
CYP2B2	0.163226	0.346126	0.008419	0.086298	0.091622	0.748876	0.618104
YC2	0.159272	1.320861	0.098872	0.08084	0.022356	0.047355	0.103389
LOC246263	0.071814	0.44832	-0.41806	-0.05155	-0.10109	-0.45503	0.029643

3.5 Conclusions

We have designed and implemented an LOC system that is close to a practical POCT hepatotoxicity assessment device, featuring semi-automation, relative ease-of-use, and sample-in-answer-out capabilities for multiplex gene expression analysis. Our LOC system embodied the integration of tissue sample preparation, multiplex RT-PCR, and real-time fluorescent detection methodology, and its efficacy was validated by multiplex gene expression analysis on two GOIs (i.e. AST and ALT) derived from Liver Function Tests' biomarkers.

The results from our microfluidic tissue sample preparation module integrated with our multiplex RT-PCR chip has yielded improved purity of mRNA extracts over manual extraction means while significantly reducing off-chip handling procedures for multiple gene targets. The GOIs derived from Liver Function Tests' protein biomarkers validated by our microfluidic RT-PCR results have illustrated up-regulations in their gene expression levels (i.e. increased in final cDNA concentration by 58.7% and 33.3% for AST and ALT respectively) in mice liver tissue samples with a 3x dosage cyclophosphamide drug treatment. Further, our fully-quantitative real-time fluorescent detection methodology has also demonstrated normalized fold changes of 1.62 and 1.31 for AST and ALT respectively, and these results were consistent with the results obtained from using bulky, conventional methodology.

We have also described our process for statistical analysis to ascertain the preliminary list of liver-critical GOIs. From our analysis, we have identified and ranked the top 25 genes that display the largest response to hepatotoxicity from extensive drug studies.

CHAPTER 4 Second Integrated LOC System Embodying Continuous-Flow PCR Approach and End-Point Detection

4.1 Introduction

The work reported in this chapter is largely extracted from our paper submitted (and currently under review) for publication in *Biomedical Microdevices* [88].

This chapter largely describes our second integrated LOC system. This LOC system serves to illustrate the feasibility of a continuous-flow microfluidic PCR system (see Section 2.2.3 for the different types of microfluidic PCR systems), wherein the main advantage is improved processing speed compared to the stationary PCR approach. Our second integrated LOC system embodied multiplex gene expression analysis and fully-quantitative detection capabilities. In this chapter, we propose, design and realize a segmented continuous-flow RT-PCR microfluidic system with a coordinate-based end-point quantitative detection for multiplex POCT gene expression analysis. This microfluidic system is largely embodies the latter two chains of NAT (i.e. nucleic acid amplification and nucleic acid detection, see Figure 1.1). Compared to the our previous stationary PCR-based LOC system delineated in Chapter 3 earlier, this microfluidic system features significantly faster diagnostic capabilities while still maintaining multiplex gene detection capabilities.

As earlier delineated in our literature review, there has been a number of reported microfluidic PCR work on continuous-flow RT-PCR systems [37, 38, 112, 113], largely due to their key advantages such as shorter thermal cycling durations arising

from the elimination of assay temperature ramping [40, 64], and requiring less complex and less efficient thermal cycling systems [22]. In particular, for gene expression analysis, most microfluidic RT-PCR systems largely embody spiral capillary-based systems [78, 101, 114, 115], wherein PCR assays are actuated through biocompatible (e.g. Teflon or PTFE) capillaries or tubings arranged in a spiral configuration around heat blocks with different isothermal regions. By using a two-phase segmented flow approach, these setups are often compatible with multiplexing [114, 116], high-throughput capabilities [115, 116], and real-time fluorescence detection [78, 101]. However, they often require large equipment footprints, and complex and expensive fluorescence detection setups that track individual fluorescent droplets during actuation through the capillaries. Simply put, they were largely incompatible with POCT, personalized healthcare and companion diagnostics [1].

To resolve these issues, in one recent notable study, Shu *et al.* [117] developed a handheld segmented continuous-flow multiplex PCR capillary-based system wherein an immiscible oil phase was used to separate PCR assays to accommodate multiplexing of different targets/samples in a single experimental run. This handheld microfluidic device featured a miniaturized fluorescent detection system where fluorescence was measured as a function of cycle number and of temperature in order to generate a qPCR melting curve. This microfluidic device was in part compatible with POCT because it still required extensive off-chip assay/analyte preparation procedures such as preparation of several multiple Eppendorf tubes in multiplexing applications.

To address the aforementioned issues in current continuous-flow PCR systems, we describe in this chapter the design and fabrication of our rapid segmented continuous-flow RT-PCR microfluidic PC chip-based system for POCT gene expression analysis. Our proposed microfluidic system is advantageous over current continuous-flow PCR systems [112, 117, 118] in terms of having a small equipment footprint, whilst retaining multiplexing capabilities and incorporating a low cost and easy-to-assemble integrated fluorescence detection module. To simplify off-chip handling procedures (such as pre-mixing of different primer sets for multiple RT-PCR assays) and to allow for ease-of-integration with potential upstream modules (such as sample preparation or nucleic acid purification) for compatibility with POCT, we demonstrate a primer preloading surface treatment procedure in dedicated GOI primer chambers on a continuous-flow microfluidic system. This primer preloading procedure allows the targeting of several GOIs concurrently with a single RT-PCR assay for multiplexing applications; its application was previously performed on stationary, chamber-based (e.g. see Chapter 3.2.5) microfluidic systems [84, 102].

Besides the inclusion of the GOI primer chambers and the preloaded primers for multiplex amplification, we adopt a microfluidic chip-based setup for continuous assay actuation to provide three distinct advantages: (i) reducing assay volume requirements and hence lowering the amount of sample material needed per experimental run; (ii) decreasing equipment footprint by using a printed-circuit board (PCB) copper-trace resistive heater instead of larger heat blocks; and most importantly, (iii) disposability of test chips to eliminate cross-contamination between experimental runs. Additionally, we adopt a segmented flow technique [114, 117] to incorporate multiplexing capabilities by using an immiscible mineral oil layer to separate different

RT-PCR assays targeting different GOIs, with a modified coordinate-based fluorescence detection for quantitative analysis.

In our experiments, we demonstrate the effectiveness of our primer preloading surface treatment procedure and of our GOI primer chamber design by obtaining an excellent cDNA amplification efficiency of 94.5% (compared to a positive control). Additionally, we illustrate the functionality of our continuous-flow microfluidic system, as well as the multiplex amplification efficiencies of 37.3 - 94.5% at different flow rates. For this exercise, we perform duplex amplification of AST and ALT to validate the applicability of our microfluidic system to POCT hepatotoxicity assessment, as well as to illustrate the potential of an extended gene list to a larger number of GOIs for a more comprehensive hepatotoxicity assessment. We thereafter carry out a gene expression analysis of said two GOIs after a short-term drug toxicity study involving the administration of cyclophosphamide. With its constitutive expression [99, 100], β -actin is selected as our housekeeping gene for normalization of data. Our experimental results demonstrate fold changes of 1.52 and 1.62 for ALT and AST respectively; indicating up-regulations of said two GOIs in response to the drug administration. The entire RT-qPCR process is completed in <20mins, i.e. 3.9-fold faster than a conventional thermal cycler and ~3.15-fold faster than our first integrated LOC system embodying the stationary PCR approach. In short, we demonstrate the efficacy of our multiplex continuous-flow RT-PCR microfluidic system, and illustrate the applicability of our microfluidic system in rapid POCT hepatotoxicity assessment.

4.2 Materials and Methods

In this section, we will first delineate the design and assembly of the different architectural features of our continuous-flow PC chip, followed by the microfluidic chip fabrication process. Secondly, we will discuss the design and assembly of our continuous-flow microfluidic RT-qPCR setup, embodying both the assay actuation setup, heating system and fluorescence detection module. Thirdly, we will delineate the surface treatment procedures carried out to prepare the microfluidic chip, followed by describing the sample preparation and assay loading protocols. Lastly, we will discuss the segmented RT-qPCR process, including the duplex amplification of said two GOIs and their ensuing fluorescence detection and quantification.

4.2.1 Microfluidic Chip Design

Our continuous-flow RT-PCR microfluidic chip comprised three different regions (see Figure 4-1a) – two chambers with preloaded primers to target different GOIs, a connected continuous serpentine microchannel for RT-PCR, and an independent reference chamber to contain an NTC.

The GOI primer chambers were designed to contain a solution volume of ~10 μ L whose dimensions were 50mm (l) x 1mm (w) x 0.2mm (d). The main purpose of these chambers was to contain the preloaded primers in dried powder form prior to their reconstitution in RT-PCR assays during the assay loading process, thereby simplifying

the off-chip handling procedures and to allow for ease-of-integration with potential upstream modules.

The connected continuous RT-PCR serpentine microchannel comprised four separate regions running through three isothermal temperature zones. The four separate regions, reverse transcription, initial denaturation, denaturation, and annealing/extension, were designed to accommodate the RT-PCR thermal cycling profile used in our experiments. To maintain a dwell time ratio compatible with our GOIs and RT-PCR master mix, a uniform microchannel depth of 50 μ m was used, with different widths for each region as follows: (i) 250 μ m wide for reverse transcription and initial denaturation; (ii) 200 μ m wide for denaturation; and (iii) 100 μ m wide for annealing and extension. The continuous microchannel passing through the denaturation, annealing and extension regions was able to accommodate 35 PCR cycles, and the total solution volume this serpentine microchannel could hold was ~20 μ L.

The independent reference chamber was designed to contain a solution volume of ~10 μ L, with dimensions of 10mm (l) x 5mm (w) x 0.2mm (d). This reference chamber was used to contain an NTC assay for intensity calibration during fluorescence detection. The NTC assay was loaded via the connected inlet microchannel.

This continuous-flow RT-PCR microfluidic chip was fabricated on a 2mm thick PC chip and sealed with a 0.25mm thick PC film on the bottom surface (see Figure 4-1b). The microfluidic chip was also secured onto a custom designed PCB copper trace

heater via thermally conductive adhesive transfer tape (3MTM, Singapore) to ensure good thermal contact and heat transfer from the heater to the PCR assay within the microchannel. NanoPortTM PEEK ferrule connectors (Upchurch Scientific[®], USA) were attached to the inlets/outlet of the microfluidic chip with 1/32" OD tubings used for RT-PCR assay actuation and collection.

Figure 4-1 Continuous-flow RT-PCR microfluidic chip design: a) Schematic layout of microfluidic chip with different regions indicated; b) Model of microfluidic chip in exploded view illustrating the PCB copper trace heater, with attached NanoPortTM ferrules and tubings

4.2.2 Microfluidic Chip Fabrication

Our microfluidic chips were patterned using a micro-milling technique. VisualMILL™ CAM software (MecSoft Corporation®, USA) was used to import and convert 2D Solidworks™ drawings to a format compatible with the micro-milling machine. Typical micro-milling settings used were ~6000rpm spindle speed and 10-20mm/min feed rate to ensure acceptable structural accuracy and minimal surface roughness.

Each PC layer was subsequently cleaned in a bath sonicator with 10% v/v isopropanol for 15mins at 40°C and dried in a vacuum oven at 80°C overnight. Finally, the PC layers were thermally bonded using a thermal press at 143°C under a 10MPa applied pressure for 15mins, followed by cooling to 50°C while maintaining the same pressure.

4.2.3 Microfluidic RT-qPCR Setup

To realize continuous-flow RT-PCR, the following setup was designed (Figure 4-2). Three programmable syringe pumps (New Era Pump Systems® Inc., USA, model number NE-1002x) were used to actuate solutions from three 100µL Hamilton® glass syringes (Sigma-Aldrich®, Singapore, catalogue number 28635-U) into the continuous-flow RT-PCR microfluidic chip via 1/32" OD tubings. The glass syringe connected to the middle inlet contained an immiscible PCR-grade mineral oil (Sigma-

Aldrich[®], Singapore), while the remaining two glass syringes connected to the GOI primer chambers held RT-PCR assays.

A 4-channel digital temperature controller (Omron[®] Corporation, Singapore, model number E5AR-T) was used to set three isothermal temperature regions (Figure 4-3) for different RT-PCR phases on the custom fabricated PCB copper trace heater (i.e. reverse transcription at 50°C, initial denaturation and denaturation at 95°C, and annealing/extension at 55°C). Thermocouples in contact with the bottom surface of the PCB copper trace heater allowed the temperature controller to maintain the required steady-state temperatures via a feedback loop.

To allow for quantitative fluorescence detection, the same fluorescence detection module delineated in Chapter 3 (Figure 4-4) was used again in our microfluidic RT-PCR setup delineated above. As aforementioned, this module comprised a detection enclosure fabricated from a high gloss black acrylic for high reflectivity and to minimize absorption of the excitation light source, as well as providing an enclosed environment to minimize light leaks and to improve thermal isolation. Other components included a 550nm longpass filter (Island Optical Systems (S) Pte. Ltd, Singapore) mounted on a 3MP CMOS camera unit, which was capable of removing >99% of light with wavelengths less than 520nm; and a blue LED array (IORodeo[™], USA) to provide an excitation light source of ~470nm. The blue LED module was affixed at an angle of ~45° to the plane of the microfluidic chip to minimize excitation light interference on the captured light pathway of the camera unit.

Figure 4-2 Schematic setup of our continuous-flow RT-PCR system

Figure 4-3 Circuit diagram for temperature control

Figure 4-4 Our fluorescence detection module with camera unit and mounted blue transilluminator

4.2.4 Surface Treatment

To render our microfluidic chip suitable for POCT gene expression analysis, we performed a two-step surface treatment procedure on the inner surfaces of the microfluidic chip microchannels. In the first step, two sets of forward and reverse primers targeting two different GOIs were preloaded through separate inlets after the PC microfluidic chip had been thermally bonded and sterilized under UV radiation for 15mins. 50 μ M of each primer (forward and reverse) was diluted in nuclease-free water to make up a final-volume of 10 μ L and final-primer concentration of 0.5 μ M. The pre-loading process was performed using separate glass syringes, and the primer assays were actuated at a flow rate of 2.0 μ L/min to fill up each serpentine GOI primer chamber.

The second step of the surface treatment procedure involved minimizing the adverse effect of the high surface area-to-volume (SAV) ratio of the continuous serpentine RT-PCR microchannel. A high SAV is undesirable as it increases the adherence of cDNA templates, Taq polymerase enzymes, and other reagents to the wall surfaces of the microchannels [38, 119] – an undesired phenomenon that largely reduces the DNA amplification efficiency of a PCR reaction [38]. A passive coating procedure was performed using 1 μ g/ μ L bovine serum albumin (BSA) (Sigma-Aldrich[®], Singapore) diluted in TE buffer [120]. With both inlets of the GOI primer chambers closed, the BSA solution was actuated at a flow rate of 2.0 μ L/min into the microfluidic chip.

Once both surface treatment steps were completed, the microfluidic chip was incubated at room temperature for 2 hours. With the inlets of the GOI primer chambers remaining closed, the BSA solution was flushed out completely with nuclease-free water, leaving the two primer assays intact in the aforesaid chambers. The treated microfluidic chip was subsequently dried in a convention oven at 65°C for 15mins to allow the water to evaporate, leaving the dried primers deposited in powder form on the GOI primer chamber walls and a BSA coating on the RT-PCR continuous serpentine RT-PCR microchannel walls.

4.2.5 Sample Preparation and Assay Loading

To demonstrate the functionality of our continuous-flow RT-PCR setup, a short-term preclinical drug toxicity study was performed on common black laboratory mice (Strain name: C57BL/6J; Age: 5-7 weeks) with the administration of

cyclophosphamide (200mg/kg body wt.) [94]. The mice were separated into two groups: a treated group with a 3x drug dosage and an untreated control group with 0.9% w/v NaCl (saline). After the drug administration, the mice were euthanized following standard carbon dioxide anaesthesia, and liver biopsy was performed to obtain liver tissue samples. Subsequently, tissue lysis and mRNA extraction were performed using the MicroHomogenizer[™] Kit (ClaremontBio[®], USA) and Dynabeads[®] mRNA DIRECT[™] Purification Kit (Ambion[®], USA).

In our RT-PCR microfluidic experiments, we targeted two GOIs (i.e. AST and ALT) from both sample groups. To prevent genomic DNA contamination during the amplification process, intron spanning primers were used. Our AST and ALT primers were designed using the PrimerQuest software (Integrated DNA Technologies[®], Singapore) and were synthesized *de novo* from the same company. For AST, a 182-bp segment was amplified using the following primer set: forward primer, 5'-GCT GAC TTC TTA GGG CGA TG-3'; and reverse primer, 5'- TCA TTC AGG AAA CCC TGG AG-3'. For ALT, a 221-bp segment was targeted with the following primer set: forward primer, 5'- CTG CAG ACC CGA ACA ACA TA -3'; and reverse primer, 5'- CAG CTC AGC GAT GTC AAG AG-3'. The β -actin primer assay was obtained from Qiagen[®], Singapore (Catalog No. PPM02945B), with a band size of 154-bp.

A single RT-PCR assay of 50 μ L was prepared with the following reagent composition: 25 μ L 2x SYBR Green RT-PCR reaction mix (BioRad[®], Singapore), 10 μ L purified mRNA extract, 1.0 μ L iScript One-Step RT-PCR reverse transcriptase (BioRad[®], Singapore) and 14 μ L nuclease-free water. This RT-PCR assay was subsequently divided equally into two portions of 25 μ L each in two glass syringes

connected to the GOI primer chambers. 25 μ L of mineral oil was loaded in the remaining glass syringe, and a 10 μ L post-RT-PCR NTC assay containing 0.5 μ M AST primers was loaded into the reference chamber.

4.2.6 Segmented RT-qPCR

To produce segmented regions in our continuous microchannel for duplex RT-PCR (see Figure 4-5), the following operational procedure was performed: 50nL of solution from each of the three glass syringes were actuated into the microfluidic chip in a repeated fixed order (i.e. 1st syringe connected to AST primer chamber, 2nd syringe with mineral oil, and 3rd syringe connected to ALT primer chamber). Each glass syringe was actuated for ~5s with a flow rate of 0.5 μ L/min before a rest phase of ~10s to produce a succession of repeated 50nL droplets.

Figure 4-5 Illustration of flow sequence in the microchannel of our segmented continuous-flow RT-PCR chip. A single RT-PCR assay is used to reconstitute the dried preloaded primers in the GOI primer chambers before entering into the central microchannel. Assay droplets with a volume of ~50nL were separated with immiscible PCR-grade mineral oil to prevent cross-contamination

In our continuous-flow RT-PCR setup, various flow rates (0.5-3.0 μ L/min, with 0.5 μ L/min being the default flow to allow for the most suitable RT-PCR dwell durations) were tested to determine the most appropriate trade-off between DNA amplification efficiency and speed of completion. Our GOIs (i.e. ALT and AST) were duplexed on a single microfluidic chip with segmented continuous-flow, while our housekeeping gene (i.e. β -actin) was separately amplified on another chip without requiring the use of the segmented flow.

The thermal cycling conditions required for RT-PCR were as follows: a reverse transcription step of 50°C for 10mins; an initial denaturation step of 95°C for 5mins;

35 PCR cycles comprising denaturation at 95°C for 10s, and annealing/extension at 55°C for 30s. To provide a control reference to our chips and to validate the PCR assay composition, 25μL RT-PCR positive controls for AST, ALT and β-actin were amplified separately on a conventional CFX96 Touch™ Real-Time PCR Detection System (BioRad®, USA) together with 25μL of the aforementioned NTC assay with AST primers.

For an integrated fully-quantitative detection methodology, a fluorescence image was captured at the end of RT-PCR using our fluorescence detection module (see Figure 4-4) and analyzed using the ImageJ image processing and analysis software. In addition, C_t values were used to quantitatively compare between different samples for gene expression analysis [108]. Rather than using a real-time droplet tracking mechanism [78, 101], we adopted an end-point quantitative detection approach, a modified form of the aforementioned melting curve fluorescent analytic method [117, 121]. The C_t values of our GOIs could be determined via a coordinate system (i.e. function of loop number and length, L of microchannel, see Figure 4-6) located at the annealing/extension region of our microfluidic chip. Effectively, the location of our assay droplets with fluorescence intensities ~20% higher than background fluorescence of the NTC assay in the reference chamber (i.e user-defined C_t threshold) would allow us to determine the C_t values of our GOIs. Our C_t threshold was defined by post-experimental results analysis wherein the number of cycles required for the fluorescence intensities to cross a pre-determined fluorescence threshold (i.e. 20% higher than background fluorescence) were consistent with the results obtained from a conventional real-time thermal cycler.

Figure 4-6 Illustration of coordinate system used by our end-point quantitative detection methodology to determine C_t values of GOIs. In this system, the loops of the annealing/extension region would determine the integer C_t value while the position of the targeted droplet along the length, $L=50\text{mm}$, of the microchannel would allow the C_t value to be resolved to 1 decimal place (i.e. C_t value is a function of loop number and length, L of microchannel)

To determine the specificity of our microfluidic PCR setup, the PCR amplicons were visualized and analyzed off-chip using conventional gel electrophoresis with a 3.0% agarose gel (TAE) prestained with ethidium bromide (BioRad[®], Singapore). The gels were typically run for 45 minutes at a constant voltage of 100V in 1X TAE buffer. After gel electrophoresis was completed, the results were viewed using a Gel Doc[™] XR+ System (Bio-Rad[®], Singapore) under trans-UV. These trans-UV images were captured and analyzed using the Quantity One[®] (Bio-Rad[®], Singapore) imaging software.

4.3 Results and Discussions

In this section, we discuss the results obtained from the microfluidic experiments performed on our continuous-flow RT-PCR setup delineated in the previous section. Firstly, we will discuss the effectiveness of the surface treatment procedure using preloaded primers and their subsequent reconstitution in actuated RT-PCR assay. Secondly, we will examine the efficiency of our segmented RT-PCR microfluidic setup in duplex cDNA amplification of ALT and AST mRNA genes, as well as the effects of increasing assay flow rates to improve the speed of reaction completion. Thirdly, we will explore our fully-quantitative detection methodology by analyzing captured fluorescence images and establishing C_t values of the different GOIs by using a coordinate-based analytic approach. Finally, we will investigate the applicability of our integrated continuous-flow RT-qPCR microfluidic setup in a duplex gene expression analysis application to assess the viability of our microfluidic prototype for rapid POCT hepatotoxicity assessment.

4.3.1 Reconstitution of Preloaded primers

In this section, we investigate the effectiveness of surface treatment using preloaded primers and their subsequent reconstitution in RT-PCR assay. To validate of the effectiveness of our primer preloading procedure, we studied three different GOI primer chamber designs (i.e. Design 1 with a 2.5 loop serpentine microchannel featured in Figure 4-1a, Design 2 with a rectangular chamber, and Design 3 with a single loop serpentine microchannel; all designs had approximately the same volume)

with varying SAV ratios. The preloaded primers were reconstituted in RT-PCR assays at a flow rate of 0.5 μ L/min using untreated mouse liver tissue samples, and the resultant solutions were amplified on-chip. The subsequent cDNA amplification efficiencies (on the ALT target gene) from the three different GOI primer chamber designs were compared with a positive control (which had the primers directly added into the assay and amplified in a conventional thermal cycler).

Figure 4-7a below depicts an agarose gel electrophoresis image illustrating the different cDNA amplification efficiencies (via the varying signal band intensities) of the different GOI primer chamber designs and positive control. The average cDNA amplification efficiencies against the SAV ratios, wherein the experiment was performed in triplicate and individual runs were normalized to a positive control amplified separately on the conventional thermal cycler, is plotted in Figure 4-7b.

Figure 4-7 a) Agarose gel electrophoresis image illustrating the effect of GOI primer chamber design (where Design 1 is a 2.5 loop serpentine microchannel; Design 2 is a rectangular chamber; and Design 3 is a single loop serpentine microchannel) on primers reconstitution and cDNA amplification efficiency, and tested using ALT as the gene of interest on the untreated control at a flow rate of $0.5\mu\text{L}/\text{min}$; b) Comparison of the effect of surface area-to-volume ratio on primer reconstitution and cDNA amplification efficiency

In Fig 4-7b, we observed that the average cDNA amplification efficiency increased linearly with the SAV ratio. With our selected GOI primer chamber design (i.e. Design 1), we were able to reconstitute majority of the dried preloaded primers coating the microchannel walls with the syringe-actuated RT-PCR assay to realize a comparable cDNA amplification efficiency of ~94.5% (to the positive control). We note that this cDNA amplification efficiency obtained from the reconstitution of preloaded primers on our continuous-flow microfluidic setup was similar to those achieved on a stationary chamber-based microfluidic setup [84, 102]. Our continuous-flow microfluidic setup nevertheless features the worthy advantage of a more rapid RT-PCR process and significantly reduced diagnostic completion time (see later section for details) – imperative merits for POCT.

Furthermore, to have adequate cDNA amplification efficiency of $\geq 80\%$ for downstream applications (such as quantitative detection), an appropriate large SAV ratio of at least 11.5mm^{-1} (at a flow rate of $0.5\mu\text{L}/\text{min}$) is recommended for use with our continuous-flow primer reconstitution procedure. We were able to, for the first time, incorporate the primer preloading surface treatment procedure in dedicated GOI primer chambers on a continuous-flow microfluidic system to allow for multiple detection of different GOIs from a single RT-PCR assay. As aforementioned, this surface treatment procedure is able to simplify off-chip handling procedures for multiplex gene detection, as well as allowing for ease-of-integration with potential upstream modules such as sample preparation or nucleic acid purification.

4.3.2 Segmented RT-PCR

The efficacy of our segmented continuous-flow microfluidic RT-PCR setup was verified by targeting said two GOIs, AST and ALT, on the untreated mice liver tissue samples, and the procedure is now briefly described. Once microfluidic RT-PCR was completed, the PCR amplicons and mineral oil were collected in an Eppendorf tube (see Figure 4-2) that was spun down in a centrifuge to separate the mineral oil from the PCR amplicons. Subsequently, the PCR amplicons were extracted using a micropipette and analyzed off-chip with gel electrophoresis. Average signal band intensities were obtained from electropherograms derived from the gel electrophoretic trans-UV images (Figure 4-8a), and subsequently the efficiency of cDNA amplification between microfluidic PCR and conventional PCR is plotted as a function of assay flow rates in Figure 4-8b.

In Figure 4-8a, we observed two clear bands on the agarose gel electrophoresis image for each flow rate, wherein the bands at 221-bp and 182-bp belonged to the ALT and AST target fragments respectively. This clear band separation largely validated the segmented continuous-flow mechanism of our microfluidic RT-PCR chip design that featured the simultaneous duplex amplification of two GOIs on a single microfluidic chip. Additionally, the absence of any band smearing indicated that our duplex RT-PCR reaction was highly specific, without the presence of any side products such as primer-dimers or non-specific DNA fragments. This was largely due to two factors – the surface treatment procedure of coating BSA on the inner microchannel wall to minimize sample cross-contamination (via adhesion on the inner wall during the

continuous-flow) between the ALT and AST droplets; and the immiscible mineral oil separation layer to prevent any assay diffusion between the duplex droplets.

In Figure 4-8b, we observed that the average amplification efficiencies of both ALT and AST decreased as the assay flow rates increased from 0.5-3.0 μ L/min. At the default flow rate of 0.5 μ L/min, the average amplification efficiencies of ALT and AST were comparable to that of the positive control, with amplification efficiencies of 94.5% and 91.4% respectively. With these high amplification efficiencies at the default flow rate of 0.5 μ L/min, we demonstrated the functionality of our duplex continuous-flow RT-PCR setup, while maintaining high specificity (with minimum unwanted side products). As expected, when the flow rate was increased 6-fold to 3.0 μ L/min, the amplification efficiencies decreased significantly, with values of 37.3% and 31.4% for ALT and AST respectively.

Figure 4-8 a) Agarose gel electrophoresis image taken under trans-UV illustrating the functionality of duplex RT-PCR targeting both ALT and AST genes of interests from untreated mice liver tissue samples, with the signal band intensities indicating the amount of cDNA amplicons synthesized at different flow rates; b) Average amplification efficiencies of continuous-flow RT-PCR at 0.5-3.0 μ L/min flow rates. Experiments were performed in triplicates, with results normalized to the positive control

Table 4-1 tabulates the required time for each RT-PCR phase at different flow rates, and Figure 4-9a plots the total time required for RT-PCR completion at different flow

rates with a positive control amplified using the conventional thermal cycler to serve as a benchmark reference. We observed that at the default flow rate of 0.5 μ L/min, the entire RT-PCR process was completed in approximately half the time required by the positive control. This illustrates one of the key advantages of continuous-flow microfluidic systems wherein the flow of PCR assays through isothermal thermal regions negate the need for any time consuming temperature ramping/cooling between PCR phases. At the fastest flow rate of 3.0 μ L/min, the entire RT-PCR reaction was completed in as short as 6.4mins, which was ~11.7-fold faster than a conventional thermal cycler and ~9.4-fold faster than our first integrated LOC system.

As a trade-off however, the average amplification efficiencies of both ALT and AST decreased as the flow rate increased, a well-known shortcoming to improving the speed of RT-PCR completion. Figure 4-9b plots the average amplification efficiencies of ALT and AST against the average rate of RT-PCR reaction (i.e. $1/(\text{□□□□□□□□□□ □□□□})$) to depict a relatively inverse linear relationship between the two properties for both ALT and AST. The expected effect of declining amplification efficiency from increased assay flow rates may be attributed to two factors critical to maintaining a high PCR efficiency: reduced time for primer reconstitution in the GOI primer chambers, and reduced dwell durations at the respective RT-PCR phases. To overcome the adverse effect of reduced time for primer reconstitution due to increased assay flow rate, a recommended approach would be to further increase the SAV ratios of the GOI primer chambers (either by increasing the overall lengths of the serpentine chambers, or increasing the number of loops of the microchannel) – hence, allowing for an increased primer reconstitution efficiency.

a RT-PCR Completion Time (mins)

Figure 4-9 a) Average PCR completion times for the conventional thermal cycler and different flow rates of our continuous-flow RT-PCR setup; b) Plot of average amplification efficiencies (%) of both ALT and AST against average rate of RT-PCR reaction (min^{-1}). Experiments were performed in triplicates, with error bars shown accordingly

In short, our results, which demonstrated significant reductions in RT-PCR completion time, illustrate the imperative pertinence of a continuous-flow RT-PCR setup in POCT where the speed of diagnosis is critical. Additionally, the inclusion of multiplex GOI amplification capability (e.g. duplexing of ALT and ALT in our experiments) promotes improved throughput capabilities to illustrate the potential of an extended gene list to detect a larger number of GOIs for a more comprehensive hepatotoxicity assessment.

Table 4-1: Different assay flow rates through our continuous-flow PCR microfluidic chip, the time taken per PCR cycle, and the total time taken to complete 35 cycles of RT-PCR

Flow Rates ($\mu\text{L}/\text{min}$)	RT-PCR Dwell Duration (s)				Total Time Taken (min)
	Reverse Transcription	Initial Denaturation	Denaturation	Annealing/ Extension	
0.5	600	300	9.6	30	38.1
1.0	300	150	4.8	15	19.1
2.0	150	75	2.4	7.5	9.5
3.0	100	50	1.6	5.0	6.4

4.3.3 Fully-Quantitative Detection with an Increased Assay Flow Rate for Rapid Gene Expression Analysis

To evaluate the applicability of our segmented continuous-flow RT-PCR microfluidic setup for duplex gene expression analysis, we adopted a fully-quantitative detection methodology by integrating a fluorescence detection module in our microfluidic setup. We performed a hepatotoxicity assessment by determining the fold changes of said two GOIs after a 3x cyclophosphamide drug administration (compared to an untreated

control) on our integrated microfluidic setup, and observed the changes in fluorescence emissions of the droplets within our microfluidic chip. To demonstrate the imperative rapid diagnostic capability of our continuous-flow microfluidic setup for POCT application, our fully-quantitative detection experiments for gene expression analysis were performed at an assay flow rate of 1.0 μ L/min (a 2-fold increase compared to the default flow rate of 0.5 μ L/min). At this particular increased assay flow rate, the entire RT-PCR process was completed in <20mins (3.9-fold faster than the conventional thermal cycler), thereby illustrating its pertinence to POCT.

A single fluorescence image (see Figure 4-10) was captured at the end of the RT-PCR process using our fluorescence detection module. The annealing/extension region of the continuous RT-PCR serpentine microchannel (maintained at an isothermal temperature of 55°C) was analyzed using the ImageJ image processing and analysis software. To track and differentiate between the ALT and AST fluorescent droplets, a reference was taken from the 1st droplet (i.e. AST assay droplet) at the terminal end of the continuous RT-PCR serpentine microchannel.

An example of a captured fluorescence image of RT-PCR performed on an untreated mouse liver tissue sample is depicted in Figure 4-10. We observed the gradual increase in fluorescence intensities along the abscissa of the RT-PCR region of the microfluidic chip. This is indicative of an increase of cDNA fragments from the PCR amplification process. The segmented continuous-flow of our microfluidic setup allowed us to distinguish between the GOI ALT and ALT droplets, which were separated by a non-fluorescent segment from the mineral oil layer. By using our coordinate-based detection methodology (see Figure 4-6), we determined the C_t values

of said two GOIs as well as our housekeeping gene β -actin. We do this by identifying the location of the assay droplets with fluorescence intensities $\sim 20\%$ (i.e. user-defined C_t threshold) higher than background fluorescence of the NTC assay in the reference chamber (tabulated in Table 4-2).

Figure 4-10 A sample fluorescence image captured by the fluorescence detection module integrated with the microfluidic RT-PCR setup illustrating the fluorescence intensity gradient along the annealing/extension region of the microfluidic chip. The non-fluorescent segments of the continuous-flow assay belonged to the mineral oil layer separating both ALT and AST RT-PCR assays

Table 4-2: Evaluation of C_t values of the different GOIs from untreated and 3x dosage mice liver tissue samples by observing the x - y positions on the annealing/extension region of the continuous-flow microfluidic chip where the fluorescence intensities are 20% higher than that of an NTC assay

GOI	Loop Number	Position	Average C_t Value
ALT (Control)	29	0.2 ± 0.3	29.2 ± 0.3
ALT (3x dosage)	28	0.7 ± 0.3	28.7 ± 0.3
AST (Control)	20	0.8 ± 0.4	20.8 ± 0.4
AST (3x dosage)	20	0.2 ± 0.2	20.2 ± 0.2
β -actin (Control)	23	0.3 ± 0.2	23.3 ± 0.2
β -actin (3x dosage)	23	0.4 ± 0.3	23.4 ± 0.3

When these results were compared with the C_t values obtained via the conventional CFX96 Touch™ Real-Time PCR Detection System (see Table 4-3), we observed that the C_t values obtained by our continuous-flow microfluidic setup exhibited a similar trend compared to those obtained by the CFX96 Touch™ system. Specifically, the ALT and AST GOIs from the untreated controls were consistently higher than the 3x drug-dosaged samples, and the β -actin housekeeping gene was constitutively expressed between the two different samples. Additionally, the differences in C_t values between the control and 3x drug-dosaged samples (i.e. ΔC_t values) remained relatively consistent between our microfluidic setup and the CFX96 Touch™ system. Collectively, our fluorescence detection results illustrated the reliability of our fully-quantitative coordinate-based detection methodology compared to a conventional real-time thermal cycler, as well as its pertinence in rapid diagnosis with an increased assay flow rate.

In our particular application of gene expression analysis for hepatotoxicity assessment, we used the Livak method [108] to first determine the ΔC_t values between a GOI and the housekeeping gene for a particular sample, before obtaining the $\Delta\Delta C_t$ values which is indicative of normalized fold change [108]. From the C_t values obtained via our microfluidic setup illustrated in Table 4-3, we demonstrated that the normalized fold changes for ALT and AST were 1.52 and 1.62 respectively. On the other hand, with the C_t values obtained from the CFX96 Touch™ system, we observed that the normalized fold changes for ALT and AST were 1.54 and 1.77 respectively. These $\Delta\Delta C_t$ values were largely comparable to those demonstrated by our microfluidic setup, and indicated that said two GOIs in the 3x drug-dosaged samples were up-regulated when compared to an untreated control.

Table 4-3: Comparison of C_t values of conventional qPCR machine (CFX96 Touch™) with our continuous-flow RT-PCR microfluidic setup

No.	GOI	CFX96 Touch™	Microfluidic Setup
1	ALT (Control)	27.28±0.10	29.2 ± 0.3
2	ALT (3x Dosage)	26.75±0.07	28.7 ± 0.3
3	AST (Control)	20.07±0.02	20.8 ± 0.4
4	AST (3x Dosage)	19.34±0.05	20.2 ± 0.2
5	β-actin (Control)	22.93±0.11	23.3 ± 0.2
6	β-actin (3x Dosage)	23.02±0.08	23.4 ± 0.3

In short, with our fluorescence detection module and coordinate-based detection methodology, we were able to demonstrate a fully-quantitative detection approach to accurately determine C_t values and changes in gene expression of multiple GOIs in response to a drug administration. With an increased assay flow rate in our continuous-flow RT-PCR setup, we were able to demonstrate the rapid diagnostic capability of our system that is imperative for POCT. Collectively, we have designed and demonstrated an integrated Lab-on-a-Chip system embodying a segmented continuous-flow multiplex microfluidic RT-PCR setup and fluorescent detection module to demonstrate the applicability of our continuous-flow microfluidic system in rapid POCT hepatotoxicity assessment.

4.4 Conclusions

We have delineated the design, fabrication and assembly of our rapid segmented continuous-flow RT-PCR microfluidic system embodying a coordinate-based quantitative detection for duplex POCT gene expression analysis. To improve on

prior-art continuous-flow microfluidic PCR systems and to incorporate imperative pertinence to POCT, we have proposed a setup using disposable polymeric-based microfluidic chips to reduce equipment footprint, reduce assay/sample requirements and minimize cross-contamination between test runs. Additionally, we have introduced here, for the first time, a primer preloading surface treatment procedure and incorporated GOI primer chambers for multiplexing capabilities via reconstitution of dried primers in a continuous-flow microfluidic setup. With this procedure, a single RT-PCR assay can be used to target multiple GOIs, thereby simplifying off-chip handling procedures, and allowing for ease of integration with potential upstream modules such as sample preparation or nucleic acid amplification. We have adopted a modified coordinate-based fluorescence detection methodology for quantitative gene expression analysis – an adopted approach that offers the advantage of requiring a less complex and low-cost fluorescence detection module.

By means of our microfluidic RT-PCR experiments, we have demonstrated the effectiveness of our GOI primer chambers design, and our primer preloading surface treatment procedure. Collectively, we have achieved an excellent 94.5% cDNA amplification efficiency (compared to the positive control). We have demonstrated the multiplex amplification efficiencies of our segmented continuous-flow RT-PCR system at different flow rates by the duplex amplification of AST and ALT, two liver-critical GOIs for hepatotoxicity. This thereby illustrated the rapid cDNA amplification capabilities (2.0 to 11.7-fold faster) of our segmented continuous-flow microfluidic system compared to conventional thermal cyclers.

We have validated the POCT applicability of our segmented continuous-flow RT-qPCR microfluidic system and coordinate-based quantitative detection methodology by performing a gene expression analysis of said two GOIs and housekeeping gene β -actin after a short-term drug toxicity study. Quantitative results obtained from end-point fluorescence images have demonstrated normalized fold changes of 1.52 and 1.62 for ALT and AST respectively, thereby indicating up-regulations of said GOIs in response to the drug administration. These results were consistent with those obtained from a conventional RT-qPCR system. The duplex gene expression analysis herein illustrated the potential of an extended gene list to a larger number of GOIs, e.g. for a more comprehensive hepatotoxicity assessment. With an assay-actuation flow rate of 1.0 μ L/min, the entire RT-qPCR process was completed in <20mins – 3.9-fold faster than the conventional thermal cycler, and ~3.15-fold faster than our first integrated LOC system embodying the stationary PCR approach.

In conclusion, our segmented multiplex continuous-flow RT-qPCR microfluidic system has illustrated the feasibility and efficacy of a rapid and low footprint system for multiplex RT-qPCR, and has demonstrated its applicability in POCT hepatotoxicity assessment.

CHAPTER 5 Conclusions and Future Work

5.1 Conclusions

We have delineated in this PhD program, work pertaining to the application of microfluidic NAT for POCT liver assessment. In this thesis, we have described a number of contributions whose embodiments served as an important step towards the realization of a practical and clinically pertinent POCT liver assessment device with *rapid, fully-automated, ease-of-use, and sample-in-answer-out* diagnostic capabilities for multiplex gene expression analysis. In particular, we have outlined our work pertaining to the design and realization of two different integrated LOC systems compatible with the entire NAT process and designed to be somewhat close to a practical POCT liver assessment device – which featured semi-automation, relative ease-of-use, and sample-in-answer-out capabilities for multiplex gene expression analysis. The conclusions arising from our studies presented in this thesis have been divided into four different chapters, and they will now be described in turn.

In Chapter 1, we have presented introductory background and motivation to this research program, both with the *Grand* and *Specific Objectives* towards a practical and clinically pertinent POCT liver assessment device based on NAT. Additionally, we have outlined the contributions of our work pertaining to two different integrated LOC systems for multiplex gene expression analysis.

In Chapter 2, we have succinctly reviewed the gold standards for liver assessment, namely histopathology and Liver Function Tests and gene expression profiling, and delineated their limitations to POCT liver assessment. We have also presented a

comprehensive literature review on current state-of-the-art PCR microfluidics research – the basis for our two integrated LOC systems embodying stationary PCR/continuous-flow PCR with fully-quantitative detection. Of particular interest, we have illustrated the largely rudimentary state of reported microfluidic devices for POCT liver assessment, and identified the real need for a practical POCT liver assessment device.

In Chapter 3, we have described, for the first time, our first integrated LOC system (stationary PCR approach) which is somewhat close to a practical POCT hepatotoxicity assessment device. Specifically, it featured *semi-automation*, relative *ease-of-use*, and *sample-in-answer-out* capabilities for multiplex gene expression analysis. Our first integrated LOC system embodied the integration and *semi-automation* of a tissue sample preparation/mRNA extraction module, a polymeric chip-compatible thermal cycler for multiplex RT-PCR, and a real-time fully-quantitative fluorescent detection methodology – encompassing, for the first time, the entire NAT process for *sample-in-answer-out* capabilities in a POCT liver assessment device.

With regard to the tissue sample preparation module (work of a colleague and not our contribution), we have illustrated the purity of the mRNA extracts yielded from said module was considerably better than the manual mRNA extracts (i.e. A260/280 ratios of ~2.17 and ~2.05 for microfluidic and manual extraction respectively). We have also outlined our proposed primer preloading surface treatment procedure and single-loading inlet mechanism to simplify off-chip handling procedures for *ease-of-use*.

To evaluate the feasibility and viability of our first integrated LOC system in POCT hepatotoxicity assessment, we have selected and validated two different liver-critical GOIs (i.e. AST and ALT) based on critical liver protein biomarkers used in Liver Function Tests. Further, we have performed a preclinical drug toxicity animal study via the administration of cyclophosphamide, and subsequent microfluidic gene expression analysis has depicted normalized fold changes of 1.62 and 1.32 for AST and ALT respectively. We have demonstrated that these up-regulations in their expression levels were consistent with results obtained from conventional methodology. In the perspective of our *Specific Objectives* to identify a group of liver-critical GOIs for a clinically pertinent liver assessment, we have described in this chapter a process to ascertain an encompassing gene list for comprehensive liver assessment. A statistical analysis based on the GEO database repository allowed us to identify and rank a series of liver-critical GOIs related to hepatotoxicity in extensive drug studies.

In Chapter 4, we have described our second integrated LOC system (continuous-flow PCR approach) which featured a small equipment footprint and embodied an easy-to-assemble fluorescent detection module. With a contact-based isothermal heating setup and a polymeric biochip embodying a segmented flow approach, we have described a method for multiplex gene expression analysis on a continuous-flow PCR platform. Using the fluorescent detection module from our first integrated LOC system, we have adopted and established a fully-quantitative coordinate-based end-point detection methodology compatible with duplex gene expression analysis of AST and ALT.

Additionally, we have described, for the first time, a primer preloading procedure in a continuous-flow PCR system to simplify off-chip handling procedures and to allow for ease-of-integration with potential upstream modules (such as the aforementioned tissue sample preparation module). On the basis of our results, an appropriate SAV ratio of $\geq 11.5\text{mm}^{-1}$ (at a flow rate of $0.5\mu\text{L}/\text{min}$) was recommended for our GOI primer chambers in order to obtain a useable cDNA amplification efficiency of $\geq 80\%$ for downstream applications.

With our second integrated LOC system embodying the continuous-flow PCR approach, we have demonstrated normalized fold changes of 1.62 and 1.52 for AST and ALT respectively from the quantitative results obtained from end-point fluorescent images. These results were comparable to those obtained by our first integrated LOC system embodying the stationary PCR approach and those obtained by conventional methodology. Additionally, we have illustrated significant reductions in RT-qPCR completion, wherein the entire process was completed in $<20\text{mins}$ – 3.9-fold faster than a conventional thermal cycler and ~ 3.15 -fold faster than our first integrated LOC system embodying the stationary PCR approach.

In general, the overall conclusions are that the work delineated in this thesis has served as a proof-of-concept for somewhat practical NAT LOC devices for POCT liver assessment. Collectively, we have demonstrated the feasibility of our two integrated LOC systems for multiplex gene expression analysis in hepatotoxicity assessment, where they featured *rapid*, *semi-automation*, relative *ease-of-use* and *sample-in-answer-out* capabilities. As aforementioned in our introduction, such a biomedical device would be congruous with the aforementioned “third revolution of medicine” for

the realization of advanced, practical and useful medical diagnostic devices in emerging applications such as theranostics, genomic medicine, companion diagnostics and personalized healthcare.

5.2 Recommendations for Future Work

In view of the *Grand Objectives* of this research program, i.e. the realization of a practical and clinically pertinent POCT liver assessment device featuring of *rapid, fully-automated, ease-of-use*, and *sample-in-answer-out* diagnostic capabilities, we recommend the following for future work.

5.2.1 “Lab-in-a-Needle” Concept Device

In Chapters 3 and 4, we described the design and realization of our two integrated LOC systems delineated in Chapters 3 and 4, and demonstrated a proof-of-principle for viable POCT hepatotoxicity assessment. We recommend future work towards the realization of a practical POCT liver assessment device to a substantially smaller and integrated form. This would require substantial improvement to our tissue sample preparation module, miniaturization the individual NAT modules, and interface the entire LOC platform with a tissue or blood sampling function. Specifically, we have envisioned such a POCT liver assessment device, and we have termed this as the “*Lab-in-a-Needle*”. This is detailed in our *Technical Disclosure* (NTU Ref: TD/270/14) filed with Nanyang Technological University on 29th September 2014 and

our *US Provisional Patent* (Application No: 62/133,014) accorded on 13th March 2015. We will now succinctly describe our “*Lab-in-a-Needle*” concept.

The proposed “*Lab-in-a-Needle*” concept device would comprise the integration of prior-art LOC microfluidic technologies and a standard core biopsy needle or a standard hypodermic needle, wherein the novelty of such a device is in the integration or combination thereof. Depending on the type of needle used, this “*Lab-in-a-Needle*” diagnostic tool is envisioned to either be compatible with tissue biopsies (i.e. liver tissue samples biopsied from the patient during a key-hole surgery using a core biopsy needle), or compatible with liquid biopsies (i.e. crude blood sampled from the patient using a hypodermic needle). We believe that the novel integration of these two components would create an entirely new class of liver diagnostic tools.

Using this integrated liver diagnostic device, clinicians would be able to perform liver assessment substantially more simply and significantly more expeditiously. By assimilating the handling procedure of an industrial-standard liver core biopsy needle (or hypodermic needle) with a fully-automated microfluidic NAT process into a single instrument, a surgeon or clinician would be able to operate or handle this device for the purpose of liver biopsy (see Figure 5-1 for an example), or blood sampling, and obtain an assessment – without the need of additional trained technicians for the diagnosis. Besides the use of such a device in the domestic market for POCT clinical applications, this “*Lab-in-a-Needle*” diagnostic device could even be extended to adverse field environments where the same simplicity in operation is required. For example, such a portable and miniaturized liver assessment device could be expanded for military use, such as to detect biotoxicity in situations like biochemical warfare.

Congruous with the typical usage and disposability of industrial-standard core biopsy needles or hypodermic needles, this “*Lab-in-a-Needle*” diagnostic device also has components which are envisioned to be detachable and disposable so as to prevent cross-contamination between usages and to adhere to medically pertinent safety protocols. The parts that are envisioned to be disposable are the penetrating needles (e.g. core biopsy needle or hypodermic needle), as well as the polymeric microfluidic chip used for NAT and gene expression analysis. The main components driving the LOC system would remain housed within the main-body of the device and are not disposable to keep operating costs low.

Figure 5-1 An example of use of “Lab-in-a-Needle” device with liver biopsy for liver assessment

In short, in the perspective of our *Grand Objectives* delineated in Chapter 1, the embodiment of our “*Lab-in-a-Needle*” concept device has all the features of a practical and clinically pertinent POCT liver assessment device – *rapid, fully-automated, ease-of-use*, and *sample-in-answer-out* diagnostic capabilities.

5.2.2 Verification of Critical Target Gene List

As aforementioned in our *Specific Objectives* in Chapter 1, there is a need to simplify and verify the list of critical GOIs for liver assessment obtained from gene expression profiling datasets. Our clinical collaborators at HMRI are of the opinion that a gene expression analysis involving a group of 6-12 critical GOIs would be needed for a practical clinical hepatotoxicity assessment device.

At this juncture, we have initiated a preliminary selection process (delineated in Chapter 2) by performing a statistical analysis on a database repository of extensive microarray gene expression profiling experiments [23, 28-31] from comprehensive animal studies. This preliminary selection process is crucial for a comprehensive liver assessment, and complements our aforementioned proposed “*Lab-in-a-Needle*” concept device for practical POCT liver assessment.

To the best of our knowledge, the validation of a specific combination of a group of critical GOIs for general hepatotoxicity assessment has yet to be determined. Hence, we recommend that future work to ascertain our preliminary list of critical GOIs using comprehensive and extensive drug studies (e.g. a range of commonly administered

chemotherapeutic drugs) on laboratory mice. Furthermore, in liver disease classification, we recommend an extension of the scope of said validation studies by investigating liver-diseased mice (vis-à-vis drug-administered mice) and correlating the more prevalent types of liver diseases such as viral hepatitis, alcoholic cirrhosis, hepatic cholestasis, etc., to changes in gene expression levels of said group of critical GOIs.

Subsequently, this ascertained group of 6-12 critical GOIs would have to be subjected to multiplex gene expression analysis on a microfluidic platform, where the overall quantitative changes in their gene expression levels could consistently and robustly identify the extent of liver damage due to hepatotoxicity and liver disease.

5.2.3 Printed Electronics in Microfluidics

Printed electronics is essentially based on organic semiconductor materials, usually in a solution form, making it possible to deposit materials on a surface using printing techniques that resemble media art printing. This printing technique allows printing of electronic circuits on nonconventional media such as paper, polymers, textiles, etc. The rapid development of printed electronics technology in recent years has begun to change the conventional electronics paradigm towards low-cost electronic components that could potentially be produced in high-volume and high-throughput [122]. These printed electronic components or devices are often small, lightweight, flexible, thin, low-cost and disposable. In our view, these characteristics are extremely amenable with microfluidic devices where miniaturization of devices, portability, low-cost and

disposability are important design factors. For example, we recommend exploration of incorporating printed electronic components such as resistive heaters, working electrodes, conductive wires, etc, onto our microfluidic biochip and system we would be able to further miniaturize our integrated LOC system. Further, the addition of miniaturized printed resistive heaters and thermistors would allow for precise, localized and direct heating – pertinent for rapid and accurate thermal cycling.

In addition, we recommend further exploration on incorporating printed electronics with ‘intelligence’ (i.e. signal processing) to ‘non-intelligent’ resistive heaters, etc. Printed electronics on flexible polymer films have been realized in literature – in one recent study, Chang *et al.* [123] described the first fully-additive printing process capable of realizing complex circuits/systems on flexible polymer films. With ‘intelligent’ printed electronics, we recommend exploration of alternative nucleic acid detection methods, such as electrochemical detection [124] of amplified gene targets.

BIBLIOGRAPHY

- [1] P. J. Santrach, *Current & Future Applications of Point of Care Testing*, Mayo Clinic, <http://www.cdc.gov>.
- [2] AdvaMedDx, "Introduction to Molecular Diagnostics," *The Essentials of Diagnostics Series*, [http://advameddx.org/download/files/AdvaMedDx_DxInsights_FINAL\(2\).pdf](http://advameddx.org/download/files/AdvaMedDx_DxInsights_FINAL(2).pdf), 2013].
- [3] American Liver Foundation. "Liver Wellness: Increasing Public Awareness of Liver Health," <http://www.liverfoundation.org/>.
- [4] National Cancer Centre. Singapore. "Treatment for Liver Cancer," 2014; <http://www.singhealth.com.sg/PatientCare/ConditionsAndTreatments/Pages/Liver-Cancer.aspx>.
- [5] K. R. Reddy, and T. Faust, *The Clinician's Guide to Liver Disease*: SLACK Incorporated, 2006.
- [6] J. Blaha, and S. Duarte. "Histology Technology and Lean Healthcare Hand in Hand," 21 September 2014; <http://www.isixsigma.com/methodology/kaizen/histology-technology-and-lean-healthcare-hand-hand/>.
- [7] J. Ho, O. Aridor, and A. V. Parwani, "Use of contextual inquiry to understand anatomic pathology workflow: Implications for digital pathology adoption," *Journal of pathology informatics*, vol. 3, 2012.
- [8] C. Zhang, J. Xu, W. Ma, and W. Zheng, "PCR microfluidic devices for DNA amplification," *Biotechnology Advances*, vol. 24, no. 3, pp. 243-284, 2006.
- [9] G. J. Kost, *Principles & Practice of Point-of-care Testing*: Lippincott Williams & Wilkins, 2002.
- [10] P. Craw, and W. Balachandran, "Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review," *Lab Chip*, vol. 12, no. 14, pp. 2469-86, Jul 21, 2012.
- [11] K. J. Shaw, E. M. Hughes, C. E. Dyer, J. Greenman, and S. J. Haswell, "Integrated RNA extraction and RT-PCR for semi-quantitative gene expression studies on a microfluidic device," *Lab Invest*, vol. 93, no. 8, pp. 961-966, 08//print, 2013.
- [12] P. M. van Midwoud, E. Verpoorte, and G. M. Groothuis, "Microfluidic devices for in vitro studies on liver drug metabolism and toxicity," *Integr Biol (Camb)*, vol. 3, no. 5, pp. 509-21, May, 2011.
- [13] S. M. Hattersley, C. E. Dyer, J. Greenman, and S. J. Haswell, "Development of a microfluidic device for the maintenance and interrogation of viable tissue biopsies," *Lab Chip*, vol. 8, no. 11, pp. 1842-6, Nov, 2008.

- [14] R. Baudoin, A. Corlu, L. Griscom, C. Legallais, and E. Leclerc, "Trends in the development of microfluidic cell biochips for in vitro hepatotoxicity," *Toxicol In Vitro*, vol. 21, no. 4, pp. 535-44, Jun, 2007.
- [15] K. Anderson, J. M. Cooper, S. J. Haswell, D. Marshall, H. Yin, and X. Zhang, "Microfluidic-based measurements of cytochrome P450 enzyme activity of primary mammalian hepatocytes," *Analyst*, vol. 135, no. 6, pp. 1282-7, Jun, 2010.
- [16] K. A. Hagan, C. R. Reedy, M. L. Uchimoto, D. Basu, D. A. Engel, and J. P. Landers, "An integrated, valveless system for microfluidic purification and reverse transcription-PCR amplification of RNA for detection of infectious agents," *Lab on a Chip - Miniaturisation for Chemistry and Biology*, vol. 11, no. 5, pp. 957-961, //, 2011.
- [17] S. Pernagallo, G. Ventimiglia, C. Cavalluzzo, E. Alessi, H. Ilyine, M. Bradley, and J. J. Diaz-Mochon, "Novel biochip platform for nucleic acid analysis," *Sensors (Switzerland)*, vol. 12, no. 6, pp. 8100-8111, 2012.
- [18] D. Chen, M. Mauk, X. Qiu, C. Liu, J. Kim, S. Ramprasad, S. Ongagna, W. R. Abrams, D. Malamud, P. L. A. M. Corstjens, and H. H. Bau, "An integrated, self-contained microfluidic cassette for isolation, amplification, and detection of nucleic acids," *Biomedical Microdevices*, vol. 12, no. 4, pp. 705-719, 2010.
- [19] J. A. Lounsbury, A. Karlsson, D. C. Miranian, S. M. Cronk, D. A. Nelson, J. Li, D. M. Haverstick, P. Kinnon, D. J. Saul, and J. P. Landers, "From sample to PCR product in under 45 minutes: a polymeric integrated microdevice for clinical and forensic DNA analysis," *Lab Chip*, vol. 13, no. 7, pp. 1384-93, Apr 7, 2013.
- [20] J. A. Lounsbury, and J. P. Landers, "Ultrafast amplification of DNA on plastic microdevices for forensic short tandem repeat analysis," *Journal of Forensic Sciences*, vol. 58, no. 4, pp. 866-874, 2013.
- [21] Y. Zhang, S. Park, S. Yang, and T. H. Wang, "An all-in-one microfluidic device for parallel DNA extraction and gene analysis," *Biomedical Microdevices*, vol. 12, no. 6, pp. 1043-1049, 2010.
- [22] C. Zhang, and D. Xing, "Miniaturized PCR chips for nucleic acid amplification and analysis: Latest advances and future trends," *Nucleic Acids Research*, vol. 35, no. 13, pp. 4223-4237, 2007.
- [23] H. Gohlmann, and W. Talloen, *Gene Expression Studies Using Affymetrix Microarrays*: Taylor & Francis, 2009.
- [24] M. Lia, R. Barouki, and S. G. Waelsch, "Chromosomal deletions around the albino locus in the mouse cause loss of hormone-inducible expression of the unlinked structural gene encoding cytosolic aspartate aminotransferase," *Proc Natl Acad Sci U S A*, vol. 92, no. 3, pp. 788-90, Jan 31, 1995.
- [25] W. J. Reagan, R. Z. Yang, S. Park, R. Goldstein, D. Brees, and D. W. Gong, "Metabolic adaptive ALT isoenzyme response in livers of C57/BL6 mice treated with dexamethasone," *Toxicol Pathol*, vol. 40, no. 8, pp. 1117-27, Dec, 2012.

- [26] L. Sosa, D. Vidlak, J. M. Strachota, J. Pavlik, and T. R. Jerrells, "Rescue of in vivo FAS-induced apoptosis of hepatocytes by corticosteroids either associated with alcohol consumption by mice or provided exogenously," *Int Immunopharmacol*, vol. 5, no. 2, pp. 301-14, Feb, 2005.
- [27] S. B. Jadhao, R. Z. Yang, Q. Lin, H. Hu, F. A. Anania, A. R. Shuldiner, and D. W. Gong, "Murine alanine aminotransferase: cDNA cloning, functional expression, and differential gene regulation in mouse fatty liver," *Hepatology*, vol. 39, no. 5, pp. 1297-302, May, 2004.
- [28] B. J. Ovando, C. M. Vezina, B. P. McGarrigle, and J. R. Olson, "Hepatic gene downregulation following acute and subchronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin," *Toxicological Sciences*, vol. 94, no. 2, pp. 428-438, 2006.
- [29] T. M. Bosch, A. D. R. Huitema, V. D. Doodeman, R. Jansen, E. Witteveen, W. M. Smit, R. L. Jansen, C. M. Van Herpen, M. Soesan, J. H. Beijnen, and J. H. M. Schellens, "Pharmacogenetic screening of CYP3A and ABCB1 in relation to population pharmacokinetics of docetaxel," *Clinical Cancer Research*, vol. 12, no. 19, pp. 5786-5793, 2006.
- [30] A. Roth, F. Boess, C. Landes, G. Steiner, C. Freichel, J. M. Plancher, S. Raab, C. de Vera Mudry, T. Weiser, and L. Suter, "Gene expression-based in vivo and in vitro prediction of liver toxicity allows compound selection at an early stage of drug development," *Journal of Biochemical and Molecular Toxicology*, vol. 25, no. 3, pp. 183-194, 2011.
- [31] B. J. Ovando, C. A. Ellison, C. M. Vezina, and J. R. Olson, "Toxicogenomic analysis of exposure to TCDD, PCB126 and PCB153: Identification of genomic biomarkers of exposure to AhR ligands," *BMC Genomics*, vol. 11, no. 1, 2010.
- [32] R. A. Standish, E. Cholongitas, A. Dhillon, A. K. Burroughs, and A. P. Dhillon, "An appraisal of the histopathological assessment of liver fibrosis," *Gut*, vol. 55, no. 4, pp. 569-578, 2006.
- [33] R. J. Andrade, M. Robles, A. Fernandez-Castaner, S. Lopez-Ortega, M. C. Lopez-Vega, and M. I. Lucena, "Assessment of drug-induced hepatotoxicity in clinical practice: a challenge for gastroenterologists," *World J Gastroenterol*, vol. 13, no. 3, pp. 329-40, Jan 21, 2007.
- [34] L. H. Augenlicht, and D. Kobrin, "Cloning and screening of sequences expressed in a mouse colon tumor," *Cancer Research*, vol. 42, no. 3, pp. 1088-1093, 1982.
- [35] M. J. Cunningham, S. Liang, S. Furman, J. J. Seilhamer, and R. Somogyi, "Gene Expression Microarray Data Analysis for Toxicology Profiling," *Annals of the New York Academy of Sciences*, vol. 919, no. 1, pp. 52-67, 2000.
- [36] M. A. Northrup, M. T. Ching, R. M. White, and R. T. Watson, "DNA Amplification with a Microfabricated Reaction Chamber," in 7th International Conference on Solid-State Sensors and Actuators (Transducers '93), Yokohama, Japan, 1993, pp. 924-26.

- [37] M. U. Kopp, A. J. De Mello, and A. Manz, "Chemical amplification: Continuous-flow PCR on a chip," *Science*, vol. 280, no. 5366, pp. 1046-1048, 1998.
- [38] J. A. Kim, J. Y. Lee, S. Seong, S. H. Cha, S. H. Lee, J. J. Kim, and T. H. Park, "Fabrication and characterization of a PDMS-glass hybrid continuous-flow PCR chip," *Biochemical Engineering Journal*, vol. 29, no. 1-2, pp. 91-97, 2006.
- [39] Q. Xiang, B. Xu, R. Fu, and D. Li, "Real time PCR on disposable PDMS chip with a miniaturized thermal cycler," *Biomedical microdevices*, vol. 7, no. 4, pp. 273-279, 2005.
- [40] N. C. Cady, S. Stelick, M. V. Kunnavaakkam, Y. Liu, and C. A. Batt, "A microchip-based DNA purification and real-time PCR biosensor for bacterial detection," *IEEE Sensors 2004*. pp. 1191-1194.
- [41] S. H. Lee, S. W. Kim, J. Y. Kang, and C. H. Ahn, "A polymer lab-on-a-chip for reverse transcription (RT)-PCR based point-of-care clinical diagnostics," *Lab on a Chip - Miniaturisation for Chemistry and Biology*, vol. 8, no. 12, pp. 2121-2127, 2008.
- [42] C. J. Backhouse, T. Footz, S. Adamia, and L. M. Pilarski, "Microfluidic chips for the molecular analysis of human cancer." pp. 377-382.
- [43] X. Qiu, D. Chen, C. Liu, M. G. Mauk, T. Kientz, and H. H. Bau, "A portable, integrated analyzer for microfluidic - Based molecular analysis," *Biomedical Microdevices*, vol. 13, no. 5, pp. 809-817, 2011.
- [44] P. Belgrader, W. Benett, D. Hadley, G. Long, R. Mariella Jr, F. Milanovich, S. Nasarabadi, W. Nelson, J. Richards, and P. Stratton, "Rapid pathogen detection using a microchip PCR array instrument," *Clinical Chemistry*, vol. 44, no. 10, pp. 2191-2194, 1998.
- [45] A. Gulliksen, L. Solli, F. Karlsen, H. Rogne, E. Hovig, T. Nordstrøm, and R. Sirevåg, "Real-Time Nucleic Acid Sequence-Based Amplification in Nanoliter Volumes," *Analytical Chemistry*, vol. 76, no. 1, pp. 9-14, 2004.
- [46] C. Ke, A. M. Kelleher, H. Berney, M. Sheehan, and A. Mathewson, "Single step cell lysis/PCR detection of Escherichia coli in an independently controllable silicon microreactor," *Sensors and Actuators, B: Chemical*, vol. 120, no. 2, pp. 538-544, 2007.
- [47] L. C. Waters, S. C. Jacobson, N. Kroutchinina, J. Khandurina, R. S. Foote, and J. M. Ramsey, "Microchip Device for Cell Lysis, Multiplex PCR Amplification, and Electrophoretic Sizing," *Analytical Chemistry*, vol. 70, no. 1, pp. 158-162, 1998.
- [48] Y. Sun, and Y. C. Kwok, "Polymeric microfluidic system for DNA analysis," *Analytica Chimica Acta*, vol. 556, no. 1, pp. 80-96, 2006.
- [49] D. Li, *Encyclopedia of Microfluidics and Nanofluidics*: Springer, 2008.

- [50] F. C. Huang, C. S. Liao, and G. B. Lee, "An integrated microfluidic chip for DNA/RNA amplification, electrophoresis separation and on-line optical detection," *Electrophoresis*, vol. 27, no. 16, pp. 3297-3305, 2006.
- [51] R. C. Anderson, X. Su, G. J. Bogdan, and J. Fenton, "A miniature integrated device for automated multistep genetic assays," *Nucleic acids research*, vol. 28, no. 12, 2000.
- [52] I. Chartier, J. Sudor, Y. Fouillet, N. Sarrut, C. Bory, and A. Gruss, "Fabrication of an hybrid plastic-silicon microfluidic device for high-throughput genotyping," *Microfluidics, BioMEMS, and Medical Microsystems*. pp. 208-219.
- [53] D. S. W. Park, M. L. Hupert, M. A. Witek, B. H. You, P. Datta, J. Guy, J. B. Lee, S. A. Soper, D. E. Nikitopoulos, and M. C. Murphy, "A titer plate-based polymer microfluidic platform for high throughput nucleic acid purification," *Biomedical Microdevices*, vol. 10, no. 1, pp. 21-33, 2008.
- [54] M. L. Hupert, M. A. Witek, Y. Wang, M. W. Mitchell, Y. Liu, Y. Bejat, D. E. Nikitopoulos, J. Goettert, M. C. Murphy, and S. A. Soper, "Polymer-based microfluidic devices for biomedical applications," *Microfluidics, BioMEMS, and Medical Microsystems*. pp. 52-64.
- [55] D. S. Lee, S. H. Park, H. Yang, K. H. Chung, T. H. Yoon, S. J. Kim, K. Kim, and Y. T. Kim, "Bulk-micromachined submicroliter-volume PCR chip with very rapid thermal response and low power consumption," *Lab on a Chip - Miniaturisation for Chemistry and Biology*, vol. 4, no. 4, pp. 401-407, 2004.
- [56] J. El-Ali, I. R. Perch-Nielsen, C. R. Poulsen, D. D. Bang, P. Telleman, and A. Wolff, "Simulation and experimental validation of a SU-8 based PCR thermocycler chip with integrated heaters and temperature sensor," *Sensors and Actuators, A: Physical*, vol. 110, no. 1-3, pp. 3-10, 2004.
- [57] J. West, B. Karamata, B. Lillis, J. P. Gleeson, J. Alderman, J. K. Collins, W. Lane, A. Mathewson, and H. Berney, "Application of magnetohydrodynamic actuation to continuous flow chemistry," *Lab on a Chip - Miniaturisation for Chemistry and Biology*, vol. 2, no. 4, pp. 224-230, 2002.
- [58] C. G. Koh, W. Tan, M. Q. Zhao, A. J. Ricco, and Z. H. Fan, "Integrating polymerase chain reaction, valving, and electrophoresis in a plastic device for bacterial detection," *Analytical Chemistry*, vol. 75, no. 17, pp. 4591-4598, 2003.
- [59] H. Becker, and C. Gärtner, "Polymer microfabrication methods for microfluidic analytical applications," *Electrophoresis*, vol. 21, no. 1, pp. 12-26, 2000.
- [60] A. T. Woolley, D. Hadley, P. Landre, A. J. DeMello, R. A. Mathies, and M. A. Northrup, "Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device," *Analytical Chemistry*, vol. 68, no. 23, pp. 4081-4086, 1996.
- [61] I. Rodriguez, M. Lesaichere, Y. Tie, Q. Zou, C. Yu, J. Singh, L. T. Meng, S. Uppili, S. F. Y. Li, P. Gopalakrishnakone, and Z. E. Selvanayagam, "Practical integration of

polymerase chain reaction amplification and electrophoretic analysis in microfluidic devices for genetic analysis," *Electrophoresis*, vol. 24, no. 1-2, pp. 172-178, 2003.

- [62] J. W. Hong, T. Fujii, M. Seki, T. Yamamoto, and I. Endo, "Integration of gene amplification and capillary gel electrophoresis on a polydimethylsiloxane-glass hybrid microchip," *Electrophoresis*, vol. 22, no. 2, pp. 328-333, 2001.
- [63] J. H. Daniel, S. Iqbal, R. B. Millington, D. F. Moore, C. R. Lowe, D. L. Leslie, M. A. Lee, and M. J. Pearce, "Silicon microchambers for DNA amplification," *Sensors and Actuators A: Physical*, vol. 71, no. 1-2, pp. 81-88, 11/1/, 1998.
- [64] P. C. Chen, W. Fan, T. K. Hoo, L. C. Z. Chan, and Z. Wang, "Simulation guided-design of a microfluidic thermal reactor for polymerase chain reaction," *Chemical Engineering Research and Design*, vol. 90, no. 5, pp. 591-599, 2012.
- [65] E. T. Lagelly, J. R. Scherer, R. G. Blazej, N. M. Toriello, B. A. Diep, M. Ramchandani, G. F. Sensabaugh, L. W. Riley, and R. A. Mathies, "Integrated portable genetic analysis microsystem for pathogen/infectious disease detection," *Analytical Chemistry*, vol. 76, no. 11, pp. 3162-3170, 2004.
- [66] C. S. Liao, G. B. Lee, J. J. Wu, C. C. Chang, T. M. Hsieh, F. C. Huang, and C. H. Luo, "Micromachined polymerase chain reaction system for multiple DNA amplification of upper respiratory tract infectious diseases," *Biosensors and Bioelectronics*, vol. 20, no. 7, pp. 1341-1348, 2005.
- [67] P. Belgrader, C. J. Elkin, S. B. Brown, S. N. Nasarabadi, R. G. Langlois, F. P. Milanovich, B. W. Colston Jr, and G. D. Marshall, "A reusable flow-through polymerase chain reaction instrument for the continuous monitoring of infectious biological agents," *Analytical Chemistry*, vol. 75, no. 14, pp. 3446-3450, 2003.
- [68] Q. Wang, and H. Gong, "An integrated software solution for real-time PCR analysis based on microfluidic biochip," *Bioengineered and Bioinspired Systems*. pp. 77-86.
- [69] R. P. Oda, M. A. Strausbauch, A. F. R. Huhmer, N. Borson, S. R. Jurens, J. Craighead, P. J. Wettstein, B. Eckloff, B. Kline, and J. P. Landers, "Infrared-Mediated Thermocycling for Ultrafast Polymerase Chain Reaction Amplification of DNA," *Analytical Chemistry*, vol. 70, no. 20, pp. 4361-4368, 1998.
- [70] B. C. Giordano, J. Ferrance, S. Swedberg, A. F. R. Hühmer, and J. P. Landers, "Polymerase chain reaction in polymeric microchips: DNA amplification in less than 240 seconds," *Analytical Biochemistry*, vol. 291, no. 1, pp. 124-132, 2001.
- [71] M. N. Slyadnev, Y. Tanaka, M. Tokeshi, and T. Kitamori, "Photothermal temperature control of a chemical reaction on a microchip using an infrared diode laser," *Analytical Chemistry*, vol. 73, no. 16, pp. 4037-4044, 2001.
- [72] Y. Tanaka, M. N. Slyadnev, A. Hibara, M. Tokeshi, and T. Kitamori, "Non-contact photothermal control of enzyme reactions on a microchip by using a compact diode laser," *Journal of Chromatography A*, vol. 894, no. 1-2, pp. 45-51, 2000.

- [73] D. Pal, and V. Venkataraman, "A portable battery-operated chip thermocycler based on induction heating," *Sensors and Actuators, A: Physical*, vol. 102, no. 1-2, pp. 151-156, 2002.
- [74] P. C. Chen, "An evaluation of a real-time passive micromixer to the performance of a continuous flow type microfluidic reactor," *Biochip Journal*, vol. 7, no. 3, pp. 227-233, 2013.
- [75] J. H. Daniel, S. Iqbal, R. B. Millington, D. F. Moore, C. R. Lowe, D. L. Leslie, M. A. Lee, and M. J. Pearce, "Silicon microchambers for DNA amplification," *Sensors and Actuators, A: Physical*, vol. 71, no. 1-2, pp. 81-88, 1998.
- [76] C. J. Easley, J. M. Karlinsey, J. M. Bienvenue, L. A. Legendre, M. G. Roper, S. H. Feldman, M. A. Hughes, E. L. Hewlett, T. J. Merkel, J. P. Ferrance, and J. P. Landers, "A fully integrated microfluidic genetic analysis system with sample-in-answer-out capability," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 51, pp. 19272-19277, 2006.
- [77] A. F. R. Huhmer, and J. P. Landers, "Noncontact infrared-mediated thermocycling for effective polymerase chain reaction amplification of DNA in nanoliter volumes," *Analytical Chemistry*, vol. 72, no. 21, pp. 5507-5512, 2000.
- [78] M. B. Sayers, T. M. Dalton, and M. R. Davies, "Real-time fluorescence monitoring of the polymerase chain reaction in a novel continuous flow reactor for accurate DNA quantification," *4th International Conference on Nanochannels, Microchannels and Minichannels, ICNMM2006*. pp. 1045-1051.
- [79] K. Han, T. Y. Lee, D. E. Nikitopoulos, S. A. Soper, and M. C. Murphy, "A vertically stacked, polymer, microfluidic point mutation analyzer: Rapid high accuracy detection of low-abundance K-ras mutations," *Analytical Biochemistry*, vol. 417, no. 2, pp. 211-219, 2011.
- [80] G. J. Zhang, J. H. Chua, R. E. Chee, A. Agarwal, and S. M. Wong, "Label-free direct detection of MiRNAs with silicon nanowire biosensors," *Biosensors and Bioelectronics*, vol. 24, no. 8, pp. 2504-2508, 2009.
- [81] Z. Zhao, D. Cui, S. Xia, and Z. Cui, "An integrated biochip design and fabrication," *Nano- and Microtechnology: Materials, Processes, Packaging, and Systems*. pp. 321-326.
- [82] M. A. Burns, B. N. Johnson, S. N. Brahmasandra, K. Handique, J. R. Webster, M. Krishnan, T. S. Sammarco, P. M. Man, D. Jones, D. Heldsinger, C. H. Mastrangelo, and D. T. Burke, "An integrated nanoliter DNA analysis device," *Science*, vol. 282, no. 5388, pp. 484-487, 1998.
- [83] S. J. Baldwin, J. L. Bramhall, C. A. Ashby, L. Yue, P. R. Murdock, S. R. Hood, A. D. Ayrton, and S. E. Clarke, "Cytochrome P450 gene induction in rats ex vivo assessed by quantitative real-time reverse transcriptase-polymerase chain reaction (TaqMan)," *Drug Metab Dispos*, vol. 34, no. 6, pp. 1063-9, Jun, 2006.

- [84] N. Ramalingam, Z. Rui, H. B. Liu, C. C. Dai, R. Kaushik, B. Ratnaharika, and H. Q. Gong, "Real-time PCR-based microfluidic array chip for simultaneous detection of multiple waterborne pathogens," *Sensors and Actuators, B: Chemical*, vol. 145, no. 1, pp. 543-552, //, 2010.
- [85] D. Summerer, D. Hevroni, A. Jain, O. Oldenburger, J. Parker, A. Caruso, C. F. Stähler, P. F. Stähler, and M. Beier, "A flexible and fully integrated system for amplification, detection and genotyping of genomic DNA targets based on microfluidic oligonucleotide arrays," *New Biotechnology*, vol. 27, no. 2, pp. 149-155, 2010.
- [86] J. Steigert, M. Grumann, T. Brenner, K. Mittenbühler, T. Nann, J. Rühle, I. Moser, S. Haerberle, L. Riegger, J. Riegler, W. Bessler, R. Zengerle, and J. Ducrée, "Integrated sample preparation, reaction, and detection on a high-frequency centrifugal microfluidic platform," *JALA - Journal of the Association for Laboratory Automation*, vol. 10, no. 5, pp. 331-341, 2005.
- [87] G. S. Lim, J. S. Chang, Z. Lei, R. Wu, Z. Wang, K. Cui, and S. Wong, "A lab-on-a-chip system integrating tissue sample preparation and multiplex RT-qPCR for gene expression analysis in point-of-care hepatotoxicity assessment," *Lab on a Chip*, vol. 15, no. 20, pp. 4032-4043, 2015.
- [88] G. S. Lim, J. S. Chang, R. Wu, Z. P. Wang, K. Cui, and S. Wong, "A Segmented Multiplex Continuous-Flow Polymeric RT-PCR Microfluidic System with a Coordinate-based Quantitative Detection for Point-of-Care Gene Expression Analysis," *Biomedical Microdevices*, 2015.
- [89] S. Schumacher, J. Nestler, T. Otto, M. Wegener, E. Ehrentreich-Förster, D. Michel, K. Wunderlich, S. Palzer, K. Sohn, A. Weber, M. Burgard, A. Grzesiak, A. Teichert, A. Brandenburg, B. Koger, J. Albers, E. Nebling, and F. F. Bier, "Highly-integrated lab-on-chip system for point-of-care multiparameter analysis," *Lab on a Chip - Miniaturisation for Chemistry and Biology*, vol. 12, no. 3, pp. 464-473, 2012.
- [90] L. VanHeirstraeten, P. Spang, C. Schwind, K. Drese, M. Ritzi-Lehnert, B. Nieto, M. Camps, B. Landgraf, F. Guasch, and A. Corbera, "Integrated DNA and RNA extraction and purification on an automated microfluidic cassette from bacterial and viral pathogens causing community-acquired lower respiratory tract infections," *LAB ON A CHIP*, vol. 14, no. 9, pp. 1519-1526, 2014.
- [91] G. Jiang, and D. J. Harrison, "mRNA isolation in a microfluidic device for eventual integration of cDNA library construction," *Analyst*, vol. 125, no. 12, pp. 2176-9, Dec, 2000.
- [92] D. D. Vakharia, N. Liu, R. Pause, M. Fasco, E. Bessette, Q. Y. Zhang, and L. S. Kaminsky, "Effect of metals on polycyclic aromatic hydrocarbon induction of CYP1A1 and CYP1A2 in human hepatocyte cultures," *Toxicol Appl Pharmacol*, vol. 170, no. 2, pp. 93-103, Jan 15, 2001.
- [93] S. C. J. Yeung, C. P. Escalante, and R. F. Gagel, *Medical Care of Cancer Patients: People's Medical Publishing House*, 2009.

- [94] E. Anton, "Delayed toxicity of cyclophosphamide in normal mice," *Br J Exp Pathol*, vol. 68, no. 2, pp. 237-49, Apr, 1987.
- [95] R. Mohan, B. R. Schudel, A. V. Desai, J. D. Yearsley, C. A. Apblett, and P. J. A. Kenis, "Design considerations for elastomeric normally closed microfluidic valves," *Sensors and Actuators B: Chemical*, vol. 160, no. 1, pp. 1216-1223, 12/15/, 2011.
- [96] H. M. Xia, Z. P. Wang, W. Fan, A. Wijaya, W. Wang, and Z. F. Wang, "Converting steady laminar flow to oscillatory flow through a hydroelasticity approach at microscales," *Lab Chip*, vol. 12, no. 1, pp. 60-4, Jan 7, 2012.
- [97] H. M. Xia, Z. P. Wang, W. Wang, W. Fan, A. Wijaya, and Z. F. Wang, "Aeroelasticity-based fluid agitation for lab-on-chips," *Lab on a Chip*, vol. 13, no. 8, pp. 1619-1625, 2013.
- [98] H. B. Liu, H. Q. Gong, N. Ramalingam, Y. Jiang, C. C. Dai, and K. M. Hui, "Micro air bubble formation and its control during polymerase chain reaction (PCR) in polydimethylsiloxane (PDMS) microreactors," *Journal of Micromechanics and Microengineering*, vol. 17, no. 10, pp. 2055-2064, 2007.
- [99] K. E. Kouadjo, Y. Nishida, J. F. Cadrin-Girard, M. Yoshioka, and J. St-Amand, "Housekeeping and tissue-specific genes in mouse tissues," *BMC Genomics*, vol. 8, pp. 127, 2007.
- [100] E. d. L. Rebouças, J. J. d. N. Costa, M. J. Passos, J. R. d. S. Passos, R. v. d. Hurk, and J. R. V. Silva, "Real time PCR and importance of housekeeping genes for normalization and quantification of mRNA expression in different tissues," *Brazilian Archives of Biology and Technology*, vol. 56, pp. 143-154, 2013.
- [101] M. B. Sayers, and T. M. Dalton, "A real-time continuous flow polymerase chain reactor for DNA expression quantification," *ASME International Mechanical Engineering Congress and Exposition, IMECE 2007*. pp. 63-69.
- [102] N. Ramalingam, H. B. Liu, C. C. Dai, Y. Jiang, H. Wang, Q. Wang, K. M Hui, and H. Q. Gong, "Real-time PCR array chip with capillary-driven sample loading and reactor sealing for point-of-care applications," *Biomedical Microdevices*, vol. 11, no. 5, pp. 1007-1020, 2009.
- [103] D. Liu, *Handbook of Nucleic Acid Purification*: CRC Press, 2009.
- [104] M. McPherson, and S. Møller, *PCR*: Taylor & Francis, 2007.
- [105] S. C. Chow, and J. Liu, *Design and Analysis of Animal Studies in Pharmaceutical Development*: Taylor & Francis, 1998.
- [106] E. Wit, and J. McClure, *Statistics for Microarrays: Design, Analysis and Inference*: Wiley, 2004.

- [107] E. A. Oblath, W. H. Henley, J. P. Alarie, and J. M. Ramsey, "A microfluidic chip integrating DNA extraction and real-time PCR for the detection of bacteria in saliva," *Lab Chip*, vol. 13, no. 7, pp. 1325-32, Apr 7, 2013.
- [108] M. T. Dorak, *Real-time PCR*: Taylor & Francis, 2007.
- [109] Life Technologies Corporation. "Real-time PCR: Understanding Ct."
- [110] J. Quackenbush, "Microarray data normalization and transformation," *Nat Genet*, 2002.
- [111] T. Iyanagi, "Molecular mechanism of phase I and phase II drug-metabolizing enzymes: implications for detoxification," *Int Rev Cytol*, vol. 260, pp. 35-112, 2007.
- [112] A. C. Hatch, T. Ray, K. Lintecum, and C. Youngbull, "Continuous flow real-time PCR device using multi-channel fluorescence excitation and detection," *Lab on a Chip*, vol. 14, no. 3, pp. 562-568, 2014.
- [113] A. Harandi, and T. Farquhar, "A microfluidic device providing continuous-flow polymerase chain reaction heating and cooling," *Journal of Micromechanics and Microengineering*, vol. 24, no. 11, pp. 115009, 2014.
- [114] B. Shu, C. Zhang, and D. Xing, "Segmented continuous-flow multiplex polymerase chain reaction microfluidics for high-throughput and rapid foodborne pathogen detection," *Anal Chim Acta*, vol. 826, pp. 51-60, May 15, 2014.
- [115] R. Hartung, A. Brösing, G. Sczcepankiewicz, U. Liebert, N. Häfner, M. Dürst, J. Felbel, D. Lassner, and J. M. Köhler, "Application of an asymmetric helical tube reactor for fast identification of gene transcripts of pathogenic viruses by micro flow-through PCR," *Biomedical Microdevices*, vol. 11, no. 3, pp. 685-692, 2009.
- [116] A. Gonzalez, D. Ciobanu, M. Sayers, N. Sirr, T. Dalton, and M. Davies, "Gene transcript amplification from cell lysates in continuous-flow microfluidic devices," *Biomed Microdevices*, vol. 9, no. 5, pp. 729-36, Oct, 2007.
- [117] B. Shu, C. Zhang, and D. Xing, "A handheld flow genetic analysis system (FGAS): towards rapid, sensitive, quantitative and multiplex molecular diagnosis at the point-of-care level," *Lab on a Chip*, vol. 15, no. 12, pp. 2597-2605, 2015.
- [118] J. Nie, Y. Zhao, and N. Peng, "Multichannel oscillatory-flow PCR micro-fluidic chip with controllable temperature gradient," *Microsyst. Technol.*, vol. 21, no. 1, pp. 41-48, 2015.
- [119] B. C. Giordano, E. R. Copeland, and J. P. Landers, "Towards dynamic coating of glass microchip chambers for amplifying DNA via the polymerase chain reaction," *Electrophoresis*, vol. 22, no. 2, pp. 334-40, Jan, 2001.
- [120] W. Schrott, Z. Slouka, P. Červenka, J. Ston, M. Nebyla, M. Přibyl, and D. Šnita, "Study on surface properties of PDMS microfluidic chips treated with albumin," *Biomicrofluidics*, vol. 3, no. 4, pp. 044101, 10/12, 2009.

- [121] I. Pjescic, C. Tranter, P. L. Hindmarsh, and N. D. Crews, "Glass-composite prototyping for flow PCR with in situ DNA analysis," *Biomed Microdevices*, vol. 12, no. 2, pp. 333-43, Apr, 2010.
- [122] E. Coatanéa, V. Kantola, J. Kulovesi, L. Lahti, R. Lin, and M. Zavodchikova, *Printed Electronics, Now and Future.*, p.^pp. 63-102: Helsinki University of Technology (TKK), MIDE, Helsinki University Print, Helsinki, Finland, 2009.
- [123] J. Chang, X. Zhang, T. Ge, and J. Zhou, "Fully printed electronics on flexible substrates: High gain amplifiers and DAC," *Organic Electronics*, vol. 15, no. 3, pp. 701-710, 2014.
- [124] H. Dong, C. M. Li, Y. F. Zhang, X. D. Cao, and Y. Gan, "Screen-printed microfluidic device for electrochemical immunoassay," *Lab Chip*, vol. 7, no. 12, pp. 1752-8, Dec, 2007.