An integrated microfluidic system for hepatotoxicity assessment based on gene expression profiling : from tissue homogenization to RT-PCR

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**AN INTEGRATED MICROFLUIDIC SYSTEM FOR
HEPATOTOXICITY ASSESSMENT BASED ON
GENE EXPRESSION PROFILING:
FROM TISSUE HOMOGENIZATION TO RT-PCR**

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SCHOOL OF ELECTRICAL AND ELECTRONIC ENGINEERING

2017

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FROM TISSUE HOMOGENIZATION TO RT-PCR**

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ABSTRACT

A practical, rapid, low-cost, and largely ‘equipment-free’ point-of-care (POC) testing device for hepatotoxicity assessment is, at this juncture, unavailable/unreported. In this thesis, we will describe our effort towards the design and realization of this POC device – two lab-on-a-chip (LOC) systems for gene of interest (GOI) expression detection with crude liver tissue input for hepatotoxicity assessment.

For the first LOC system, we describe our *semi-integrated* 2-chip (a tissue homogenization/mRNA extraction chip and a stationary multiplex microfluidic reverse transcription-polymerase chain reaction (RT-PCR) chip) LOC system with real-time fluorescent detection. We demonstrate that our tissue homogenization/mRNA extraction chip (based on our verified modification of a selected conventional manual tissue homogenization and mRNA extraction method (for smaller sample input and less chemical reagents)) has an mRNA extraction efficiency comparable to our said modification of selected conventional manual protocol. With liver tissue samples from our pre-clinical drug toxicity study on mice administrated with different dosages of cyclophosphamide, we demonstrate that our tissue homogenization/mRNA extraction chip is applicable for semi-quantitative multiplex gene expression studies. Specifically, the gene expression level of GOIs (AST, AST1, and ALT1) derived from our tissue homogenization/mRNA extraction chip elevates with increasing dosages of cyclophosphamide and is consistent with that obtained from the conventional laboratory procedures in reported studies. We perform multiplex RT-PCR for GOIs (AST and ALT) on our stationary multiplex RT-PCR chip with the mRNA extracted from our tissue homogenization/mRNA extraction chip. The electrophoresis gel image

and real-time fluorescent images of our on-chip multiplex RT-PCR process indicate elevations of expression level of both GOIs (AST and ALT) with increasing dosages of cyclophosphamide. These results are consistent with that obtained from the conventional laboratory procedure.

For the second LOC system, we describe a *fully-integrated* LOC system comprising a tissue homogenization/mRNA extraction component and a continuous-flow RT-PCR component for rapid processing within an enclosed microfluidic environment. We demonstrate that our continuous-flow RT-PCR chip is able to amplify a specific gene (ALT1) rather rapidly – within 45 minutes, which is equivalent to 50% time reduction of the conventional RT-PCR process. Nevertheless, our on-chip RT-PCR only achieves 60% amplification efficiency of the conventional RT-PCR. By integrating a tissue homogenization/mRNA extraction chip and a continuous-flow RT-PCR chip, we realize a *fully-integrated* microfluidic system. This system features rapid processing – 33% time reduction of our first LOC system. However, our integrated LOC system only achieves ~50% amplification efficiency of the conventional manual procedure.

In short, our work herein contributes towards the continuing development of a new generation of a practical, rapid, low cost POC testing device with “sample-in-answer-out” capability for hepatotoxicity assessment as part of liver disease diagnosis.

AUTHOR'S PUBLICATIONS

Provisional Patent and PCT

- [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”
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- [2] 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”
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- [3] J. S. Chang, G. S. Lim, **L. Zhang**, Z. P. Wang, R. Wu, K. Cui, and S. Wong
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- [4] G. S. Lim, J. S. Chang, **L. Zhang**, R. Wu, Z. P. Wang, K. Cui, and S. Wong
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- [5] **L. Zhang**, J. S. Chang, Z. P. Wang, G. S. Lim, W. Yang, R. Wu, K. Cui, and S. T. C. Wong
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LIST OF ABBREVIATIONS

mRNA	Messenger Ribonucleic Acid
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
LFTs	Liver Function Tests
LB	Liver Biopsy
ALT	Alanine Aminotransferases
GGT	Gamma-Glutamyl Transferase
ALP	Alkaline Phosphatase
AST	Asparatate Aminotransferase
LOC	Lab-on-a-Chip
HCC	Hepatocellular carcinoma
SAGE	Serial Analysis of Gene Expression
MPSS	Massive Parallel Signature Sequencing
AGPC	Acid Guanidinium Thiocyanate– Phenol–Chloroform
MEMS	MicroElectroMechanical Systems
μTAS	Micro-Total Analysis Systems
PDMS	Polydimethylsiloxane
COC	Cyclic Olefin Copolymer
COP	Cyclo Olefin Polymer
PMMA	Polymethylmethacrylate

PC	Polycarbonate
MHD	Magneto Hydrodynamic
PZT	Lead Zirconate Titanate
EDTA	Ethylenediaminetetraacetic acid
PTFE	Polytetrafluoroethylene
POC	Point-of-Care
GOI	Gene of Interest
PCB	Printed Circuit Board
CAD	Computer Aided Design
ANOVA	Analysis of Variance

Chapter 1 Introduction

This chapter delineates the background/motivation, objectives and contributions of our PhD research program. It also introduces the structure of this thesis.

1.1 Background and Motivation

Modern medicine is now in its ‘third revolution’ – the “convergence of the life science, physical sciences, mathematics and engineering” [1, 2]. This paradigm shift, embodying multi-disciplinary research and technology integration, inspires the development of the next generation of medical diagnostic devices and procedures. One such device is the point-of-care (POC) device for liver disease diagnosis, that would “allow patient diagnoses in physician’s office, an ambulance, the home, the field, or in hospital. The results of care are timely, and allow rapid treatment to the patients” [3]. Of specific interest, these POC devices allow diagnosis outside the hospital setting, and in some sense, “bring the hospital to the patient” instead of the conventional approach of the “patient going to the hospital”.

Diagnostic testing is a growing market with a commensurable increasing healthcare expenditure. The market for diagnostic testing was valued at US\$45.6 billion in 2012, and expected to reach US\$64.6 billion in 2017 [4]. In USA, annual diagnostic testing was tested approximately 7 billion times in 2016, and is expected to continue growing rapidly. Some of these tests are based on microfluidics, i.e., lab-on-a-chip (LOC) in

part because of its capability of manipulating small volume liquid samples, and performing complex biochemical experiments in an enclosed integrated system.

Liver disease diagnosis is one of the major applications for diagnostic testing due to the pervasiveness of liver disease globally where approximately 240 million people are infected by hepatitis B and 170 million people infected by hepatitis C. In particular, 20% to 30% of the infected population will continue to develop cirrhosis and liver cancer [5]. In USA, liver cancer is the fifth most common cause of cancer mortality among male [6]; in China, 13% to 19% of cancer mortality is caused by liver cancer [7]; whereas in Singapore, it is the fourth leading cause of cancer [8]. Early diagnosis and regular monitoring of the disease are vital to provide appropriate medical treatment.

At this juncture, there are two general methods for diagnosing liver disease and assessing the condition of the liver: Liver Function Tests (LFTs) and Liver Biopsy (LB). These two diagnosis methods will now be described in turn.

LFTs are routine blood tests conducted in most pathological laboratories to screen critical liver biomarkers. In many hepatic illnesses, abnormalities in these biomarkers often infer the condition of the liver [9]. Specifically, these biomarkers provide a means for the hepatologist to infer the specific liver disease type and the condition of the liver.

Table 1-1 tabulates the common biomarkers used in LFTs as well as the possible diagnosis of the liver disease inferred thereto.

Table 1-1 Common biomarkers used in LFTs

Biomarkers	Possible Diagnosis
Serum albumin	Liver function disorder, liver cirrhosis
Serum bilirubin	Hepatic steatosis
Alanine aminotransferases (ALT)	Acute viral hepatitis A or B, profound liver damage inflicted by toxins, prolonged collapse of the circulatory system
Gamma glutamyl transferase (GGT)	Early alcoholics, hepatitis
Alkaline phosphatase (ALP)	Alcoholic hepatitis
Asparatate aminotransferase (AST)	Acute viral hepatitis A or B, profound liver damage inflicted by toxins, prolonged collapse of the circulatory system, alcoholic liver damage, alcoholic hepatitis

Despite the prevalent application of LFTs, it is generally acknowledged that there are limitations to their accuracy in liver diagnosis [10]. For example, as LFTs are largely unable to detect some liver diseases during their initial stages, there is reduced possibility for the detection of acute liver diseases with a short duration of detectable phase due to the phenomenon of ‘length bias’ [11]. Further, the results of LFTs may not necessarily be of liver origin. For example, the elevation of AST may be due to muscle tissue damage owing to intensive exercise, which is not related to liver disease [12]. Furthermore, LFTs may have low sensitivity in some conditions. For example, patients with chronic hepatitis C typically have only slight AST and ALT concentration elevations despite having severe liver damage [11]. These shortcomings may not be unexpected as LFTs are derived from blood samples (vis-à-vis liver tissue) where they

are largely ‘indirect and inferred’ assessments of the liver condition. Put simply, LFTs may be unreliable, and the pathologist’s assessment (based on LFTs) of a patient’s liver is expected to have some degree of uncertainty.

LB involves extracting several small liver tissue samples, which are thereafter prepared for the pathologist’s visual examination [13]. Figure 1-1 depicts the typical procedure (‘supply chain’) for LB. In Step 3, the visual examination provides an assessment of the stage of the fibrosis as well as the severity of the liver disease, necessary for ensuing therapeutic management [14]. As the samples include actual liver tissue, the diagnosis from LB features a lower degree of uncertainty and consequently, higher accuracy (than LFTs). It is hence not unexpected that, at this juncture, the ‘gold standard’ for liver assessment is a diagnosis by means of LB.

Figure 1-1 Typical procedure for liver biopsy

Despite LB being the ‘gold standard’ for liver disease diagnosis, it is well established that this diagnosis nevertheless suffers from several shortcomings [15]. Firstly, as the procedure is invasive, it is at times a high risk procedure, particularly for hemophiliacs or patients suffering from severe liver disease. Secondly, it is a relatively expensive procedure due to the complex infrastructure (medical equipment and expertise) and the longer processing time (to prepare the sample for the aforementioned pathologist’s visual examination, and complex logistics, see Figure 1-1). Thirdly, the results of the diagnosis largely depend on the pathologist’s interpretation of the biopsy specimen [14].

It is easily to appreciate that given the present conventional LFT and LB procedures, they are largely incompatible with the aforementioned POC liver disease diagnosis device to provide rapid and accurate diagnostic results. A possible alternative for liver disease diagnosis is gene expression profiling. Gene expression profiling is gaining growing acceptance in medical applications largely due to the fact that it provides a detailed perspective of the global or specific gene expression level (represented by the concentration of messenger ribonucleic acid (mRNA) within a biological sample) and elucidates the underlying molecular mechanism for the disease pathogenesis [16]. Many studies on gene expression profiles have been reported [17-19] for different diseases, and its application for disease diagnosis and prognosis.

At this juncture, there are reported research efforts to exploit gene expression profiling for liver diagnosis [20, 21] and these studies demonstrate the potential of gene expression profiling as a promising alternative approach to hepatotoxicity assessment for liver disease diagnosis. Specifically, instead of relying on biomarkers (in LFTs) or visual examination of liver tissue (in LB), the classification and stages of the liver disease could be determined by the gene expression profiling of the liver tissue sample.

Based on these reported work, Figure 1-2 depicts a possible procedure for gene expression profiling as an alternative for liver disease diagnosis; details are delineated in Chapter 2.

Figure 1-2 Possible procedure for gene expression profiling as an alternative for liver disease diagnosis

This alternative is attractive as it may possibly provide detailed, quantitative and objective assessment of the condition of the liver from a relatively small (amount of tissue) sample. Put simply, gene expression profiling offers the potential of an alternative diagnosis method that does not suffer from the limitations of both LFTs and LB – where analysis is conducted on actual liver tissues (compared with the ‘indirect’

‘inferred’ assessment for LFTs) and the amount of tissue samples required is small (relative to a large amount of samples required of LB) [22]. The latter is important because the risk to patients is mitigated. In short, gene expression profiling is potentially an improved new ‘gold standard’ for liver disease diagnosis; it is nevertheless worthy to note that the validity of the gene expression profiling for liver disease diagnosis is yet to be established.

Despite the aforementioned advantages and potential, gene expression profiling nevertheless suffers from drawbacks. These include the need for a liver tissue sample (albeit a substantially smaller amount compared to LB and vis-à-vis the ideal case where a tissue sample is not required), and the relatively complex and tedious procedure for the gene expression profiling (especially for tissue preparation and mRNA extraction in Step 3 in Figure 1-2). Further, the quality of the mRNA is easily affected by many factors in Step 3, including such as human error.

At this juncture, a number of microfluidic chips and LOC systems have been reported that performed experiment procedures involved in gene expression profiling, such as sample preparation, mRNA and ribonucleic acid (RNA) extraction and reverse transcription-polymerase chain reaction (RT-PCR). Nevertheless, an integrated microfluidic device capable of performing the entire gene expression profiling process is yet unreported – this is because the embodiment of the entire gene expression profiling process into a microfluidic device is extremely challenging [23], such an integrated microfluidic device with “sample-in-answer-out” capability requires an integration of different microfluidic functional units to perform complex biological experimental procedures with the result comparable to the conventional method. In

fact, this complete embodiment remains one of the ‘holy grails’ of microfluidic chips and LOC systems, particularly the integration of an effective tissue homogenization component and detection result analyzing unit within the same microfluidic chip and/or LOC system. The tissue homogenization component could potentially serve as the missing interface between the practical medical world and the microfluidic devices capable of handling substantially smaller inputs such as proteins, cells, bacteria and other bodily fluids.

Despite the extreme challenge of such an integrated device, it is definitely interesting and rewarding to investigate this possibility, particularly that gene expression profiling within the same microfluidic chip and/or LOC system is potentially a much sought-after answer for the next generation of POC liver disease diagnosis device. This POC device would embody the functionalities involved in the gene expression profiling based liver diagnosis method: tissue homogenization, mRNA extraction, RT-PCR and target gene expression detection and analysis. It would be able to automatically perform a standardized process directly with raw liver tissue input, in a short time.

The desired attributes of such a POC device include:

- (a) A minimal amount of tissue sample input;
- (b) Short processing time, ideally less than 1 hour;
- (c) Compact form factor (size);
- (d) Automatic operation with minimum human intervention; and
- (e) Simplified design for mass-production and relatively low cost (disposable).

1.2 Objectives

In view of the aforesaid in the preceding section, we will in our PhD program attempt an ambitious project – the *Grand Objective* is to develop a low-cost, rapid, “sample-in-answer-out” POC liver disease diagnosis device that requires a minimal amount liver tissue sample for detecting multiplex gene expression profile and that provides information for rapid and accurate diagnosis. This is congruous to POC diagnosis service, constituting the foundation of a “Lab-in-a-Needle” system described in our technical disclosure (NTU Ref: TD/270/14), and PCT (PCT/SG2016/050116). The envisioned device is an integrated LOC system comprising three parts:

- (I) Tissue preparation and mRNA extraction;
- (II) RT-PCR; and
- (III) Target gene expression detection and data analysis.

The *Specific Objectives* of this PhD program pertain to part (I) and part (II) of the envisioned POC device, and are stipulated as follows:

- (A) To select a conventional (established) method for tissue homogenization and mRNA extraction, as well as to modify the selected tissue homogenization and mRNA extraction protocol for the application to the envisioned microfluidic device;
- (B) To design and fabricate a microfluidic chip for tissue homogenization and mRNA extraction, verify the efficacy of this microfluidic chip with liver tissue samples from a pre-clinical drug toxicity study, as well as to demonstrate the feasibility of this microfluidic chip to detect and analyze

multiple genes for gene expression profiling for hepatotoxicity assessment;

- (C) To design, fabricate and verify a microfluidic chip for continuous-flow RT-PCR; and
- (D) Finally, to integrate (by means of fluidic interconnecting two chips) the tissue homogenization/mRNA extraction chip in (B) above and the RT-PCR chip in (C), hence to collectively demonstrate the feasibility of this *fully-integrated* LOC system with “sample-in-answer-out” capability to detect specific genes with raw liver tissue sample input.

From a broad perspective, the objectives of this PhD program are a ‘proof-of-principle’.

1.3 Contributions

Congruous to our *Specific Objectives*, we made a number of contributions towards the *Grand objectives* – the development of a low-cost, rapid, “sample-in-answer-out” POC liver disease diagnosis device. More specifically, our contributions are the development of a tissue homogenization/mRNA extraction microfluidic chip and continuous-flow RT-PCR microfluidic chip, and the demonstration of the feasibility of our *fully-integrated* LOC system comprising these two chips. Our contributions will now be succinctly delineated in turn.

For *Specific Objective (A)*, on the basis of a comprehensive literature review and preliminary experiments, tissue grinding (by means of a simple inexpensive grinder or

rotor-stator homogenizer) was selected as our primary approach to tissue homogenization. The established magnetic bead-based mRNA extraction method was selected/adopted for the mRNA extraction due to its easy separation of magnetic beads from waste solution and its ability to extract mRNA directly from lysate; thereby simplifying the ensuing design of the envisioned integrated microfluidic device. The protocol for the selected tissue homogenization and mRNA extraction method was thereafter modified so that only a small amount of tissue sample and a small amount of chemical reagents were required.

The protocol modification is the critical step for the design and fabrication of the first integrated tissue homogenization/mRNA extraction microfluidic chip to handle raw liver tissue directly for hepatotoxicity assessment. To meet the requirements/constraints of a microfluidic system, we have to modify the original protocol to substantially reduce the amount of chemical reagents without compromising the quality of the mRNA extracted.

For *Specific Objective (B)*, our microfluidic chip for tissue homogenization and mRNA extraction was designed, fabricated and validated for its efficacy and sensitivity using mouse liver tissue samples from pre-clinical drug toxicity study with different dosages of drug administered. The results obtained from on-chip tissue homogenization and mRNA extraction were compared against the results from the said modified conventional protocol. The comparison depicted the efficacy and sensitivity of our microfluidic chip, i.e., our chip was able to homogenize raw liver tissue and extract mRNA for multiplex gene expression profiling.

Our tissue homogenization/mRNA extraction chip is, to the best of our knowledge, the first integrated microfluidic chip with the capability of preparing raw liver tissue directly. This “liver tissue sample in” capability has not been realized in reported LOC devices for hepatotoxicity assessment. At the same time, our integrated microfluidic chip serves as the missing interface between the practical medical world and microfluidic devices available to perform various testing procedures.

For *Specific Objective (C)*, we designed and fabricated a microfluidic chip for continuous-flow RT-PCR. We thereafter validated the feasibility and efficiency of our microfluidic chip to amplify specific genes by comparing its functionality against the conventional RT-PCR process with the same batch of liver tissue samples. We were able to amplify a specific gene (ALT1) on our continuous-flow RT-PCR chip within 45 minutes, i.e., ~50% time reduction compared to the conventional RT-PCR process. Nevertheless, the amplification efficiency of our chip is inferior (~60%) to the conventional RT-PCR process. This continuous-flow RT-PCR microfluidic chip is a critical component for developing the *fully-integrated* LOC system in specific objective (D).

Finally, for *Specific Objective (D)*, we constructed a *fully-integrated* LOC system by connecting (fluidic means) our tissue homogenization/mRNA extraction chip to our RT-PCR chip. We successfully demonstrated the feasibility of our *fully-integrated* LOC system to detect amplification product of gene of interest (GOI) from raw liver tissue samples, i.e., we were able to conduct the tissue homogenization, mRNA extraction, and RT-PCR on our *fully-integrated* LOC system for less than one hour and successfully amplified the GOI (ALT1) directly from raw liver tissue input.

Nevertheless, the amplification efficiency of our integrated system was inferior (~50%) to the conventional laboratory procedure.

To the best of our knowledge, this is the first *fully-integrated* LOC system capable of performing the entire tissue homogenization, mRNA extraction, RT-PCR process. It demonstrates the ‘proof-of-principle’ for our grand objective, and the feasibility of developing a POC diagnosis device for hepatotoxicity assessment with a *fully-integrated* microfluidic system featuring a true “sample-in-answer-out” capability.

1.4 Organization/Structure

This thesis is organized /structured as follows:

Chapter 1 presents the background for the POC diagnostic devices, the imperativeness for such device for liver related diseases, and limitations for current diagnostic methods for liver assessment, followed by the motivation, objectives, and contribution of this project.

In Chapter 2, a comprehensive but critical literature review of gene expression profiling for disease diagnosis, tissue homogenization and mRNA/RNA extraction methods is presented. In addition, a literature survey on relevant state-of-the-art microfluidics technology is delineated in this chapter.

In Chapter 3, the details of the proposed modification of the selected conventional method for tissue homogenization and mRNA extraction are presented. This chapter largely describes our work towards *Specific Objective (A)*. We verify the efficacy of

the selected conventional method (tissue homogenization by simple tissue grinder and bead-based mRNA extraction) by the method of tissue homogenization (tissue grinder) and column-based RNA extraction. This is followed by a description of our proposed modification of the selected conventional method with the objective of requiring only a small amount of tissue sample (10 mg vis-a-vis ~30 mg) and reagents (~40% less of magnetic beads, and ~76% less of the washing buffers).

In Chapter 4, we present a *semi-integrated* 2-chip Lab-on-a-Chip (LOC) system comprising three components: tissue homogenization/mRNA extraction microfluidic chip, stationary multiplex microfluidic RT-PCR component and real-time fluorescent detection setup. This *semi-integrated* 2-chip LOC system serves as a proof-of-concept for our *Grand Objective* delineated in Chapter 1. This chapter provides an overview of the collective work of our research group. The emphasis of this chapter is our work on the tissue homogenization/mRNA extraction chip and its contribution to the entire LOC system.

First, we describe our tissue homogenization/mRNA extraction microfluidic chip, where the details relating to the design, fabrication and verification of its efficacy are delineated. We verify and evaluate the quality and yield of the on-chip tissue homogenization/mRNA extraction process with raw mouse liver tissue samples from pre-clinical drug toxicity study. We thereafter demonstrate the feasibility of applying our microfluidic tissue homogenization/mRNA extraction chip to ascertain the changes in the expression levels of GOIs (AST, AST1, and ALT1) for hepatotoxicity assessment. This is largely our work towards *Specific Objective (B)*.

Second, a brief description on the design, fabrication, and experiment on multiplex microfluidic RT-PCR chip and real-time fluorescent detection setup (the work contributed by our colleague) is presented to demonstrate the capability of our microfluidic system. This system performs real-time, quantitative gene expression level detection which indicate elevation of gene expression level for GOIs (normalized fold changes for AST and ALT were 1.62 and 1.31 respectively) with increased drug dosage administrated (with 3x drug dosage administrated). The result is consistent with gene expression level change of AST and ALT observed in the reported studies [24-26]. Collectively, this chapter presents the work of our research group towards *the Grand Objective*.

In Chapter 5, we present the design, fabrication of our continuous-flow RT-PCR microfluidic chip and compare the efficacy of our on-chip RT-PCR against the conventional means. This is largely our work towards *Specific Objective (C)*.

In this chapter, we also describe our *fully-integrated* LOC system embodying our tissue homogenization/mRNA extraction chip connected (by fluidic means) to our continuous-flow RT-PCR chip. We validate its feasibility for a practical POC hepatotoxicity assessment by successfully amplifying a target gene (ALT1) from raw liver tissue samples (i.e., “sample-in-answer-out”) within an hour (i.e., rapid diagnosis). This is largely our work towards *Specific Objective (D)*.

Finally, in Chapter 6, conclusions are drawn on the basis of the research presented in this thesis. Future recommended work is also described.

Chapter 2 Literature Review

In this literature review, we will first provide a brief overview of gene expression profiling, including a delineation of the potential advantages and disadvantages of this biomolecular diagnostic approach over conventional LFTs and LB. This introduction provides the preamble and framework for the basis of our POC liver disease diagnosis device.

Secondly, we will delineate a succinct review on current tissue preparation and RNA/mRNA extraction methods to identify the most appropriate approach to transpose onto a microfluidic platform. Emphasis will be placed on factors such as ease-of-use, robustness, efficiency, cost-effectiveness, etc.

Thirdly, we will provide a comprehensive review on current microfluidic technologies and elaborate on the concept of LOC. This literature review serves to identify crucial microfluidic modules/components used in LOC systems, and facilitates the design of our POC liver disease diagnosis device.

This chapter is in part extracted from the author's Qualifying Examination Report submitted to Nanyang Technological University in 2012.

2.1 Gene Expression Profiling: A Review

In Section 1.1, we described the potential advantages of gene expression profiling as an alternative method to the conventional LFTs and LB for liver disease diagnosis. This alternative method constitutes the basis of our attempt to develop a novel POC liver disease diagnosis device. We will now review gene expression profiling.

One of the major outcomes of the human genome project in 2003 [27] was the immense opportunity for researchers to investigate the vast amount of data (from said project) to study the mechanisms of gene expression [16, 28] and the regulation of complex biochemical activities [29, 30]. As the central dogma of molecular biology [31] depicts in Figure 2-1, genetic information stored in deoxyribonucleic acid (DNA) is expressed in two steps. In the first step, the genetic information stored in DNA is passed to RNA via the transcription process. In the second step, mRNA (a type of RNA with a string of adenosines (poly(A) tail) attached at the 3' end) directs the proteins synthesis in the translation process.

DNA → RNA → Protein

Figure 2-1 The central dogma of molecular biology

Proteins are the final product of gene expression, and they play a major role in various functions and biochemical activities essential to living organisms [31]. Due to the significance of proteins, the field of ‘proteomics’ was established in 1997 to study the “protein properties (expression level, post-translational modification, interaction, etc.)

on a large-scale that results in a global, integrated view of disease processes, cellular programs and networks at protein level” [32]. A conventional approach to proteomics is to measure the expression of the proteins within a cell or tissue, and to study the quantitative changes under different conditions [33]. This is potentially applicable to disease diagnosis and prognosis [34], such as the study of the molecular response to toxicity [35], the discovery of tumor markers [36], etc.

mRNA is the necessary precursor for synthesizing proteins as depicted in Figure 2-1. Any alternation in the expression of proteins can be reflected by the change of concentration of mRNA. Instead of detecting and analyzing the protein expression patterns, detecting mRNA offers a better alternative. This is because it is substantially easier to amplify and quantify the small amount of mRNA with technologies such as RT-PCR, real-time RT-PCR and microarray platforms [37-39]. The information of gene expression patterns can hence be obtained with relatively high or equal accuracy (with respect to expression of proteins) [40] by measuring the mRNA concentration in the cells or tissue samples. This process of obtaining and analyzing the gene expression patterns via measurement of the concentrations of mRNA is known as gene expression profiling [41]. In short, gene expression profiling provides a powerful tool for surveying genetic alterations as well as identifying novel markers of practical significance in disease assessment [20].

In the case of liver disease, substantial differences in terms of gene expression between normal and diseased liver tissues have been reported by gene expression profiling analysis [42, 43]. For example, mutations of p53 [44] and β -catenin [45] have been reported in hepatocellular carcinoma (HCC); some proteins like c-myc [46] and cyclin

D1 [47] are frequently over-expressed in hepatocarcinogenesis; while the expression of cytoplasmic dynein light chain 1 gene is enhanced in chronic hepatitis B or C [9, 48]. The gene expression levels of AST and ALT (which are the GOIs we employ in this PhD program) are typically significantly elevated after a drug treatment or liver injury [24-26]. With a yet to be established system of biomarkers for liver disease diagnosis, gene expression profiling appears to provide a possible alternative for liver disease diagnosis as aforementioned in Chapter 1.

Several general gene expression profiling techniques have been reported to study gene expression activities in cells or tissue samples. These techniques encompass serial analysis of gene expression (SAGE) [49, 50], massive parallel signature sequencing (MPSS) [51], and microarray [39, 52, 53]. Among these techniques, the microarray is probably the most commonly used technique for gene expression profiling because it is capable to profile thousands of gene expressions simultaneously [53].

Figure 2-2 depicts a typical methodology involving microarrays for gene expression profiling. The first two steps involve tissue preparation and mRNA/RNA extraction, followed by microarray gene expression detection (the PCR process is required when the amount of complementary deoxyribonucleic acid (cDNA) is insufficient for the ensuing step of microarray) and data analysis [54].

Figure 2-2 Outline of one methodology of gene expression profiling involving cDNA and oligonucleotide microarray [18]

Although gene expression profiling is now a prevalent laboratory practice to study gene activities, there are two main shortcomings which restrict its acceptance as a better alternative for liver disease diagnosis method in clinical settings:

- (i) It requires complex and tedious procedures (time consuming), and it is performed in laboratory with specific/specialized equipment and trained technicians; and
- (ii) The quality of mRNA is easily influenced by many factors, including the method of tissue preparation, mRNA or RNA extraction, human error, the surrounding laboratory environment, etc.

As suggested in Chapter 1, LOC-based POC liver disease diagnosis device can potentially circumvent these shortcomings of the gene expression profiling and transform this complex, tedious manual experiment procedure into a compact, efficient, standardized automatic operation with less unpredictable variables that influence the final result. In other words, an LOC system for gene expression profiling could greatly simplify the gene expression profiling as alternative liver diagnosis method for POC medical service.

2.2 Tissue Preparation and RNA/mRNA Extraction: A Review

2.2.1 Tissue Preparation

Tissue preparation is the first and probably the most critical step for gene expression profiling [17]. Without breaking down the tissue structure to release the cellular contents, it is not possible to proceed with the subsequent steps [55]. Liver tissue preparation for the ensuing RNA/mRNA extraction typically involves two steps. In step 1, tissue is pulverized/homogenized. In step 2, the pulverized/homogenized tissue sample is treated with lysis buffer to break down the cell membrane, thereby releasing the cellular contents (including mRNA, DNA, and proteins).

Generally, there are four tissue preparation methods reported in laboratory practice:

- (a) The liver tissue sample is homogenized by cryogenic grinding [56]. The tissue sample is first frozen by liquid nitrogen, and then pulverized in a pre-cooled

clean mortar. During this process, the tissue sample is fully submerged in liquid nitrogen. The fine powder produced is subsequently mixed with lysis buffer.

- (b) Raw liver tissue sample is manually homogenized in a tissue grinder with lysis buffer [56]. Additional incubation time is generally needed to complete the lysis process. The sample is homogenized and lysed at the same time.
- (c) A rotor-stator system is used to homogenize liver tissue sample with lysis buffer [56]. The rotation of the metal tip of the rotor-stator system generates a strong mechanical force to rapidly homogenize the tissue sample.
- (d) A bead beater system is used to homogenize liver tissue sample [56, 57]. The tissue sample is placed in a plastic tube, together with lysis buffer and beads (made of metal, ceramic, or glass). The tissue is homogenized by beads when the plastic tube is placed into the bead beater system for orbital or vertical motion.

Figure 2-3 depicts the various instruments used for these four tissue preparation methods. There are merits and shortcomings for each of these methods. The first homogenization method (method (a)) involving liquid nitrogen, is often used for liver tissue sample preserved under -80 °C, and the tissue can be pulverized easily into fine powder. Despite the perceived ease, liquid nitrogen is physically hazardous due to its low temperature, also the usage and storage of liquid nitrogen requires specific equipment and training. The second homogenization method (method (b)) involving a tissue grinder, is an easy method. However, it requires a large amount of lysis buffer, and the result of homogenization is not consistent for its manual process. The third

homogenization method (method (c)) involving a rotor-stator system, is able to homogenize tissue samples efficiently and automatically. However, the instruments involved are expensive. The fourth homogenization method (method (d)) involving a bead-beating system, is more appropriate for processing multiple samples simultaneously. Nevertheless, the centrifuge instrument is required.

Figure 2-3 Instruments used for the four tissue preparation methods: (a) liquid nitrogen cooled mini mortar and pestle set [58], (b) Pyrex® Potter-Elvehjem tissue grinder with Polytetrafluoroethylene (PTFE) pestle [59], (c) hand-held rotor-stator homogenizer [60], and (d) Precellys®24 bead beating tissue homogenizer/grinder [61]

It is evident that all four tissue preparation methods are incompatible with a rapid, portable POC liver disease diagnosis device. Amongst these four methods, methods

(a) and (d) are arguably the least compatible with a microfluidic device due to the hazardous-to-handle liquid nitrogen and the need for centrifugation equipment.

To identify the appropriate tissue homogenization method for our LOC device embodying tissue homogenization and mRNA extraction (Chapter 4), we will explore the conventional manual tissue homogenization method (method (b) or (c)) involving respectively either a plastic disposable tissue grinder or a rotor-stator tissue homogenizing system for the ease of operation and large mechanical force. However, these methods need to be compatible to an effective microfluidic approach for a small amount of tissue sample input for the envisioned integrated microfluidic device.

2.2.2 RNA/mRNA Extraction Methods

There are several reported methods for mRNA/RNA extraction from cells or tissue samples for gene expression profiling. These methods can be generally categorized into the phenol phase (aqueous phase) [62] and the solid phase methods [63]. In general, mRNA extracted by phenol phase methods has relatively high purity and high yield concentration compared to mRNA extracted by the solid phase methods [64], but the solid phase methods have the advantage of being a simple, rapid RNA/mRNA extraction process. These two categories of extraction methods will now be reviewed in turn.

2.2.2.1 Phenol Phase (Aqueous Phase) Extraction Methods

The phenol extraction is an efficient nucleic acid purification method [65, 66]. During phenol extraction, proteins and other impurities are removed from the lysate (homogenized tissue sample in lysing solution) to the inter-phase or the phenol phase, where the nucleic acids remain in the aqueous phase. There are two common methods based on this principle: Acid guanidinium thiocyanate–phenol–chloroform (AGPC) extraction method [67] and TRIzol[®] method [64].

The limitations of the phenol phase methods include:

- Time consuming method;
- Complex procedure, which involves a long centrifugation time; and
- Hazardous solvents (phenol, chloroform) [68], etc.

Due to the limitation stated above, we consider that the phenol phase methods are not compatible to our envisioned microfluidic device featuring simple chip design and short operating time.

2.2.2.2 Solid Phase Methods

Many commercial RNA purification kits are based on the silica (solid phase) adsorption of nucleic acids mediated by guanidinium [69]. For example, the Qiagen RNeasy[®] Mini Kit (for RNA) provides fast and efficient purification of RNA from cells and tissues using a silica-membrane RNeasy[®] column [64]. This kit utilizes the mesoporous

silica membrane layers to extract RNA while washing away unwanted waste through the centrifugation washing process. The typical procedure for RNeasy® Mini Kit is shown in Figure 2-4.

Figure 2-4 The typical procedure for RNeasy® Mini Kit [64]

The advantage of this column-based method is the rapid extraction process with satisfactory RNA extraction quality. However, the centrifugation step is undesirable for the envisioned integrated microfluidic device.

A reported alternative method involves covalently coating the oligo(dT) – a short sequence of deoxy-thymine nucleotides onto the surface of the solid base, such as small beads [70, 71]. By exploiting the existence of the poly(A) tail in most of the eukaryotic mRNA by means of hybridization of oligo(dT) and poly(A) tail of mRNA, mRNA can be directly extracted from the sample lysate; we will also exploit this phenomenon in Chapter 3. By utilizing magnetic beads coated with oligo(dT), the mRNA can be directly selected from the crude lysate through hybridization process. The separation

of magnetic beads from unwanted waste can be easily achieved by a magnet. Figure 2-5 shows a typical procedure for Dynabeads® mRNA DIRECT™ Kit, a well-known commercial kit based on this method [72].

Figure 2-5 The typical procedure for Dynabeads® mRNA DIRECT™ Kit [72]

In the context of mRNA extraction component within the microfluidic device, this method is a possible ‘gold standard’ to be benchmarked against. It is because it provides an easy, rapid separation of mRNA from unwanted waste solution as well as direct extraction of mRNA from the sample lysate.

From the aforesaid review on tissue preparation and mRNA extraction, we conclude that its realization in an LOC device to perform the equivalent of the time consuming and tedious bench-top process is largely unavailable and is very challenging. This LOC

device embodying tissue homogenization and mRNA extraction is however necessary for our envisioned POC liver disease diagnosis device.

2.3 Microfluidics: A Review

The first reported microfluidic device is probably a simple gas chromatography built on a silicon wafer in the 1970s [73]. From there, a new research field was spawned to perform chemical and biochemical experiments on the micro-scale to the nano-scale. With the development of Micro Electro Mechanical Systems (MEMS) technology [74], a range of actuation and valve systems can be built on the microfluidic devices for sophisticated fluid flow control. All these developments are critical towards the integration of different functional components into a single miniaturized system, otherwise known as Micro-Total Analysis Systems (μ TAS) [75] or LOC [76]. These μ TAS or LOC devices are capable of performing complicated tasks for practical chemical and biological applications [77-79], and present the possibility of an LOC liver disease diagnosis device based on gene expression profiling.

This review of microfluidics includes three aspects, which will be reviewed in turn:

- (i) Common substrate materials in microfluidic devices;
- (ii) Microfluidic components for different functions; and
- (iii) LOC devices.

2.3.1 Common Substrate Materials in Microfluidic Devices

It is imperative to select the appropriate material for the design and fabrication of microfluidic devices. Several factors influence the choice of substrate materials: intended application, fabrication method, production volume, etc. Sometimes, a group of materials might be selected for an integrated microfluidic system with multifarious functional components. We summarize in Table 2-1 several common substrate materials used in microfluidic devices, and their advantages and limitations.

Table 2-1 Summary of common substrate materials in microfluidic devices

Material	Advantages	Disadvantages	Fabrication	References
Polydimethylsiloxane (PDMS)	Ease of fabrication, transparent, low toxicity, flexibility	Not appropriate for mass production	Casting	[80, 81]
Cyclic olefin copolymer (COC)	Solvent resistance, higher optical clarity, large scale fabrication	Useful temperature range is low	Hot embossing, injection molding	[82, 83]
Cyclo olefin polymer (COP)	Optical properties, low water uptake, higher signal to noise ratio, higher electrophoresis efficiency	Fabrication temperature might be too high	Hot embossing, injection molding	[84]
Polymethylmethacrylate (PMMA)	Mechanically stable, inexpensive, transparent, bio-compatible	Resistance to solvent is moderate	Hot embossing, injection molding, milling, laser cutting	[85, 86]
Polycarbonate (PC)	Inexpensive, mechanically stable, high glass transition temperature, transparent	Resistance to solvent is moderate	Milling, laser cutting, hot embossing, injection molding, milling	[87, 88]
Silicon	Structures with higher accuracy, well established fabrication process	Opaque for UV and visible light, relatively expensive fabrication process, incompatible with biomaterials	Photolithography, anisotropic etching	[73, 74]
Glass	Structures with higher accuracy, well established fabrication process	Relatively expensive fabrication process	Ultrasonic lapping, micro-blast	[89]
Paper	Inexpensive	Limited applications	Lithography	[90, 91]

In the nascent stage of the development of microfluidic devices, silicon was the material of choice [73]. This is largely due to the leveraging on the mature semiconductor fabrication process. The success of silicon-based microfluidic devices was limited largely because of the incompatibility of silicon with bio-molecules as well as the opacity limitations of the silicon-based microfluidic devices in biochemical processes. Glass was the subsequent material [89] and is a better alternative to silicon, because it is biocompatible, transparent yet the fabrication process thereto is similar to silicon. The major limitation of both silicon and glass is their high fabrication cost. Consequently, they are unacceptable for low-cost disposable devices.

Owing to the characteristics of low-cost, biocompatibility, fabrication process and optical property, polymers (such as PC, PMMA, PDMS and COP) are now more commonly used as the substrate materials for biochemical applications. In the perspective of this project for an integrated disposable microfluidic device used for tissue preparation and mRNA extraction, PC and PMMA are the possible candidates. Their attractive attributes include relatively moderate resistance to chemical solvents, glass transition temperature above 95 °C (for withstanding the temperature of the RT-PCR process), mechanical stability, biocompatibility, low cost.

2.3.2 Microfluidic Components for Different Functions

2.3.2.1 Mixing Component

The mixing of different chemical reagents is a critical step for various chemical processes. At this juncture, this remains a challenging function for microfluidic devices because of the laminar nature of the liquid flow in microfluidic devices. Put simply, it is difficult to generate sufficient turbulent flows to achieve adequate mixing. We summarize in Table 2-2, the currently available mixing components in microfluidic devices reported in literature.

Table 2-2 Summary of different mixing components in microfluidic devices

Mixer	Principle	Advantages	Disadvantages	References
Magneto hydrodynamic (MHD) mixer	Chaotic mixing effects are generated by coupling magnetic and electric fields for electrolyte solution	Effective mixing process	For electrolyte solution only	[92, 93]
Acoustic mixer	Use acoustic actuator to stir the liquid inside chamber or create air bubble to mix the liquid	Fast, powerful	Temperature rise may be harmful for samples due to cavitation	[94, 95]
Multistage lamination mixer	Parallel lamination splits the inlet stream into sub streams and then joins them into one stream as laminae.	Mixing in a short range, effective	Complex design, difficult to fabricate	[96]
Chaotic advection mixer	Complicated structures of microfluidic channel to achieve high mixing rate when fluid passes through the channel.	Mixing in short distance	Complex design, difficult to fabricate	[97]
Droplet mixer	Forms a droplet of the mixed sample liquid, and the movement of droplet produces internal flow field and makes the mixing possible inside droplet	Effective mixing process	Complex design	[94]
Fixed-volume microfluidic mixer	Uses pressurized air using pneumatic actuation to drive part of the fluid in chamber to mix	Fast, fixed volume of reagent, simple design	External air pressure control unit	[98]

In the perspective of mixing where the volume of reagent is fixed for the process of mRNA extraction, mixing with the continuous fluidic flow may not be appropriate. Hence, of the various mixing methodologies mentioned in Table 2-2, the fixed-volume microfluidic mixer with external air pressure control unit (fast mixing process and

simple design) is a potential candidate for mixing component in a microfluidic device with mRNA extraction function. This is the methodology we will explore in Chapter 4 for our LOC system for tissue homogenization and mRNA extraction. A simplified passive mixing channel (with finite volume of reagents) based on chaotic advection mixer will be explored in Chapter 5 for our continuous-flow RT-PCR microfluidic chip.

2.3.2.2 Valve Component

For an integrated microfluidic system to perform complex chemical or biochemical processes, an effective flow control of different reagents is imperative. This requires an efficient, effective and simple valve component with several key attributes. Firstly, the valve should be able to withstand the pressure generated during the operation. Secondly, it should be easy to switch between on/off stages. Thirdly, it should have a small dead volume. Finally, it should be very easy to fabricate. We summarize in Table 2-3 our review of different reported types of valve components used in microfluidic devices.

Table 2-3 Summary of different valve components in microfluidic devices

Valve	Principle	Material	Disadvantages	Advantages	References
Hydrogel valve	Volume change of hydro gel in response to different input parameter change	Water, paraffin, pluronic's sol-gel material	Leakage problem	Simple chip design	[94]
Hydrophobic valve	Hydrophobic interface to prevent aqueous solution from passing through the channel	Hydrophobic chemicals, air-permeable hydrophobic membrane	Leakage problem	Simple chip design	[94, 99]
Thermo pneumatic valve	Volumetric thermal expansion causes membrane to deflect and block the channel	Elastic membrane	Heating device, addition control chamber, dead volume	Tight seal	[94]
Shape memory alloy valve	The deformation of shape memory alloy under different temperature to control the valve	Shape memory alloy	Heating device, dead volume	Compact design, high work output	[94]
Pneumatic membrane valve	The deflection of flexible membrane under air pressure to seal off the channel.	Silicone, PDMS, thin latex sheet	External air pressure control unit, dead volume	Simple chip design, tight seal	[100]
Mechanical membrane valve	Mechanical force to deform the flexible membrane	Silicone, PDMS, thin latex sheet	External motor system, dead volume	Simple chip design,	[94]

From the review above and given the consideration of the attributes required of the envisioned integrated microfluidic device delineated in Chapter 1, the mechanical membrane valve may be a possible choice. This is largely because this type of valve is simple and easy to fabricate. We could integrate the mechanical control system into a base station of the POC disease diagnosis device, and the microfluidic chip is a disposable part of the device.

2.3.2.3 Pump Component

The pump is a critical component to actuate chemical reagents in a microfluidic channel structure and to facilitate various chemical experiments. The in-situ micro-pump and external pump are the available options for various microfluidic chips and we summarize our review of the various pumps used in microfluidic devices in Table 2-4.

Table 2-4 Summary of different pump components in microfluidic devices

Pump	Principle	Material	Advantage	Disadvantage	References
Piezoelectric pump	Diaphragm and peristaltic micro-pump based on piezoelectric actuation	Lead zirconate titanate (PZT) materials	High stroke volume, high actuation force, fast mechanical response	External power supply and control unit, noise issue	[101]
Pneumatic pump	When a series of pneumatic valves are controlled in the sequence of on-off stage, it is a pneumatic peristaltic pump	Elastic membrane PDMS, silicone etc.	Easy integration into the microfluidic chip,	External pumping system	[94]
Thermo-pneumatic pump	Air-filled chamber is used. When air is heated, the change of volume cause membrane to deform and the liquid is driven	Elastic membrane, PDMS, silicone etc.	Compact chip design, operating as low voltage condition	Low pumping rate, complicated fabrication	[94]
Electro-kinetic pump	Electro-osmosis: movement of molecules in electrical field due to their own change	PMMA	No moving component, simplified design	Low flow rate	[94, 102]
Pressure driven by an external pump	Flow is driven by pressure applied by external pump	PC, PMMA, etc.	No moving component, simple design	External pumping system	[103]

Given the need for simple chip design and inexpensive substrate materials for the low-cost and disposable microfluidic POC device, the pressure driven method by an external pump may be a possible option – at least at the early juncture of the project. This

method eliminates the need for the in-situ pump component – thereby simplifying the design and fabrication for the microfluidic chip.

2.3.2.4 Sample Homogenization Component

Design and techniques for homogenization component vary with different types of input samples. Compared to blood, bacteria, and cells, the structure matrix of fibers within the tissue sample makes it difficult to break down the tissue sample. The first step of tissue preparation would be a complete homogenization of tissue samples. In the context of microfluidics, the task of tissue homogenization is substantially more formidable than the conventional manual procedure. This is largely due to the difficulty of disrupting the tissue samples in a small space within microfluidic devices.

We will review the available state-of-art techniques and designs for sample homogenization, and select the suitable design for our microfluidic chip.

Table 2-5 Summary of different sample homogenization components in microfluidic devices

Homogenizer	Principle	Samples	Advantage	Disadvantage	References
Piezo diaphragm homogenization chamber	Mechanical, piezo diaphragm induced vibration	Cells, tissue	Rapid, strong mechanical force	Heat generation, power consumption	[104]
Centrifugal microfluidic system	Mechanical force by centrifugation	Cell, tissue	Easy fabrication, fast process	External centrifuge machine, hard to integrated	[105]
Sandwich-type microfluidic biochip for cell lysis	Chemical lysis	Blood cell	Easy integration, simple design	Long processing time, only for simple structured sample like cells, multiple chemical reagents involved	[106]
Dielectrophoresis microfluidic channel	Electrical field	Cells, bacteria	Small size, easy integration	Low fabrication repeatability, short life of electrode, individual cells	[107, 108]

From the Table 2-5 above, we infer that the mechanical method is the better option to homogenize tissue sample. This is largely because it provides a large mechanical force to break down the tissue structure within a relatively short time. In this project, we will explore the possibility of incorporating a miniaturized blender into a customized chamber within an integrated microfluidic device for the easy integration process. The large mechanical force provided by the miniaturized blender could possibly homogenize the tissue sample completely for subsequent processes.

2.3.2.5 PCR/RT-PCR Component

In order to detect the specific GOI or study the gene expression level, it is essential to conduct the PCR (DNA) or RT-PCR (RNA) to amplify the small traces of GOI from the samples. In the microfluidic adoption of PCR/RT-PCR process, researchers have reportedly exploited the merits of microfluidics such as large surface to volume ratio for rapid heat transfer, feasibility of integration with other microfluidic functional components, and very small amount of sample input. We will now summarize our review of the reported microfluidic PCR/RT-PCR devices in the Table 2-6.

Table 2-6 Summary of different PCR/RT-PCR components in microfluidic devices

PCR/RT-PCR Chip	Samples	Advantage	Disadvantage	References
Microfluidic continuous-flow PCR/RT-PCR	DNA, RNA, influenza virus	Fast heat transfer, uniform temperature, rapid processing time	Adsorption from the channel surface, carryover contamination, external pump system	[109]
Capillary-driven, self-propelled continuous flow microfluidic PCR	DNA	Fast heat transfer, small chemical consumption and no external pump system	Difficult to create hydrophilic surface, high cost, relatively complex fabrication process	[110]
Droplet microfluidic PCR/RT-PCR	DNA, cDNA, mRNA	Massive parallelization, generate droplet on demand, small discrete volume	Difficult flow control of oil or water and droplet, difficult separation of droplet from oil for subsequent analysis, external pump system	[111]
Stationary PCR / RT-PCR	DNA, mRNA	Easy procedure, simple design, easy to fabricate chip	Long processing time, relatively slow speed	[109]

In project, we need a RT-PCR component with short processing time to meet the demand of POC device for rapid “sample-in-answer-out” capability, at the same time, this RT-PCR component should require only a small sample and reagent input to lower the cost. On the basis of our review, the microfluidic continuous-flow RT-PCR technique with water or oil as the flow median to carry reaction mixture through the microfluidic device could be the appropriate choice.

2.3.3 Lab-on-a-Chip (LOC)

In 1990s, 20 years after the first microfluidic device, the concept of μ TAS was introduced [112]. The microfluidic device was for the first time shown to have a great potential for executing chemical as well as biological analytical processes. This is because of its ability to transfer the entire processes otherwise carried out manually in the laboratory environment to the chipset-like system. Such μ TAS devices were subsequently named LOC [76], and were indeed shown to be applicable to the chemical and biological applications related to cell, protein, and DNA analysis [79, 113]. In these cases, congruous to the small target, the system is commensurably small. These include the appropriately designed channel structures and actuation systems to perform tasks such as sorting different cells from blood sample, drug test, isolating specific cell, extracting and manipulating the DNA fragments from single human chromosome [75, 79], etc. When compared to conventional laboratory procedures, the LOC could be advantageous in the sense it provides for delicate and accurate control not easily achieved in the conventional procedures.

The advantages of LOC devices over the conventional lab experiment procedures in biological and chemical applications include:

(i) Small Amount of Sample and Chemical Reagents Input

When biological and chemical analytical tasks are conducted in the conventional way, a large amount of sample is normally needed for accurate analysis or for repeated experiments. For example, in liver biopsy, specimens of length ranging from 18 mm to 25 mm are typically extracted from patients

[114, 115]. Conversely, for microfluidic devices, a substantially smaller amount of specimen (envisioned to be less than 1 mm) is sufficient for the whole analytical task, congruous to the size of the device. For the same reason, a small amount of chemical reagents is needed in the LOC devices.

(ii) Standardized Process

Although the conventional manual protocols are well established, the human factor is omnipresent in experiment procedures. Not unexpectedly, this often affects the result, particularly if there is human error. In the case of the LOC device, as the entire process can be largely automated. Consequently, the human factor aspect has likely a lesser effect. For example, Broyles *et al.* [116] demonstrated an integrated system with sample filtration, concentration and separation functions in a single device.

(iii) Short Processing Time

As most/all steps can be carried out within one LOC system in an automatic manner, the time for transferring and storing is reduced. Furthermore, as the microfluidic system is compact (form factor), the process therein is able to be processed faster than the conventional counterpart. For example, Li *et al.* [117] fabricated an integrated microfluidic device to perform RT-PCR process for rotavirus within 1 hour, compared to 1.5 hours in conventional methods.

(iv) Portability

Within a microfluidic device, the experiments can be carried out largely without any requirement of laboratory facilities, such as the portable i-STAT[®] analyzer for clinical blood tests [76]. This provides the microfluidic device with

enhanced portability, potentially allowing the physician/medical care personnel to carry microfluidic devices and perform the test in-site. Put simply, microfluidic devices are deemed as potentially ‘bring the hospital to the patient’ instead of the ‘patient going to the hospital.’

(v) Low Cost

The small amount of the input material needed for microfluidic devices potentially reduces the cost of analytical task (in terms of reagents such as primers, reaction buffers, dye, etc.). The use of inexpensive polymer materials for fabrication makes the device relatively low cost, thereby disposable.

From the attributes of an integrated microfluidic device, it is potentially possible to develop foresaid parts (I and II) in Chapter 1 of the envisioned POC device embodying tissue homogenization, mRNA extraction, and RT-PCR functionalities (based on LOC technology). To date, although some microfluidic devices have been reported to perform these functionalities, an integrated microfluidic system capable of performing the entire process (tissue homogenization, mRNA extraction, and RT-PCR) remains unavailable. These reported microfluidic devices include the following, Buser *et al.* [118] presented a small portable audio device for the lysis of bacteria samples, *Mycobacterium marinum*. Ilyin *et al.* [105] utilized a modified tube and centrifugation to homogenize brain tissue samples. Guo *et al.* [104] developed a piezo-diaphragm based tissue homogenization chamber. Hughes-Chinkhota *et al.* [119] fabricated a microfluidic chip to perform mRNA isolation and cDNA construction from cells. Tachibana *et al.* [110] built an autonomous microfluidic PCR device to detect pathogen genes.

Besides mono-functional microfluidic devices, researchers have also explored the possibilities of an integrated microfluidic system with multi-functionalities – but not the integrated system embodying tissue homogenization, mRNA extraction and RT-PCR as delineated in Chapter 1. For example, David *et al.* [120] developed a “Lab-on-a-Disk” microfluidic system capable of processing ocular samples and performing protein measurement. Jose *et al.* [113] reported an array-type microfluidic platform capable of processing 1024 serum samples for protein measurement. Jiang *et al.* [121] reported an integrated system for airborne pathogens capture, enrichment and RT-PCR for gene analysis. Gan *et al.* [91] reported a filter-paper based microfluidic device for DNA extraction and amplification from samples like whole blood, swab, and saliva.

In the perspective of our work in this PhD program, there are several reported LOC devices for liver disease diagnosis and hepatotoxicity assessment. These include devices designed to simulate the *in-vivo* environment for liver cell/tissue growth and to study the response of liver tissue or hepatocyte cells under the influence of different drugs [122, 123]. Of note, Shaw *et al.* [124] developed an integrated microfluidic device for specific gene expression level which was able to perform RNA extraction and RT-PCR from homogenized liver tissue samples prepared off-chip. Nevertheless, in the perspective of POC liver disease diagnosis device based on gene expression profiling, these integrated devices are impractical because some of the functionalities need to be performed off-chip. These off-chip processes limit the effectiveness of their “sample-in-answer-out” capabilities. Put simply, as delineated in Chapter 1, the practical POC devices with “sample-in-answer-out” capability are yet to be developed.

2.4 Conclusions

This chapter has provided a review of gene expression profiling, tissue preparation and mRNA/RNA extraction methods, followed by a review of state-of-the-art microfluidics technology in three aspects: substrate materials, different microfluidic functional components, and LOC. On the basis of this literature review, we have identified a potentially suitable conventional tissue homogenization and mRNA extraction method as reference protocol and benchmark to validate the efficacy of the proposed modification of the selected conventional protocol for the ensuing microfluidic adoption.

Chapter 3 Proposal for the Modification of a Conventional Tissue Homogenization and mRNA Extraction Method for Microfluidic Adoption

In Chapter 1, we suggested a possible alternative procedure for liver disease diagnosis based on gene expression profiling (Figure 1-2) and envisioned a POC device to perform the gene expression profiling based liver disease diagnosis. Specifically, in this PhD program, our objective is to develop a *fully-integrated* LOC system comprising two primary components pertaining to the two steps in the alternative liver disease diagnosis procedure (Figure 1-2): Step 3 (tissue homogenization, mRNA extraction) and part of Step 4 (RT-PCR).

In this chapter, we will delineate our proposal for the modification of a conventional method of tissue homogenization and mRNA extraction (Step 3) for microfluidic adoption. The effort for this proposal includes the following.

First, we identify an appropriate conventional method of tissue homogenization and mRNA extraction, with which our proposed modification of the selected conventional method will be verified against. Our identification of the appropriate conventional method of tissue homogenization and mRNA extraction involves the comparison of the efficacy of the selected tissue homogenization (with a tissue grinder) and bead-based mRNA extraction method with the tissue homogenization (with a tissue grinder) and column-based RNA extraction method.

At the outset, the key consideration is that the selected tissue homogenization and mRNA extraction method needs to be realizable in a microfluidic device with simple chip design and simple operating procedure. This key consideration is formidable because, as delineated in Chapter 1 and reviewed in Chapter 2, there is no microfluidic device at this juncture that is capable to homogenize raw liver tissue and extract mRNA from the lysate within the same microfluidic device. Viewed differently, our selection is in part ‘an act of faith’ where the selection chosen involves processes that we ‘think’ that we are able to realize (i.e., raw tissue homogenization and mRNA extraction) in a simple and efficient microfluidic device. Undoubtedly, there are many unknowns and ensuing challenges – these will be delineated in this chapter and in Chapter 4.

The steps involved in our identification include the following:

- Step 1. We extract mRNA from mouse tail and mouse liver tissue samples by the selected tissue homogenization (with a plastic tissue grinder) and the magnetic bead-based mRNA extraction method.
- Step 2. We perform the tissue homogenization (with a plastic tissue grinder) and the column-based RNA extraction with the same batch of tissue samples in Step 1, by a commercial RNA extraction kit (Qiagen RNeasy® Mini Kit).
- Step 3. The purity and yield of extracted mRNA from Step1 will be compared against the purity and yield of extracted RNA in Step 2. This is to demonstrate the merits (and shortcomings) of the selected tissue homogenization and mRNA extraction method as an appropriate

reference and benchmark for our modification of the selected conventional method.

Second, after said identification, we will propose a modification of the selected tissue homogenization and bead-based mRNA extraction method for microfluidic adoption, which would require smaller amount of tissue sample input and less reagents for its realization in a microfluidic device. The efficacy of the modified method will be verified by the selected conventional tissue homogenization and mRNA extraction method. This modified tissue homogenization and mRNA extraction method also serves as a benchmark, with which our fabricated microfluidic device (embodying the modified protocol) will be verified against in Chapter 4.

3.1 Identification of the Conventional Tissue Homogenization and mRNA Extraction Method

We choose tissue grinding (a simple inexpensive tissue grinder) as the manual tissue homogenization method for its capability of generating sufficient mechanical force to homogenize liver or tail tissue sample easily. During the tissue homogenization process, the tissue sample is fully homogenized with lysis buffer (by the tissue grinder), and the sample lysate is obtained. The magnetic bead-based mRNA extraction method (Dynabeads[®] mRNA DIRECT[™] Kit) (Figure 2-5) is chosen for the selected mRNA extraction method.

The bead-based mRNA extraction is chosen for its two advantages:

- i. Direct extraction of mRNA out from the sample lysate; and
- ii. One step separation of magnetic beads from the residual lysate (unwanted waste solution).

For sake of brevity, we will henceforth term the aforesaid as the ‘conventional method’ for selected tissue homogenization and bead-based mRNA extraction method.

To verify the efficacy of the magnetic bead-based extraction method, we will conduct the conventional column-based RNA extraction method (Qiagen RNeasy[®] Mini Kit) depicted in Figure 2-4, with the same batch of tissue samples. This verification provides us with a high level of confidence that the selected bead-based mRNA extraction method is efficacious.

3.1.1 Biological Samples

Two types of tissue samples (mouse liver and mouse tail) were used for the experiments of tissue homogenization and mRNA/RNA extraction for the purpose of identifying the appropriate conventional method. Tail samples were supplied by our collaborator, Houston Methodist Research Institute (HMRI, Cornell University), USA. The liver tissue samples of common laboratory mouse, on the other hand, were obtained from the National University of Singapore (NUS), Singapore.

3.1.2 Tissue Homogenization and mRNA Extraction (Conventional Method): Experiment

A disposable plastic tissue grinder (15 ml Fisherbrand Closed Ultra Tissue Grinder System) was used to homogenize tissue samples to obtain the lysate solution for mRNA extraction. Thereafter, we conducted mRNA extraction with the Dynabeads® mRNA DIRECT™ Kit (the protocol is provided by the manufacturer, Thermo Fisher Scientific) [72]. The experiment was conducted within a bio-safety level 1 cabinet to minimize the contamination risk of RNase, which is a nuclease catalyzing the degradation of mRNA and abundant in the surrounding environment [125].

The detailed protocol for tissue homogenization and mRNA extraction is presented below. This protocol is largely a preamble to identify the critical steps as well as its evaluation for the protocol modification and the microfluidic adoption:

- (i) A tissue sample (either 3 mm long tail tissue or ~30 mg liver tissue) was homogenized in a plastic disposable tissue grinder (15 ml Fisherbrand Closed Ultra Tissue Grinder System) with 1000 µl Lysis/Binding Buffer (Dynabeads® mRNA DIRECT™ Kit). After full homogenization of the sample, the mixture was kept for 1 minute for tissue lysis.
- (ii) 250 µl magnetic beads solution (Dynabeads® mRNA DIRECT™ Kit) was transferred into a 1.5 ml micro-centrifuge tube, suspended by a vortex machine for 30 seconds. A magnet was used to attract the beads to the bottom of the tube for separation.

- (iii) After removing the supernatant, the beads were washed with 250 μ l Lysis/Binding Buffer (Dynabeads[®] mRNA DIRECT[™] Kit), followed by the separation step.
- (iv) 400 μ l lysate in the tissue grinder was transferred to a new 1.5 ml micro-centrifuge tube and a 20-gauge needle with 1 ml syringe was used to shear DNA in the lysate for 4 times.
- (v) The lysate was transferred to the micro-centrifuge tube containing the beads. The beads/lysate mixture was subsequently resuspended by the vortex machine for 1 minute for the hybridization of oligo(dT) (coated on the surface of beads) and poly(A) (tail portion of mRNA).
- (vi) The beads were separated from the unwanted solution by the magnet, the supernatant was removed, and the beads were resuspended in 1000 μ l Washing Buffer A (Dynabeads[®] mRNA DIRECT[™] Kit). This washing step was repeated twice.
- (vii) The mRNA attached beads were washed twice with 1000 μ l Washing Buffer B (Dynabeads[®] mRNA DIRECT[™] Kit).
- (viii) 25 μ l Elution Buffer (Dynabeads[®] mRNA DIRECT[™] Kit) was added in the tube containing the beads. The mixture was incubated at 65 °C in a water tank for 2 minutes, and followed by the separation of the beads from rest of the solution. The supernatant, which contained the mRNA, was transferred by pipetting to a pipette tube.
- (ix) mRNA was hence extracted and stored at -80 °C.

3.1.3 Tissue Homogenization and RNA Extraction: Experiment

Column-based RNA extraction was conducted with the same batch of tissue samples (mouse liver and mouse tail) used for the aforesaid tissue homogenization and mRNA extraction. The tissue sample was homogenized by the plastic tissue grinder as well to provide the lysate for subsequent RNA extraction. As delineated earlier, the intention is to verify the efficacy of the bead-based mRNA extraction method in the selected ‘conventional method’. The protocol for tissue homogenization and RNA extraction is provided by the manufacturer of the commercial RNA extraction kit (Qiagen RNeasy® Mini Kit) [126].

This protocol serves to allow a direct comparison with the selected ‘conventional method’ extraction method, allowing us to identify the advantages and shortcomings of the latter. The detailed protocol of tissue homogenization and RNA extraction is presented below:

- (i) A tissue sample (3mm long tail tissue or ~30 mg liver tissue) was homogenized in a plastic disposable tissue grinder (15 ml Fisherbrand Closed Ultra Tissue Grinder System) with 1000 µl RLT Buffer (Qiagen RNeasy® Mini Kit). After full homogenization of the sample, the mixture was kept for 1 minute for tissue lysis.
- (ii) 400 µl lysate was transferred to a 1.5 ml micro-centrifuge tube. A 20-gauge needle with 1 ml syringe was used to shear DNA within lysate for 2-4 times.

- (iii) The lysate was centrifuged at 10000 rpm for 2 minutes and the supernatant was transferred to a new 1.5 ml micro-centrifuge tube for later processing.
- (iv) 400 µl of ethanol solution (70% ethanol solution for tail tissue sample and 50% ethanol solution for liver tissue sample) was added into the supernatant and was mixed well by pipetting.
- (v) 700 µl of mixed solution was transferred into the RNeasy[®] spin column (Qiagen RNeasy[®] Mini Kit) placed in a 2 ml collection tube. The collection tube (with the lid gently closed) was placed into a centrifuge machine for 15 seconds at 10000 rpm in order to wash the spin column and the flow-through was discarded.
- (vi) 700 µl Buffer RWI (Qiagen RNeasy[®] Mini Kit) was added to the RNeasy[®] spin column, the spin column placed in the collection tube was centrifuged for 15 seconds at 10000 rpm to wash the column membrane, and the flow-through was discarded.
- (vii) 500 µl Buffer RPE (Qiagen RNeasy[®] Mini Kit) was added to the RNeasy[®] spin column, the spin column placed in the collection tube was centrifuged for 15 seconds at 10000 rpm to wash the column membrane, and the flow-through was discarded.
- (viii) 500 µl Buffer RPE (Qiagen RNeasy[®] Mini Kit) was added to the RNeasy[®] spin column, the spin column placed in the collection tube was centrifuged for 2 minutes at 10000 rpm to wash the column membrane again, and the flow-through was discarded.

- (ix) The RNeasy[®] spin column was placed in a new 1.5 ml collection tube, 50 μ l RNase-free water was added to the spin column, and the spin column placed in the collection tube was thereafter centrifuged for 1 minute at 10000 rpm to collect the RNA.
- (x) The flow-through containing extracted RNA was kept in 1.5 ml collection tube and was stored at -80 °C.

3.1.4 Assessment of mRNA and RNA Extraction Results and Discussion

After mRNA was extracted (using the selected ‘conventional method’) from the tissue samples, the efficacy of the ‘conventional method’ was evaluated by two parameters (of the mRNA extracted): the purity (A260/A280 ratio), and the yield (extracted mRNA/RNA concentration). This efficacy was thereafter verified by comparing the RNA extracted by the tissue grinder homogenization and column-based RNA extraction method from the same batch of tissue samples. The purity and yield of mRNA and RNA extracted from tissue samples were assessed by the NanoDrop[™] 2000 (Thermo Fisher Scientific, Singapore) spectrophotometer (Figure 3-1), where 1 μ l of the extracted mRNA solution or 1 μ l of extracted RNA solution was loaded on the NanoDrop[™] 2000.

Figure 3-1 NanoDrop™ 2000 [127]

It is well established that pure mRNA/RNA has A260/A280 ratio of approximately 2 [127]. The concentration of extracted mRNA/RNA was quantified by the value of A260 (A260 is the absorbance of 260 nm wavelength light by mRNA/RNA or DNA), and the quality of the mRNA/RNA extracted was ascertained by their A260/A280 ratios; A280 is the absorbance of 280 nm wavelength light by proteins. On this basis, the A260 and A280 determined by the spectrophotometer (NanoDrop™ 2000) can be used to assess the purity and yield of the extracted mRNA as well as RNA. We use this purity of the mRNA or RNA as the indicator to decide whether further processing, such as RT-PCR, should be conducted on the mRNA/RNA extracted. The yield of the mRNA and RNA extraction method is the indicator that ascertains which method has a higher efficiency for extracting sufficient mRNA from a small amount of liver tissue.

We now present and discuss the results of the two extraction methods.

The values of the A260/A280 ratio for mRNA and RNA extracted from liver/tail tissue samples are plotted in Figure 3-2.

Figure 3-2 A260/A280 for mRNA and RNA extracted from the same batch of tail and liver tissue samples (n = 3 for each extraction method)

In the case of the mouse tail tissue sample, we obtained the A260/A280 ratio for the extracted mRNA ($A_{260}/A_{280} = 1.72$) to be below 2 (indicated as a red line in Figure 3-2). With the same batch of tail tissue samples, we also obtained the A260/A280 ratio for the extracted RNA to be below 2 ($A_{260}/A_{280} = 1.79$). Hence, both RNA and mRNA extracted were insufficiently pure for further processing, indicating the degradation of the mouse tail tissue samples used for the mRNA and RNA extraction. The result was not unexpected as the mouse tail tissue samples (shipped from USA)

were in poor state due to long time storage at room temperature during transportation. Although not delineated earlier, this was the reason why an alternative tissue sample, the liver tissue sample, was obtained and the experiments were repeated.

On the other hand, the A260/A280 ratio for mRNA extracted from liver tissue sample was, as expected, ~2. This A260/A280 ratio indicated that the extracted mRNA was sufficiently pure for further processing. This measured value was also in agreement with the A260/A280 ratio result for RNA extracted with the same batch of liver tissue sample.

For each extraction method, the A260/A280 ratio of the two groups of tissue samples is consistent with the other extraction method. On this basis, we have a good level of confidence in the two methods – the efficacy of the bead-based mRNA extraction method has been verified with the column-based RNA extraction method.

Besides the purity of the mRNA and RNA extracted, the efficiency of the bead-based mRNA extraction method was, in a sense, further evaluated by comparing the concentration of mRNA extracted by the mRNA extraction method with the concentration of mRNA within the extracted RNA obtained from the column-based RNA extraction. The concentrations of extracted RNA and mRNA are tabulated in Table 3-1.

Table 3-1 Concentrations of mRNA and RNA extracted from liver tissue samples

Extraction Methods	Concentration (ng/ μ l)
mRNA Extraction Method	76.2 (mRNA)
RNA Extraction Method	501.5 (RNA)

To interpret the results of Table 3-1, first consider the following. In typical mammalian cells and tissues, 1% - 5% of RNA content is mRNA [128]. Hence, the concentration of mRNA in the extracted RNA (501.5 ng/ μ l) is expected to range between 5.0 ng/ μ l and 25.1 ng/ μ l. With the consideration of different final volumes of extraction product (25 μ l for mRNA extraction, and 50 μ l for RNA extraction), the concentration of mRNA in the extracted RNA would be expected to range between 10 ng/ μ l and 50.2 ng/ μ l (if we use 25 μ l instead of 50 μ l as the final volume in order to compare with the result from mRNA extraction method). This range is, however, lower than the concentration of the extracted mRNA (76.2 ng/ μ l) by the bead-based mRNA extraction method with same amount of tissue sample input. We, thus, infer that the bead-based mRNA extraction method is more efficient of extracting mRNA from same amount of tissue sample. This inference is not unexpected as the column-based RNA extraction method extracted RNA only. However, bead-based mRNA extraction method is mRNA specific, with beads directly binding the mRNA in the lysate.

In short, we have verified the efficacy of the selected bead-based mRNA extraction method by comparing it against the column-based RNA extraction method. We have also inferred that the bead-based mRNA extraction method has higher efficiency than the column-based RNA extraction method. Collectively, we have identified the

‘conventional method’ is the appropriate selection as the benchmark and reference for developing a modified ‘conventional method’ protocol for a microfluidic adoption.

3.2 Modification of Tissue Homogenization and mRNA Extraction Protocol

3.2.1 Biological Samples

Liver tissue samples were obtained from common black laboratory mice (Strain name: C57BL/6J; Age: 5-7 weeks) which were administrated with different dosages of cyclophosphamide (a drug commonly used in chemotherapy) [129]. All experiments were conducted in accordance with the National Institutes of Health Guide for care and use of laboratory animals (NIH Publication No. 80-23, revised 1996) as well as with the approval of the Institutional Animal Care and Use Committee of The Methodist Hospital Research Institute (TMHRI).

3.2.2 Modified Tissue Homogenization and mRNA Extraction Protocol

Based on the literature review in Chapter 2 and the comparison with the efficacy of the column-based RNA extraction process, we selected the plastic tissue grinder and magnetic beads-based mRNA extraction method (Dynabeads[®]mRNA DIRECT™ Kit, Thermo Fisher Scientific, Singapore) as our tissue homogenization and mRNA extraction method. Nevertheless, this ‘conventional method’ is incompatible to the

envisioned integrated microfluidic device which featuring a minimum amount of tissue sample and small amount of reagents, congruous to a practical POC device for hepatotoxicity assessment. To this end, we propose a modification of the tissue homogenization and mRNA extraction protocol and this will now be discussed:

Step 1. Homogenize a liver tissue sample (10 mg of liver tissue sample) in a 5ml Eppendorf™ tube via a mechanical rotor-stator homogenizer (Bio Gen. PRO 200) with 300 µl Lysis/Binding Buffer (Dynabeads®mRNA DIRECT™ Kit, Thermo Fisher Scientific, Singapore), and keep the mixture for 5 minute for tissue lysis.

Note that a 10 mg liver tissue sample (instead of 30 mg) is used to reduce sample input. The rotor-stator homogenizer instead of plastic tissue grinder is used for the reduced Lysis/Binding Buffer (from 1000 µl to 300µl). 5 minutes instead of 1minute incubating time for the desired lysis result with less Lysis/Binding Buffer used (300 µl).

Step 2. Transfer the lysate to a 1.5 ml micro-centrifuge tube and use a 20-gauge needle with 1 ml syringe to shear DNA inside the lysate for 4 times.

Step 3. Suspend 150 µl magnetic beads in a pipette tube by a vortex machine for 30 seconds and use a magnet to attract the beads for separation.

Note that instead of 250 µl beads solution, only 150 µl is used. This is congruous to the smaller amount of lysate, from which we extracted the mRNA.

Step 4. After removing the supernatant, wash the beads with 150 µl Lysis/Binding Buffer (Dynabeads®mRNA DIRECT™ Kit, Thermo

Fisher Scientific, Singapore), followed by the separation step.

Note that 150 µl Lysis/Binding Buffer is used, instead of 250 µl in the conventional method.

Step 5. Transfer the lysate to the pipette tube containing the beads and resuspend beads/lysate mixture by the vortex machine for 5 minutes.

Note that 5 minutes incubating time (instead of 1 minute) allows higher mRNA extraction yield after reducing the solvent used.

Step 6. Separate the beads from the unwanted solution by the magnet and remove the supernatant. Resuspend the microbeads in 150 µl Washing Buffer A (Dynabeads®mRNA DIRECT™ Kit, Thermo Fisher Scientific, Singapore) and wash beads with 150 µl Washing Buffer A 3 times.

Note that the total amount of Washing Buffer A is reduced to 450 µl (from 2000 µl in the conventional method) and it is sufficient to extract mRNA from lysate with good purity.

Step 7. Wash the mRNA attached beads 3 times with 150 µl Washing Buffer B (Dynabeads®mRNA DIRECT™ Kit, Thermo Fisher Scientific, Singapore).

Note that the total amount of Washing Buffer B used is reduced to 450 µl (from 2000 µl in the conventional method) and the smaller volume of Washing Buffer B is sufficient to extract mRNA with good purity.

Step 8. Add 70 µl Elution Buffer (Dynabeads®mRNA DIRECT™ Kit, Thermo Fisher Scientific, Singapore) in the pipette tube containing the beads,

incubate the mixture at 70 °C in a water tank for 2 minutes, followed by the separation of the beads from the rest of the solution. Transfer the supernatant, which contained the mRNA, by pipetting to a pipette tube.

Note that the total amount of Elution Buffer used is increased to 70 μ l (from 25 μ l in the conventional method). The increase in the extracted mRNA solution is desired for the ensuing multiplex gene expression detection process.

Step 9. Finally, apply the extracted mRNA to the subsequent RT-PCR process or store the extracted mRNA at -80 °C for long term storage for later use.

3.2.3 Validation of Modified ‘Conventional Method’

We used the aforesaid proposed modified protocol of the ‘conventional method’ to extract mRNA from 10 mg raw liver tissue sample and will now verify this modified protocol against the original manual protocol with 10 mg of the same batch of liver tissue samples. In order to directly compare the concentration of extracted mRNA from two different protocols, the final volume of extracted mRNA from original manual protocol is increased to 70 μ l (by adding 45 μ l more Elution Buffer).

Figure 3-3 Concentration of extracted mRNA from the same batch of liver tissue samples by different extraction protocols: original manual protocol, and modified manual protocol (n = 3 for each protocol)

Figure 3-3 is a graph of the extracted mRNA using the original manual protocol and modified manual protocol for 10 mg of the same batch of liver tissue samples. This figure indicates that the yield of extracted mRNA from the modified protocol was ~ 10 ng/μl and is comparable to the yield of mRNA extraction from the original protocol (~11 ng/μl). The results indicate that the amount of Dynabeads® and Lysis/Binding Buffer used in original protocol was excessive for extracting mRNA from 10 mg liver tissue sample and that the 150 μl of Dynabeads® solution (60% of the original 250 μl Dynabeads® solution) and 300 μl of Lysis/Binding Buffer (30% of the original 1000 μl Lysis/Binding Buffer) were sufficient for 10 mg liver tissue sample. It also demonstrated the flexibility of magnetic bead-based mRNA extraction method, in which the amount of magnetic beads input can be adjusted accordingly to different amount of sample input.

Figure 3-4 A260/A280 ratio of extracted mRNA from the same batch of liver tissue samples by different protocols: original manual protocol, and modified manual protocol (n = 3 for each protocol)

Figure 3-4 depicts the A260/A280 ratio of extracted mRNA from the original manual protocol and from the modified manual protocol for the same batch of liver tissue samples. This figure depicts that the modified protocol could extract mRNA with A260/A280 ratio above 2 and this is consistent with the original manual protocol. This indicates that lower consumption of washing buffers (~23% of original manual protocol) was sufficiently effective to process the same amount of liver tissue sample input, washed away any unwanted impurity, and extracted mRNA without compromising the purity.

This modification of the ‘conventional method’ protocol is a critical step to develop an efficient microfluidic system with small chemical usage of footprint. We will employ

this modified tissue homogenization and mRNA extraction method for our microfluidic system in the next chapter.

3.3 Conclusions

By performing the bead-based mRNA extraction with mouse tail and mouse liver tissue samples, and comparing the purity and yield of mRNA within the RNA extracted with the column-based commercial RNA extraction kit, we have conclusively identified that the tissue homogenization by a simple plastic tissue grinder and the magnetic bead-based mRNA extraction method as the appropriate reference and benchmark for the modification of the selected conventional method for microfluidic adoption. The purity (A260/A280 ratio was above 2 for liver tissue, and below 2 for tail tissue) of the extracted mRNA has been shown to be consistent with the purity of RNA extracted. The concentration of mRNA extracted has been shown to be higher than the concentration of mRNA within the RNA extracted by column-based RNA extraction method.

We here also proposed the modification of the selected tissue homogenization and mRNA extraction method with less beads (60% of the conventional method) and less chemical reagents (~26% of the conventional method). With this proposed modified method, we have successfully extracted mRNA from 10 mg raw liver tissue, where the purity and yield of extracted mRNA were comparable with results obtained from the conventional tissue homogenization and mRNA extraction method, the purity of mRNA sample extracted from modified method was good, and the yield of mRNA

concentration was ~ 10 ng/ μ l, over 90% of the yield of the ‘conventional method’. We have demonstrated that the modified tissue homogenization and mRNA extraction protocol is as efficient as the conventional protocol.

In conclusion, we have proposed and validated the modification (with smaller tissue input and less chemical consumption) of the selected ‘conventional method’. This modified ‘conventional method’ will serve as the appropriate reference and benchmark for our (to be designed and fabricated) microfluidic device.

Chapter 4 A Semi-Integrated 2-Chip LOC System Embodying Liver Tissue Homogenization, mRNA Extraction and Multiplex RT-PCR for Hepatotoxicity Assessment Based on Gene Expression Profiling

4.1 Introduction

This chapter is, in part, extracted from our published manuscript [130] and a manuscript [131] under review. In the former manuscript, we presented, arguably for the first time ever, a *semi-integrated 2-chip* (a tissue homogenization/mRNA extraction chip and a stationary multiplex microfluidic RT-PCR chip) LOC system embodying liver tissue homogenization, mRNA extraction and multiplex RT-PCR for hepatotoxicity assessment based on gene expression profiling. We also described its design, fabrication, and feasibility of multiplex gene expression analysis from raw liver tissue input. In the latter manuscript, we presented, in detail, the design, fabrication and testing of the tissue homogenization/mRNA extraction chip – arguably the first-ever microfluidic device with the capability of processing raw liver tissue within a microfluidic chip for subsequent on-chip mRNA extraction process.

The *semi-integrated 2-chip* LOC system presented in this chapter is largely a proof-of-concept for our *Grand Objective* – to develop a low-cost, rapid, “sample-in-answer-out” POC liver disease diagnosis device that requires a minimal (amount) liver tissue sample for multiplex gene expression profiling for rapid and accurate diagnosis. Our LOC system comprises three components: tissue homogenization/mRNA extraction microfluidic chip, stationary multiplex microfluidic RT-PCR chip and real-time

fluorescent detection setup. Compared to reported microfluidic devices [79, 108, 124, 132], our *semi-integrated* 2-chip LOC system is arguably the first-ever microfluidic system with “sample-in-answer-out” capability for hepatotoxicity assessment applications involving raw liver tissue samples. This capability is one of the primary attributes of our envisioned practical POC liver disease diagnosis device. Our LOC system is able to homogenize the raw liver tissue sample directly (“sample-in”) and extract mRNA from the lysate with good purity within a single microfluidic chip for subsequent RT-PCR process, and its efficacy is comparable with the conventional tissue homogenization and mRNA extraction method. The stationary multiplex microfluidic RT-PCR component and real-time detection setup provide ‘real-time’ information (“answer-out”) to analyze the cDNA concentration of multiple target genes during the RT-PCR process for hepatotoxicity assessment.

With regard to our contribution to our *Specific Objective (B)* in Chapter 1, the first chip of our 2-chip LOC system involves the design and fabrication of a tissue homogenization/mRNA extraction microfluidic chip featuring two critical functional units: a tissue homogenization chamber and a micro-reactor for mRNA extraction. The tissue homogenization chamber incorporated into this microfluidic chip enables the microfluidic chip to handle raw liver tissue directly and to provide lysate for subsequent mRNA extraction process. The integration of the tissue homogenization chamber and the micro-reactor in a single microfluidic chip reduces off-chip intervention; in this case, off-chip transfer of the sample lysate. With raw liver tissue sample from the pre-clinical drug toxicity study, we demonstrate that the tissue homogenization/mRNA extraction chip is capable of providing extracted mRNA from raw tissue sample with high purity,

and the yield concentration is comparable with the conventional and modified manual methods. We also demonstrate the feasibility of applying our tissue homogenization/mRNA extraction chip to detect the changes in the expression level of GOIs (AST, AST1, and ALT1) for hepatotoxicity assessment [24, 133]. Our results illustrate that the final cDNA concentration of AST, AST1, and ALT1 after conventional RT-PCR process increased by 81%, 21.5% and 51% respectively for 3x dosage drug administration. This indicates an increase in gene expression level of GOIs (AST, AST1, and ALT1).

The second chip of our *semi-integrated* 2-chip LOC system involves a stationary multiplex microfluidic RT-PCR chip with primer preloading treatment that simplifies the off-chip RT-PCR reagents preparation process for multiple GOIs. The real-time fluorescent detection setup provides a practical way to assess the cDNA concentration of GOIs in real-time during the RT-PCR process. With mRNA extracted from our tissue homogenization/mRNA extraction chip, the final cDNA concentration of AST and ALT after on-chip multiplex RT-PCR are increased by 58.7% and 33.3% respectively for 3x dosage drug administration. With images obtained from our real-time fluorescent detection setup during RT-PCR process, we demonstrate normalized fold changes of 1.62 for AST and 1.32 for ALT for the gene expression profiling analysis.

The results of gene expression level changes for GOIs (AST, ALT, AST1, and ALT1) from our on-chip experiments are largely consistent with that obtained from conventional laboratory procedure and reported observation [24-26, 133], and

demonstrate the feasibility of our *semi-integrated* 2-chip LOC system for POC hepatotoxicity assessment.

Collectively, we demonstrate in this chapter the feasibility of our *semi-integrated* 2-chip LOC system to perform the entire process including tissue homogenization, mRNA extraction, stationary multiplex RT-PCR and real-time fluorescent detection – our microfluidic system features “sample-in-answer-out” capability. This LOC microfluidic system is an imperative step towards the development of a truly practical rapid POC liver diagnosis device.

For sake of depicting our contribution to this *semi-integrated* 2-chip LOC system, we declare that the stationary multiplex microfluidic RT-PCR chip and real-time fluorescent detection are the work of our colleague in the same research group. Nevertheless, to depict the significance of this LOC system as a collective effort towards our grand objective and to demonstrate the contribution of our integrated tissue homogenization/mRNA extraction microfluidic chip to this endeavor, we will describe the entire LOC system in this chapter.

4.2 Materials and Method

In this section, we will first delineate the protocol used in our preclinical drug toxicity study to establish the feasibility of our proposed microfluidic system. After that, we will discuss the design and fabrication of the tissue homogenization/mRNA extraction chip, the design fabrication and setup of the multiplex RT-PCR and real-time

fluorescent detection unit. We will then describe the operation of the tissue homogenization/mRNA extraction chip and assessment of the quantity and quality of the mRNA extracted, and demonstrate the efficacy of our microfluidic tissue homogenization/mRNA extraction chip with raw liver tissue samples. Thereafter, we will perform a multiplex gene expression study comprising three liver-critical genes (i.e. AST, AST1, and ALT1) and monitor their changes in expression level under different dosages of cyclophosphamide with mRNA extracted on-chip; the set of genes (AST1 and ALT1) are recommended by our collaborator, Houston Methodist Research Institute (HMRI, Cornell University), USA, for their excellent performance for gene expression detection in the mouse liver toxicity study. Subsequently, we will briefly describe the operation of stationary microfluidic multiplex RT-PCR and real-time fluorescent detection. Collectively, we will demonstrate the feasibility of our integrated microfluidic system by performing the entire testing process (i.e., tissue sample homogenization, mRNA extraction and multiplex RT-PCR and real-time fluorescent detection) on our microfluidic system to detect liver-critical GOIs (ALT and AST) for POC hepatotoxicity assessment.

4.2.1 Biological Samples

Liver tissue samples were obtained from common black laboratory mice (Strain name: C57BL/6J; Age: 5-7 weeks) which were administered with different dosages of cyclophosphamide. All experiments were conducted in compliance with the National Institutes of Health Guide for care and use of laboratory animals (NIH Publication No.

80-23, revised 1996), and with approval by the Institutional Animal Care and Use Committee of Houston Methodist Research Institute (HMRI).

The mice subjects were divided into two groups, with 4 mice in each group. Within each group, one mouse was chosen as the control and administered with saline (concentration of 0.9% weight/volume NaCl), while the remaining three mice were administered with cyclophosphamide (200 mg/kg of body weight) through the intraperitoneal route. The first group of mice had a single dose of saline or cyclophosphamide administered on the 1st day only while the second group had a dose of saline or cyclophosphamide administered on the 1st, 3rd, and 5th days for a total of three dosages. The animals were subsequently euthanized by standard carbon dioxide anesthesia procedure on the 5th day after final drug administration. The liver tissues were harvested, stored in RNeasy[®] RNA Stabilization Reagent (Qiagen, USA) under -80 °C to prevent RNA degradation for long term storage.

There were two batches of liver tissue sample from pre-clinical drug toxicity study used in this project. The first batch was used for the verification of efficacy of the tissue homogenization/mRNA extraction microfluidic chip. The second batch was used for demonstrating the feasibility that the tissue homogenization/mRNA extraction microfluidic chip could provide mRNA extracted from raw liver tissue sample for multiplex gene expression study comprising three GOIs (i.e., AST, AST1, and ALT1).

4.2.2 Design and Fabrication of Microfluidic Chip Embodying Tissue

Homogenization and mRNA Extraction

The two critical components in our microfluidic chip in the operation flow chart are indicated in Figure 4-1(a), and the Computer Aided Design (CAD) drawing of the microfluidic chip with each functional unit labeled is presented in Figure 4-1(b). The photograph of the fabricated microfluidic chip in the real experiment setting is depicted in Figure 4-1(c).

(a)

(b)

(c)

Figure 4-1 (a) The operation flow of our microfluidic tissue homogenization/mRNA extraction chip embodying two critical components: tissue homogenization chamber and micro-reactor for mRNA extraction procedures; (b) CAD drawing of the microfluidic chip, R1: reservoir for Lysis/Binding Buffer, R2: reservoir for Washing Buffer A, R3: reservoir for Washing Buffer B, R4: reservoir for Elution Buffer, V: valve, M: micro-reactor, H: homogenization chamber; and (c) Photograph of the microfluidic chip

The microfluidic chip (Figure 4-1) performing tissue homogenization and mRNA extraction comprises two critical functional components and four reservoirs as well as fluidic control valves. These two functional components are now described in turn.

(a) Tissue Homogenization Chamber

The tissue homogenization chamber (depicted in Figure 4-1(b), structure H) embodied three components: a miniaturized tissue blender (microHomogenizer™ from ClaremontBio, USA) with dimensions of 7 mm (diameter) x 15 mm (height); a homogenization chamber; and an O-ring. This chamber was designed for simple and tight seal after mounting the miniaturized blender on the chamber, lower power consumption and portability of the chip. The O-ring was placed at the top of the chamber. When the tissue blender was mounted, the motor portion of the blender pressed and deformed the O-ring towards the wall of the chamber and created a tight seal between the blender and O-ring. Thus, the chamber could hold the blender properly during the operation with minimum air bubble generation. The miniaturized tissue blender was powered by 3 AAA batteries, this external power supply unit could be easily attached to our integrated microfluidic chip and improved the portability of the device.

(b) Micro-Reactor

All mRNA extraction steps were performed in the micro-reactor, including binding of mRNA to microbeads, and washing beads with Washing Buffer A and Washing Buffer B. The micro-reactor (M in Figure 4-1(b)) comprised a cylindrical chamber with a silicone elastic membrane (HT6240-Roger Corp,

0.25 mm thick) as the bottom layer. The schematic drawing of the micro-reactor is depicted in Figure 4-2.

Figure 4-2 A schematic drawing of the micro-reactor

In the micro-reactor, rapid mixing is achieved through the induced vibration of silicone elastic membrane by air flow agitation [78]. It is arguably the first microfluidic mixing chamber based on air flow agitation mechanism for mRNA extraction application. A nozzle is placed against the silicone elastic membrane to allow for membrane deformation when air pressure is released from the nozzle. When the membrane deforms convexly, the air pressure at the end of nozzle decreases, resulting in a corresponding decrease in upward pressure on the membrane. Concurrently, an internal elastic force is generated as the deformation of the membrane increases convexly, and this resultant elastic force prevents the membrane from deforming further, thereby pulling it back to its original position (i.e., un-deformed state) to block the air flow path again. The

deformation and un-deformation process then repeats. This collective interaction of the aerodynamic force, elastic force and the inertial force of the membrane creates a constant vibration state of the silicone membrane to agitate the liquid sample inside the micro-reactor for rapid mixing.

Figure 4-3 depicts the rapid mixing in the micro-reactor where the Figure 4-3(a) depicts the aggregation of magnetic beads at the boundary of the micro-reactor's bottom before the mixing induced by vibration. Figure 4-3(b) depicts the ensuing uniform dispersion of magnetic beads within the solution, only after 2 – 3 seconds of mixing process. This finite volume (160 μ l) micro-reactor is imperative for the mRNA extraction procedure, including small and finite reagent consumption for each step, and fast and robust mixing to facilitate uniform suspension of beads in washing buffers. Reagents are actuated into and out of the mixing chamber via a programmable syringe pump. An external air compressor provides the necessary pressurized air flow ($\sim$ 1.2 atm) to drive the silicone membrane vibration, yielding fast mixing of small volume reagents in less than 3 seconds.

Figure 4-3 Rapid mixing process of the micro-reactor, illustrating full suspension of beads in liquid. (a) Before mixing, beads aggregate at the boundary of micro-reactor's bottom;
(b) After mixing, beads disperse uniformly in the solution.

The mixing process in the micro-reactor is critical for the extraction of mRNA with good quality for downstream experiment and analysis. The quality of mRNA is usually assessed by two indicators: A260/A280 value for purity and the extracted mRNA concentration for yield. A pure mRNA has an A260/A280 ratio of about two [127].

Figure 4-4 depicts the change of A260/A280, and concentration of mRNA extracted by our microfluidic chip with and without the mixing process in the micro-reactor. The results depict significant improvement in the quality of mRNA extracted with the efficient mixing process. The A260/A280 value increased from 1.5 to 2, indicating that a pure extracted mRNA sample was obtained from on-chip mixing. The extracted mRNA concentration increased by 60%, from ~5 ng/μl (without mixing process) to ~8 ng/μl (with mixing process).

(a)

(b)

Figure 4-4 Quality of mRNA extracted by on-chip tissue homogenization/mRNA extraction without or with effective micro-reactor mixing. (a) A260/A280 ratio, (b) concentration of mRNA extracted

The CAD drawing of the microfluidic chip was depicted in Figure 4-1(b). The fabricated chip comprising eight PMMA layers, depicted in Figure 4-1(c), was fabricated as follows. First, 8 PMMA sheets (6 sheets with 3 mm thickness each; and 2 sheets with 1 mm thickness each) were cut with a 35 W CO₂ laser (VersaLASER VLS2. 30, Universal[®] Laser Systems, USA). These PMMA parts were subsequently cleaned in a bath sonicator with 10% V/V isopropanol for 10 to 15 minutes and dried in vacuum oven at 80 °C overnight. Finally, all 8 PMMA layers were clamped between two pieces of borosilicate glass (5 mm thick) and thermally bonded in an oven at 128 °C under 1 atm pressure for 2 hours, before cooling down to room temperature naturally.

4.2.3 Design and Fabrication of Multiplex RT-PCR Chip

The PMMA based multiplex RT-PCR chip comprised 12 reaction chambers, which as depicted in Figure 4-5 were connected to a central microchannel. Structurally, the RT-PCR chip was fabricated from 3 PMMA layers: 1 mm thick middle layer for reaction chambers, two 0.5 mm thick PMMA layers for the top and bottom layer enclosing the chamber cavity in the middle layer. This design ensured minimum rough surface finishing within the reaction chambers due to fabrication cutting. The rough surface finishing would otherwise induce the unwanted bubble formation during the PCR thermal cycling process.

Figure 4-5 Schematic design of multiplex RT-PCR chip [130]

The chip design also included a single assay loading inlet with NanoPort™ PEEK ferrule connectors and 1/32 inch OD tubing. The RT-PCR reagent mixture containing mRNA extracted from the upstream tissue homogenization/mRNA extraction chip was actuated to be equally distributed into the 12 reaction chambers via this inlet. Each reaction chamber had a volume of ~10 μ l, with microchannel of 5 mm by 0.2 mm to allow the RT-PCR reagent mixture to be loaded.

4.2.4 Design and Setup of Fluorescent Detection System

To enable real-time fluorescent detection of amplified cDNA during the RT-PCR process, a fluorescent detection system was developed. This is depicted in Figure 4-6.

Figure 4-6 Fluorescent detection setup [130]

It comprised a camera module with a 470 nm blue excitation LED (IORodeo[®], USA) to be mounted on the chip-compatible stationary thermal cycler. A 3MP CMOS camera with 550 nm longpass optical filter (Island Optical Systems[®], Singapore) was fitted on top of a black opaque acrylic frame. The blue LED module was affixed at $\sim 45^\circ$ angle to the plane of the microfluidic chip to provide an excited source for the SYBR[®] Green 1 fluorescent dye present in the RT-PCR assay. The angle was chosen to minimize excitation light interference on the captured light pathway of the camera unit.

4.2.5 On-Chip Tissue Homogenization and mRNA Extraction

150 μ l of magnetic beads (Dynabeads[®]mRNA DIRECT[™] Kit, Thermo Fisher Scientific, Singapore) solution was loaded into the mixing chamber and washed with 150 μ l Lysis/Binding Buffer (Dynabeads[®]mRNA DIRECT[™] Kit, Thermo Fisher Scientific, Singapore) before the liver tissue sample was loaded into the homogenization chamber. An external magnet was used to separate the beads from the unwanted waste after washing, by placing the magnet close to the bottom of the mixing chamber. The liver tissue sample (~10 mg in weight) was loaded directly into the tissue homogenization chamber and sealed inside the chamber once the miniaturized blender (microHomogenizer[™] from ClaremontBio, USA) was fitted into the chamber opening by the rubber O-ring. 300 μ l of Lysis/Binding Buffer was subsequently actuated into the homogenization chamber. The liver tissue was fully homogenized by the blender (powered by AAA batteries), and the sample/buffer mixture was incubated for 4 minutes to allow for completion of the lysis process. The next sub-step involved actuating the lysate (~150 μ l) to be mixed with the beads, followed by 5 minutes of incubation for mRNA/beads binding process. The mRNA-bound beads were washed with Washing Buffer A (Dynabeads[®]mRNA DIRECT[™] Kit, Thermo Fisher Scientific, Singapore) three times (150 μ l each time) and with Washing Buffer B (Dynabeads[®]mRNA DIRECT[™] Kit, Thermo Fisher Scientific, Singapore) three times (150 μ l each time). Finally, the beads were resuspended with 70 μ l Elution Buffer (Dynabeads[®]mRNA DIRECT[™] Kit, Thermo Fisher Scientific, Singapore) and collected at the outlet in an Eppendorf[®] tube. The collected beads solution was incubated at 70 °C for 2 minutes, followed by the separation of the beads from the rest

of solution containing mRNA. This mRNA solution was used for the subsequent RT-PCR process. The on-chip tissue homogenization and mRNA extraction processing time was approximately 12 – 14 minutes. To evaluate the feasibility and efficiency of on-chip tissue homogenization and mRNA extraction, we compared the results against that obtained from the aforementioned modified manual tissue homogenization and mRNA extraction method for the same batch of liver tissue samples.

4.2.6 mRNA Quantification and Quality Assessment

As delineated in Chapter 3, the mRNA extracted from manual and on-chip methods was evaluated by two parameters (of the extracted mRNA): the purity (A260/A280 ratio) [128], and the yield (the concentration of the extracted mRNA) [134]. The A260/280 ratio and the concentration of extracted mRNA were assessed by the NanoDrop™ 2000 (Thermo Scientific™) spectrophotometer, where 1 µl of the extracted mRNA solution is loaded on the NanoDrop™ 2000.

Also, as delineated in Chapter 3, the extracted mRNA, with the A260/A280 ratio approximately equal to 2, is considered to be pure [127, 134]. This purity of the mRNA is essential indicator to decide whether the subsequent process, i.e., RT-PCR, should be conducted with the mRNA/RNA extracted. The concentration will indicate the efficiency of the on-chip mRNA extraction process.

4.2.7 Conventional RT-PCR and Gel Electrophoresis

Table 4-1 Primers of GOIs

GOI	Primer
AST	Forward: GCT GAC TTC TTA GGG CGA TG
	Reverse: TCA TTC AGG AAA CCC TGG AG
ALT	Forward: CTG CAG ACC CGA ACA ACA TA
	Reverse: CAG CTC AGC GAT GTC AAG AG
ALT1	Forward: TGA GGG CTC TCA GTT CCA TT
	Reverse: ACC TCG GCA TCC ATG TTT AC
AST1	Forward: TCT TCG GAT TGG AGC TGA CT
	Reverse: TAG CAA TAG GGC CGA ATG TC

Table 4-1 depicts the intron spanning primes used in this project for four GOIs (AST, AST, ALT1 and AST1). Intron spanning primers were used to ensure the amplification of the specific targeted mRNA fragments as well as to prevent any PCR side-product amplified from genomic DNA contamination [135]. The primers were designed using the PrimerQuest software (Integrated DNA Technologies®, Singapore) and synthesized *de novo* from the same company.

20 µl RT-PCR reagent was prepared in an Eppendorf® tubes according to the protocol of iScript™ One-Step RT-PCR Kit SYBR® Green [136] using the following: 2x

SYBR[®]Green RT-PCR reaction mix (12.5 μ l), nuclease free water (6 μ l), 0.5 μ M forward/reverse primers (forward primer: 0.5 μ l, backward primer: 0.5 μ l) for specific targeted gene, extracted mRNA (6 μ l), and iScript[™] reverse transcriptase (0.5 μ l). This RT-PCR mixture was subsequently placed in a thermal cycler using the following sequence: reverse transcription (50 °C for 10 minutes, 95 °C for 5 minutes), PCR (95 °C for 10 seconds, 57 °C for 45 seconds), 35 cycles.

To evaluate the efficacy of our microfluidic tissue homogenization/mRNA extraction chip and the feasibility of applying this microfluidic chip to study the expression level of multiplex genes under different drug dosages, the conventional gel electrophoresis was performed with 3.0% agarose gel (Tris-acetate-EDTA or TAE) pre-stained with ethidium bromide (BioRad[®], Singapore). The mixture for gel electrophoresis comprised 10 μ l RT-PCR product, 2 μ l loading dye and 1 μ l SYBR[®] Green dye (10,000x stock solution). The gel was first put into a gel electrophoresis container filled with 1x TAE buffer, and thereafter the mixture was loaded into the wells of the gel. Constant voltage of 100 V was applied for 45 minutes to the electrodes at the two sides of container in order to create an electrical field for electrophoresis. The images of the gel were taken for further analysis.

4.2.8 On-Chip Tissue Homogenization/mRNA Extraction for Multiplex Gene Detection on Hepatotoxicity Assessment

The microfluidic chip for tissue homogenization and mRNA extraction was used to extract mRNA from liver tissue obtained from pre-clinical drug toxicity study to assess the gene expression profiling change of targeted genes (AST, AST1, and ALT1) under different dosages of cyclophosphamide.

The mRNA extraction from liver tissue samples was carried out on the microfluidic chip. Subsequently, from the extracted mRNA, conventional RT-PCR was conducted to detect the GOI. The RT-PCR products were loaded in the gel for gel electrophoresis and images of gel were taken by the Gel Doc™ XR System (Bio-Rad®, Singapore) [137]. The intensity of the signal bands were measured with Quantity One® 1-D Analysis Software (Bio-Rad®, Singapore) [138]. The intensity of different bands of targeted genes was normalized against the intensity of 500 bp band of the DNA ladder (PCR 100 bp Low Ladder, Sigma-Aldrich®, Singapore) on the same gel image. With this normalized value, we are able to ascertain the relative change of the final cDNA (as the product of RT-PCR process) concentration of the GOI. We can determine the expression level of the target gene under different drug dosages and study the effect of the drug on the gene expression level.

4.2.9 Multiplex Microfluidic RT-PCR

The 12 chambers (Figure 4-5) were separated into three groups for the AST, ALT, and β -actin. Before loading the RT-PCR mixture containing extracted mRNA, both forward and reverse primers [130] (10 μ l of 0.5 μ M) of the two GOIs (AST and ALT) and the housekeeping gene β -actin [139] were preloaded through the vent outlet/primer loading inlets to the designated reaction chambers. Subsequently, the chip with preloaded primers was dried in an oven at 65 °C for 15 minutes for the primers to form powders on the surface of each chamber. The RT-PCR chip with preloaded primers was ready for subsequent multiplex RT-PCR process.

150 μ l RT-PCR mixture was prepared with the following composition: 30 μ l of mRNA extracted on-chip, 75 μ l 2x SYBR[®] Green RT-PCR reaction mix (Bio-Rad[®], Singapore), 3 μ l iScript[®] One-Step RT-PCR reverse transcriptase (Bio-Rad[®], Singapore) and 42 μ l nuclease-free water. This RT-PCR mixture was transferred into a 250 μ l Hamilton glass syringe connected to the RT-PCR microfluidic chip via an 1/32 inch OD tubing. The mixture was actuated into the 12 reaction chambers using a programmable syringe pump at a flow rate of 50 μ l/min. To minimize the undesirable effect of evaporation and the formation of bubbles, the vent outlets were sealed with transparent adhesive PCR film during the RT-PCR process. 30 μ l immiscible PCR-grade mineral oil (Sigma-Aldrich[®], Singapore) was subsequently actuated into the chip to remove the excess RT-PCR mixture remaining in the central microchannel. The multiplex RT-PCR chip was mounted to a chip-compatible aluminum block thermal cycler based on the open-source OpenPCR (Chai Biotechnologies, USA) platform. The sequence of thermal cycling of this thermal cycler is as follows: dehybridization of the captured

mRNA from the microbeads (70 °C for 2 minutes), reverse transcription (50 °C for 10 minutes), denaturation (95 °C for 5 minutes), and 35 cycles of PCR process (denaturation at 95 °C for 10 seconds, annealing/extension at 55 °C for 30 seconds).

To evaluate the specificity of multiplex RT-PCR process, the amplified PCR products were visualized and analyzed off-chip using conventional gel electrophoresis [124].

4.2.10 Real-Time Fluorescent Detection of Amplified cDNA

The schematic setup for this experiment is depicted in Figure 4-7. During the real-time fluorescent detection for multiplex RT-PCR process, the fluorescent detection module was placed over the thermal cycler to capture the fluorescent images of the chip under the blue LED light during annealing/extension step (55 °C) of each PCR cycle. The mean intensity from each reaction chamber was measured subsequently. The fluorescence intensity results were finally plotted into real-time PCR amplification curves for gene expression analysis on the GOIs (AST and ALT) and β -actin.

Figure 4-7 Schematic setup of the multiplex microfluidic RT-PCR with real-time fluorescent detection setup, featuring a syringe pump, PMMA microfluidic chip, RT-PCR thermal cycler, blue LED, and 3MP CMOS camera (with 550 nm longpass filter) connected to a computer [130]

4.3 Results and Discussion

In this section, we will first discuss the feasibility of our tissue homogenization/mRNA extraction chip by comparing the purity and concentration of its extracted mRNA against that obtained from the original and modified manual protocol in Chapter 3. Secondly, we will discuss the efficacy of our tissue homogenization/mRNA extraction chip by extracting mRNA from multiple tissue samples with different drug dosages. Thirdly, we will demonstrate the feasibility of using on-chip extracted mRNA to detect multiple GOIs (AST, AST1, and ALT) for gene expression profiling and study the relative change of gene expression level under different drug dosage. With the mRNA

extracted from our tissue homogenization/mRNA extraction chip, we will demonstrate the efficacy of our multiplex RT-PCR chip by amplifying the GOIs (AST and ALT) and the housekeeping gene β -actin on chip and analyze the final cDNA amplification product by conventional gel electrophoresis method. Finally, we will demonstrate the feasibility of real-time fluorescent detection during on-chip multiplex RT-PCR process to analyze the expression level change of GOIs (AST and ALT) and housekeeping gene β -actin.

Collectively, we shall demonstrate a *semi-integrated* 2-chip LOC system embodying tissue homogenization, mRNA extraction, multiplex microfluidic RT-PCR, and real-time fluorescent detection for hepatotoxicity assessment directly from raw liver tissue sample.

4.3.1 Feasibility of On-Chip Tissue Homogenization and mRNA Extraction

Figure 4-8 depicts the purity of extracted mRNA, in terms of A260/A280 ratio, by three different experimental processes (original manual protocol, modified manual protocol and on-chip extraction) for the same batch of liver tissue samples.

Figure 4-8 A260/A280 ratio for mRNA extracted by three different processes with the same batch of liver tissue samples: original manual protocol, modified manual protocol and on-chip extraction based on modified protocol (n = 3 for each process)

As indicated in Figure 4-8, the values of A260/A280 ratio of the extracted mRNA were consistent for different experimental processes. Specifically, the values of A260/A280 were above 2 for all three processes, thereby indicating a good purity. The results depict that it is feasible to replace the manual process with our microfluidic chip that is capable to directly homogenize liver tissue samples and provide mRNA extracted from the samples without compromising the yield (more than 90% of original manual protocol) and quality (A260/A280 is above 2) of mRNA, and with smaller chemical consumption.

Figure 4-9 Concentration of extracted mRNA from same batch of sample by different processes: original manual protocol, modified manual protocol and on-chip process based on modified protocol
(n = 3 for each process)

Figure 4-9 depicts the mRNA extraction yield, in terms of concentration, by three different experimental processes for the same batch of liver tissue samples: original manual protocol, modified manual protocol and on-chip process.

The yield of extracted mRNA (Figure 4-9) and A260/A280 ratio (Figure 4-8) showed that lower volume usage of chemical buffer (~26% of the original protocol) and magnetic beads (60% of the original protocol) were sufficient to conduct on-chip tissue homogenization and mRNA extraction from 10 mg of liver tissue sample without compromising the yield as well as the quality of extracted mRNA, which was critical to develop an efficient microfluidic chip with small chemical consumption. The on-chip tissue homogenization and mRNA extraction process generated more than 90% of

the yield of extracted mRNA as compared to the manual processes for the same batch of liver tissue samples.

4.3.2 Purity and Concentration of Microfluidic Tissue Homogenization/mRNA Extraction

Figure 4-10 depicts the values of the A260/A280 ratio of extracted mRNA from liver tissue samples (under different drug dosages: untreated control group, 1x dosage group, and 3x dosage group) by using our manual and on-chip process.

Figure 4-10 Purity of extracted mRNA from liver tissue sample (under different drug dosages) obtained from manual process and from our on-chip process (n = 3 for each process)

We observed that the on-chip tissue homogenization/mRNA extraction yielded consistent results with A260/A280. There was no statistically significant effect of the different liver tissue groups and different extraction processes on the A260/A280 value, because p-values for both factors (liver tissue groups and experimental processes) are >0.3 from the 2-way analysis of variance (ANOVA) statistical analysis [140]. Specifically, the A260/A280 value was approximately 2 for all 3 groups of liver tissue samples, and this A260/A280 ratio agreed with that obtained from manual procedure. This demonstrated that our microfluidic adaption of tissue homogenization and mRNA extraction yielded similar efficiency of multiple manual washing steps used to purify the extracted mRNA. Simply put, the reduced washing buffers volume used was still sufficiently effective in removing unwanted materials from tissue lysate within the microfluidic device.

Figure 4-11 depicts the yield concentration of extracted mRNA from liver tissue samples under different drug dosages (untreated control group, 1x dosage group, and 3x dosage group) by using the manual and on-chip processes.

Figure 4-11 Concentration of extracted mRNA from liver tissue samples (under different drug dosages) obtained from the manual process and from our on-chip process
(n = 3 for each process)

Figure 4-11 depicts that the mRNA extraction yield for the three groups of liver tissue samples were consistent, with an average mRNA concentration of ~ 7 ng/ μ l for both the manual process and the on-chip process. There was no statistically significant effect of the different liver tissue groups and different extraction processes on the yield of extraction, because the p-values for both factors (liver tissue groups and experiment processes) were >0.7 from 2-way ANOVA statistical analysis [140]. This consistency in mRNA concentration demonstrated that the microfluidic adaption of the manual means did not reduce the efficiency of mRNA extraction. From hepatotoxicity POC perspective, our microfluidic chip for liver tissue homogenization and mRNA extraction could potentially replace the otherwise lengthy, manual procedure.

To demonstrate the feasibility of applying our tissue homogenization/mRNA extraction chip to detect the multiple genes for gene expression profiling, we used the mRNA extracted by the on-chip extraction process to conduct the conventional RT-PCR and gel electrophoresis for three GOIs (i.e., AST, AST1, and ALT1), the gel electrophoresis of the conventional RT-PCR product from mRNA extracted by the manual process was also performed for the verification purpose. The images of gel electrophoresis were depicted in Figure 4-12.

Figure 4-12 Gel electrophoresis image depicting the RT-PCR product for target genes AST1 (147 bp), ALT1 (165 bp) and AST (182 bp) with a 100 bp DNA ladder as reference: (a) manual process, and (b) on-chip process

Figure 4-12 depicts the gel electrophoresis images of the conventional RT-PCR amplification product from the extracted mRNA by the manual process (Figure 4-12(a)) and that by the on-chip process (Figure 4-12(b)). The signal bands of our three GOIs (AST, AST1, and ALT1) were well defined with strong fluorescent intensities in both images. The difference of the intensities in Figure 4-13(a) and Figure 4-13(b) was due

to different exposure times when the gel images were taken, but the relative intensity against ladder band in both images was similar.

Figure 4-12(b) depicts that the mRNA extracted from our tissue homogenization/mRNA extraction microfluidic chip is of sufficient quality and yield for subsequent genetic related experiments. It also depicts that our homogenization/mRNA extraction chip is a viable alternative for gene expression studies for on-the-spot hepatotoxicity assessment.

4.3.3 On-Chip Tissue Homogenization and mRNA Extraction for Multiplex Gene Expression Level Detection

Figure 4-13 depicts images of gel electrophoresis of amplified cDNA (the RT-PCR product) for target genes (AST1, AST, and ALT1) with mRNA extracted from the tissue homogenization/mRNA extraction chip.

Figure 4-13 Gel electrophoresis image showing RT-PCR product for target genes AST1(147 bp), ALT1 (165 bp) and AST (182 bp): (a) for liver tissue sample under no drug treatment, (b) for liver tissue sample under 1x dosage drug treatment, and (c) for liver tissue sample under 3x dosage drug treatment

All three target genes (AST1, ALT1, and AST) were successfully amplified after conventional RT-PCR process. The signal bands were clearly identified for respective target genes, 147 bp band for AST1, 165 bp band for ALT1, and 182 bp band for AST. These images also indicated a high quality of mRNA with little impurity.

From the intensity of the signal bands, we can analyze final cDNA concentration difference of the GOIs for the liver tissue samples under different drug dosages. With this information, we can determine the change of the mRNA concentration level for the GOIs from liver tissue samples under an increased cyclophosphamide dosage.

As the concentration of 100 bp DNA ladder (PCR 100 bp Low Ladder, Sigma-Aldrich[®], Singapore) used for gel electrophoresis was standardized, we chose the intensity of 500 bp band from ladder as the common base scale, and normalized the GOIs band intensity with the intensity of 500 bp band of DNA ladder at each respective image. The normalized intensity would allow us to compare the intensity value across different images, and study the change of the normalized intensity of target genes under different drug dosages.

Figure 4-14 depicts the relative intensity of the signal bands for RT-PCR products of target genes (AST1, ALT1 and AST) for the liver tissue samples under different drug dosages.

From the 2-way ANOVA (analyzing the data of relative intensity from the three replicates of each treatment group) [140], we concluded that the variations of the

relative intensity for GOIs were statistically significant (p-value <0.0001) for the different drug dosages.

Figure 4-14 The relative intensity of target genes for liver tissue samples under different drug dosages.

(n = 3 for on-chip process and gel electrophoresis)

From Figure 4-14, we note that there was no significant increase in final cDNA for all three target genes from liver tissue samples after 1x dosage drug administration. The result might be attributed to the low metabolic reaction of the liver towards the drug at 1x dosage. However, a relatively significant increase of intensity level for the 3 targeted genes (AST1, ALT1, and AST) was observed for tissue samples under 3x dosage drug administration. ALT1 and AST1 showed a 51% and 21.5% increase in the final cDNA concentration respectively, and of particular interest, the final cDNA concentration of AST almost doubled, with 81% increase after 3x dosage drug administration. The increase in final cDNA concentration of target genes indicated an increase in gene expression levels of the liver tissue samples with 3x dosage drug administrated. These results agreed with the increased concentration level of protein

biomarkers of AST and ALT under the drug cyclophosphamide [129], and the result for AST also agreed with the results we obtained from on-chip multiplex RT-PCR process and real-time fluorescent detection.

The gene expression profile change, indicated in Figure 4-15, suggested that the gene expressions for AST and ALT1 were more easily elevated when liver tissue was exposed to toxic drugs. Hence, they are appropriate for early toxic detection.

The result also showed that our tissue preparation and mRNA extraction microfluidic chip can provide high quality mRNA with acceptable sensitivity for subsequent gene expression profiling in order to detect biomarker changes under different drug dosages.

4.3.4 Microfluidic Multiplex RT-PCR

Multiplex RT-PCR was successfully performed on the microfluidic chip to amplify the target GOIs (AST, ALT) and β -actin. To ascertain the specificity of the multiplex RT-PCR amplification result, the product of RT-PCR was analyzed using conventional gel electrophoresis. The gel electrophoresis image is depicted in Figure 4-15.

Figure 4-15 Agarose gel electrophoresis image illustrating the specificity of multiplex RT-PCR for GOIs (AST and ALT), and β -actin housekeeping gene from 3x dosage liver tissue samples.

The 12 reaction chambers provided four replicate groups, three target fragments for each group

The three bands are clearly identified on the gel image for replicate groups in Figure 4-15 where each band corresponds to one gene (221 bp band for ALT, 182 bp band for AST, and 154 bp band for β -actin). This clear band separation with minimal band smearing indicated a highly specific RT-PCR result, and that mineral oil was verified as an effective separation medium to eliminate cross-contamination between reaction chambers.

Figure 4-16 depicts relative intensity of signal bands in the gel electrophoresis image for RT-PCR products of target genes (AST, ALT, and β -actin) from liver tissue samples under different drug dosages. From the relative intensity of signal bands, we can determine the differences in the final cDNA concentrations from different liver tissue

samples with different drug dosages. A statistically significant difference (p-value <0.0001) in the normalized signal intensity of GOIs (AST, ALT) for the different drug dosages was observed.

Figure 4-16 Comparison of the differences in AST, ALT and β -actin cDNA concentration after varying levels (1x and 3x) of drug treatment. Experiments were performed in triplicates with results normalized to the untreated control [130]

Specifically, we observed no significant difference in final cDNA concentration of target genes at 1x drug dosage largely when compared to the untreated control samples. The lack of increase in the gene expression levels at 1x drug dosage could be attributed to low (or delayed) drug metabolism of the administered drug in the mice. There was significant increase in final cDNA concentration for both AST (58.7% increase) and ALT (33.3% increase) at 3x drug dosage. These results support the feasibility of our proposal-use gene expression profiling approach as an alternative for conventional LB and LFTs for hepatotoxicity assessment. Particularly, the elevations in gene expression levels were consistent with reported research studies [24-26] of gene expression

profiling for liver samples suffered toxin or severely damaged. We also observed that β -actin bands had no significant change in the intensities after 1x and 3x drug dosage, and verified β -actin as an appropriate choice as a housekeeping gene and positive control for the microfluidic system for hepatotoxicity assessment.

4.3.5 Real-Time Fluorescent Detection and Gene Expression Analysis

Figure 4-17 plots the real-time fluorescence emission intensity curves for AST and ALT and the house keeping gene (β -actin) obtained from the images captured by our fluorescent detection setup. We were able to estimate the initial mRNA concentrations of the extracted mRNA via the average C_t values; the C_t values are often used as an indicator of starting concentration of the nucleic acid template for quantitative PCR. The C_t threshold value (the number of cycles required to exceed the background intensity) is determined by the number of cycles required for the fluorescence intensity to exceed the background level (120% of No Template Control fluorescence intensity). With the C_t values obtained, the Livak method [141] was deployed to determine the difference in C_t values between a target gene (AST or ALT) and housekeeping gene (β -actin). The normalized fold change of the target gene's expression level can be obtained.

Figure 4-17 Quantification of real-time fluorescent detection for

a) AST, b) ALT and c) β -actin at 3 different drug dosages using ImageJ with images captured at the annealing/extension phase of PCR cycles [130]

From the microfluidic experimental data, we conclude the normalized fold changes for AST and ALT were 1.62 and 1.31 respectively. These values indicated that the 3x drug dosage samples had a higher mRNA concentration related to the target gene's expression when compared to an untreated control group. We also observed that ALT had a relatively lower increase in the gene expression levels compared to AST. This might be due to the lower ALT response to cyclophosphamide administration. Nevertheless, our analysis indicated a significant elevation in gene expression level when dosage of administrated drug increased from no dosage to 3x dosage. These results were consistent with the gene expression changes from our previous experiment results. In short, we demonstrated the efficacy of the fully-quantitative fluorescent detection method with our microfluidic RT-PCR system.

Collectively, we have designed and demonstrated an integrated LOC system embodying tissue sample homogenization. Multiplex RT-PCR and real-time fluorescent detection for POC hepatotoxicity assessment.

4.4 Conclusions

We have developed an LOC microfluidic system embodying two LOC chips (tissue homogenization/mRNA extraction chip and a stationary multiplex RT-PCR chip) with real-time fluorescent detection set-up. Collectively, its feasibility has been verified by the amplification and qualitative and quantitative analysis of the gene expression level on selected GOIs (AST and ALT).

Specifically, we have validated the efficacy of the tissue homogenization/mRNA extraction chip (first LOC chip) based on the modified protocol (discussed in Chapter 3), where the modified tissue homogenization/mRNA extraction protocol was comparable with the conventional manual protocol. We have also demonstrated the feasibility of applying our microfluidic tissue homogenization/mRNA extraction chip to ascertain the changes in the expression levels of GOIs (AST, AST1, and ALT1) for hepatotoxicity assessment. These changes were largely consistent with the reported research studies [24-26, 133].

We have successfully amplified the GOIs (AST and ALT) on our multiplex microfluidic RT-PCR chip, and images from conventional gel electrophoresis illustrated their elevations in expression level. The fully-quantitative real-time fluorescent detection demonstrated the normalized fold changes of GOIs, consistent with the reported studies by conventional laboratory procedure [24-26, 133].

Chapter 5 Integrated Functions of Tissue Homogenization, mRNA Extraction and Continuous-Flow RT-PCR in a Fully-Integrated LOC System for Point-of-Care Hepatotoxicity Assessment

The work in this chapter is largely extracted from our manuscript submitted on May 31, 2016 (and currently under review) for publication in *Sensors: Special issue “Micro/Nano Fluidic Devices and Bio-MEMS”*.

5.1 Introduction

In Chapter 4, we demonstrated the feasibility of utilizing two microfluidic chips (tissue homogenization/mRNA extraction chip, and multiplex stationary RT-PCR chip) to create a *semi-integrated* LOC system for handling raw liver tissue and conducting multiplex RT-PCR. However, there are shortcomings of our *semi-integrated* LOC system, which include:

- (i). The off-chip transfer of mRNA extracted by our tissue homogenization/mRNA extraction chip;
- (ii). The off-chip RT-PCR reaction mixture preparation, manual transfer of the mixture to the multiplex RT-PCR chip; and
- (iii). The relatively long processing time of stationary multiplex microfluidic RT-PCR.

In this chapter, we present our integrated LOC system to circumvent these shortcomings, specifically a *fully-integrated* LOC system with practical “sample-in-answer-out” capability for hepatotoxicity assessment from raw liver tissue samples. This integrated LOC system comprises a tissue homogenization/mRNA extraction component and a continuous-flow RT-PCR component, and it is capable of performing all experiment procedures (i.e., tissue homogenization, mRNA extraction and continuous-flow RT-PCR) within an enclosed microfluidic environment, and amplifying GOI (ALT1) for gene expression detection. This integration is a contribution towards the endeavor to develop a truly practical POC device for hepatotoxicity assessment as our *Grand Objective* aforementioned in Chapter 1. We circumvent the aforesaid shortcomings (i)-(iii) as follows.

As reviewed in Chapter 2, there are several well-established microfluidic continuous-flow RT-PCR techniques reported [109-111] for rapid RT-PCR to amplify targeted cDNA. Based on these microfluidic techniques, we could design and fabricate a continuous-flow RT-PCR microfluidic component to overcome the aforesaid shortcomings (ii) and (iii).

In our continuous-flow RT-PCR chip, mineral oil was utilized as the main fluidic medium to push a finite volume of RT-PCR reagent mixture to flow through the RT-PCR channel for amplification of the GOI in a relatively short time, compared to the stationary microfluidic RT-PCR process we employed in our previous system. By this means, we can circumvent the shortcoming (iii). Furthermore, we incorporate the reservoirs (for RT-PCR reagents and extracted mRNA) and a passive mixer into the continuous-flow RT-PCR chip. The integration of these two components eliminates

off-chip manual handling procedures for RT-PCR reaction mixture preparation and transfer, thereby circumventing the shortcoming (ii).

By integrating the continuous-flow RT-PCR microfluidic chip (via a 1/8-inch tube) with our tissue homogenization/mRNA extraction microfluidic chip described in Chapter 4, we realized an integrated LOC system with an enclosed environment. The extracted mRNA can be directly transferred to the continuous-flow RT-PCR component within the same system, and this integration eliminates the off-chip transfer of extracted mRNA to circumvent the aforesaid shortcoming (i). Compared to other reported microfluidic devices for continuous-flow RT-PCR [109-111], our *fully-integrated* LOC system is advantageous for its capability to take crude liver tissue sample directly for mRNA extraction, and eliminate any off-chip steps (i.e., reagents transfer, mixing) during the process.

In this chapter, a continuous-flow RT-PCR component is designed and fabricated, and tested for its feasibility to amplify a specific gene (ALT1) from the extracted mRNA within a short time (~45 minutes), ~50% of the processing time required for the conventional RT-PCR. We also describe the assembly of an integrated LOC system by connecting (via fluidic means) our tissue homogenization/mRNA extraction chip connected to our continuous-flow RT-PCR chip to eliminate the off-chip mRNA sample transfer and reagent preparation processes. We validate its feasibility for a practical POC hepatotoxicity assessment by successfully amplifying a target gene (ALT1) from crude liver tissue sample (“sample-in-answer-out”) within 1 hour (rapid process). At this juncture, although the amplification efficiency of our integrated LOC system is ~50% that of conventional laboratory procedures, we demonstrate that the

complete procedures can be completed within an hour on our integrated LOC platform, which is only half of that required for conventional laboratory procedures (~120 minutes).

5.2 Material and Method

In this section, we will first delineate the design and fabrication of our continuous-flow RT-PCR chip, and the fabrication of our integrated LOC system comprising the tissue homogenization/mRNA extraction component and continuous-flow RT-PCR component. Secondly, we will describe the operation of the continuous-flow RT-PCR chip and verify the on-chip continuous-flow RT-PCR amplification efficiency by conducting the conventional RT-PCR with the same batch of mRNA extracted and comparing the results of gel electrophoresis images from the conventional and on-chip RT-PCR product. Finally, we will demonstrate the feasibility of our *fully-integrated* LOC system by performing the entire process (i.e., tissue sample homogenization, mRNA extraction and continuous-flow RT-PCR) on our system to detect a GOI (ALT1) for hepatotoxicity assessment, the result will be compared with the conventional manual process.

5.2.1 Design and Fabrication of Microfluidic Chip for Continuous-Flow RT-PCR

Figure 5-1(a) depicts the schematic CAD drawing and Figure 5-1(b) depicts the photograph of the fabricated microfluidic RT-PCR chip.

Figure 5-1 Figure5(a), CAD drawing of the continuous-flow RT-PCR microfluidic chip comprising three main regions: (i) reservoirs (a1 and a2), (ii) mixing unit (b), and (iii) RT-PCR region (c and d); Figure5(b), fabricated RT-PCR microfluidic chip

This chip consists of three major functional regions and are described below.

(a) Reservoir Area

There were two reservoirs: RT-PCR reagent reservoir (a1 in Figure 5-1(a)) and mRNA sample reservoir (a2 in Figure 5-1(a)). In both reservoirs, a 30 μ l volume channel (depth of 170 μ m) was used to store RT-PCR reagent mixtures and a 10 μ l volume channel reservoir was used to store the sample solution from tissue homogenization/mRNA extraction chip.

(b) Mixing Unit

The mixing unit (b in Figure 5-1(a)) was utilized to mix the RT-PCR reagents with the extracted mRNA before the on-chip RT-PCR process. The expanding and contracting width (maximum width: 0.4 mm, minimum width: 0.2 mm) of the zig-zag channel along its entire length created turbulent flow for passive mixing of the two liquids [142], RT-PCR reagent mixture and mRNA solution.

Figure 5-2 depicts the COMSOL Multiphysics[®] simulation results (type of mesh: physics controlled free-tetrahedral mesh; number of elements: 39665 domain elements, 9318 boundary elements, and 1649 edge elements) of the mixing unit to evaluate the mixing efficiency of two input flows. The simulation results indicated a 100% mixing efficiency (the 2 input flows were uniformly mixed to form a homogeneous solution at the outlet) was achieved with ~36 mm passage of the mixing channel. Compared to an active mixer, this passive mixer design simplified the overall chip structure.

Simulation with COMSOL Multiphysics®:

Flow rate: Inlet 1 = 11.98 $\mu\text{l}/\text{min}$

Inlet 2 = 47.90 $\mu\text{l}/\text{min}$

Corresponding Re number is less than 0.1

Base solvent: Water

Diffusion constant: $5 \times 10^{-12} \text{ m}^2/\text{s}$

Corresponding molecular weight = $9.88 \times 10^5 \text{ g/mol}$

Figure 5-2 Simulation results of the mixing unit with two inlets. The flow rate ratio for the two inlets was Inlet 1(mRNA): Inlet 2 (RT-PCR reagent) = 1:4

(c) RT-PCR

The continuous-flow RT-PCR region embodied two main sections with a depth of $170 \mu\text{m}$. The first section was the RT region (c1 in Figure 5-1(a)) which was used to conduct reverse transcription process to generate cDNA of GOI from extracted mRNA. The second section was the PCR region (d in Figure 5-1(a)) for the amplification process of cDNA of GOI, by flowing through two temperature zones: 97°C zone (d1 in Figure 5-1(a)) and 57°C zone (d2 in Figure 5-1(a)) to form a PCR thermal cycle.

The fabrication process for our RT-PCR chip was largely similar to our tissue homogenization/mRNA extraction chip described in the Chapter 4. Specifically, the two PC layers were machined from PC sheets with a micro-milling machine to form channel structures, then cleansed in a bath sonicator, and dried in a vacuum oven. Subsequently, the two layers were clamped between two pieces of 5 mm thick borosilicate glass pieces and thermally bonded in an oven at 160°C under 1 atm for 1.5 hours, and finally cooled to room temperature.

5.2.2 Fabrication of Fully-Integrated LOC System for Tissue Homogenization, mRNA Extraction and Continuous-Flow RT-PCR

Figure 5-3 depicts the *fully-integrated* LOC system comprising the tissue homogenization/mRNA extraction chip and a continuous-flow RT-PCR chip.

Figure 5-3 Photograph of the *fully-integrated* LOC system comprising the tissue homogenization/mRNA extraction and continuous-flow RT-PCR microfluidic chips

The tissue homogenization/mRNA extraction chip and continuous-flow RT-PCR chip were connected via 1/8 inch tubes from the mRNA sample outlet of tissue homogenization/mRNA extraction chip to the mRNA sample inlet of continuous-flow RT-PCR chip. This formed an enclosed environment for all procedures to be conducted within the *fully-integrated* LOC system.

5.2.3 Experiment Procedures

The experimental procedure largely comprises three steps as described below.

Step 1: RT-PCR Reagent Mixture Preparation and Microfluidic Continuous-Flow RT-PCR

20 μ l RT-PCR reagent was prepared in an Eppendorf[®] tube according to the protocol [136] of iScript[™] One-Step RT-PCR Kit SYBR[®] Green as followings: 2x SYBR[®] Green RT-PCR reaction mix (12.5 μ l), nuclease free water (6 μ l), 50 μ M forward/reverse primers (forward primer: 0.5 μ l, backward primer: 0.5 μ l) for the GOI (ALT1), and iScript[™] reverse transcriptase (0.5 μ l). This assay mixture was pre-loaded into the reagent reservoir chamber for subsequent on-chip RT-PCR. 10 μ l mRNA/bead mixture obtained from our tissue homogenization/mRNA extraction chip was loaded directly via a 1/8-inch tube into the sample reservoir channel of our continuous-flow RT-PCR chip. After 2-minute incubation at 70 °C, the bound mRNA molecules were separated from the microbeads, and eluted in the surrounding buffer solution to form a purified mRNA solution. Subsequently, 20 μ l of assay mixture and 5 μ l of mRNA solution were mixed through the micromixer with different flow rates: RT-PCR reagents: 14.4 μ l/min and mRNA solution: 3.6 μ l/min.

After passing through the micromixer, a total volume of 30 μ l of the resultant mixture was actuated with mineral oil using a syringe pump into the RT region for conversion of mRNA to cDNA (50 °C for 10 minutes, 97 °C for 2 minutes). Thereafter, with a flow rate of 3.6 μ l/min, this cDNA mixture was actuated through the PCR channel for

cDNA amplification (97 °C for 10 seconds, 57 °C for 45 seconds, for a total of 35 cycles). The product of RT-PCR was collected and stored in an Eppendorf® tube for subsequent analysis.

Step 2: On-Chip RT-PCR Product Assessment

To evaluate the feasibility and efficiency of our on-chip continuous-flow RT-PCR process to detect GOI (ALT1), gel electrophoresis was performed with 3.0% agarose gel (Tris-acetate-EDTA or TAE) pre-stained with ethidium bromide (BioRad®, Singapore). The mixture for gel electrophoresis comprised 10 µl RT-PCR product, 2 µl loading dye and 1 µl SYBR® Green dye (10,000x stock solution). The gel was first put into a gel electrophoresis container filled with 1x TAE buffer, and thereafter the mixture was loaded into the wells of the gel. A constant voltage of 100 V was applied for 45 minutes to the electrodes at the two sides of container to create electrical field for electrophoresis. Conventional RT-PCR was performed using the mRNA extracted from the same batch of liver tissue sample to allow for cross-comparison of results.

Step 3: Feasibility Test of Fully-Integrated LOC System to Perform Tissue Homogenization, mRNA Extraction and RT-PCR with Liver Tissue

After demonstrating the feasibility and efficiency of our microfluidic continuous-flow RT-PCR chip, we tested the feasibility of detecting GOI (ALT1) with raw liver tissue sample by conducting every procedure on-chip with our *fully-integrated* LOC system. The mRNA extracted from tissue homogenization/mRNA extraction chip was directly transferred, through a connecting tube, to the sample reservoir region on our RT-PCR chip to conduct subsequent procedures (Step 1). Finally, a gel image was taken and

analyzed to determine the band of the GOI (ALT1) for validation of a successful GOI amplification with our integrated microfluidic system. The gel image result was compared with that from a conventional manual mRNA extraction and RT-PCR process to determine the efficacy of our *fully-integrated* LOC system.

5.3 Results and Discussion

In this section, we will demonstrate the efficacy of our continuous-flow RT-PCR chip by amplifying one GOI (ALT1) on-chip and comparing the result against that obtained from the conventional RT-PCR. With the microfluidic system comprising our microfluidic tissue homogenization/mRNA extraction chip and continuous-flow RT-PCR chip, we will validate the entire integrated LOC system by performing on-chip liver tissue homogenization, mRNA extraction and RT-PCR, and determine the efficacy of our *fully-integrated* LOC system by comparison against the manual procedures.

5.3.1 Efficiency of Microfluidic Continuous-Flow RT-PCR for ALT1

Figure 5-4 depicts the gel electrophoresis image indicating the signal bands of GOI (ALT1) band for the manual RT-PCR and on-chip RT-PCR from the same batch of extracted mRNA. This depicts that our modified continuous-flow microfluidic RT-PCR chip is largely successful for amplifying specific gene. In the perspective of amplification efficiency, it can be seen by comparing the fluorescent intensities of the

signal bands that the amplification efficiency of our microfluidic continuous-flow RT-PCR was lower (~60%) than that of with the conventional RT-PCR process carried out on thermal cycler. Nevertheless, our on-chip RT-PCR required only ~45 minutes while the conventional RT-PCR required twice the duration (~90 minutes). We attribute the lower 60% amplification efficiency for on-chip RT-PCR to the air bubbles generated during the injection of reagents and the heating during RT-PCR process. We conjecture that an optimized control procedure for sample injection and flow will mitigate the problem of air bubble, and improve the efficiency of RT-PCR.

Figure 5-4 Gel electrophoresis image depicting the on-chip and manual RT-PCR product for GOI
ALT1 (165 bp)

5.3.2 Gene Amplification for the Fully-Integrated LOC System for Tissue Homogenization, mRNA Extraction, and Continuous-Flow RT-PCR

Figure 5-5 depicts the gel electrophoresis image with the signal bands of the product from our on-chip and manual process of tissue homogenization, mRNA extraction and

RT-PCR to detect gene ALT1. The results depicted that the ALT1 target gene was successfully amplified in our *fully-integrated* LOC system from liver tissue sample, and that the amplification result was validated by the result obtained from the manual process. However, the efficiency of our integrated LOC system is, at this juncture, ~50% of amplification efficiency of the manual procedure. We attribute this lower amplification efficiency of our *fully-integrated* LOC system to the air bubble issue discussed earlier for the on-chip RT-PCR process. Nevertheless, the results demonstrated the feasibility of an integrated microfluidic system capable of performing all processes within an enclosed/integrated LOC system under an hour (~33 % faster than our previous work [130]) to perform liver tissue homogenization, mRNA extraction, mixing, and RT-PCR for specific gene detection. Our *fully-integrated* LOC system is hence potentially practical POC device for hepatotoxicity assessment.

Figure 5-5 Gel electrophoresis image depicting the product of on-chip and manual process of tissue homogenization, mRNA extraction and RT-PCR for GOI ALT1 (165 bp)

5.4 Conclusions

We have presented a *fully-integrated* LOC system with a practical “sample-in-answer-out” capability for POC hepatotoxicity assessment. This integrated LOC system, which embodies a tissue homogenization/mRNA extraction chip and a microfluidic continuous-flow RT-PCR chip, features rapid crude tissue homogenization, mRNA extraction, and RT-PCR. We have demonstrated that said *fully-integrated* LOC system was able to detect the target gene within an hour, albeit with 50% amplification efficiency of the GOI compared to conventional and lengthy (~120 minutes) manual procedures. The current version of on-chip RT-PCR, nevertheless, has achieved only about 60% efficiency of the conventional RT-PCR.

Chapter 6 Conclusions and Future Work

6.1 Conclusions

We have presented research effort towards our *Grand Objective* - to develop a low-cost, rapid, “sample-in-answer-out” POC liver disease diagnosis device that requires a minimal amount liver tissue sample for detecting multiplex gene expression profile and that provides information for rapid and accurate diagnosis. In particular, we have developed an LOC microfluidic system embodying tissue homogenization, mRNA extraction, multiplex RT-PCR, and real-time fluorescent detection for qualitative and quantitative analysis of the gene expression level on selected GOIs (AST and ALT); a continuous-flow RT-PCR microfluidic chip and the integrated microfluidic system comprising of the tissue homogenization/mRNA extraction chip and the continuous-flow RT-PCR chip to detect GOI (ALT1) directly with raw liver tissue samples for hepatotoxicity assessment.

In Chapter 1, we have presented the background and motivation to this project, and have delineated the grand and specific objectives towards developing a practical POC liver disease diagnosis device based on gene expression profiling. We also have unambiguously defined for the development of an LOC system comprising a tissue homogenization/mRNA extraction microfluidic chip and a continuous-flow RT-PCR microfluidic chip to perform functions of tissue homogenization and mRNA extraction (Part I) and RT-PCR (Part II).

In Chapter 2, we have presented a literature review on gene expression profiling, as an alternative diagnosis method to present-day LFTs and LB. We also have presented a comprehensive review on conventional tissue preparation and mRNA/RNA extraction method. This is to facilitate our selection of the suitable conventional manual method as the reference and benchmark for their ensuing modification of the selected conventional method for microfluidic adoption. Lastly, we reviewed the state-of-art microfluidic technology pertinent to the critical functionalities for our integrated microfluidic system embodying tissue homogenization, mRNA extraction and RT-PCR functionalities, and the merits of an LOC device for our envisioned POC liver disease diagnosis device.

In Chapter 3, we have identified the tissue homogenization by simple plastic tissue grinder and the magnetic bead-based mRNA extraction method as the appropriate selection by performing the mRNA extraction from mouse tail and mouse liver tissue samples, and comparing the purity and yield of mRNA extracted from the commercial RNA extraction kit. This selected conventional method serves as the reference and benchmark for our proposed modification of the said selected conventional method. We also have proposed the modification of the selected tissue homogenization and mRNA extraction method with less magnetic beads (60% of the conventional method) and less chemical reagents (~26% of the conventional method). With this proposed modified method, we have successfully conducted mRNA extraction from 10 mg raw liver tissue where the purity (A_{260}/A_{280} was above 2) and yield of extracted mRNA (mRNA concentration was ~ 10 ng/ μ l) were comparable with results obtained from the conventional tissue homogenization and mRNA extraction method. We have

demonstrated that our modified tissue homogenization and mRNA extraction is as efficient as the conventional method, and it is an appropriate reference for our ensuing designed and fabricated microfluidic device.

In Chapter 4, we have developed a *semi-integrated* 2-chip LOC microfluidic system embodying tissue homogenization, mRNA extraction, multiplex RT-PCR, and real-time fluorescent detection. Collectively, its functionalities have been verified by amplification and qualitative and quantitative analysis of the gene expression level on selected GOIs (AST and ALT).

We have demonstrated that efficacy of the tissue homogenization/mRNA extraction chip was comparable with conventional and modified manual protocol where the purity of the extracted mRNA from raw tissue sample was high (A260/A280 ratio is ~2) and yield concentration was adequate (concentration is ~10 ng/μl). Furthermore, with the second batch of liver tissue samples from the pre-clinical drug toxicity study, we have demonstrated the feasibility of applying our microfluidic tissue homogenization/mRNA extraction chip to ascertain the changes in the expression levels of GOIs (AST, AST1, and ALT1) for hepatotoxicity assessment. Specifically, after conventional RT-PCR process with mRNA extracted on the microfluidic chip, the final cDNA concentration of GOIs increased with liver tissue samples with 3x dosage drug administration (51% increase for ALT1, 21.5% increase for AST1, and 81% increase for AST). The results, as expected, indicated elevated expression level of GOIs, which were largely consistent with the conventional experiment procedure and the gene expression level result we obtained from our multiplex microfluidic RT-PCR chip.

We have successfully amplified the GOIs (AST and ALT) on our multiplex microfluidic RT-PCR chip, and images from conventional gel electrophoresis have illustrated elevation in expression level (58.7% increase for AST, 33.3% increase for ALT) in mouse liver tissue samples under 3x dosage drug administration. Further, the fully quantitative real-time fluorescent detection has demonstrated the normalized fold changes of 1.62 and 1.31 for AST and ALT respectively. This result was consistent with the results we obtained previously using the conventional RT-PCR and gel electrophoresis detection method.

In Chapter 5, we have presented a *fully-integrated* LOC system, embodying a tissue homogenization/mRNA extraction module and a continuous-flow RT-PCR module that featured rapid tissue homogenization, mRNA extraction, and RT-PCR for POC hepatotoxicity assessment. We have demonstrated that said integrated LOC system was able to detect the target gene within one hour, albeit with 50% amplification (i.e., undesirably lower) efficiency of the GOI compared to the conventional and lengthy manual procedures. Our current version on-chip RT-PCR achieved about 60% efficiency (i.e., undesirably lower) of the conventional RT-PCR.

In summary, our work presented in this thesis have demonstrated a ‘proof-of-principle’ for a practical POC device for hepatotoxicity assessment by means of our two integrated LOC systems, specifically, our tissue homogenization/mRNA extraction microfluidic chip demonstrated the “sample-in” capability for our LOC system embodying tissue homogenization, mRNA extraction, stationary multiplex RT-PCR and real-time

fluorescent detection for multiplex gene expression profiling in hepatotoxicity assessment. Our *fully-integrated* LOC system incorporated the continuous-flow RT-PCR component into our tissue homogenization/mRNA extraction chip, and there is no need for troublesome off-chip procedure. This served as a ‘proof-of-principle’ for a fully enclosed microfluidic system featuring a true “sample-in-answer-out” capability without requiring off-chip intervention. Put simply, our *fully-integrated* microfluidic devices have contributed towards the continuing development of a new generation of a practical, rapid, low cost POC liver disease diagnosis device with “sample-in-answer-out” capability.

6.2 Recommendations for Future Work

The *Grand Objective* of our project is to develop a low-cost, rapid, “sample-in-answer-out” POC liver disease diagnosis device that requires a minimal amount liver tissue sample for detecting multiplex gene expression profile and that provides information for rapid and accurate diagnosis. In view of this *Grand Objective*, we recommend the following future work

6.2.1 Recommendation 1: Integration and Miniaturization of Fluorescent Detection and Analysis Unit

At this juncture, we have demonstrated the feasibility of a *semi-integrated* approach to complete the entire process from tissue homogenization to multiplex gene expression

detection for hepatotoxicity assessment. Nevertheless, in the more rigorous sense, our “answer-out” capability is somewhat incomplete at this juncture, hence *semi-integrated*. Specifically, we were able to obtain images from the real-time fluorescent detection unit, the images were analyzed on a computer to interpret the gene expression profiling of GOIs.

In view of this, we recommend the development of an integrated unit embodying real-time fluorescent detection and data analysis, whose form factor is miniaturized to be compatible to our microfluidic system.

6.2.2 Recommendation 2: Automation Control System for Integrated Microfluidic System

Although we have demonstrated the feasibility of a *fully-integrated* LOC system embodying tissue homogenization, mRNA extraction and RT-PCR by incorporating a continuous-flow RT-PCR microfluidic component into the existing tissue homogenization/mRNA extraction microfluidic chip, the control of the mechanical valve and air pump system were conducted and monitored manually. In view of this manual mechanical pump and air-control system, we suggest research work to enable them to be fully automatic, hence closer to a truly practical POC device.

6.2.3 Recommendation 3: Specific GOIs List for Liver Disease Classification for POC Liver Disease Diagnosis Device

It is imperative to select a group of specific GOIs and thereafter establish accurate correlations between the GOI expression patterns and the specific liver diseases/conditions. In this fashion, from crude liver tissue sample inputs, the POC device would provide rapid and accurate interpretation of the GOI expression patterns pertinent to liver disease classification and liver condition assessment.

In this end, we recommend a study to select a group of specific GOIs (11-12 genes as suggested by our collaborator in USA). This study would involve comprehensive gene expression profiling experiments to establish the specific correlations between the targeted GOIs expression patterns obtained from liver tissue samples of mice and different liver diseases and conduction (e.g., hepatitis A or B, profound liver damage inflicted by toxins, prolonged collapse of the circulatory system, hepatic steatosis, liver cancer, etc.). The outcome from this study can be used to establish a standard reference of GOIs expression patterns.

6.2.4 Recommendation 4: “Lab-in-a-Needle” as the ‘Next Generation’ POC Devices

We have demonstrated, in Chapter 4 and 5, a *semi-integrated* 2-chip LOC system as a ‘proof-of-principle’ for performing entire hepatotoxicity assessment on a microfluidic platform, and the feasibility of integrating the two microfluidic chips for an enclosed, rapid-processing LOC system.

As future work, we recommend the design and construction of a practical LOC based POC device with an even smaller form-factor. Specifically, we have filed a patent [143] for an unprecedented “Lab-in-a-Needle” POC device. This envisioned device incorporates an integrated LOC system within the handle of standard core biopsy needle and/or standard hypodermic needle. In this “Lab-in-a-Needle” POC device, the tissue/blood samples obtained from the needle is automatically transferred to the said integrated LOC system for rapid “sample-in-answer-out” diagnosis

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