Development of RNA Microchip for Pathogen and Cancer Direct Detection

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ABSTRACT

Development of a simple, specific, sensitive and rapid RNA microchip for detection of Head and Neck Cancer (HNC) mRNA, pathogenic bacteria and dengue virus (DENV) RNA is reported. By use of nucleases and polymerases specific RNAs are selectively labeled and detected without separation, reverse transcription and or polymerase chain reaction. This is accomplished by designing specific Hybrid probes consisting of DNA-2’-O-Me-RNA-DNA regions to target the RNA of interest. Upon hybridization with the target RNA, RNase H digestion is used to remove the 3’- RNA sequences which exposes the template for Klenow extension with reporter molecules such as hapten or fluorophore labels. This novel RNA microchip is fast (ca. 1 h detection time), selective as individual RNAs are detected in a synthetic mixture and total RNA mixtures, specific for single nucleotide polymorphisms (SNPs) discrimination and sensitive up to attomole level for chemiluminescence detection and lower femtomole for gold nanoparticles (AuNPs) and silver staining method. Using chemiluminescence, HNC biomarkers, VCAM1 and
IL8 are specifically labeled and detected in the presence of thousands of other mRNAs in cancer cell lines and human colon cancer total RNA without interference. Furthermore, the method is highly specific as shown with DENV SNPs discrimination.

Moreover, we report rapid (ca 1 hour), selective, specific multi-marker detection of pathogenic mRNAs and HNC mRNAs using AuNPs-silver staining on the RNA microchip. Streptavidin gold nanoparticles technology has a potential in the analysis of specific mRNAs in a wide array of field including infectious diseases diagnosis, viral infections, food safety, gene expression profiling and cancer detection. A simple and rapid NaOH RNA extraction procedure was developed for *E. coli* total RNA extraction with specific results on the RNA microchip using both chemiluminescence and AuNPs silver staining. This extraction avoids the use of commercial RNA purification kits thus reducing the cost. Furthermore, visual detection on the RNA microchip is simple, does not require electricity or special equipment, and therefore is a good candidate for field diagnostics with minimum resources.

**INDEX WORDS:** Microarray, RNA microchip, gold nanoparticles, Head and neck cancer, Pathogenic bacteria detection, Dengue, single nucleotide polymorphism, NaOH lysis
DEVELOPMENT OF RNA MICROCHIP FOR PATHOGEN AND CANCER DIRECT DETECTION

by

LILIAN WAIRIMU KAMAU-GATOGO

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University 2013
DEVELOPMENT OF RNA MICROCHIP FOR PATHOGEN AND CANCER DIRECT DETECTION

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DEDICATION

This work is dedicated to four important people who laid a very good foundation in my life, and though gone, every lesson you all instilled on me were well appreciated. My Mom, Susan Njambi you invested heavily into good grades and ensured I did not slip past your expectation every other term. My Dad, Douglas Kamau and my brother, Stanley Muchanga, you were willing to give it all for the benefit of each one of us. Finally, to my brother, Charles Karagu whose mentorship and encouragement gave me courage to pursue graduate education. You put all your effort to see the best in others came to fruition. It was sad to see cancer claim you so early. Your loss was a major blow to us but the good you all did and the presence of the Lord have carried me this far.
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1. **NUCLEIC ACID DETECTION METHODS**

1.1 **Introduction**

Nucleic acid detection methods have become an important aspect in molecular biology to understand the status of the cells. Since the introduction of genome wide nucleotide sequence determination, nucleic acid detection methods have seen unprecedented growth. Nucleic acids assays find application in molecular biology methods including gene expression profiling, pathogen detection, medical, forensic and environmental field. Advances in nucleic acid technology have yielded many analytic procedures that allow quantification of rare transcripts to qualitative analysis of the status of the cell by analyzing thousands of transcripts. The current qualitative tools can also allow analysis of the human genome in one microarray experiment. The range and the scope of nucleic acids applications continue to expand with more sophisticated methodologies being developed to cater for the more increasing biological questions.

1.2 **Biological background**

A cell is the basic structural and functional unit of all known living organism. The cell contains the nucleus where hereditary information is stored on the chromosome. The chromosomes are made up of deoxyribonucleic acids (DNA) which codes the hereditary information in a double stranded helix. The genetic information is encoded as a sequence consisting of four nucleotides; guanine, adenine, thymine, and cytosine normally abbreviated as using the letters, A, G, C and T. All organisms except some RNA viruses are built from these four components. The common use of DNA and the genetic code by all organisms underlies one of the most powerful discoveries of the past century: organisms are remarkably uniform at the molecular level. Each chromosome contains between several hundred and many thousands of genes. A gene is a DNA fragment that codes for a protein. Each cell contains same set of chromosomes and technically same set of genes. Irrespective of these, there is a wide variety in appearance.
This is explained by the central dogma of molecular biology which explains the flow of genetic information. First, the gene is transcribed into ribonucleic acids (RNA) and to be specific, messenger RNA (mRNA; Figure 1.1). The next step involves translation of mRNA to a protein. Most proteins undergo modification after translation and before becoming functional proteins. This coupled with different level of transcription among cell types, pre-translational processes on the mRNA and thus different levels of translation among cell types lend the differences observed in the appearances of the cell, tissues, organs and finally organisms. Detection of proteins would therefore indicate the difference between cells but most proteins undergo modification after translation and before becoming functional. These post translational modifications render direct relationship of the amount of proteins and the cell status difficult. Protein based approaches are also more difficult, less sensitive and have lower throughput. The mRNA detection therefore bears a close relationship to the state of the cell thus making the transcriptome worth of measurement. Through mRNA detection, one can understand the cell state, changes in mRNA abundance as a result of protein levels as well as monitoring gene expression profiles changes as a result of cellular perturbations. This can lead to better understanding of what goes on in normal cells and will find an application in the study of cancerous cells which have been linked to changes in the cell environment.
1.3 Nucleic acid hybridization

Technically, all nucleic acid methods in molecular biology exploit a potent feature of the DNA; the ability of two complimentary strands or sequence to come together to form a duplex. It is remarkable that a molecule of such great structural complexity can reassemble to form a hybrid with perfect fidelity from separated complimentary strands under the appropriate conditions i.e. temperatures and salt. This process is referred to as nucleic acid hybridization. Hybridization requires the two sequences involved to have a degree of complementarity which also determines the stability of the duplex. Nucleic acid hybrids can be formed between two strands of DNA, two strands of RNA, or a hybrid between one strand of DNA and one of RNA. The extent and the rate of hybridization are influenced by several factors including the composition of the sequences, the length of the sequences, degree of complexity, as well as hybridization conditions. Presence of mutations also lowers the stability of the duplex formed. The stability of duplex formation is often determined from the melting temperature ($T_m$) of the hybrid which is the
temperature at which half of the DNA strands are in the random coil or single-stranded DNA (ssDNA) state.

Nucleic acid hybridization provides a means for detecting any isolated nucleic acid such as a viral genome, pathogens genome, cancer mRNA, microRNA or RNA by designing a complementary DNA or RNA sequences that are complementary to the target. By fine tuning the factors affecting hybridization, any sequence can be specifically detected. There are various methods taking advantage of hybridization for detection of nucleic acids, specifically mRNA including northern blots, S1 nuclease protection assay, reverse transcription-polymerase chain reaction, serial analysis of gene expression, nucleic acid sequence based amplification and of recent, DNA microarrays. The methods tries to answer a few questions; how much mRNA is transcribed, abundance of mRNAs and other types of RNAs, the function, the presence of mutation and how the variations in that sequence lead to increased risk for diseases. This chapter outlines methods specifically designed for RNA detection

1.4 Northern blot

Northern blot analysis is a classical method for analysis of the size and steady-state level of a specific RNA in a complex sample. Northern transfer is the equivalent of southern transfer procedure for DNA analysis. Once the target RNA is extracted, it is separated by denaturing gel electrophoresis, then transferred to a nitrocellulose filter (Figure 1.2). The specific RNA species are detected by hybridization with a radioactively-labeled probe. The intensity of the autoradiographic signal is a measure of the concentration of the target RNA while the migration distance is a measure of its molecular weight. The first northern blot was first performed on a diazotized paper.

Northern blot is frequently used in gene expression studies. The method is relatively simple to perform, inexpensive, and not plagued by artifacts. The membranes can also be stored and reused after many years. With the availability of the northern blots publications in Blot Base, sharing of information is possible which facilitates research in the area. Initially, only
one gene could be analyzed at a time. The problem is that the method provides no information as to the copy number of RNA species hybridizing to the probe. Northern blot usually look at one or a small number of genes making it unsuitable for large scale analysis.

![Image](image.jpg)

**Figure 1.2:** The detection of RNA from *D. melanogaster* using $^{32}$P labeled probe (Thomas et al., 1980).

### 1.5 Nuclease protection assays (NPAs)

Nuclease protection assays (S1 nuclease and RNase protection) are sensitive procedures for detection and quantification of mRNA species. They are well suited for 5' and 3' mapping, determining the positions of external and internal junctions in RNA and can be used to discriminate between closely related targets. The method was initially used to determine the location and size of intervening sequences in eukaryotic mRNA. NAPAs uses a probe DNA/RNA to protect the target RNA from nucleases. When the probe is a DNA molecule, S1 nuclease is used and when the probe is RNA, any single stranded specific RNases e.g. RNase H can be used. The choice of the probe depends on the nuclease available.

The first step in an NPA experiment is the choice of target RNA followed by extraction of the RNA from a biological sample. The RNA is then hybridized to a complimentary probe of choice (Figure 1.3). The probe that spans the predicted RNA terminus or splice point can be
prepared by cloning part of the gene of interest. The restriction fragment is then purified and end-labeled with $^{32}$P. The probe is then denatured and hybridized with the RNA of interest. After hybridization, the reaction mixture is incubated with nuclease that specifically cleaves the single stranded segments of the probes leaving double stranded fragments. The products are separated on a denaturing gel electrophoresis and detected by autoradiography. The intensity of the band is proportional to the concentration of the complimentary RNA if the probe was used in excess. Non-isotopic probes can also be used, in which case the samples are visualized by transferring the gel to a membrane and performing secondary detection.

NPA analysis is easy to use and can allow multiple target assays. The method is more sensitive than the Northern blotting and is more tolerant to partially degraded RNA. The main problem is the non-specific cleavage observed as nuclease S1 may cut at AT-rich region in the probe causing spurious bands down the lower region of the gel. Also multiple bands are often seen which may be attributed to heterogeneity at the 5'- and 3'- ends of the RNA or exonucleolytic cleavage of the DNA-RNA hybrid.

Figure 1.3: The principle of nuclease protection assay using 5’- end-labeled probe.
1.6 Polymerase chain reaction (PCR)

PCR is a biochemical technology for amplification of a single or a few copies of DNA in a mixture. Developed in 1983 by Kary Mullis at Cetus Corporation in California, PCR has become a common technique in biochemical sciences with a wide range of applications.\(^\text{10}\) Basically, PCR consists of repetitive cycles of denaturation, hybridization, and polymerase extension resulting in an enrichment of a DNA sequence up to a factor of \(10^6\). PCR amplification involves two oligonucleotides also referred to as, primers, which flank the DNA fragment to be amplified. These primers hybridize to opposite strands of the target sequence allowing doubling of target by the polymerase after each cycle. The thermal denaturation step regenerates the primers and templates setting in a motion of a chain reaction with an exponential accumulation of targets, approximately \(2^n\) copies.

One major drawback of the invention was the thermal instability of the DNA polymerase causing inactivation on heating. This required separation of the primers and addition of more polymerase at the start of each cycle thus reducing the efficiency of the amplification. But discovery of \textit{Thermus Aquaticus} (Taq), a thermostable DNA polymerase purified from the thermophilic bacterium revolutionalized the technique.\(^{11}\) Taq polymerase is a stable enzyme at heat denaturing temperature i.e. 95\(^\circ\)C. Taq polymerase therefore allowed the chain reaction to be done continuously. This modification not only increased the yield, sensitivity and specificity but also paved way for automation. Amplified sequences can be detected by a variety of methods including gel electrophoresis and ethidium bromide staining. Usually, final detection of PCR products rely on hybridization methods using DNA probes.\(^{14}\) \(^{15}\)

The method that formed the cornerstone of the human genome project has created an imaginable opportunities, with many developments and several patents. Quantitative PCR (qPCR), also quantitative real time PCR (RT-PCR), one of the most common variant of PCR allows the amplified products to be detected as the reaction progresses in real time i.e. after every cycle. Detection is accomplished by use of intercalating dyes e.g. fluorescent dyes or use
of specific reporter probes.\textsuperscript{16 17 18 19} qPCR eliminates post-PCR manipulations that add time to the analysis and may also introduce errors.

\textbf{1.6.1 Reverse transcription PCR (RT-PCR)}

RT-PCR, one of the many variant of PCR is used to qualitatively measure RNA expression levels.\textsuperscript{20} For detection of RNA sequences, a preceding reverse transcriptase (RT) step is required (Figure 1.4). RT translates RNA into a cDNA sequences which can serve as a template for PCR amplification. Although this RT step complicates the procedure considerably, RT-PCR has been used successfully for the detection of many RNA sequences in the biological and biomedical sciences. Currently, real-time RT-PCR analysis is the gold standard for RNA detection in the fields of fundamental research, drug discovery and molecular diagnostics. Quantitative RT-PCR can also be used for RNA quantification.\textsuperscript{21 18}

The technique is highly sensitive and has a wide dynamic range. Even though RT-PCR allows analysis of several genes, this requires designing of amplification standards for each gene and characterization of each reaction kinetics. In addition, RT-PCR usually requires two separate reactions, RT and PCR, in different buffers and temperatures. This step not only increases the handling time but also introduces the risk of cross-contamination among samples. Although several one-step RT-PCR reagents are commercially available and allow both RT and PCR in a single tube without sample dilution or transfer between the reactions, two-step RT-PCR is generally more sensitive and specific than the one-step reaction format. One of the major problems with the one-step reaction format is primer dimerization or mis-priming formation during the RT step, which deteriorates the sensitivity and accuracy of the following PCR reaction. This is problematic especially with real-time PCR using SYBR Green.\textsuperscript{22}
1.7 Nucleic acid sequence based amplification (NASBA)

Developed in 1991 by J. Compton, NASBA is a method in molecular biology that is used to amplify RNA sequences. Compton defined it as a primer-dependent technology that can be used for the continuous amplification of nucleic acids at constant temperature. Unlike PCR which uses repeated thermal denaturation of double-stranded DNA thus requiring specialized thermal cyclers, NASBA is accomplished at an isothermal temperature, normally 41 °C (Figure 1.4).

NASBA makes use of simultaneous enzymatic activities of avian myeloblastosis virus reverse transcriptase, RNase H and T7 RNA polymerase. The method requires two short single stranded DNA primers derived from opposite sides of the target RNA. Once RNA is introduced into the reaction mixture, reverse transcriptase synthesizes the complimentary DNA strand, fol-
allowed by the RNase H cleavage of the RNA template. Consequently, T7 RNA polymerase produces a complementary RNA strand that can be re-used in step 1 thus initiating a cyclic amplification process. NASBA can allow amplification of up to a billion fold in two hours. The isothermic temperature control allows each step to proceed as soon as an intermediate becomes available. Products of NASBA are single stranded and, thus can be applied to detection formats using probe hybridization without further treatment.

Immediately after invention, NASBA was applied for rapid diagnosis of HIV-1 which enabled detection of 10 molecules of target RNA.\textsuperscript{24} Van Der Meide et. al., comparison of NASBA, Real-Time RT-PCR and Real Time PCR favored qRT-PCR over NASBA.\textsuperscript{25} Nevertheless, the method has undergone various modifications since discovery to allow even multiplex analysis as reported by Mader et al., in the detection of microRNAs coupled with biochips.\textsuperscript{26}

![Figure 1.5: Schematic presentation of NASBA method.](image-url)
1.8 Serial analysis of gene expression (SAGE)

SAGE was developed in mid 1990 by Velculescu et al., for the analysis and identification of many genes simultaneously. This came at a time when determination of genomic sequences required a method that would allow rapid identification of many genes and their abundance. Technically, SAGE allows quantitative and simultaneous analysis of a large number of transcripts without any prior information.

First the mRNA of the target of interest is isolated followed by sequence tags selection from a defined position of each mRNA (Figure 1.6). Two tags are then ligated, followed by ligation with many tags, as many as 70 to 100 tags per concatemer. The concatemer sequences are cloned and later sequenced in parallel which allows the identification of several sequences in one shot. Thereafter, SAGE software is used to extract the tags from the sequences of concatemer clones. These tags are then matched to the genome which identifies the corresponding gene. The output reflects the gene expression patterns and the abundance of each tag in the RNA population. SAGE generates nucleotide sequence tags with sufficient information to match the genome and identify a transcript. The results can also give information for potentially undiscovered genes or exons.

Originally, short tags, 9 to 10 base pairs were used which allowed efficient analysis of thousands of transcripts in a serial manner but several variants have been developed since. Most notably, a more robust version developed by Saha et al., allows production of longer transcripts tags even though it is limited to lower cloning efficiency and small inserts. Robust-long SAGE, an improvement of long SAGE can generate over 4.5 million tags from a small amount of mRNA using just 20 ditag PCR products.

The major advantage of SAGE is that the output is digital so the data obtained can be directly compared between labs and researchers. SAGE also allows both qualitative and quantitative evaluation of thousands of genes without any prior information thus it is a powerful global approach for analyzing gene expression profiles and discovery of genes. On the other hand,
SAGE is relatively time consuming and expensive thus it is generally not the preferred method for large scale analysis. The technique is similar to expressed sequence tags (EST) sequencing except the sensitivity is high.

![Schematic presentation of serial gene expression analysis.](image)

**Figure 1.4:** Schematic presentation of serial gene expression analysis.

### 1.9 Molecular beacons

Molecular beacons (MBs), first proposed in 1996 by Tyagi and Kramer are a hairpin-structured oligonucleotide probes containing an internally quenchable fluorophore and a quencher at different ends of the strand (Figure 1.7). MBs consists of a loop which is complementary to the target sequence, a stem at both termini of the loop made of 5-7 nucleotide residues complimentary to each other, a fluorophore at the 5'-end and a quencher at the 3'-end. The stem keeps the quencher and the fluorophore in close proximity to each other causing the fluorescence to be quenched by either fluorescence energy transfer (FRET) or collisional quenching. Detection occurs when a target RNA sequence hybridizes with the loop causing
dissociation of the fluorophore from the quencher thus resulting in restoration of fluorescence emission. Since unhybridized MBs are dark, MBs can be used in homogeneous assays and in living cells without separation. MBs have been widely used in many areas such as the detection of PCR products, mutational analysis, clinical diagnosis, genotyping and SNPs discrimination.\textsuperscript{32}

The first reported MBs consisted of an 18-base loop section and a 5-base-pair stem with 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS) as a fluorophore and 4- (4'-dimethylaminophenylazo) benzoic acid (DABCYL) as the quencher.\textsuperscript{30} Incomplete quenching often reduced the sensitivity of MBs thus requiring increased research to improve MBs technology. Organic dyes, quantum dots and pyrene excimer have been envisioned as better performing MB components. Quantum dots are nanocrystal structures made of CdSe and CdTe cores. They have high quantum yields, narrow, symmetric and stable photoluminescence, tunable adsorption and emission which makes them attractive as fluorophores.\textsuperscript{32} Yeh et al described use of CdSe-ZnS core-shell QDs and gold nanoparticles quenchers for detection of infectious virus RNA.\textsuperscript{33}

![Molecular beacons principle](image)

**Figure 1.5:** Molecular beacons principle. In the presence of a complementary target RNA sequence, the beacon unfolds, hybridizes with the target, and exhibits fluorescence that is easily detectable.
1.10 Detection on microchip

Detection on the chip also referred to as DNA microarray is a very attractive method for RNA analysis. This is accomplished by use of cDNA to indirectly detect RNA. This method enables detection of thousands of mRNAs in an RNA mixture. With the unparalleled high density detection, the method was a great tool in the discovery of human genomes among other genomes.
2. DNA MICROARRAY

2.1 Overview of DNA microarray technology

The completion of human genome among other genomes provided a lot of data with many intriguing questions on how it functions. These generated a great interest in the study of functions and expression profiles of the many genes discovered. But, many of the nucleic acid methods available at the time worked on “one gene one experiment” principle, thus limiting the impact of the results. SAGE and NASBA, the currently available multiplex technologies offered great promise but they had limited development and acceptance. The methods required the identified genes to be sequenced and analyzed in a serial fashion. These procedures were time and labor intensive and prone to false positives. There was therefore a challenge to find a cost effective platform that would enable analysis of thousands of genes simultaneously, and still maintain a high level of sensitivity and specificity. The development of DNA microarray technology in the mid 1990s was therefore a promise to help monitor the whole genome as well as elucidate the purposes of the genes discovered.

A DNA microarray also referred to as DNA microchip or hybridization array can be thought of as a miniaturized gene detection assay that allow monitoring of expression levels of thousands of genes simultaneously. As the name suggests, DNA microarray represents DNA fragments attached to a solid surface in an ordered manner at a high density. The high density is made possible by miniaturization of detection from macro to micron level. For example, the DNA fragments spot size range from 20 to 200 µm as opposed to more than 5000 µm observed in micro titer plates experiments. This enables printing of thousands of micron spots on a microscope slide. Thus it is possible to place the entire human genome compliment on one microscope slide.
The principle behind DNA microarray technology is that RNAs from a given cell or organism is labeled and hybridized to DNA immobilized on a solid support. As with many other nucleic acid detection methods, microarray takes advantage of the formation of duplex in the presence of complementary sequences. The presence and abundance of specific target sequences within the sample of interest are indicated by the intensity of the hybridization signal. The RNA sample whose identity is being sought is referred to as a ‘target’ while the DNA sequences tethered on the surface are referred to as ‘probes’.

Microarrays detection is a complex process involving many experimental and analysis steps; sample collection, total RNA or mRNA extraction, cDNA /cRNA synthesis and labeling, fabrication, hybridization, image capture, data processing, statistical analysis and biological verification. Microarray technology therefore is an example of the convergence of several technologies, including automated DNA sequencing, PCR, oligonucleotide synthesis, nucleic acid labeling chemistries and bioinformatics. The early days of microarray technology were filled with a lot of excitement due to the ability to detect many genes or samples simultaneously which was a departure from the current nucleic acid methods. This saw emergence of many platforms and variations but now the focus is in the applications and data quality. This chapter discusses the most important aspects in the DNA microarray technology.

2.2 Preparation of target RNA

DNA microarray detection of RNA is an example of an indirect method for RNA detection. Even though the main target is RNA, indirect detection through cDNA is favored due to the complications associated with the nature of RNA. RNA is easily degraded by nucleases and has the capability to form complex secondary structures. This complicates handling and detection of RNA. Target preparation therefore involves reverse transcription of RNA into cDNA after RNA extraction from tissues or cells. A tag or label can be incorporated at this step.
2.3 Probe design

The success of microarray experiment is determined by the design of the probes. Each probe presents a gene or a transcript of interest and is complimentary to the target. The objective of this step is to find target specific probes that hybridize under similar condition. The completion of several genomes provided a lot of information that opened a wide array of research in this field. The genome sequences are housed at the national center for biotechnology information (NCBI) in Genbank. The database is available online through Entrez. Gene information can be obtained from these databases. Probes are designed to give a good representation of the target of interest, afford the highest hybridization signal and must be as specific as possible to minimize cross hybridization. The first and most widely used approach is to choose a probe sequence that is complimentary to your target and has little sequence similarity to all other available sequences. This requires screening of the millions of raw data with search engines such as basic local alignment search engine (BLAST). There are essentially two main types of probes: PCR products and oligonucleotide probes.

2.3.1 PCR products

Microarrays are mostly differentiated by the size of the probes. cDNA microarrays consists of cDNA of about 500-2000 sequences obtained from cloned PCR products. The PCR products are purified to remove unwanted salts, detergents and proteins and run on gel to verify the clone size and purity to avoid cross contamination. The concentration is also determined to flag the suboptimal clones. The main drawback lie on the requirement to prepare a large number of cDNA or PCR products, which need to be purified, quantitated, and catalogued before spotting on the solid surface. The process can be labor intensive and is prone to many errors. This were the probes of choice when the technology was first reported.

2.3.2 Oligonucleotide probes

Oligonucleotide probes are normally shorter and can be obtained either by solid phase or in situ synthesis. The probes can be home made using standard solid phase
phosphoramidites chemistry on a DNA synthesizer. This comes with flexibility and affordability but control of the size among researcher is not standardized, thus data comparison might not be easy. Generally the size of the probes ranges from 25-70 bases long but shorter probes with 10 bases have been used with enough specificity.\textsuperscript{42} \textsuperscript{43} \textsuperscript{44} The length of the probes is usually restricted by the efficiency of the standard solid phase phosphoramidite chemistry.\textsuperscript{45}

\textbf{2.3.3 In situ synthesis of oligonucleotide probes}

\textit{In situ} synthesis methods are the most efficient method for high density oligonucleotide microarrays for gene expression profiling although can be limited in terms of flexibility and affordability. The methods permit combinatorial synthesis of oligonucleotides on the solid surface nucleotide by nucleotide. This involves several repetitive steps of i.e.; deblocking, coupling, capping and oxidation. This is repeated until the full length probe is synthesized. Two major platforms exist; the Agilent Inkjet and the Affymetrix photolithography method. The Agilent Inkjet synthesis uses hydrophobic surfaces to block unreactive areas leaving exposed OH groups compatible with nucleic acid synthesis.\textsuperscript{46} Probes are synthesized using standard phosphoramidite chemistry to generate 60 bases long oligonucleotides. Comparison of spotted 60mer microarrays and the Agilent cDNA microarrays gave highly consistent results.\textsuperscript{42}

\textbf{2.3.4 Affymetrix photolithographic synthesis}

The Affymetrix GeneChip is the most widely used for gene expression profiling. The combinatorial technique combines solid phase oligonucleotide synthesis and photolithographic computer chip technology.\textsuperscript{41} \textsuperscript{47} \textsuperscript{48} The platform comprises of high-density standardized microarrays and instrumentation. Affymetrix method requires irradiation with UV light through holes of photographic masks on the surface to remove photo-labile protecting groups. Flooding of the surface with the four bases ensures coupling at the specific locations (Figure 2.1). The probes do not exceed 25 nucleotides due to reaction efficiencies limit. The method allows production of highly miniaturized microarrays with current arrays having a capacity of 1.3 million unique features per array of 5 in x 5 in. Specificity is compensated by synthesizing many oligonucleo-
tides for each gene sequence paired with mismatched oligonucleotides that differs at the centre of the probe with one nucleotide. Multiple probes are designed to represent each transcript. The technology offers platforms for exon, gene or whole genome analysis. The method is highly specific and reproducible but custom arrays are needed for each organism.

![Diagram of Affymetrix In-situ synthesis](image)

**Figure 2.1: Affymetrix In-situ synthesis (Fodol et al., 1991)**

### 2.4 Probe immobilization

Irrespective of the type of probes used, they must be immobilized on a solid support for specific detection. The main distinction between dot blots and DNA microarrays is in the use of an impermeable, rigid substrate such as glass which are advantageous over porous membranes or gel pads. As liquid cannot penetrate the support surfaces, surface hybridizations is enhanced. These speeds up the washing step thus improving reproducibility as well as reducing the background. The flatness and rigidity improves automation which facilitates image capturing and processing.
Suitable surfaces should have low intrinsic fluorescence, achieve high density and high hybridization signal. The surface should allow homogenous chemical functionalization with high resistance to non specific adsorption. Planar solid supports allow accurate scanning and imaging. The rigid impermeable quality of glass or silicon support enables automated manufacture with accurate distances and increased precision. The surfaces are amenable to chemical modifications via well known silanization chemistries. Amongst all these surfaces, glass has been widely used due to its great chemical resistance and is easily available. Other surfaces include nylon/filter membranes, plastic materials and poly(methyl methacrylate) (PMMA).

Immobilization on the planar surface can be achieved either by printing pre-synthesized molecules or by in situ synthesis. Probes require suspension in appropriate buffer before printing. The purpose of the buffer is to promote uniform delivery, increase printing efficiency, reduce sample evaporation, increase visibility of the spots and provide the right condition for immobilization chemistry. Nucleic acids form a monolayer on the solid surface. Interactions of the solution phase with the immobilized probes are much faster thus accelerating hybridization and washing procedures. These not only improves reproducibility but also reduces background.

Various microarraying robots deposit the probes in an ordered grid with predetermined columns and rows. Printing can be accomplished by either contact or non-contact printing. The former uses pin in contact with the surface while the latter accomplishes deposition without direct contact with the surface. Non contact printing applies ink-jet printer’s principle to dispense single drops of the probes (Figure 2.2). The main drawback with non contact printing is the occurrence of droplets outside the grid. Factors that influence the fabrication of DNA on modified surfaces include surface chemistry, probe concentration and spotting buffer. The probes should also be well spaced to enable maximum hybridization signal and avoid electrostatic interactions.
2.5 Surface coupling chemistry

Oligonucleotides cannot be coupled directly to the surface silanol groups of silicon or glass. It is therefore necessary to functionalize the surface with a group that can easily be coupled with an oligonucleotide probe or from which it is easy to initiate the growth of an oligonucleotide chain in case of *in situ* synthesis. Tethering one end of an oligonucleotide to a surface leads to a loss of degree of freedom. Some of the drawbacks associated with the loss include, sterical hindrance, non-uniform coupling of the chemical layer, low probe density and inhomogeneous probe morphology. Thus a highly specific and efficient coupling reaction is required. There are myriad of coupling approaches including simple adsorption approaches and covalent modifications.
2.5.1 Adsorptive coupling techniques

Adsorption immobilization is a noncovalent coupling method based on electrostatic, Van der Waals interactions, hydrogen bonding and hydrophobic interactions. With adsorption technique, there is no need to modify the probes thus reducing the cost of arrays. A widely used chemistry is avidin adsorption on the glass surface. Avidin modified surface affords immobilization of biotinylated probes by the formation of electrostatic interactions, hydrogen bonds, Van der Waals and hydrophobic interactions. Avidin-Biotin interaction is one of the strongest noncovalent interactions. This method is however quite expensive due to the high amount of avidin and specific biotinylated probes required. Also avidin is susceptible to desorption at high temperatures and high ionic strength solutions.

Poly-L-lysine chemistry also affords electrostatic interaction of DNA on the surface. A positively charged lysine is grafted onto the surface. The DNA is bound on the surface through noncovalent interaction between the negatively charged phosphodiester groups of the DNA backbone and the positively charged amino functions of surface-bound lysine side-chains. The binding however is reversible thus resulting in decreased sensitivity. In addition, the electrostatic interactions reduce the conformational freedom of the bound cDNA thus reducing their affinity and specificity to the target molecules. Poly-L-lysine also desorbs on exposure to high or low pH solutions thus requiring pH control on the surface. The surface also ages fast thus affecting the hybridization yields. The DNA molecules can be cross-linked using UV light to form extra bonds which enhance the stability. This was the first chemistry to immobilize PCR products. Due to the high background resulting from non-specific adsorptions and low stability, non-covalent adsorption methods are not widely used.

2.5.2 Covalent coupling techniques

The need for efficient chemistries for attachment of oligonucleotides on the surfaces led to great interest in covalent chemistries. Covalent coupling involves formation of a covalent bond between the probe and the solid surface. Technically, this will also involve the activation of
probes with functionalities that easily react with the modified surface. Immobilization on un-modified is also possible with silanized nucleic acid or propanolamine-derivatized oligonucleotides. While use of unmodified glass afforded good results, the methods did not gain popularity with surface activation being the preferred method.

An efficient coupling chemistry should be chemically stable; the linker should be long enough to avoid steric interference and well defined on the surface. A great number of attachment chemistries have been developed. Generally, the substrates are usually subjected to silanization chemistries such as alkoxy silanes derivatives before introduction of the coupling chemistry. Silylation is a very attractive method for glass and silicon surface activation and is also an important step in further modification of surface. The most commonly used silanes are aminopropyl triethoxysilane, aldehyde-modified silanes, epoxy silanes and mercaptosilanes.

Amino-modified oligonucleotides can be bound to aldehyde-activated surfaces epoxide or isothiocyanate functionalized surfaces. Aldehyde modification involves amination of glass with a consequent step of glutaraldehyde which binds DNA by a Schiff base reaction. The amino silane linker lifts the DNA probes from the surface making it more accessible for incoming target. Epoxy functionalization is also a very attractive method but printing of probes must be conducted in strongly alkaline solutions which convert amine to free base. This might lead to hydroxide formation during evaporation and siloxane degradation.

Thiol-modified oligonucleotides, also commonly used can easily be immobilized on surfaces functionalized with maleimide groups such as heterobifunctional cross-linker succinimidyl 4-[p-maleimidophenyl]butyrate (SMPB). Halo carbonyl compounds such as bromoacetamide or α-halo acetyl are also good surfaces for –SH modified oligonucleotides. Disulfide–modified oligonucleotides on the other hand, can be immobilized on a mercapto-silanised glass support through a thio/disulfide exchange reaction. The disulphide bridges were found to be stable during contact with biological samples, hybridization conditions and PCR reactions.
Use of dendrimeric structures have been reported to allow homogenous functionalization and increased binding capacity as a result of increased binding sites. However preparation of the three dimensional-linker is time consuming and often times generate a mixture of dendrimers. 50b, 63 70

2.6 Detection methods

Accurate signal transduction is imperative for gene expression studies. This mostly involves introduction of a label in the system either using reverse transcriptase during preparation of cDNA or direct chemical labeling. Detection methods can be divided into two categories; radioactive and non radioactive. Radio-isotopic detection, a more traditional method, involves detection of radio-isotopes. Non radioactive methods include chemiluminescence, fluorescence and electrical methods. With the advent of gold nanoparticles (AUNPs) technology, various detection methods are feasible.

2.6.1 Radioactive detection

Radioactivity detection involves use of radioactive isotope in conjunction with an imaging plate/phosphoimaging system yielding a digital image of the radioactivity. X-ray film can also be used; however it requires long exposure times and the output is not digitalized. 71 72 73 The probe of interest is labeled with an isotopic molecule, 32P which is imaged by radiographic film or phosphor imaging. Radioactive labeling is easy and sensitive but the labels are expensive to prepare and quite toxic. 74 75 The method battle signal spreading from high spot densities which can complicate the dynamic range. Radioactive labels are also limited due to their short half life. 76 77

2.6.2 Fluorescence detection

Target RNAs can be labeled during reverse-transcription using dUTPs with tagged fluorescent dyes e.g. fluorescein and cyanine. 39, 78 79 The cyanine derivatives, i.e. Cy3- and Cy5-dUTP affords one or two channels detection using one or both dyes respectively (Table 1). Two channel involves labeling RNAs from two groups e.g. control and test samples with different flu-
orescent markers. This improves precision and enables simultaneous detection and comparative analysis of both test and control targets. Detection is accomplished by use of a confocal scanning microscope or charge coupled device (CCD) with different filters. The main drawback with confocal scanners is the use of expensive lasers for each excitation wavelength. CCD detectors on the other hand can use a single light source with different filters to afford different excitation wavelength. Agilent technologies comparison of the single and dual channel comparison showed high concordance. The MammaPrint, first FDA approved microarray-based gene expression assay is based on Agilent two-color platform.

2.6.3 Chemiluminescence detection

Chemiluminescence detection is also an attractive method for microarray detection. The method uses an enzyme to catalyze a chemical reaction in the presence of a substrate. The Applied Biosystems microarrays employs digoxigen label and alkaline phosphatase-antibody conjugate for chemiluminescence detection. The addition of a substrate with an initiator and enhancer leads to chemiluminescence signal which can be captured with a CCD camera. The method has a high sensitivity and a wide dynamic range.

2.6.4 Electrochemical detection

Electrochemical detection couples bio-recognition step directly to the detector that generates a useful electrical signal. The devices can be cost effective and can be easily miniaturized. Direct detection can be accomplished via the oxidation of the guanine bases. In contrast, indirect methods rely on interactions between nucleic acids and other molecules i.e. metal nanoparticles to generate a detectable signal. Tagging DNA with gold nanoparticles leads to conductivity changes associated with target-probe binding events. Silver deposition facilitated by gold nanoparticles leads to conductivity measurable signals.

2.6.5 Nanoparticles based detection

Nanomaterials feature unique physicochemical properties that can be greatly utilized in creating new signal transduction processes in microarray technology. Gold nanoparticles
(AuNPs) have unique physical chemical properties that make them excellent reporter molecules. Nucleic acid detection methods take advantage of the high AuNPs affinity for sulfur as well as their intense surface plasmon resonance associated with thiolated oligonucleotides functionalized with AuNPs. Upon hybridization with the target, AuNPs afford various read out methods. Another approach is the use of functionalized protein probes e.g. streptavidin. Streptavidin labeled AuNPs offers an advantage over oligonucleotide labeled AuNPs since the latter involves preparation of specific probes for each application. Use of AuNPs provides highly specific detection method of nucleic acids with various readout methods. Various applications include global gene expression profiling, pathogen detection, drug development and cancer therapeutics.

Table 2.1: Microarray gene expression platforms

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2.7 Applications

DNA microarray has been a major tool in helping decode the genomes of various organisms including pathogens and viruses. DNA microarray data have shown it is possible to group genes on the basis of expression patterns from which functional relationships can be deducted. This technology has therefore been applied in the study of expression patterns in disease diagnosis, prognosis, treatment outcome and progression studies. Simultaneous detection has been used in point mutations and single nucleotide polymorphisms (SNPs) analysis. The method has
steadily advanced and soon it might be a corner stone in medical applications and personalized medicine; e. g. MammaPrint for breast cancer detection. The major genetic risk factors for diseases such as diabetes, cancer, bipolar illness, Alzheimer’s and many others may be identified with array technology years to come. Other applications have been reported in food safety analysis and forensics.

2.8 MicroRNA detection

MicroRNAs (miRNAs) constitute a class of short RNAs discovered recently. miRNAs are initially transcribed as long precursor RNA molecules and successfully processed by Drosha and Dicer complexes into their mature forms of ~22 nucleotides. Only a few have a known function in the control gene expression post transcriptionally which is important for gene regulation and proteins. The qualitative and quantitative expression levels of miRNAs are therefore expected to exert a profound regulatory influence on the transcriptome. Accurate detection of miRNAs is therefore important to investigate physiological states as well as elucidate their functions.

Different methodologies have been used to profile miRNA expression, including miChip with locked nucleic acids, surface Plasmon resonance imaging (SPRI), gold nanoparticles. By contrast to mRNA profiling technologies, miRNA profiling must address the short nature or probes and should be able to discriminate SNPs. Detection using microarrays is therefore problematic requiring inclusion for highly specific probes. Use of locked nucleic acids (LNA) that bind more strongly to miRNA not only increase sensitivity but specificity on the microarrays.
3. RNA MICROCHIP TECHNOLOGY

3.1 Introduction

Culture based detection methods and biochemical techniques can be quite time consuming taking 2-3 days and often can be inconclusive. In an effort to increase the sensitivity, speed and reliability of RNA detection, various nucleic acid based methods have been developed including but not limited to; RT-PCR, NASBA, differential display and cDNA microarrays. Though these genotypic approaches have allowed highly sensitive and specific RNA detection, they only offer an indirect measurement of RNAs requiring conversion to cDNA by RT-PCR followed by labeling. The cDNA synthesis not only increases detection time but introduces multiple biases and artifacts that could interfere with both the proper characterization and quantification of RNA. cDNA synthesis is also not suitable for the analysis of short and degraded RNA which are not captured by the primers thus making them difficult to amplify and label.

The direct detection of RNA therefore remains an important and challenging research goal. This work is based on the development of a simple, easy to use, rapid and cost effective mRNA detection chip. Other than pathogen detection, the RNA microchip will be a great tool in functional genomics studies and the analysis of how mutations produce genetic disorders or increase risks for a disease. The RNA microchip could also be adapted for point of care diagnostics (POCs).

Development and validation of an RNA biosensor was investigated. Chapter 4 outlines application in head and neck cancer detection as our model using chemiluminescence detection. Chapter 5 discusses detection of E. coli total RNA, Chapter 6 discusses the application in dengue virus serotyping. Development of visual RNA chip facilitated by gold nanoparticles and silver staining is outlined in chapter 7.
3.2 Target selection

There is a wide range of fields that require molecular diagnostics to determine the status of the cell, causative agents or the effects thereof. These include but are not limited to food safety, recreational water safety, animal health care, pathology, clinical diagnostics, forensics research and bio-defense. Infectious diseases and cancer to mention a few account for >25% of all causes of death (Figure 3.1). Development of rapid, sensitive and specific assays for cancer and infectious diseases is therefore an important task. Bacterial and pathogens epidemics pose a significant threat to human, animal and agricultural health. For example the highly pathogenic strain of *Escherichia coli* (*E. coli*) (O157:H7) is the main cause of food borne illness, therefore epidemic monitoring and detection of *E. coli* in food production and recreational water is very important.

![Figure 3.1: Infectious diseases and cancer diseases accounts for >25% of all illnesses.](image)

3.2.1 Probe sequence selection

Gene selection is an important step to ensure selective and specific information is obtained from the experiments. The gene should be specific and should give empirical evidence of the status of the target. An example is lethal factor of the *Bacillus anthracis* (*B. anthracis*). Once
the gene of interest has been selected, the target sequence of the genes can be obtained from the NCBI which houses the genome sequencing data in Genbank. This is followed by probe design.

Our probe sequences are mostly between 15 - 40 nucleotides. This length has been shown to be long enough to be target specific. This sequence length is well suited for solid phase phosphoramidite synthesis and gel electrophoresis purification. Shorter probes have been shown to increase hybridization yields too. Once a region is selected, we use BLAST to search for sequence similarity. BLAST compares the sequence against all the sequences available at Genbank and rates them according to their similarity. A choice is made on the sequence with the highest specificity to our target to avoid non specific hybridization. Figure 3.2 is a BLAST analysis of *B. anthracis* lethal factor gene. Our choice and design of target is easy and any researcher can be able to use it. One can also use other search engine such as Entrez or ExPASy. Entrez allows users to search many discrete health sciences databases at the NCBI website. Entrez efficiently retrieve gene and protein sequences, structures and chromosomal maps. There also exist other probe design tools for designing optimal oligonucleotides such as OligoArray, OligoWiz, Array designer and Agilent eArrayv4.5 to name a few. This come in handy if one is dealing with a large number of probes.
3.3 Oligonucleotide probe design

The design of probes is an important step due to the complex physicochemical constraints resulting from immobilization of probes on the chip surface. Highly selective and specific probes are required to attain high sensitivity. The probe should be long enough to be specific for a region of interest but not too long to introduce steric interactions especially when RNA is the target. RNA has the ability to form complex secondary structures which may interfere with hy-
bridization.\textsuperscript{50a} Other parameters to consider in probe design include base composition, sequence length and specificity. We have designed novel hybrid/chimera probes that allow direct detection of total RNA without prior enrichment, amplification, labeling and separation. The hybrid probes consists of three regions, i.e. 3'-DNA-2'-O-MeRNA-DNA (Figure 3.3). The probes are amino modified at the 3' end to afford surface immobilization.

Tethering one end of an oligonucleotide to a solid surface is expected to affect the kinetics of duplex formation and other reactions in the solution. Various studies have shown tethering one end of an oligonucleotide probe to a microarray surface affects the accessibility of the oligonucleotide near the surface than the one farthest away.\textsuperscript{50a} The base nearest to the surface is less accessible than those further away. Due to steric constraints imposed by molecular interactions on microarrays, a molecular spacer is normally introduced in the probe. The spacer separates the hybridization region from the microarray surface thus reducing steric constraints introduced which sometimes have a destabilizing effect on the chip surface. Hybridization yields have been shown to increase up to two orders of magnitude by introducing spacers.\textsuperscript{50a} \textsuperscript{106}

Guo et al. did extensive work to study the importance of a spacer by introducing a non-specific poly dT spacer sequence at the end of the oligonucleotide immobilized on the surface.\textsuperscript{76} They observed that experiments without spacers yielded no hybridization signals while inclusion of 6 or less poly dT yielded very low signal. But they observed an increase in signal with length up to 15 nucleotides. Poly or oligo (ethylene glycol) grafted surfaces have also been used as passivation layers to provide a variable distance between the surface and the oligonucleotides to avoid non-specific adsorption.\textsuperscript{64} \textsuperscript{107} The hydrophilic polymeric chains helps avoid steric hindrance in linker less DNA hybridization. This can also help resist the nonspecific adsorption of DNA on the chip surface. A dense PEG layer might also mask the inhomogeneities of the underlying surface caused by inconsistent surface functionalization. A study in our laboratory confirms inclusion of a spacer yields higher signal. Normally we use 10-15 dNTPs which is part of the gene of interest.
The probes are designed to enable both RNase H and Klenow specificity. RNase H is an endonuclease capable of selectively digesting RNA in an RNA/DNA duplex. This is made possible by incorporating specific 2’-O-Me-methylated RNA into the probes. The 2-O-methylated RNA probes were reported to have increased T_m, faster kinetics of hybridization and increased specificity against De-oxy probes. The methylated RNA component stabilizes the RNA probe against RNases. Finally, the last DNA portion enhances the performance of our probes on the microarray. This DNA portion serves both as a guiding sequence for RNase digestion and Klenow extension. The hybrid probes are amino alkylated at the 3’-terminus upon reaction with a commercially available amino modifier phosphoramidite derivative during solid phase synthesis.

\[3’-\text{NH}_2\text{-TAGAAATCTTCTGTAUAGACUUCUAUUCTTTTTTTAG-5’}\]

**Figure 3.3:** *B. anthracis* hybrid probe with the three specific regions outlined.

### 3.4 Microchip surface chemistry

A high specific reaction and efficient coupling of probes is required due to drawbacks associated with microarray technology such as steric hindrance, non-specific interactions and inhomogeneous spot morphology. The substrates/microchips/chips are first silanized with aminopropyl trimethoxysilane. This is a fast and efficient silanization procedure to introduce amino group on the surface. Alkox silanes provide a surface with a myriad of straightforward synthesis solutions for further modification. Further functionalization with homobifunctional cross linker 1, 4-phenylene diisothiocyanate (PDITC) introduces NHS groups that readily reacts with amino modified hybrid probes via N-hydroxysuccinimide (NHS) displacement. The bound probes are stable and can be kept for several months in -4 °C. Figure 3.4 illustrates the
procedure to obtain NSH chemistry on the glass or silicon surface. Prior to functionalization, glass microscope chips are usually activated by treatment with strong sulphuric acid to remove organic residues as well as hydroxylate the surfaces.

The amino modified probes can also be immobilized on other surfaces such as aldehyde, epoxy or chinone surfaces. Aldehyde modification has been used in our lab with positive results even though the spot morphology was not well defined in comparison with phenylene diisothiocyanate surfaces. Also coupling on the aldehyde surface required longer times, up to 16 hours compared with NHS displacement chemistry which takes 2 hours. Also post immobilization reduction for the aldehyde modification was necessary to reduce background.

![Activation of silicon or glass chip surface](image)

**Figure 3.4:** Activation of the silicon or glass chip surface. Surface chemistry enables immobilization of amino modified sequences.

### 3.5 Probe printing and immobilization

Printing of the oligonucleotide was accomplished using a Cartesian Technologies robot with a stealth pin that deposited 0.7 nL/spot on the substrates. The sizes of the spots are ~75 µm in diameter. The pins work by drawing probes into a small channel by capillary action like a fountain pen, also referred to as quilling. The microarray robotic system can accommodate 10
standard microscope slides with a capacity to operate 48 pins (Figure 3.5). One 384-well plate can be printed in ~7.5 minutes with over 100,000 features per slide. The pins are easy to handle and only require periodic cleaning using a buffer. In house printing is widely used in research laboratories because it is relatively cheap and easily modified to fit changing research problems.

Figure 3.5: Omnigrid microarray robot and an SMP3 pin.

3.5.1 3'-Labeling technique

Our lab has developed a method that affords labeling and detection of mRNAs in an RNA mixture. Our system is based on RNA 3’-labeling approach where a DNA polymerase incorporates hapten labeled dNTPs directly on a DNA template immobilized on the chip surface.\textsuperscript{112,113} Upon hybridization, 3’-RNA portion hybridized to the DNA probe is digested by the RNase H. RNase H offers an advantage since it doesn’t digest RNA in an RNA duplex. This helps stabilize the RNA probe and the target RNA. The 2’-O-methylation of the RNA probe also stabilizes the probe RNA sequence against RNases.\textsuperscript{109,108} RNase H digestion exposes a DNA
template which acts as a guiding sequence for DNA polymerase labeling with biotin dNTPs (Figure 3.6). With this method, one or all the biotin labeled dNTPs can be used. The method allows specific labeling of the captured target RNAs at the 3'-end. Detection of specific RNAs in total RNA or in an RNA mixture is possible without separation, purification, reverse transcription and PCR. The fact that short primers of up even 5 nucleotides can be used indicates the method can be used for short and degraded RNAs. The biotinylated probes are converted to enzymatic labels through the specific interaction of a binder enzyme conjugate. Further steps can afford desired detection methods i.e. radioactive, chemiluminescence, colorimetric or fluorescence detection.

\[-3' - d(TAGAAATCTTCGT) - r(AAUAGACUUUCAUUC) - d(TTTTTTTAG) - 5'\]

\[\text{RNA hybridization}\]

\[5' - AUCUUUAGAAGCAUUAUCUGAAGAUAGAAAAAAA - 3'\]

\[-3' - TAGAAATCTTCGTAUAGACUUCUAAUCTTTTTTAG - 5'\]

\[\text{RNase H digestion}\]

\[5' - UUAUCUGAAGAUAG - 3'\]

\[-3' - TAGAAATCTTCGTAUAGACUUCUAAUCTTTTTTAG - 5'\]

\[\text{Klenow extension with hapten labels}\]

\[5' - UUAUCUGAAGAUAGAAAAAAA - 3'\]

\[-3' - TAGAAATCTTCGTAUAGACUUCUAAUCTTTTTTAG - 5'\]

Figure 3.6: Flow chart for the 3’-labelling principle

3.6 Detection methods

3.6.1 Radioactive detection

Preliminary work to identify the right enzyme and the effectiveness of labeling was accomplished by use of radioactive detection. By labeling the target with $\alpha$-$^{32}$P dNTPs during
Klenow extension, radioactive detection was achieved even at the attomole level. The labeled nucleotides were also found to be stable.\textsuperscript{103b} The method was used for specific detection of mRNA in a mixture of RNAs.\textsuperscript{114} While this method has high sensitivity, the radioactive probes are expensive with a short lifetime and the fact that they are toxic led to limited application. Radioactive labeling method has also not gained acceptance in microarray technology.

### 3.6.2 Chemiluminescence detection

Klenow extension with hapten labels e.g. biotin followed by enzyme conjugation will yield a chemiluminescent signal in the presence of a substrate. Enzymes such as horseradish peroxidase and alkaline phosphatase are common reporter molecules for chemiluminescence detection. Alsaida et al., reported use of alkaline phosphate which yielded a chemiluminescent signal in the presence of dioxetane substrate.\textsuperscript{102} The main disadvantage with alkaline phosphatase catalyzed detection was longer exposure time (5h) which increased the background. Another option is to use HRP with streptavidin binding molecules. This is generally popular since HRP is less expensive than alkaline phosphatase and maximum intensity can be achieved in seconds. HRP detection has been demonstrated to the higher attomole level.

### 3.6.3 Gold nanoparticles detection

The recent advancement in AuNPs technology has led to development of diagnostics approaches which take advantage of the attractive physicochemical properties of the gold nanoparticles. The nanoparticles can be easily fine tuned to afford different detection methods for example, electrochemistry and spectroscopy. AuNPs absorb and scatter light with extraordinary efficiency. Their strong interaction with light occurs because the conduction electrons on the metal surface undergo a collective oscillation when they are excited by light at specific wavelengths. This oscillation, known as a surface plasmon resonance (SPR), causes the absorption and scattering intensities of gold nanoparticles to be much higher than identically sized non-plasmonic nanoparticles. AuNPS absorption and scattering properties can be tuned by controlling the particle size, shape, and the local refractive index near the particle surface.
3.6.4 Fluorescence detection

Fluorescence detection, one of the most developed detection technique with the DNA microarray can also be applied on this RNA microchip. One way to generate signal is by use of Enzyme Labeled Fluorescence probes (ELF). The biotinylated targets are bound to streptavidin conjugated to alkaline phosphatase. Addition of an ELF substrate produces a fluorescent crystal that adheres to the slide surface by the action of alkaline phosphatase. The crystal can be excited by UV light upon which the emission is corrected using a wide pass filter. Use of a secondary antibody conjugated to a fluorophore will yield a fluorescent emission. Preliminary work with fluorescence detection was done using gold nanoparticles.

3.7 Design, development and validation of RNA microchip

Development of a rapid, sensitive accurate and cost-effective RNA chip is investigated. Our lab has developed a method that affords labeling and detection of mRNAs in an RNA mixture. This method applied in cancer detection and pathogen detection. The method is direct and easy to use thus can easily be adapted as a POC devices or an easy to use micro fluidic system.

3.8 Experimental

3.8.1 The hybrid probe synthesis and purification.

The RNA probes were synthesized using standard solid phase phosphoramidite chemistry on ABI 3400 DNA synthesizer. The standard DNA phosphoramidites and commercial 2-O-Me RNA phosphoramidites were purchased from Glen Research (www.glenresearch.com) and Azco Biotech (www.azcobiotech.com). The RNA probes were modified with amino groups at the 3'-ends for immobilization on the chip surface. The RNA probes were deprotected in aqueous ammonium hydroxide solution (30%) at 55 °C overnight, followed by n-butanol precipitation. Then polyacrylamide gel electrophoresis (PAGE) was used to purify the RNA probes, followed by NaCl/ethanol precipitation. The quality of the probes was assessed by analytical PAGE, followed by quantification with UV-Vis spectrophotometer.
3.8.2 Chip activation

The glass chips (0.5 x 0.5 cm) were first degreased in CH$_2$CL$_2$ (Sigma Aldrich, St. Louis, MO) with gentle shaking for 30 min. The chips were subsequently cleaned in concentrated sulfuric acid (Sigma Aldrich, St. Louis, MO) for 45 min with shaking. The chips were rinsed with deionized water several times until the last wash had a pH 7. Silylation and amino activation was done by incubating the chips for 45 min with shaking in a mixture of 3% 3-aminopropyltriemethoxysilane (APTS; Sigma Aldrich, St. Louis, MO) in aqueous ethanol (95% ethanol and 5% water). Once silylation was complete, the chips were washed three times, first with ethanol, water and a final rinse with ethanol and air dried. The activation to obtain amino-reactive surfaces was accomplished by incubating the chips in a mixture of 1, 4-phenylene diisothiocyanate (PDITC; Sigma Aldrich, St. Louis, MO) and 1% pyridine (Fluca) in CH$_2$Cl$_2$ for 2 hr. The chips were then washed three times in CH$_2$Cl$_2$ and air dried before storage at room temperature in a desiccator. A step-wise representation of the activation process is shown in Figure 3.4.

3.8.3 Spotting and probe immobilization

The synthesized hybrid probes were diluted to appropriate concentration using sodium phosphate buffer (100 mM, pH 8.5) followed by printing on the activated microchip using a Cartesian Technologies PixSys spotting robot (OmniGrid® Micro Digilab Inc, Holliston, MA) with a stealth pin that deposited 0.7 nL/spot on the substrates. The sizes of the spots were ~75 µm in diameter. The chips were then incubated in a water bath at 37 °C for 30 min for coupling. The aldehyde chips were incubated for 2 hours at 37 °C followed by 16 hours at room temperature.

3.8.4 Imaging system: microscope, camera and imaging software.

The imaging system consisted of a microscope (Nikon Eclipse 80i) equipped with an ultrasensitive charge coupled device camera (CCD, CXp-5500 XL, VersArray System; Princeton Instruments, Princeton, NJ) controlled by ImagePro Plus 6.1 computer software for image ac-
quisition, processing and analysis (Media cybernetics, Inc., Silver Spring, MD). The detection is normally performed by using 2 x 2 images binning, 2X lens and an object distance of 1.5 cm.
4. CANCER BIOMARKERS DIRECT DETECTION ON THE RNA MICROCHIP

4.1 Cancer biology

Cancer is a major health problem in the United States and around the world resulting in over 7.6 million deaths per year. Cancer is a group of diseases characterized by uncontrollable growth and spread of abnormal cells. The transformation from a normal cell into a cancerous cell is a multistage process defined by three steps; initiation, promotion and progression. The initiation step is characterized by a permanent DNA mutation. Once the cell has been mutated by an initiator or a trigger, the mutated cell is susceptible to promotion. Promotion refers to the process of growth and proliferation of the mutated cells resulting in a small tumor which in the progression stage undergoes further transformation to a neoplasm and could potentially lead to malignancy. Sometimes progression may lead to metastasis when cancer cell acquires the ability to travel through the blood stream to other tissues of the organism. The transformation changes are a result of interaction between internal and external causative factors which may act together in sequence to initiate or promote cancer. Couple this with genetic differences among patients and the result is a disease characterized by great complexity and heterogeneity.\textsuperscript{115}

4.2 Head and neck cancer

Annually about 15,800 deaths are attributed to head and neck squamous cell carcinoma or referred to simply as head and neck cancer (HNSCC, HNC),\textsuperscript{116} making it the sixth most common form of cancer. HNC usually begins in the squamous cells that line the moist, mucosal surfaces inside the salivary glands of the head and neck. HNC therefore comprises of all tumors originating from the mucosa of the oral cavity, pharynx, larynx and cervical esophagus. The known risk factors include smoked and smokeless tobacco and excessive drinking of alcohol. Human papillomavirus (HPV) infection has also been linked with some types of HNSCC.\textsuperscript{117,118}
In previous studies, gene expression profiles have shown correlations with tumor development and progression.\textsuperscript{119} Therefore by monitoring gene expression, the altered gene patterns can be identified long before the physical characteristics are observable. These patterns offer a platform for the development of a molecular diagnostic tool for early detection of cancer. Analysis of mRNA and microRNA in precancerous or cancer cells could afford a breakthrough in HNC detection and disease management.\textsuperscript{117a} The goal of this research project is the search for a precise and early diagnosis method for early diagnosis of tumorous malignancies allowing for a tailored management of cancer.

\textbf{4.2.1 HNC biomarker detection}

A biomarker is any characteristic that can be objectively measured and evaluated as an indicator of the status of the normal biological processes and or pharmacologic responses to a therapeutic intervention.\textsuperscript{120} 121 122 There are various sources of biomarkers such as body fluids, primary tissues and cell lines, surgical margin tissue and secondary tumors.\textsuperscript{118} Cancer status can be identified using different molecules i.e. DNA, RNA, proteins or metabolites of processes such as apoptosis, angiogenesis or proliferation. These also affords different diagnosis methods.\textsuperscript{118} 121 123 122 The main goals of biomarker studies is to provide information for diagnosis, prognosis and therapeutics. An ideal biomarker should achieve all these goals which require extensive research and validation.

The sources of biomarkers include the growth factors, angiogenic factors, cytokeratines, chemokines and metalloproteinases to name a few.\textsuperscript{124} 125 126 127 While breast cancer has an independent biomarker for screening i.e. epidermal growth factor receptor (EGFR) or the recently approved 76 gene MammaPrint, there is no independent biomarker or profile for HNC.\textsuperscript{128} 81 Linkov et al., (2007) studied many factors such as hormones, proteases, growth and angiogenesis factors, cytokine, chemokines and adhesion molecules to identify biomarkers for a HNC multi-marker system using serum immune-based assays.\textsuperscript{129} From a panel of 60 biomarkers,
they identified 25 biomarkers with significant expression patterns between normal and cancer cells. From this panel, we selected three biomarkers for the development of a model HNC detection RNA microchip. Among them were insulin-like growth factor binding protein 1 (IGFBP1) and interleukin 8 (IL8) which were found to be higher in cancer groups than in normal cells. Indeed, serum levels IL8 are consistently elevated in patients with HNC. Earlier studies by Li et al., showed that RNA isolated from cancer patients saliva had high levels of IL8. IL8 is also an important marker in other forms of cancers i.e. ovarian, gastric, pancreatic, prostate, melanoma and colon cancer.

Recently, infectious diseases have been linked to causing cancer. Many infectious diseases cause the body system to respond by causing inflammation. During a persistent infection, chronic inflammation can lead to continual damage and accumulation of genetic mutation. On the other hand, interaction of viruses with host DNA can lead to activation of genes that promote tumor growth. Of main importance are HPV and Epstein-Barr virus (EBV) viral infections which have been associated with several forms of cancers. HPV is a non-enveloped, circular DNA virus that has been implicated in a variety of diseases ranging from genital warts to cervical cancer. These viruses infect cells in the basal layer of squamous epithelium. HPV involvement with HNC was first proposed in 1983 by Syrjanen et al and confirmed later by others. Currently, more than 320 HPV types have been identified but the most important ones in HNC are HPV 16 and HPV 19. As a biomarker, HPV is becoming a major prognostic and predictive marker in HNC. HNC diagnostic will therefore greatly benefit from discrimination of HPV positive and HPV negative tumors (HPV+ and HPV- respectively).

HPV tests include southern blot, in situ hybridization, immunostaining an immunohistochemistry PCR and DNA microarray. Although PCR is a reliable and sensitive marker for HPV RNA and DNA detection, it cannot localize HPV to the area of neoplasia thus lowering the ability to distinguish clinically relevant HPV infection. In situ hybridization specificity is high and have commercially available kits with the Hybrid Capture 2 approved by FDA for pap smears but
application in HNC detection is limited due to the need to classify HPV positive and HPV-negative tumors. It is therefore hypothesized that the RNA microchip can be used to distinguish between HPV+ and HPV- tumors.

### 4.2.2 HNC markers target and probe design

The target RNA should be gene specific to avoid non-specific hybridization. The probe should be long enough to be specific for a region of interest but not too long to introduce steric interactions especially when RNA is the target. RNA has the ability to form complex secondary structures. Other parameters to consider in probe design include base composition and sequence. Once the biomarkers were identified, a 40 nucleotide sequence of the genes was selected from NCBI followed by BLAST search for sequence alignment for the three genes.

Hybrid probes, DNA-RNA-DNA were thereby designed as complimentary sequence to the target. The probe had less the 10 nucleotides which compensated for the spacer. The RNA component consists of 15 2’-O-Me-methylated RNA which has an important function in stabilizes the RNA against RNases. Finally, the last portion of the probe consists of 15 dNTPs which acts as a guiding sequence for Klenow DNA polymerase incorporation with labels.

### 4.2.3 Probe synthesis

The hybrid probes were synthesized by solid phase phosphoramidite method on 3400 DNA/RNA synthesizer (Applied Biosystems). Elution from the column was accomplished with 30% NH$_4$OH followed by incubation at 55 °C for deprotection. The hybrid probes were purified by on an acrylamide preparative gel electrophoresis (PAGE) and eluted by soaking in water overnight. After ethanol/NaCl precipitation, the oligonucleotides were redissolved in water and concentrations determined spectrophometrically. The quality of the oligonucleotides was confirmed by use of analytical PAGE. Below is the oligonucleotide library for detection of HNC on the RNA microchip.
IGFBP1 RNA [Homo sapiens insulin-like growth factor binding protein 1 (IGFBP1), mRNA, 251-290 nt]: 5'-GCAUCGGCCC CUGUCUGCUG CUCGCGCCUG-3'

IGFBP1 probe: 5'-d (CAGGCGCGAG CAGCA)-2'-O-Me-(GACAG GGGCGAUGC) d (TCTCTGGGCT) - NH₂-3'

VCAM1 RNA [Homo sapiens vascular cell adhesion molecule 1 (VCAM1), transcript variant 1, mRNA, 2051-2090 nt]: 5'-AUCUAUAGAU GGCGCCUAUA CCAUCCGAAA-3'

VCAM1 probe: 5'-d (TTTCGGATGG TATAG)-2'-O-Me-(CGGCC AUCUAUAGAU) d (TTTAGTACTG)-NH₂-3'

IL8 RNA [Homo sapiens interleukin 8 (IL8), mRNA, 251-290 nt]: 5'-GUGAUUGAGA GUGGACCACA CUGCGCCAAC-3'

IL8 probe: 5'-d (GTTGGCGCAG TGTGG)-2'-O-Me-(UCCAC UCUCAAUCAC)-d (TCTCAGTTCA)-NH₂-3'

### 4.3 3'-labeling strategy

mRNA is mostly detected indirectly using cDNA coupled with PCR or RT-PCR. Signal detection therefore is time consuming and partially degraded RNA is not accounted for. Labeling a specific RNA in an RNA mixture is also difficult. Our lab has developed a method that affords direct labeling and detection of mRNAs in an RNA mixture.  

The system is based on 3'-labeling approach where a DNA polymerase incorporates hapten labeled dNTPs directly on a DNA template immobilized on a microchip. Before incorporation of the labels, the 3'-region is removed by RNase H in the presence of a guiding sequence. RNase H is an RNA endonuclease capable of selectively cutting RNA in an RNA/DNA duplex. Also, RNase H offers an advantage since it doesn’t digest RNA/RNA duplex. This prevents target RNA form digestion. The 2'-O-methylation of the RNA probe also stabilizes the probe RNA sequence against RNases. Once the biotin labeled dNTPs are incorporated by use of Klenow polymerase, they are converted to enzymatic labels through the specific interaction of a binder enzyme conjugate that catalyze chemiluminescent reactions in the presence of substrates (Figure 4.1).
Streptavidin-horseradish peroxidase binds specifically to the biotin labels and serves as an enzymatic label for the HNC mRNA. The bound horseradish peroxidase catalyzes an oxidation-reduction reaction that produces chemiluminescence signal in the presence of enzymatic substrate. This signal can be detected using a CCD camera. Here we successfully demonstrate this approach by selective detection of HNC biomarkers (Figure 4.2).

Figure 4.1: Flow chart for the rapid and direct RNA microchip. RNA (red) binds via complementary base pairing to the last two section of the hybrid probe. RNase H then digests the RNA portion of the RNA/DNA duplex. The resulting single stranded portion of the DNA acts as a guiding sequence for Klenow incorporation with biotin labeled dNTPs. Once streptavidin HRP binds to biotin, HRP enzyme interaction with substrate give the desired signal.

4.4 Selectivity of the detection method

Selectivity on the RNA microchip is critical to ensure accurate results are obtained. Selectivity also helps in planning experiments to avoid wastage of time and precious samples. Real samples or patient samples for research purposes can be difficult and costly to obtain so it is important to obtain accurate results when conducting microchip experiments. Selectivity is also important to check for viability as well as cross hybridization which will help avoid false positives that can be detrimental in human life.
4.4.1 Selectivity on the microchip

First, selectivity assay was done by testing each RNA on the microchip. Four different chips were spotted with four different probes VCAM1, IGFBP1, IL8 and Avian flu (AF) respectively (Figure 4.2). On each chip, a different RNA was applied. For example, on chip A, only VCAM1 RNA was applied while the other probes acted as negative control. As observed, there was no interference or cross hybridization from other mRNAs.

The main challenge as observed in Figure 4.2 is signal consistency, for example IL8 on chip C had a strong signal compared to IGFBP1 on Chip B. The RNA chip needed for gene expression profiling requires a consistent signal transduction method for effective signal comparison. Such a method should also be able to report accurately the status of the cell without any bias. Optimization of experimental conditions on the RNA microchip was therefore needed to ensure similar signal transduction across all probes. A good comparison can only be made if signal transduction is the same.

Hybridization is most efficient at 25 °C below \( T_m \).\(^{142}\) The GC content plays a major role in duplex strength with high GC percentage resulting in high \( T_m \). As observed with the probes, IGFBP1 had a high GC content which would have increased the \( T_m \). Hybridization at room temperature must have compromised the signal due to the few hybrid duplexes formed. This temperature must have been efficient for IL8 hybridization as observed with the slightly higher signal among the three. It is advised that the predicted \( T_m \) should be between 58-62 °C for all probes to be used in one chip to ensure similar behaviour.\(^{143}\) Hybridization at higher temperatures was therefore envisioned as a way to improve the signal (Table 4.1). Other changes included increasing the time required for the enzyme-conjugate binding step. Previously washing was done with lots water which introduces variations if not well handled. But introduction of a PBS buffer washing step significantly reduced the background therefore improving signal to noise ratio (Figure 4.3).
4.4.2 Experimental

Four different chips were printed with four probes; VCAM1, IGFBP1, IL8, and AF for selectivity studies. After immobilization, the chips were incubated with StartingBlock blocking buffer for 20 min. Subsequently, each chip was flooded with a different RNA in 5x SSC buffer (20 µL, 0.75M NaCl, 75 mM sodium citrate, pH 7.0) and incubated at room temperature for 15 min. The unbound RNAs were washed twice with 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, pH 7.4). The chips were then incubated with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) at room temperature for 15 minutes. The digested RNAs were then washed twice with 1x PBS buffer followed by polymerase extension step with Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and 1 µL of 0.4 mM biotin labeled dNTPs; biotin 14-dATP (Invitrogen, Carlsbad, CA), biotin 14-dCTP (Invitrogen, Carlsbad, CA), biotin 11-dGTP (PerkinElmer, Waltham, MA) and biotin 11-dUPT (PerkinElmer, Waltham, MA). The chips were incubated at 37 °C for 30 minutes followed by washing (3 x) with PBS buffer. The chips were then incubated with Poly-HRP Streptavidin (Thermo Scientific, Rockford, IL) diluted 5000x in TBS buffer for 5 minutes. The chips were washed with a lot of water to remove unbound enzymes. SuperSignal ELISA Femto Maximum sensitivity substrates (Invitrogen, Carlsbad, CA) were applied on the chips. The chemiluminescence signal was captured with a CCD camera.
VCAM1 RNA: 5’-AUCUAUAGAUGGCAGC CUAUACCAUCCGAAA-3’
VCAM1 Probe: 3’-NH2-d(GTCACTGATT)-r(UAGAAUCUACCGG)-d(GATATGGTAGGCTTT)-5’

Figure 4: Selectivity on the RNA microchip. The RNA chip was immobilized with similar probes, VCAM1, IGFBP1, IL8, and AF respectively. Only one RNA was applied on each chip i.e. VCAM1, IGFBP1, IL8, and AF on chips A, B, C and D respectively.

Table 4.1: Condition optimization on the RNA microchip. Room temperature (RT)

<table>
<thead>
<tr>
<th>Step</th>
<th>Conditions</th>
<th>New Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridization</td>
<td>RT</td>
<td>37 °C, Wash 3 x PBS buffer</td>
</tr>
<tr>
<td>RNase H digestion</td>
<td>RT</td>
<td>37 °C, Wash 3 x PBS buffer</td>
</tr>
<tr>
<td>Blocking</td>
<td>RT</td>
<td>37 °C</td>
</tr>
<tr>
<td>Streptavidin HRP</td>
<td>Water Washing</td>
<td>Wash 5 x PBS buffer</td>
</tr>
</tbody>
</table>

4.5 RNA microchip for multiple HNC biomarker identification

Since there is no independent biomarker approved for HNC diagnosis, a microchip that allows for simultaneous detection of a number of biomarkers may present a promising approach for early diagnosis of HNC. The RNA chip can also be an important tool in the search of biomarkers due to the unparalleled detection of many samples. Simultaneous detection enables one to get as much information relating to gene expression from one sample. A single hybridization event in a microarray set up can help elucidate thousands of mRNA and thereafter help un-
derstand many cell processes. In this way, identification of biomarkers and thereafter cancer detection is more simplified in comparison with RT-PCR where multiplexing is limited. Indeed a few markers are enough to give a disease status but only after they have been studied from a pool of markers and validated to give enough information that can aid in diagnostic and prognosis with a high confidential level. But since cancer is a result of many cellular processes gone berserk, it is hypothesized that a multi-marker detection will aid in elucidating and better understanding the processes.

**4.5.1 Simultaneous detection of HNC cancer biomarkers on the RNA microchip**

For simultaneous detection, five different chips were immobilized with BA, VCAM1, IGFBP1 and IL8 respectively (Figure 4.3). Chip A and B acted as positive control where only one target RNA was applied. On chip C, IGFBP1 and IL8 RNAs were applied while BA and VCAM1 acted as negative control. The RNAs applied on chips D and E are as indicated on the table below each chip. Figure 4.3 shows that the method is specific and simultaneous detection is possible with consistent results for different target RNAs.

**4.5.2 Experimental**

Five different chips were printed with four different probes; BA, VCAM1, IGFBP1, and IL8 respectively. After immobilization step, the chips were incubated with StartingBlock (TBS, Pierce, Rockford, IL) blocking buffer for 20 min. The chips were then flooded with target RNA samples (500 fmol) in 5x SSC buffer (20 µL, 0.75M NaCl, 75 mM sodium citrate, pH 7.0) at 37 °C for 15 min. The unbound RNAs were then washed (2x) with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, pH 7.4). The chips were flooded with RNase H (0.5 µL, 5000 U mL⁻¹) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) followed by incubation at 37 °C for 15 minutes. The digested RNAs were then washed (2 x) with 1x PBS buffer followed by incubation with Klenow fragment (0.5 µL, 5000 U mL⁻¹) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and biotin labeled dNTPS (1 µL, 0.4 mM); biotin 14 dATP (Invitrogen, Carlsbad, CA), biotin 14-dCTP (Invitrogen, Carlsbad, CA), biotin 11-dGTP
(PerkinElmer, Waltham, MA) and biotin 11-dUPT (PerkinElmer, Waltham, MA) at 37 °C for 30 minutes followed by washing (3 x) with PBS buffer. The chips were then incubated with Poly-HRP Streptavidin (Thermo Scientific, Rockford, IL) diluted 5000x in TBS buffer for 15 minutes. The chips were rinsed with PBS buffer (5 x) to remove unbound enzymes. SuperSignal Elisa Femto Maximum sensitivity substrates (Invitrogen, Carlsbad, CA) were applied on each chip. The chemiluminescence signal was captured with a CCD camera.

![Chip Image](image)

<table>
<thead>
<tr>
<th>Chip</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VCAM1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 4.2: Specificity and simultaneous detection of different RNAs. The chips were immobilized with similar probes, BA, VCAM1, IGFBP1 and IL8 respectively. Different mRNAs were applied on the chips as shown on the table below each chip. (Positive sign indicates presence of the RNA (+); negative sign indicates absence of the RNA). (Positive (+); negative (-)
4.6 HNC cancer cell lines mRNA detection on the RNA microchip

4.6.1 mRNA direct detection from head and neck cancer cell lines total RNA

It has been shown that gene expression profiles between normal and cancer cells can be statistically different. Changes in the transcriptome have been shown to correlate with onset and progression of cancer with some genes over expressed while others are under regulated. This pattern can be identified by direct analysis of the transcriptome to identify which genes have been affected by the uncontrolled growth characteristic of cancer. Research targeting genes/mRNA as indicator of the cell status has opened new avenues not only in cancer diagnostics but also therapeutics where some of the identified genes have been marked as relevant for drug targeting. As a matter of fact, mapping of the transcriptome have been used to diagnose cancer, identify the stage and predict the future trend. Several treatment options have been also developed to target some biomarkers. Biomarker studies therefore offer a wide array of benefits in cancer management.

To test the effectiveness of the RNA microchip on real RNA, we used total RNAs extracted from different cancer cell lines i.e. TU 686, LN 686 and LN 212. The chips were printed with the three cancer probes; IL8, IGFBP1 and VCAM1. Initially, the total RNAs were first heated at 65 °C for 2 minutes and then hybridized on the surface with slow cooling at controlled humidity. But method development and evaluation has shown treatment with NaOH at elevated temperatures give good and consistent results. The hybridization is also reduced to 30 minutes. The heating was necessary to disrupt any secondary structures that may interfere with target RNA duplex formation. Figure 4.4 is an indication that the RNA chip can be used to selectively and specifically detect mRNA from total RNAs. Selectivity and simultaneous detection was demonstrated by the ability to detect only the present RNAs which can be correlated to the mRNA expression level. TU 686 and LN 212 had two biomarkers detected i.e. IL8 and VCAM1 while LN 686 had only VCAM1 detected. IGFB1 was not detected in any of the three cell lines. Even with the presence of thousands of mRNAs in thousands of nucleotides, there was no
cross hybridization among target RNAs as observed with different signal profiles. Specificity on the other hand was demonstrated by the fact that different cell lines showed different expression levels as observed on the chips. Discrimination at this level is a clear indication that the RNA microchip is specific to detect mRNA from different cell lines.

The RNA microchip is sensitive enough to enable detection without RT-PCR, the amplification method used in DNA microarray experiments even when total RNA amounts is lower than the starting material in DNA microarray experiments (10 µg). The RNA microchip offers direct RNA detection which easily gives a direct status of the cell. The method is rapid since there is further treatment needed on the RNA after extraction. RT-PCR requires reverse transcription and thereafter PCR amplification. These steps can be time consuming and often are marred by contamination. In addition, high hybridization yields were achieved even at short hybridization time in comparison to the DNA microarray which are normally done at longer hybridization times (16 hrs for hybridization step only).

4.6.2 Experimental

The cell lines total RNA was prepared at Emory University. Four different chips were immobilized with three different probes; IL8, IGFBP1 and VCAM1 respectively. After immobilization step, the chips were incubated with StartingBlock (TBS, Pierce, Rockford, IL) blocking buffer for 20 min. Chip A was flooded with synthetic RNA samples in 5x SSC buffer (20 µL, 0.75M NaCl, 75 mM sodium citrate, pH 7.0) and incubated at 37 °C for 15 min. The cancer cell lines total RNA was heated for 5 minutes at 92 °C in NaOH, neutralized, added to 5x SSC and applied the remaining chips and incubated at 37 °C for 30 minutes. The unbound RNAs were then washed (2 x) with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, pH 7.4). The chips were then flooded with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) followed by incubation at 37 °C for 15 minutes. The digested RNAs were then washed (2 x) with PBS buffer followed with incubation with Klenow fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and
biotin labeled dNTPs (1 µL, 0.4 mM); biotin 14-dATP (Invitrogen, Carlsbad, CA), biotin 14-dCTP (Invitrogen, Carlsbad, CA), biotin 11-dGTP (PerkinElmer, Waltham, MA) and biotin 11-dUPT (PerkinElmer, Waltham, MA) at 37 °C for 30 minutes followed by washing (3 x) with PBS buffer. The chips were then incubated with Poly-HRP streptavidin (Thermo Scientific, Rockford, IL) diluted 5000x in TBS buffer for 15 minutes. The chips were rinsed five times with PBS buffer to remove unbound enzyme. SuperSignal Elisa Femto Maximum sensitivity substrates (Invitrogen, Carlsbad, CA) were applied on each chip. The chemiluminescence signal was captured with a CCD camera.

![Image of chip A to D with biomarkers IL8, IGFBP1, and VCAM1]

<table>
<thead>
<tr>
<th>Chip</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IGFBP1</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VCAM1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 4.3:** Detection of mRNAs in total RNAs from different HNC cancer cell lines. The chips were immobilized with similar probes, i.e. IL8, IGFBP1 and VCAM1 respectively. Chip A acted as positive control while on chip B and C had 4.4 µg of RNA applied while 2.2 µg of total RNA was applied on chip D. The detected biomarker is as indicated below each chip.
4.7 Human papilloma virus as a marker for head and neck cancer

4.7.1 HPV related cell lines mRNA detection on the microchip

HPV-related head and neck cancers occur primarily in the oropharynx (tonsils and the back of the tongue). Oropharyngeal cancers are more common in white men. Researchers believe that up to 80% of oropharyngeal cancers in the U.S. are due to infection with the HPV virus. HPV is therefore an important HNC marker and its detection will help understand its role and implication in cancer progression. In this case study, two different sets of cancer cell lines, set 1 with HPV+ DNA and set 2 with HPV- DNA were hypothesized to have different biomarker expression.

The relevance of HPV infection on the cancer cell lines was studied using our markers on our novel RNA microchip. The chips were immobilized with different markers, i.e. IL8, IGFBP1 and VCAM1 (Figure 4.5). Chip A and B acted as positive controls while on chips C and D, two different cell lines i.e. SSC90 (HPV positive) and M4-GFP (HPV negative) applied on them respectively. Selectivity was highly achieved on the chips as observed with different levels of expression levels for the biomarkers. Specific is clearly demonstrated by the difference in the expression levels of the two cell lines. M4-GFP had higher expression of IL8 compared with SSC90. Also, there is a trace IGFP1 signal for M4-GFP on chip D. This is the first time IGFP1 is selectively detected from the cell lines, even though the signal is marred by artifacts. Even though we are dealing with only one sample comparison, these results combined with other biomarkers results are a clear indication that the RNA microchip can be used for expression profiling studies.

4.7.2 Experimental

The HPV cancer cell lines total RNAs were prepared at Emory University. Four different chips were immobilized with three different probes; IL8, IGFBP1 and VCAM1 respectively. After
immobilization step, the chips were incubated with StartingBlock (TBS, Pierce, Rockford, IL) blocking buffer for 20 min. Chip A and B were flooded with synthetic RNA samples in 5x SSC buffer (20 µL, 0.75M NaCl, 75 mM sodium citrate, pH 7.0) and incubated at 37 °C for 15 min. The HPV cancer cell lines total RNA was fragmented with 50 mM NaOH solution by heating for 5 minutes at 92 °C. The fragmented RNA samples were cooled down in ice, centrifuged and neutralized using 1N acetic acid. Hybridization buffer was added to the fragmented RNA, applied on the remaining chips and incubated at 37 °C for 30 minutes. The unbound RNAs were then washed (2x) with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, pH 7.4). The chips were then flooded with RNase H (0.5 µL, 5000 U mL⁻¹) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) followed by incubation at 37 °C for 15 minutes. The digested RNAs were then washed (2 x) with PBS buffer followed by incubation with Klenow fragment (0.5 µL, 5000 U mL⁻¹) in Klenow buffer (20 µL, 1x New England Biolabs, MA) and biotin labeled dNTPs (1 µL, 0.4 mM); biotin 14-dATP (Invitrogen, Carlsbad, CA), biotin 14-dCTP (Invitrogen, Carlsbad, CA), biotin 11-dGTP (PerkinElmer, Waltham, MA) and biotin 11-dUPT (PerkinElmer, Waltham, MA) at 37 °C for 30 minutes followed by washing (3 x) with PBS buffer. The chips were then incubated with Poly-HRP Streptavidin (Thermo Scientific, Rockford, IL) diluted 5000x in TBS buffer for 15 minutes. The chips were thereafter rinsed five times with PBS buffer to remove unbound enzyme. SuperSignal Elisa Femto Maximum sensitivity substrates (Invitrogen, Carlsbad, CA) were applied on each chip. The chemiluminescence signal was captured with a CCD camera.
Figure 4.4: Detection of mRNAs in total RNAs from HPV related cancer cell lines. The chips were immobilized with similar probes, i.e. IL8, IGFBP1 and VCAM1 respectively. Chip A and B had synthetic RNAs applied. Chip A had IL8 and IGFBP1 applied to it while VCAM1 acted as negative control. Chip B had all RNAs applied. On chip C, 2.75 µL of SSC 90 (2.42 µg), HPV positive cell line was applied and chip D had 2 µL M4-GFP (3.32 µg), a HPV negative cell line applied. The detected signal is as indicated in the table below each chip.

<table>
<thead>
<tr>
<th>Chip</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td>-</td>
<td>-</td>
<td>SSC 90</td>
<td>M4-GFP</td>
</tr>
<tr>
<td>HPV+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

4.8 Human colon cancer

4.8.1 Introduction

Colorectal cancer, commonly known as colon cancer or bowel cancer, is a cancer from uncontrolled cell growth in the colon or rectum (parts of the large intestine) or in the appendix. Colon cancer is the fourth most common type of cancer in Western society. Symptoms of colorectal cancer typically include rectal bleeding and anemia which are sometimes associated with weight loss and changes in bowel habits. Colon cancer correlates strongly with genetic al-
iterations that occur during the progression from adenoma to carcinoma to metastatic disease.\textsuperscript{147}\textsuperscript{148}\textsuperscript{149} Molecular diagnostics therefore offer the promise of precise and systematic cancer diagnostics and classification but current marker and molecular diagnostic methods are not recommended for clinical use due to diversity of results among different platforms.\textsuperscript{149} There is therefore ongoing research to better molecular diagnostics methods for better performance in cancer diagnostics. It was therefore hypothesized that the RNA microchip can be used as a tool for human RNA detection on the chip. Even though the method was designed for HNC markers, there is a tendency for markers to be over expressed in different cancers especially because they have a unique common role in the body. As stated earlier, some cancer biomarkers play an important role in more than one cancer. IL8 a chemokine, has been detected in many human tumors including colorectal cancer.\textsuperscript{150}\textsuperscript{151} In addition, the behavior of RNAs should be the similar if present in the colon tumor.

\textbf{4.8.2 Detection of human colon cancer mRNA on the RNA microchip}

Detection of real RNAs from colon cancer was evaluated on the RNA microchip. Figure 4.6 is an indication that the microchip can be used to detect mRNAs from human total RNAs. Chip A acted as a positive control while others were test controls from different batches of colon samples. As observed, VCAM1 and IL8 were detected on chip B while only VCAM1 was detected on the other chips. Not surprising because biomarker studies on colon cancer have reported higher VCAM levels in cancer patients.\textsuperscript{152} Detection of the two mRNAs in a mixture of thousands of genes without separation or RT-PCR is a promising result for the RNA microchip. Different total RNAs were applied on the chip due to the availability but even at 1.4 µg the signal is strong enough for chip A. Also different expression levels can be observed among the batches which is an indication the of the microchip exceptional specificity.

\textbf{4.8.3 Experimental}

The colon cancer total RNA samples were provided by Georgia Health Sciences University. Four different chips were immobilized with three different probes; VCAM1, IL8 and IGFBP1
respectively. After immobilization step, the chips were incubated with StartingBlock (TBS, Pierce, Rockford, IL) blocking buffer for 20 min. Chip A was flooded with synthetic RNA samples in 5x SSC buffer (500 fmol, 20 µL, 0.75M NaCl, 75 mM sodium citrate, pH 7.0) and incubated at 37 °C for 15 min. The colon cancer total RNAs were fragmented by heating for 5 minutes at 92 °C. The fragmented RNA samples were cooled down in ice, centrifuged for 30 seconds, diluted in 5x SSC buffer and applied on the remaining chips. The chips were incubated for 30 minutes at 37 °C. The unbound RNAs were then washed (2 x) with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, pH 7.4). The chips were then flooded with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) followed by incubation at 37 °C for 15 minutes. The digested RNAs were then washed with PBS (2x) buffer followed by incubation with Klenow fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and biotin labeled dNTPS (1 µL, 0.4 mM); biotin 14-dATP (Invitrogen, Carlsbad, CA), biotin 14-dCTP (Invitrogen, Carlsbad, CA), biotin 11-dGTP (PerkinElmer, Waltham, MA) and biotin 11-dUPT (PerkinElmer, Waltham, MA) at 37 °C for 30 minutes followed by washing with PBS buffer (3 x). The chips were then incubated with Poly-HRP Streptavidin (Thermo Scientific, Rockford, IL) diluted 5000x in TBS buffer for 15 minutes. The chips were rinsed five times with PBS buffer to remove unbound enzyme. SuperSignal Elisa Femto Maximum sensitivity substrates (Invitrogen, Carlsbad, CA) were applied on each chip. The chemiluminescence signal was captured with a CCD camera.
Figure 4.5: Detection of mRNAs in colon tumor total RNAs. The chips were immobilized with similar probes, VCAM1, IL8 and IGFBP1 respectively. Chip A acted as a positive control while different amount of colon tumor RNA from different batches were applied on the other chips. Chip B had 1.4 µg of colon RNA, chip C, 1.4 µg and Chip C had 2.2 µg respectively. The detected marker is as indicated below each chip.

<table>
<thead>
<tr>
<th>Chip</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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</thead>
<tbody>
<tr>
<td>Colon tumor</td>
<td>-</td>
<td>Batch 1</td>
<td>Batch 2</td>
<td>Batch 3</td>
</tr>
<tr>
<td>VCAM1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL8</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>IGFBP1</td>
<td>+</td>
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4.9 Summary

A rapid, selective, specific and sensitive RNA microchip was demonstrated. High selectivity was observed with short synthetic RNAs which gave us the confidence to test total RNAs extracted from cancer cell lines. Detection of head and neck cancer total RNAs non specific cancer cell lines and HPV related cell lines on the RNA microchip was reported. The RNA microchip had high selectivity and specificity for the biomarkers selected. Results show a repeatable specific signature from the total RNA which is an indication that there was no cross hybridization or nonspecific interaction even when we target only a few mRNAs i.e. VCAM1, 1L8, and IGFBP1 from a mixture of thousands of RNA. 3’-labelling technique enabled selective and spe-
specific detection of only the three markers and only if present in the system. This was accomplished without prior extraction of mRNAs or selection using primers, reverse transcription and amplification as commonly observed with DNA microarrays. The method is highly sensitive as observed with the low amount of starting material (5 µg) in comparison with RT-PCR recommended starting material (10 µg) for DNA microarray experiments.

The different signal intensity observed with HPV related cell lines as well as other cell lines suggest that the RNA microchip can also be used as reporter for gene expression profiling. By using the RNA microchip, we are also able to detect the cancer mRNA from human colon cancer total RNA without amplification or prior mRNA extraction. The RNA probes are highly specific to capture the targeted sequence if present. The performance of the few genes indicate no requirement of other mismatched probes as observed with Affymetric system due to high level of cross hybridization on their chips even though the size of probes is the same. This direct RNA microchip is simple with minimal step and thus is well adaptable for a POC device.

Development of a rapid, sensitive accurate and cost-effective RNA chip for early detection of HNC is reported. A breakthrough in this field will also find other applications in drug development, efficacy optimization and toxicity analysis. It is important to note that even with different mRNAs in the cell lines, it was possible to detect the three RNAs simultaneously without any visible interference or cross hybridization. Application of the RNA chip on different cancers is an indication for application as a universal tool for expression profiling as well as cancer diagnosis. The RNA microchip is a good candidate for the detection of any kind of RNA, i.e. mRNA, small RNAs, rRNAs or microRNAs as long as specific probes are designed. The length of the RNAs is also not a limitation as fragmentation experiments will denature secondary structures. Upon hybridization, RNase H will digest all other RNAs thus enhancing the performance of other subsequent steps. The method is easy, rapid, specific and quite sensitive.
5. NOVEL RNA MICROCHIP FOR RAPID AND DIRECT DETECTION OF PATHOGENIC BACTERIA

5.1. Introduction

Pathogen identification and detection is an important part of research in many fields e.g. food safety, water safety, animal health care, pathology, diagnostics, forensics and biodefense. Of great importance is food borne illnesses arising from improper handling of food during preparation and storage which are becoming a major challenge to human survival. Man seems helpless against these unpredictable epidemics even with constant preventive and control measures. One of the main causative agents of food borne infections diseases includes pathogenic E. coli, salmonella and Campylobacter jejuni. Although most E. coli bacteria are important component of the biosphere and are harmless, others are notorious for causing food borne infections. Pathogenic E. coli strains are responsible for infections of the enteric, urinary, pulmonary and nervous systems. For example, the shiga toxin producing E. coli (STEC) is responsible for food-borne outbreaks from contaminated food or water. The strain O157:H7 of E. coli is considered one of the most dangerous food-borne pathogens and has been associated with several outbreaks.153 154 155

Current methods rely upon conventional clinical microbiology monitoring which suffer considerable drawbacks. Indeed the most sensitive and reliable method is the detection of endotoxins via coagulation of horseshoe crab blood using the limulus amebocyte lysate (LAL).156 157 But the standard culture and susceptible test are laborious, time consuming and expensive and are plagued by many interferences. Besides, the methods do not characterize the virulence factors and can only handle a small set of samples at a time.

There is therefore a need for reliable and accurate tools for pathogen detection. Molecular diagnostics is one of the most attractive areas since one can determine the pathogen, virulence, and serotypes by taking advantage of the unique genomic signature (DNA or RNA) for
each organism. Advances in this field have been made possible by the availability of genomic information. Invention of PCR and thereafter qPCR was seen as a major breakthrough in microbe detection but reverse transcription inclusion not only increases detection time but also errors could be amplified in the process. DNA microarrays are able to combine the powerful amplification strategies with the multiplex screening resulting in high sensitivity and throughput. But they are limited by the cost and the organizational complexity of performing large number of PCR. This indirect measurement requires a further validation step which among other aforementioned problems has reduced the utility of this technique. There is therefore an ongoing search for a rapid and sensitive method for pathogenic detection.

To meet this challenge, our lab has developed a method for direct RNA detection on a microchip. The RNA microchip affords a direct and sensitive method for detection of *E. coli* total RNA without reverse transcription and thereafter PCR amplification. Further, we have developed a very fast, simple and reliable method for direct extraction of total RNA using NaOH. By treating bacteria cells directly using NaOH to extract RNA, we are able to detect *lacZ* mRNA directly from the supernatant. Direct extraction of total RNA and detection on the RNA chip overcomes the long RNA purification process using kits. This not only reduces operation cost but also the RNA handling time. The RNA chip is a good POC diagnostic candidate for *E. coli* monitoring and detection in food and recreational water pathogens as well as any other field that require RNA detection.

### 5.2. Hybrid probe design

Probe design is an important aspect in a microarray experiment. This will ensure selectivity, hybridization specificity as well as sensitivity. We adapted the hybrid probes as discussed in chapter 3. The hybrid probes have three regions as mentioned but in this case, the target RNA was chosen from a genome region with multiple Ts. This allows incorporation of biotin dATP as the only hapten label. Three different probes *lacZ* probes were designed with a consecutively shorter 2-O-Me RNA (Figure 5.1). The behavior of the probes was tested on the chip.
The experimental assay was as outlined in chapter 3. The 3’-labelling principle can be applied to any RNA by designing specific probes. As shown in Figure 5.1, probe with 10 nucleotides in the region gave a higher signal followed by 15 nucleotides. It can be observed that a probe as short as 5 nucleotides is enough to stabilize the region and or anchor the RNase H. this is an indication that as short as 5 nt probes can be detected on the chip. This method can therefore be applied for degraded RNAs. The signal was slightly lower than the other probes due to the hybridization competition but on its own, 5M probe gives a strong signal.

Even though 10M gave the highest signal, further experiments using total RNA extracted from E. coli had non-specific hybridization with 10M which led to the use of 35.1 (lacZ) probe. This study will enable better design of probes in future to give the highest hybridization yields. Below are the lacZ hybrid probes which target the same RNA. The naming is derived from the shorter 2’-O-Me-RNA region except for the 35.1 probe.

lacZ mRNA (lacZ, E. coli lacZ mRNA: 724-748 nt): 5’-AUGUGGAUUGGCGAUAAAAACAA-3’
35.1 probe: 5’-d(GTTGTTTTT)-2’-O-Me-RNA (AUCGCCAAUCCACAU)-d (CTGTGAAAGA)-NH₂-3’
10M probe: 5’-d(GTTGTTTTT)-2’-O-Me-RNA (AUCGCCAAUCCAC)-d (CTGTGAAAGA)-NH₂-3’
5M probe: 5’-d(GTTGTTTTT)-2’-O-Me-RNA (AUCG)-d (CTGTGAAAGA)-NH₂-3’

5.2.1 Experimental

The chip was spotted with three lacZ probes; 35.1, 10M and 5M. After immobilization, the chips were incubated with StartingBlock TBS blocking buffer for 20 min. Subsequently, the chip was flooded with a 500 fmol RNA in 5x SSC buffer (20 µL, 0.75M NaCl, 75 mM sodium citrate, pH 7.0) and incubated at 37 °C for 15 min. The unbound RNAs were washed twice with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, pH 7.4). The chips were then incubated with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer 20 µL, 1x New England Biolabs® Inc, Ipswich, MA) followed by incubation at 37 °C for 15 minutes. The digested RNAs were then
washed twice with PBS buffer followed by incubation with Klenow large fragment (0.5 µL, 5000 U mL⁻¹) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and 1 µL of 0.4 mM; biotin labeled 14-dATP (Invitrogen, Carlsbad, CA). The chip was incubated at 37 °C for 30 minutes followed by washing three times with 1x PBS buffer. The chips were then incubated with Poly-HRP Streptavidin (Thermo Scientific, Rockford, IL) diluted 5000x in TBS buffer for 15 minutes followed by washing 5x with PBS to remove unbound enzymes. SuperSignal ELISA Femto Maximum sensitivity substrate (Invitrogen, Carlsbad, CA) was applied on the chips and the chemiluminescence signal captured immediately with a CCD camera.

Figure 5.1: Probe design specificity results. The chip was immobilized with 3 different lacZ probes i.e. 35.1, 10M and 5M respectively. lacZ RNA was applied to the chip.

5.3. *E. coli* cells generation

The wild type strain (MG1655) approximates wild-type *E. coli* but with only minimal genetic manipulation. The wild type lac operon has two distinct mechanisms of regulation; one stimulates transcription and the other down-regulates transcription. Inducers of lac operon i.e. isopropyl β-D-1-thiogalactopyranoside (IPTG) permit transcription by binding to lac repressor. This process is also controlled by the presence of cyclic AMP and its receptor protein which binds to the lac promoter thus stimulating transcription. The cAMP levels are strongly influenced by the amount of carbon in the medium. Addition of glucose as a source of carbon therefore leads to low cAMP which consequently leads to low transcription level. By using these two
agents, we are able to generate both lacZ induced and lacZ repressed cells which acts as a positive and negative control in the experimental system.\textsuperscript{159, 160}

The bacteria strain; an \textit{E. coli} K-12 MG1655 was a gift from Scarab Genomics LLC (Madison, WI). Growth of MG1655 on LB medium was previously reported to give good results. Generation of the bacteria was done by first inoculating on the plate or directly on a starter culture. In our experiments, inoculation was done directly in the starter culture. Streaking on the plate was only considered as a control method. Initially, growth was monitored by UV spectrophotometer with induction done at OD 600 of at least 0.6. This was generally achieved after \(~2.30\) hrs thus requiring only OD periodic measurements. Different induction timings were tested with 6 hours giving the best results on the chip. Four hours induction time was probably too short as the signal on the chip was very weak while more than 6 hrs did not improve the signal. Different bacteria dilution ratio was also tested with 1:50 giving the best results.

\textbf{5.3.1 Experimental; \textit{E. coli} cell preparation}

The cells (5 \(\mu\)L) of the wild-type \textit{E. coli} bacteria strain (K-12 MG1655) culture (or a colony) were used to inoculate 6 mL Luria Broth (LB, Sigma Aldrich, St. Louis, MO) and the culture was incubated overnight at 37 °C with shaking at 220 RPM. The bacteria culture was then added to a 1L-Erlenmeyer flask containing 300 mL of sterilized LB culture and incubated for 2.5 hr at 37 °C. The culture was equally divided, followed by adding isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG, Sigma Aldrich, St. Louis, MO) to one portion (final concentration: 1 mM; inducing LacZ mRNA expression) and adding D-(+)-glucose (Sigma, St Louis, MO) to the other flask (final concentration: 1 mM; suppressing LacZ mRNA expression). Both cultures were incubated for additional 6 hr at 37 °C. The \textit{E. coli} cells were then harvested by centrifugation. Total RNAs of \textit{E. coli} cells were extracted and purified using MasterPure Complete RNA Purification Kit (Epicenter Biotechnologies Madison, WI). The final RNA samples were dissolved in 100 \(\mu\)L of TE buffer [10mM Tris-HCl (pH 7.5), 1mM EDTA]. The quantities of the IPTG-induced
and glucose-suppressed total RNAs were determined by UV-Visible spectrophotometer (Varian Inc., Sana Clara, CA).

5.4. Target RNA extraction and purification

Proper RNA detection is preceded by RNA extraction process. This step must be handled with a lot of caution due to the sensitive nature of RNAs. There are several procedures for RNA extraction depending on the source of the RNA. Bacteria do not have poly-A tail therefore it is difficult to separate mRNAs from the total RNA. On the other hand, total RNA requires less handling and even works better therefore there is usually no need for mRNA isolation procedures unless need be. Traditional RNA extraction protocol involves use of phenol/chloroform extraction which has been criticized due to the use of potentially dangerous procedure and its laborious.\(^{42}\)\(^{161}\)\(^{162}\) A variety of total RNA purification kit are commercially available from different commercial sources i.e. Invitrogen, Qiagen, Ambion and TRIzol which is based on phenol extraction method.

The RNA from both IPTG-induced and glucose-suppressed E. coli cells was extracted using the MasterPure RNA Purification protocol. The salt based-RNA extraction protocol effectively removes proteins including enzymes. Removal of DNA by RNase-free DNase 1 not only increases sensitivity but also helps reduce nonspecific binding and background. Total RNA is precipitated using isopropanol with a final rinsing done with ethanol (2 x). The RNA is dissolved in TE buffer followed by UV measurement to determine the concentration and purity. Purity was determined by calculating 260/280 with a ratio of 2 considered to be pure RNA. High purity RNA was required in these experiments with a value <1.9 affecting the signal. Further purification of lower ratio RNA was done using phenol-chloroform extraction but this did not improve the purity of the RNA.
5.4.1 Experimental; total RNA extraction and purification from E. coli cells

Total RNA of E. coli cells was extracted and purified according to MasterPure Complete RNA Purification kit with some modifications (Epicenter Biotechnologies Madison, WI). After purification, the purity and concentration of the total RNA were determined by measuring absorbance using UV-Visible spectrophotometer (Varian Inc., Sana Clara, CA). The RNA was dissolved in TE buffer (10mM Tris-HCl [pH 7.5], 1mM EDTA) and stored in -80 °C.

5.5. Detection of lacZ RNA from E. coli total RNA.

Detection of specific RNAs from total RNA presents a number of challenges. For one, the RNAs tend to be long and have ability to form secondary structures and tertiary structures. This may hinder efficient probe-target duplex formation especially because microarray experiments use shorter probes. This may lead to reduced hybridization efficiencies and therefore low signals. Proper measures are therefore required to reduce the size of the RNA as well as the intrinsic secondary structures. One method to accomplish this is by fragmentation of RNAs to shorter sequences which has been shown to improve hybridization signals. Fragmentation can often be done via chemical, enzymatic or thermal mediation.\textsuperscript{163 164 165}

Secondly, it is not easy to label specific RNAs in an RNA mixture. This led to increased use of DNA microarrays which normally use transcribed and amplified RNA either as cRNA or cDNA. We therefore present a method for detection of mRNA from total RNA without reverse transcription. In our experimental procedure, we are able to label specific RNAs, only the ones captured by our specific probes. This is made possible by internal hydrolysis of all non hybridized RNAs using RNase H. Also, RNase H digests RNA/DNA duplex thus exposing the Klenow guiding sequence which enables internal labeling of the target RNA. In this way, we are able to directly label and detect mRNA in a total RNA mixture without amplification.
5.6 Alkali assisted fragmentation of *E. coli* total RNA

The covalent backbone of RNA is subject to slow, nonenzymatic hydrolysis of the phosphodiester bonds to yield a mixture 2’- and 3’-nucleoside monophosphates. This also leads to reduction in size into shorter, less structured fragments which can easily be available for hybridization. Hydrolysis can be mediated by bases which help cut the RNA into small fragments. Liu et al., found that heating rRNA with NaOH produced 20-100 shorter RNA which improved hybridization efficiency on the microarray.$^{163}$

In an effort to improve sensitivity, optimization of the fragmentation parameters was necessary. The chips were immobilized with three different probes; negative control (BF), *lacZ* and positive control (BA, 25 fmol). For each of the concentration, four different time variations were done to determine the best fragmentation condition time with the temperature held at 92 °C. The NaOH assisted hydrolysis of total RNA improved the signal as shown on Figure 5.2. Thermal denaturation alone did not have any signal (Chip A). While 20 mM NaOH had low signal except at longer timings, 100 mM NaOH was marred with a lot of background with a positive signal only observed at 5 min fragmentation. 50 mM NaOH was the best overall fragmentation concentration with a signal observed in all timings, i.e. 1, 5, 7 and 10 minutes. It was observed that 5 min gave a significantly better signal in all the three NaOH concentrations. Next, 5 min fragmentation time was tested for all the condition in one set-up to identify the best concentration using 30 µg total RNA (Figure 5.2). Chip A (0 mM) and chip B (20 mM) had barely any signal while chip D (100 mM) had a lot of background to a point where the blocking effect of the spotted probes can be observed. Also, this condition did not afford a consistent signal. Chip C which was treated with 50 mM NaOH had the best signal compared to the others and the signal was consistent too. Therefore, this concentration was chosen for other experiments. This concentration has been reported in literature but at lower temperature and longer timings.
5.6.1 Experimental

The chips were immobilized with three different probes; BF probe, lacZ probe and BA probe. RNA fragmentation was carried out by either heating in absence or presence of NaOH. For alkaline-catalyzed fragmentation, 10 µL of RNA (30 µg) of total RNA was digested with different concentrations of sodium hydroxide (NaOH) at 92 °C. Chip A sample was only heated in TE buffer, B; 20 mM NaOH, C; 50 mM NaOH and D; 100 mM NaOH. Heating was accomplished at 92 °C for 5 minutes. Previous experiments had involved different time course at all the concentrations to identify the right time. The hydrolysis reaction was stopped by adding 1N acetic acid. After cooling down, 5x SSC was added to the RNA sample, applied on the chip and hybridization done for 20 minutes. Thereafter, BA RNA was added and hybridization done for further 15 minutes. The unbound RNAs were washed twice with pre-warmed PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, pH 7.4). The chips were then incubated with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) at 37 °C for 15 minutes. The digested RNAs were followed by incubation with Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and 1 µL of 0.4 mM; biotin labeled 14-dATP (Invitrogen, Carlsbad, CA) at 37 °C for 30 minutes followed by washing three times with PBS buffer. This was followed by incubation with Poly-HP streptavidin (Thermo Scientific, Rockford, IL) diluted 5000x in TBS buffer for 15 minutes followed by washing (5x) with PBS to remove unbound enzymes. SuperSignal ELISA Femto Maximum sensitivity substrate (Invitrogen, Carlsbad, CA) was applied on the chips and the chemiluminescence signal captured immediately with a CCD camera.
Figure 5.2: Alkaline assisted fragmentation conditions optimization results. Similar amounts of total RNA (30 µg) were fragmented for 5 minutes and applied on each chip BA acted as positive control (25 fmol) and BF as a negative control.

5.7 Metal assisted *E. coli* total RNA fragmentation

Metal cations have been used as candidates to assist in degradation of nucleic acids to shorter fragments. Metal catalyzed fragmentation can generate fragment in the region of 50-100 nucleotides. Research by Browns (2002) indicated Mg$^{2+}$, Zn$^{2+}$ and Ce$^{3+}$ performance increased with decreased concentration of the three metals with the size of the RNA ranging between 50 – 100 nucleotides. Fragmentation was shown to substantially result in a significant enhancement of hybridization signal. Zn$^{2+}$ and Mg$^{2+}$ are also the main components of commercially fragmentation buffers.

Generally, lower temperatures and longer times are used for fragmentation methods but with an aim to develop rapid methods, higher temperatures and shorter times were investigated in our experimental set up with good results. Thermal and metal catalyzed, i.e. ZnCl$_2$ and MgCl$_2$ fragmentation were investigated in this set-up. All fragmentation was done at ~92 ºC and the metal ion concentration was 20 mM. Even though thermal denaturation has been shown to improve signal, in our case, there was barely any signal observed as well as 20 mM MgCl$_2$ (Figure 5.3). As shown in figure 5.3, comparison with alkali catalyzed fragmentation indicates similar
results for 20 mM Zn$^{2+}$ and 50 mM NaOH but Zn$^{2+}$ catalyzed fragmentation was not consistent among batches which led to the use of 50 mM NaOH fragmentation as our method of choice. The positive control signal is very high even at lower femtomole.

5.7.1 Experimental

The chips were immobilized with three different probes; BF probe, lacZ probe and BA probe respectively. For metal ion-catalyzed fragmentations, the total RNA was incubated with 20mM of MgCl$_2$ and ZnCl$_2$ in 25 mM of Tris-HCl (pH 7.4) at 92 °C for 3 min each. Thermal fragmentation was done for 2 minutes while NaOH fragmentation was done for 5 minutes after which neutralization was necessary. After cooling down and centrifuging, 5x SSC was added to the fragmented total RNA and later applied to the chips. The chips were incubated at 37 °C for 20 min after which 1 µL (10 nM) BA was added. This was further incubated for 15 minutes after which hybridization was stopped by washing the chips with mildly warm water. Further experimental procedure on the chips was followed as outlined in alkali catalyzed fragmentation experimental section (Section 5.6.1).

Figure 5. 3: Metal assisted fragmentation. Similar amounts of total RNA (10 µg) was fragmented and applied on the chips. BF acted as a negative control.

5.8. Specificity of detection

Specificity was also illustrated using IPTG-induced *E. coli* and glucose-suppressed *E. coli* at 50 mM NaOH concentration. The two works in the opposite direction. IPTG is able to
mimic lactose as a carbon source which bind to the lac repressor which leads to transcription of the gene (Figure 5.4, chip A). The presence of E. coli can be detected by targeting lacZ gene. On the other hand, glucose indirectly inhibits the lac expression by reducing cAMP, therefore there is no cAMP-CAP complex that activates transcriptome by binding to promoter. In this case, the gene is not transcribed (Chip B). The RNA was fragmented at 92 °C in 50 mM NaOH for 5 minutes.

![Image of chip A and B](image)

**Figure 5.4: Specificity of detection using E. coli total RNA.** A; had IPTG-induced total RNA applied to it while B glucose-expressed total RNA.

### 5.9 Direct detection of lacZ RNA from alkali lysed bacteria

Direct lysis of cells with NaOH and sodium dodecyl sulphate (SDS) has been reported previously for the extraction of plasmid DNA from bacteria. Normally, SDS, an ionic detergent lyses the membrane by dissolving the phospholipids and protein components of the cellular membrane while NaOH loosens the cell walls and releases both the plasmid and chromosomal DNAs. NaOH also denatures the cells. The plasmid extraction protocol relies on potassium acetate to precipitate the bacterial proteins, broken cell walls and nucleases. Upon centrifugation, plasmid DNA and lower molecular weight RNA molecules remain in the supernatant.

NaOH lysis of bacteria cells was envisioned as a simple alternative which may provide adequate RNA for the direct detection on the RNA microchip. This method will enable a simple, easy and rapid RNA extraction procedure devoid of enzymes, potentially hazardous organic solvents or commercial kits. After the simple workout, the supernatant should contain the nucleic acids i.e. RNA and DNA for detection on the chip or other detection methods. RNA handling
time is reduced by several hours or days thus reducing degradation. The method is simple and cost-effective requiring only NaOH and acetic acid for neutralization purposes. The method is even easier than phenol/chloroform extraction that requires proteinase K for protein digestion before extraction among other reagents.\textsuperscript{161}

It can be observed that this simple NaOH treatment worked as shown on Figure 5.5. The \textit{E. coli} cells were treated with 25 µL of 50 mM, 100 mM, 200 mM of NaOH for chips A, B, and C respectively. This was followed by neutralization after which the RNA was diluted in 5x SSC and applied to the chips. Chip B had the best signal followed by chip A. Precipitation on the surface was observed on chip C during hybridization step. Even washing with gently hot water could not help remove the white precipitate. This is an indication that the NaOH concentration was quite high to cause precipitation. Any kind of precipitation on a chip surface leads to a lot of background. Chip C indicates that BA which was added 20 minutes later can penetrate through the white precipitate. This can be attributed to the fact that BA synthetic RNA is short compared to the \textit{E. coli} total RNA.

Further conditions optimization was necessary to ensure no precipitation on the surface. Normally in all the other chips, 25 µL of NaOH was used for fragmentation but no more than 15 µL was applied on the chip. Reduction of the NaOH volume and increase in concentration to give a final concentration of 200 mM was found to improve the results.

\textbf{5.9.1 NaOH treatment of \textit{E. coli} cells and extraction of total RNA}

\textit{E. coli} was generated as explained in the procedure above (section 5.3). For the optimization of NaOH fragmentation, the chips were spotted with three probes; a negative control (IL8), positive control (BA) and \textit{lacZ}. Similar amount of pelleted \textit{E. coli} (1 mL) was used in chips A, B and C. Three different NaOH concentrations were used; A; 50 mM; B; 100 mM and C; 200 mM. The volume used on A, B, and C was 25 µL. The \textit{E. coli} pellet was vortexed to mix and later heated at 92 °C for 5 minutes. The sample was cooled in ice, centrifuged and the precipitate was discarded. The supernatant was neutralized with 1N acetic acid and centrifuged briefly.
5.9.2 Detection on the microchip

The NaOH extracted RNA (12.5 µL) was added to 5x SSC and applied on chips A, B and C respectively. The positive control was added 20 minutes afterward at 5 fmol. The other experimental procedure is as outlined in the alkali fragmentation section (Section 5.6.1).

![Image of chips A, B, C with probe signals]

Figure 5.5: Conditions optimization for NaOH *E. coli* total RNA extraction. Lysis and fragmentation accomplished at 92 °C for 5 minutes.

5.10 Specificity of NaOH bacteria lysis and fragmentation on the microchip

Specificity was tested using both IPTG-induced and glucose-suppressed NaOH extracted total RNA. As explained above, glucose suppressed is a good negative control model which mimics the bacteria cell other than the synthetic RNA normally used. Figure 5.6 shows specificity results. Chip A had IPTG induced RNA while chip B had glucose suppressed RNA. Only a very small signal was observed on Chip B which means the extraction is quite specific. It is also good to note that the negative control did not show up in any of the chips.

5.10.1 Experimental

*E. coli* was generated as explained in the procedure above (Section 5.3). For the optimization of NaOH extraction, the chips were spotted with three probes; a negative control (IL8), positive control (BA) and *lacZ*. 25 µL of 100 mM NaOH was added to two tubes with 1 mL of harvested *E. coli* pellet and vortexed to mix. Tube A contained IPTG-induced *E. coli* while tube B
contained glucose-suppressed *E. coli*. The tubes were incubated for 5 min at 92 °C. After cooling, the tubes were centrifuged; the supernatant was removed and neutralized with 1N acetic acid. The supernatant (12.5 μL) was mixed with 5x SSC and applied on chip A and B respectively. BA (5 fmol) was applied to the chips after 20 minutes. The rest of the procedure was followed as indicated in alkali fragmentation section (Section 5.6.1).

![Image](image_url)

**Figure 5.6: Specificity of NaOH lysis and *E. coli* fragmentation.**

### 5.11 Detection of lacZ mRNA on the microchip without neutralization

Interestingly, it was observed that the NaOH extracted RNA could be directly detected on the chip without neutralization (Figure 5.7). These unexpected results could have been possible because the handling time is less therefore the RNA was not hydrolyzed to destruction as is normally believed. Even RNA as short as 40 nt (BA) could be detected by incubation at this harsh alkali conditions.

As previously noted, fragmentation was done using 25 μL of NaOH but not more than 12.5 μL of the supernatant was added on the chip. There was therefore more sample left over render direct measurement of the *E. coli* used void. Further fragmentation conditions were designed to reduce the volume of NaOH used but at the same keep the amount of NaOH the same. Different concentrations were therefore tested with 400 mM (6.5 μL) giving the best signal. This amount gives the final NaOH concentration as 200 mM. More *E. coli* (1.5 mL) was also
used to avoid the uneven hybridization on the chip as represented by the black holes at the centre of the spot. With this condition, there was no precipitation and the signal is better and cleaner as compared to the others. Further simplification involved elimination of neutralization step which also gave a positive signal (Figure 5.7). It can be concluded that NaOH not only improved extraction but also fragmentation as observed with the high hybridization signal.

5.11.1 Experimental

*E. coli* was generated as explained in the procedure above (Section 5.3). For the optimization of NaOH extraction, the chips were spotted with three probes; a negative control (IL8), positive control (BA) and *lacZ*. Total RNA from *E. coli* cells (harvested form 1.5 mL) were heated with 6.5 µL of 400 mM at ~92 °C for 5 minutes. This was followed by cooling down, centrifuging for a few minutes. The RNAs were mixed with 5x SSC and applied on each chip. The other procedure on the chip is as indicated in alkali fragmentation section (Section 5.6.1).

![Image of chips with probes IL8, BA, and lacZ](image)

*Figure 5.7: Direct detection of NaOH lysed bacteria RNA without neutralization*
5.12 Summary

Direct and rapid detection of lacZ gene mRNA from E. coli total RNA was reported with high sensitivity. IPTG was used to induce the expression of lacZ mRNA while glucose repression afforded a negative control. The RNA microchip was specific for detection of lacZ from the bacteria total RNA. By using NaOH (50 mM), fragmentation of total RNA and detection thereafter gave the best results. Detection was accomplished to lower femtomole as indicated by the positive control signal. RNA probes with as low as 5 nt (5M) was also detected on the chip and discriminated from others. This is an indication that the RNA microchip can be used even with degraded RNAs; only 5 nt are required at the RNase H specific region. This is an important aspect since labeling of degraded probes with the current methods is not easy.

A simple and rapid total RNA extraction protocol was also developed and validated. By treating bacteria cells directly with NaOH for 5 minutes, we are able to directly and specifically detect lacZ form IPTG induced bacteria cell lines only. The method is rapid with minimum RNA handling steps thus any person can be able to use it. Elimination of commercial RNA purification kits ensures cost effectiveness and results in a more rapid detection method. Detection was accomplished at $10^6$ CFUs and even as low as $10^4$ CFUs could be detected without amplification. NaOH lysis of bacteria is an easy and rapid method for extraction of total RNA and even gives better results.
6. DENGUE FEVER SEROTYPING ON THE RNA MICROCHIP

6.1 Introduction

Virus infections have been one of the most dangerous threats to human, animal and agricultural health. An example is the influenza pandemic, commonly referred to as Spanish flu of the early 20th century that caused more than 20 million deaths worldwide. Although the recent pandemic of the H1N1 influenza strain was far less dramatic, the current H7N9 is feared to be more deadly (WHO updates; 2013). Such infectious pathogens can cause much human suffering and widespread economic loss.\textsuperscript{170, 171} The other most common pandemic viral infectious disease was HIV/AIDS which was clinically observed in 1981 in the US and by 2009, it had led to 30 million deaths. Currently it is estimated there are \textasciitilde30 million living with AIDS.\textsuperscript{172} Others include, West Nile virus, an arboviral infection prevalence in the US, the tropics and subtropics,\textsuperscript{173} chikungunya virus, a positive RNA alpha virus\textsuperscript{175} and dengue virus, an arthropod-borne viral disease. The situation is complicated by re-emergence of the infectious diseases and a tendency to adapt to new environment.\textsuperscript{104}

Dengue is a viral disease transmitted by \textit{Aedes aegypti} mosquito. It is estimated that greater than one-third of the world’s population lives in areas at risk for transmission.\textsuperscript{176} Dengue poses significant health risks for individuals living in the tropics and subtropics. As many as \textasciitilde50 million people are infected annually mainly in the Southeast Asia, the pacific and the Americas. Africa and Eastern Mediterranean are also having increased transmission.\textsuperscript{177} Sporadic cases of locally acquired disease have been reported in the US since 1980. Outbreaks have been reported in Texas\textsuperscript{178} and most recently Florida.\textsuperscript{179, 180} Dengue related infections are becoming the most common cause of fever among travelers in the United States. Dengue is therefore becoming the most rapidly spreading vector-borne disease in the world. The world health organization (WHO) has been designated dengue as a major international public health problem.

Dengue virus (DENV) belongs to the \textit{Flaviviridae} family and consists of 4 related serotypes, DENV 1-4. DENV is a spherical, positive single-strand RNA virus with a \textasciitilde11 kb genome.
All DENV share around 65% of the genome. Despite this, the clinical manifestations range from asymptotic infections to a severe disease characterized by dengue hemorrhagic fever and/or dengue shock syndrome (DHF/DSS). Infection by one of the serotype provides a lifelong immunity against that particular strain but not the remaining three, with secondary infection leading to DHS/DSS. The asymptomatic nature of dengue makes it difficult to isolate the case in the early phase of infection.

Dengue virus detection can be accomplished by testing either the viable virus, viral RNA, circulating viral antigens or host antibodies. Virus can be detected in the patient’s blood stream 4-5 days after onset of illness. The early diagnosis methods include culture based method, antigen detection and RT-PCR. After this incubation time, antibody detection offers the most sensitive results. Antibody detection can be accomplished using five serological tests: hemagglutination inhibition (HI), complement fixation (CF), neutralization test (NT), immunoglobulin M (IgM) and immunoglobulin IgG capture ELISA assays. While HI is the gold standard due to high sensitivity and reliability, the method lacks serotype specificity. ELISA antibody detection kits are more developed with commercially available thus they are widely used. But detection is complicated by a number of factors including the availability of a large pool of antigens, acquired immune response to infection, specimen type, the virus serotypes, presence of cross-reacting co-circulating flavivirus antibodies as a result of prior infection or vaccination as well as antigens used in ELISA. Both the IgM and IgG test also responds to antibodies for other flavivirus including, yellow fever and West Nile virus thus resulting in cross contamination. Therefore a researcher should put all these entire factors into consideration to achieve a correct ELISA diagnosis. Furthermore, the antibodies are better detected around the fifth day of disease onset making this unfeasible for rapid detection.

Molecular based detection methods have an advantage of targeting the unique genome of the virus. Nucleic acid hybridization assays e.g. RT-PCR, real-time PCR and NASBA have also been developed for dengue diagnosis. These tests could offer early diagnosis there-
fore lowering the risk of developing a severe disease. However, none of these tests have been approved by the Food and Drug Administration (FDA). WHO only considers PCR and RT-PCR as experimental methods for dengue detection. Besides, the two methods are limited in serotyping the four dengue viruses and often requiring extensive work to identify one or all of them.

There is therefore a need for a rapid, low cost and reliable diagnosis method that can identify the serotypes in the early phase. The molecular based detection method can also identify biomarkers that can help understand and predict the progression from classic dengue fever to the severe forms of the disease and better understanding of the pathogenesis of the disease. In addition, dengue diagnosis will be of importance for research in host, virus and vector characteristics, epidemiological conditions as well as antiviral drug and vaccine development. The RNA microchips are sensitive, rapid and have greater power in discrimination of SNPs which is an important aspect since the DENV viruses differ by single or a few mutations in their genome. Rapid and early detection will help in patient management which can lower the risk of developing severe disease. Development of a rapid, sensitive, accurate and cost-effective RNA microchip for DENV viral serotyping is investigated.

6.2. Results and discussion

6.2.1 DENV probe design and immobilization on the microchip.

The target RNAs and hybrid probes were designed from the DENV RNA genome for serotyping on the RNA chip. Four target RNAs were chosen from specific regions of the four DENV genomes to enable specific detection of each one of them. There was an inclusion of a general RNA that could target all four of them. The general RNA was same in all four serotypes. The hybrid probes are complimentary to the target RNAs. All the probes were modified with an amino group which affords immobilization on the chip surface. The probes have two regions; RNA region that is 2′-O-methylated and the other portion is a DNA region which serves as a guiding template for polymerase extension. The 2′-O-Methylation stabilizes the RNA region and
protects it against RNases. Figure 6.1A is a general probe that can target all the serotypes while Figure 6.1B is the four DENV serotype probes with SNPs highlighted against DENV-1. All four serotypes maintain their names in this dissertation while the general probe is named DENV-5.

A. DENV-1 RNA (Accession no.: U88536.1; 10627-10648 nt): 5'-AAACAGCAUAUUGA-CGCUGGGA-3'
DENV-2 RNA (Accession no.: U87411.1; 10617-10638 nt): 5'-AAACAGCAUAUUGA-CGCUGGGA-3'
DENV-3 RNA (Accession no.: AY099336.1; 10599-10620 nt): 5'-AAACAGCAUAUUGA-CGCUGGGA-3'
DENV-4 RNA (Accession no.: AF326825.1; 10541-10562 nt): 5'-AAACAGCAUAUUGA-CGCUGGGA-3'
DENV-5 (General probe) of all four dengue serotypes: 5'-TCCCAGCG-[2'-Me-\(r(UCAAUAUGCUU)\)]-NH\(_2\)-3'

B. DENV-1 RNA (Acc. no.: U88536.1; 10484-10504 nt): 5'-GGAAGCUGUACGC-AUGGGGUA-3'
DENV-2 RNA (Acc.: U87411.1; 10470-10490 nt): 5'-GGAAGCUGUACGC-AUGGGGUA-3'
DENV-3 RNA (Acc. no.: AY099336.1; 10457-10477 nt): 5'-GGAAGCUGUACGC-AUGGGGUA-3'
DENV-4 RNA (Acc. no.: AF326825.1; 10393-10413 nt): 5'-GGAAGCUGUACGC-AUGGGGUA-3'
DENV-1 Probe 1: 5'-TACCCCAT-[2'-Me-\(r(GCGUACAGCUU)\)]-NH\(_2\)-3'
DENV-2 Probe 2: 5'-TACGCCAT-[2'-Me-\(r(GCGUACAGCUU)\)]-NH\(_2\)-3'
DENV-3 Probe 3: 5'-ATACCCGT-[2'-Me-\(r(GCGUACAGCUU)\)]-NH\(_2\)-3'
DENV-4 Probe 4: 5'-TATGCCAC-[2'-Me-\(r(GCGUACAGCUU)\)]-NH\(_2\)-3'

Figure 6.1: The probes were designed using different regions of viral RNA genomes. (A) for dengue general probe detection using regions with identical RNA sequences across all serotypes; (B) for individual serotype detection. Underlined sequences are viral RNAs complementary to italicized 2'-O-Me-RNAs of RNA probes.

6.2.2 Chemiluminescence detection of DENV serotypes using 3'-labeling technique

Chemiluminescence detection on the RNA microchip can afford a fast, sensitive and direct RNA detection. The specific probes are able to capture the target RNAs on the RNA microchip. Internal RNase H digestion exposes the Klenow template which allows incorporation of biotin dNTPs. Use of streptavidin HRP followed by ELISA substrates generates the desired chemiluminescence signal (Figure 6.2).
First, all the probes were tested to ensure they worked on the chip. Figure 6.3 is an example of DENV-3 probe concentration optimization results. DENV-3 probe was immobilized at four different concentrations i.e. 50-300 µM. 500 fmol RNA was applied on the chip with hybridization, RNase H digestion and Klenow polymerization performed at 37 °C. Results indicate an increasing level of signal with increase in the concentration of the probe. This phenomenon was observed across all DENV RNAs with the exception of DENV-4 which showed only slight differences. A choice was made to keep 100 µM as the working probe concentration which would ensure low level of cross contamination in the presence of other probes. Therefore, 100 µM probe concentration was used in all the experiments. It is important to note that 50 µM also works with a high signal but the hybridization competition in this case reduced the signal.

-3’ -NH₂-2-O-Me- (CCUUCGACAU<sup>CG</sup>C) TGCCACAT-5’

RNA hybridization

5’-GGAAGCUGUACGC-ACGGUGUA-3’
-3’ -NH₂-2-O-Me- (CCUUCGACAU<sup>CG</sup>C) TGCCACAT-5’

RNase H digestion

5’-GGAAGCUGUACGC
-3’ -NH₂-2-O-Me- (CCUUCGACAU<sup>CG</sup>C) TGCCACAT-5’

Klenow extension with biotin labels

5’-GGAAGCUGUACGC-ACGGTGTA-biotin-3’
-3’ -NH₂-2-O-Me- (CCUUCGACAU<sup>CG</sup>C) TGCCACAT-5’

6.2: Flow chart for the direct DENV serotyping RNA microchip. DENV-3 probe was immobilized on the chip. RNA (red) binds via complimentary base pairing to the probe. RNase H then digests the RNA portion of the RNA/DNA duplex. The resulting single stranded portion of the DNA acts as a guiding sequence for Klenow incorporation with biotin labeled dNTPs. Streptavidin HRP binds to biotin which produces chemiluminescence signal upon ELISA substrate addition.
Figure 6.3. DENV-3 probe condition optimization. Denv-3 Probe: 3'–NH$_2$–2-O-Me–(CCUUCGACAU)$\text{G}$CG–TGCCACAT–5'. DENV-3 RNA; 5'–GGAAGCU$\text{G}$GUACGC–ACGGUGUA–3'. RNA hybridization, RNase H digestion and Klenow incorporation were done at 37 °C.

6.2.3 RNA concentration optimization

Further optimization of RNA was done by spotting all the probes as shown on Figure 6.4 but only DENV-4 RNA was applied on both chips; 500 fmol on chip A and 100 fmol on chip B. RNA hybridization (15 min), RNase H digestion (15 min) and Klenow polymerization (30 min) were all performed at 55 °C. As shown on both chips, DENV-4 signal was comparable at both RNA concentrations but 100 fmol RNA had better specificity. Chip B had relatively low cross hybridization as observed with DENV-1 and DENV-2 signal but this can be eliminated with further conditions optimization. This led to choice of 100 fmol RNA as the working concentration for all other experiments. The experiments were done at elevated temperatures to improve stringency which also improved the signal.
DENV-4 RNA: \[5' - \text{GGAAGCUGUCGC-GUGGCAUA-3'}\]

DENV-4 Probe: \[3' - \text{NH}_2-2\text{-O-Me-(CCUUCGACAUGCG)CACCGTAT-5'}\]

Figure 6.4. DENV-4 conditions optimization. Five DENV probes (100 µm) were spotted on the chip. Different DENV-4 RNA concentrations were applied on each chip; chip A had 500 fmol while chip B had 100 fmol. RNA hybridization, RNase H digestion and Klenow were done at 55 °C.

6.2.4 DENV-2 detection on the RNA microchip

As shown with the RNA genome, DENV serotypes only differs with a few mutations. It is important to note that while the symptoms are similar and asymptotic, secondary infection can be quite severe. This spurs the need for DENV serotyping on the RNA chip as a valuable indicator for clinical diagnosis. This will help in infection management and disease therapy which could prevent occurrence of DHF/DSS thereby helping save lives. But there is lack of a simple, reliable and easy to use molecular detection method that can differentiate all the four serotypes. The RNA microchip could be a versatile tool for serotyping the virus.

SNPs often introduce perturbations to the double helix which in turn reduces the binding interaction and stability of the duplex which is commonly characterized by lower T_m. By taking advantage of this unique duplex stability, one can be able to differentiate between closely related species. Conditions optimization will eliminate detection resulting from cross hybridization which can result in false positives. False positives could be detrimental in life threatening situations especially when one gets a second infection with a different serotype.\[176\] \[186\]
By taking advantage of the SNPS effect on hybrid stability on the RNA microchip, we can be able to detect all four serotypes specifically. These requires conditions optimization to ensure only the perfect match are able to form a hybrid duplex. As shown in Figure 6.1B above, all four DENV target RNA differ from DENV-1 by 1, 2, 3 respectively, but taken individually, each DENV differ from each other by different SNPs, therefore, it is important to be able to differentiate each one of them from the others. As shown in Figure 6.4, DENV-4 was easily differentiated from each other but DENV-2 proved a formidable task with various different conditions tested. Below is the probe sequence with SNPs underlined in relation to DENV-2 while Figure 5 shows some of the conditions tested on the chip to ensure DENV-2 RNA specific detection.

DENV-2 RNA: 5’-GGAAGCUGUACGCUGGA-3’
DENV-2 Probe: 3’-NH2-2-O-Me-(CCUUCGCAUGCG)TACCGCAT-5’
DENV-1 Probe: 3-NH2-2-O-Me-(CCUUCGCAUGCG)TACCCCAT-5’
DENV-3 Probe: 3’-NH2-2-O-Me-(CCUUCGCAUGCG)TGCCACAT-5’
DENV-4 Probe: 3’-NH2-2-O-Me-(CCUUCGCAUGCG)CACCGTAT-5’

Figure 6.5: Optimization of conditions for DENV-2 target RNA. All the probes were printed on the chip at 100 µm. The conditions for RNA hybridization, RNase H digestion and Klenow incorporation are as shown below each chip.

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Temp</th>
<th>Time</th>
<th>Temp</th>
<th>Time</th>
<th>Temp</th>
<th>Time</th>
<th>Temp</th>
<th>Time</th>
<th>Temp</th>
</tr>
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<tbody>
<tr>
<td>RNA</td>
<td>10</td>
<td>65</td>
<td>10</td>
<td>70</td>
<td>10</td>
<td>75</td>
<td>5</td>
<td>70</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>RNase H</td>
<td>5</td>
<td>65</td>
<td>5</td>
<td>70</td>
<td>5</td>
<td>75</td>
<td>5</td>
<td>70</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>Klenow</td>
<td>10</td>
<td>65</td>
<td>10</td>
<td>70</td>
<td>10</td>
<td>75</td>
<td>5</td>
<td>70</td>
<td>2</td>
<td>65</td>
</tr>
</tbody>
</table>

As shown in the Figure above, DENV-2 RNA detection had notable hybridization competition with DENV-1 and DENV-4. From the sequences, there is only one single nucleotide between DENV-2 RNA and DENV-1 RNA. It appears that a C:C is well tolerated during hybridization and enzymatic incorporation. This could be explained by the fact that the C:C is small thus
don’t afford very high perturbation but with temperature control, specificity can be achieved. DENV-4 was consistently observed even though DENV-2 and DENV-4 differs by two SNPs and hybridization will afford A: C and G: T mispairing (chip B and D). It is amazing how this two are tolerated even by RNase H and Klenow. Specificity was only afforded at high temperatures and the reduction of time with RNA hybridization, RNase H digestion and Klenow incorporation done at 2 minutes (Chip E). While detection at two minutes indicates the possibility of development of a very rapid RNA chip, the signal was not consistent. Lower salt concentrations for hybridization step has been shown to increase stringency by forcing the partially complimentary sequences to remain apart, but in our case, 2x SSC and 1x SSC did not increase specificity, in fact only DENV-1 and DENV-4 had a higher and comparable signal than DENV-2 (data not shown). Even though the enzymes used are not thermostable, they seemed to work well up to 70 °C with very low signal above this temperature (Chip B). This can be attributed to the short reaction times for the enzymatic steps. It is also important to note that high degree of caution is required when doing these experiments to avoid cross contamination attributable to human error.

### 6.2.5 RNA microchip for DENV serotypes detection

Conditions optimization was done for all DENV target RNAs to find a condition that could afford the highest specificity against other RNAs. Figure 6.6 shows the signal and the conditions for each probe which afforded the best specificity. Since DENV-5 is a general probe, optimization was not necessary but the most common condition was used in this case. Except for DENV-2, all the others there serotypes (DENV-1, DENV-3, DENV-4) were well discriminated in the presence of other probes at a very high confidential level. DENV-3 was easily discriminated at mild conditions compared with the others but the high stringency conditions should be okay. As noted above, DENV-2 specificity was also achieved detected but it requires high stringency conditions for specific results. Even then, signal spreading was observed. Figure 6.6 shows the final DENV serotyping results on the RNA microchip.
Figure 6.6: Similar probes were printed on each chip but different serotype specific RNA was applied on each chip. T °C stands for temperature while t stands for time in minutes.

By targeting the genome which is specific for each serotypes, we are able to avoid the limitations observed with the serological tests as mentioned in the introduction, i.e. HI, CF, NT, IGM –ELISA test. These limitations include cross-reactivity thus requiring a comprehensive pool of antigens, the four serotypes and other flaviviruses causing similar clinical manifestations. In addition, the test can only be done five days after infection. IgM levels have been shown to vary in patients, infection period and the serotypes as well a consequent secondary infection. This complicates results interpretation. The RNA microchip will therefore be a great tool since the genome can be detected as early as the symptoms are observed with specific results by use of target specific probes. Detection of two different DENV RNAs can also be an indication of secondary infection and proper measures can be taken to reduce severe form of dengue fever.

Molecular methods such as nested PCR and RT-PCR have also been used for detection and serotyping studies. Generally, these methods report dengue infection without an indication of which serotypes further method modification can allow multiplex analysis that allow serotyping. The amplification methods can be time consuming because of the rigorous steps required from primer design and synthesis, RNA extraction, RT-PCR and fractionation. In addi-
tion, nested PCR involves two PCR reactions and only one serotype can be identified. On the other hand, RT-PCR can be used for serotyping but this requires labeling each probe with a specific signal reporter and the subsequent signal analysis is time consuming. Besides, PCR amplifications are prone to errors even though the primers are designed from regions without mutations. But in our case, only four probes are required to serotype the viruses. The RNA microchip is more superior in single nucleotide polymorphism, sensitive, fast and offers a direct detection method without amplification.

6.3. Experimental

6.3.1. DENV RNA probe design and immobilization on the RNA microchip.

The target RNAs and hybrid probes were designed from the RNA genome with inclusion of a general probe that was identical in all four serotypes. The probes were synthesized in our lab by using solid phase phosphoramidites chemistry and thereafter purified using Glen Pak column. The hybrid probes were diluted to 100 µM with sodium phosphate buffer (100 mM, pH 8.5) followed by printing on the functionalized microchip using Omnigrid Micro (Cartesian Technologies Inc., Irvine, Ca, U.S.A). Immobilization was achieved by incubation at 37 °C for 30 min.

6.3.2. DENV serotypes detection on the RNA microchip

After immobilization, the chips were incubated with StartingBlock (TBS, Pierce, Rockford, IL) blocking buffer for 20 min. The chips were then flooded with 100 fmol target RNA in 5 x SSC buffer (0.75 mM NaCl, 75 mM sodium citrate, pH 7.0) followed by incubation at elevated temperatures and time. The unbound RNAs were pipette off followed by incubation with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) at elevated temperatures with different time courses. The digested RNAs were then pipette off followed by incubation with Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and biotin labeled dNTPS (0.25 µL per chip, 0.4 mM); biotin 14-dATP (Invitrogen, Carlsbad, CA), biotin 14-dCTP (Invitrogen, Carlsbad, CA), biotin 11-dGTP (PerkinElmer, Waltham, MA) and biotin 11-dUTP (PerkinElmer, Waltham, MA) at
elevated temperatures followed by washing with PBS buffer. Thereafter, the chips were incubated with Poly-HRP streptavidin (5000x) in TBS buffer for 15 minutes (Thermo Scientific, Rockford, IL). The unbound enzyme conjugates were rinsed five times with PBS. SuperSignal ELISA Femto Maximum sensitivity substrate (Invitrogen, Carlsbad, CA) was then applied to the chip and the chemiluminescence signal captured using a CCD camera.

6.4. Summary

Our study describes rapid, specific and easy to use method for early detection of dengue viruses. Even though the genome differs by only small regions, we are able to discriminate the serotypes by designing DENV serotype specific probes. The probes target regions that are similar but differ with a few mutations. Specificity studies indicate each of the DENV serotypes can be detected individually in the presence of other DENV probes. This was made possible designing DENV serotype specific probes to target each probe. Further specificity is afforded by RNase H and Klenow incorporation to ensure only the present RNA was detected in the presence of the DENV probes. This method is highly specific both at the 2-O-Me region and the Klenow template.113
7. RNA MICROCHIP BASED ON GOLD NANOPARTICLES DETECTION

7.1 Introduction

Gold nanoparticles (AuNPs) based nucleic acid detection methods have become the basis of an increasing number of diagnostic applications. Methods utilizing oligonucleotide functionalized gold nanoparticles takes advantage of the Au affinity for sulfur and their intense surface plasmon resonance and the capabilities for controlling particle size and composition. Upon hybridization with the target, AuNPs afford various read out methods such as optical detection methods; light scattering, spectroscopy i.e. surface-enhanced Raman (SERS) as well as electrochemical methods.

Another technique is the use of gold nanoparticles functionalized protein probes e.g. streptavidin. The target is labeled with biotin which upon binding with streptavidin provides a reporter system for signal generation. Silver staining relies on the gold autocatalytic nature which reduces silver ions into metallic silver. The silver shell around AuNPs also autocatalyzes further depositions resulting in a signal enhancement with a growth of nanoparticles. The signal generated by this methods result from precipitation of silver on labeled AuNPs. Silver enhancement is electron dense thus the images can be detected by naked eye or by use of a visible light scanner or an instrument that measures scattered light from the silver spots.

Streptavidin labeled AuNPs offers an advantage over oligonucleotide labeled AuNPs since the latter involves preparation of specific probes for each application. The silver staining process is an autocatalytic process allowing signal amplification up to 100 times. The metallic deposition is not quenched and does not decompose thus can be stored for a long time whereas fluorescence intensity is sensitive to a variety of quenchers including glass surfaces and the molecules tend to decompose. Also, the same visual detection chip can be used as a light reflection-based chip since silver strongly reflects/scatters light in a visible spectrum. A single nanoparticle can scatter as much as $10^6$ fluorescein molecules and are resistant to photo bleaching. The method can be used for various support surfaces other than glass e.g. acrylic layer.
in comparison with fluorescence detection for which plastic arrays cannot be used due to the strong auto fluorescence of polymers. Silver deposit is stable and can be stored for a long period of time. Use of AuNPs provides a rapid, specific and low cost detection of nucleic acids. The visual detection RNA chip will be a powerful tool in pathogen global gene expression profiling,\textsuperscript{38} pathogen detection,\textsuperscript{202} drug development and cancer therapeutics.\textsuperscript{36a}

To take advantage of this technology and tackle RNA detection challenge, we have developed a rapid RNA detection on RNA microchip based on AuNPs and silver staining. The method takes advantage of nucleases and polymerase activities which allows direct detection of mRNA using biotin labeled dNTPs and streptavidin AuNPs. Silver staining leads to autocatalytic deposition of zinc metal on the AuNPs. The signal is captured using a CCD camera. The colorimetric silver detection can be used as a rapid, easy and sensitive tool for pathogen detection. The method specificity will be investigated by use of DENV virus RNA and lacZ mRNA. Development of a multi-marker detection sensor will be investigated using HNC RNAs and different pathogenic RNAs. Sensitivity and rapidity which are also desired properties for a biological sensor will also be investigated using lacZ. Biological sample detection and application into pathogen detection will be evaluated using lacZ mRNA.

7.2. Pathogen mRNA detection

7.2.1 Introduction

Bacterial and pathogens pose a significant threat to human, animal and agricultural health. \textit{B. anthracis} has been posed as a biological weapon for a long time.\textsuperscript{203} \textsuperscript{204} More recently, the 2001 anthrax attacks lead to 22 infections, 5 deaths, and over $1 billion damages. More hoaxes were reported which cost the United States government millions of dollars. Predominant techniques for pathogen detection rely on conventional microbiology approaches which are laborious, time-consuming and require labile natural products. Detection of pathogens through nucleic acid based technologies is a promising approach especially with our multi-marker platform. Development of rapid, sensitive and specific assays for these organisms is necessary in a
number of fields including food safety, animal health care, diagnostics, pathology and clinical research, bio-defense and forensics.

### 7.2.2 Target selection

Infectious disease has been a major challenge to human development and survival. Epidemics of old and emerging infectious diseases coupled with unpredictable outbreaks continue to be a global burden.\(^{104}\) Also rapid and direct detection of infectious agents feared as bioterrorism agents such as *B. anthracis* and H1N1 influenza is needed to reduce the effect of such outbreaks. Morens et al., have outlined a wide range of infectious diseases occurring globally which include viral and bacterial infections.\(^{104}\) Once the target has been selected, the next step is selection of gene of interest from the targeted organism. The gene or protein chosen should be species specific e.g. the lethal factor (LF) gene for *B. anthracis* is not present in any other member of the *Bacillus* family.\(^{204}\) Table 6.1 shows the species and the gene targets selected for this study. The target mRNAs sequence were selected from a region that had several concurrent A`s. An alignment with BLAST ensures the sequences are targets specific.

### Table 7.1: Gene selection table. The gene is selected from a protein or gene which encodes for a particular toxin or virulence factor

<table>
<thead>
<tr>
<th>Target</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (Wild type)</td>
<td><em>LacZ</em></td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> (BA)</td>
<td>Lethal factor (LF)</td>
</tr>
<tr>
<td>Bird Flu (H5N1; BF)</td>
<td>Matrix protein (M1)</td>
</tr>
<tr>
<td>Avian Flu (H5N1; AF)</td>
<td>Matrix protein (M2)</td>
</tr>
<tr>
<td>Dengue fever</td>
<td>Viral RNA</td>
</tr>
</tbody>
</table>
7.2.3 Probe design and oligonucleotide library

Probes were thereby designed from the target mRNA. Hybrid probes were designed as outlined in chapter 3 with the last DNA portion consisting of multiple Ts to enable incorporation of biotin labeled dATPs only which is a departure from HNC and DENV probes. The versatility of probe design enables a wide application of this method. Four different pathogens; *E. coli*, bird flu, avian flu and *bacillus anthracis* were synthesized for the model visual detection RNA microchip. Below is the oligonucleotide library for multi-pathogen with the RNA genomic region indicated in brackets.

*lacZ* mRNA (*lacZ*, *E. coli lacZ* mRNA, 724-748 nt): 5’-AUGUGGAUUGGCGAUAAAAAACAA-3’

*lacZ* probe: 5’-d(GTTGTTTTTT)-2’-O-Me-RNA(AUCGCAAUCCACAU)-d(CTGTGAAAGA)-NH₂-3’

*Bacillus anthracis* RNA (BA, *B. anthracis* lethal factor mRNA, 855-892 nt): 5’-

AUCUUUAGAAGCAUAUCUGAAGAUAAGAAAAAAA-3’

BA probe: 5’-d(GATTTTTTTT)-2’-O-Me-RNA(CUUAUCUUCAGAUAA)-d(TGCTTCTAAAGAT)-NH₂-3’

Bird Flu RNA [BF, Avian Influenza (H5N1) matrix protein 1 (M1) mRNA, 692-729 nt]: 5’-

AAUCUUCUUGAAAAUUUGCAGACCUCACAAACACGA-3’

BF probe 1: 5’-d(TCGTTTTTT)-2’-O-Me-(GGUAGGUCGAAAAUUUU)-d(CAAGAAGATT)-NH₂-3’

Avian Flu RNA [AF; Avian Influenza (H5N1) matrix protein (M2) mRNA, 838-872 nt]: 5’-CAU UUAUCGUCGC CUUUAAAUAACG GUUUG AAAAGAG-3’

Avian Flu Probe: 5’-d(CT CTTTT)-2’-O-Me-(CAAC CGUAUUAAAG)-d(GCGACGATAA ATG)-NH₂-3’

Biotin labeled DNA for rapid detection (*lacZ* DNA): 3’-NH₂-

AGAAAGTGTCTACACCTACCGCTATTTTGTGG-biotin-Cy3-5’
7.3 3'-labelling technique

Our lab has developed a method that affords labeling and detection of mRNAs in an RNA mixture. Our system is based on RNA 3'-labeling approach where a DNA polymerase incorporates hapten labeled dNTPs directly on a DNA template immobilized on a microchip.\textsuperscript{112, 113} Before incorporation of the labels such as biotin labeled dNTPs (Figure 7.1) the 3'-region is removed by RNase H in the presence of a guiding sequence. This is followed by antibody conjugate binding using streptavidin AuNPs. Upon addition of silver staining solution (silver ions in the presence of a reducing agent), gold nanoparticles autocatalyze precipitation of silver metal. The images of the dark spots can easily be detected with the naked eye.

![Figure 7.1: Flow chart for the direct and visual detection RNA microchip. RNA (red) binds via complimentary base pairing to the last section of the chimeric probe. RNase H then digests the RNA portion of the RNA/DNA duplex. The resulting single stranded portion of the DNA acts as a guiding sequence for Klenow incorporation with biotin labeled dNTPs. Streptavidin labeled gold nanoparticles (AuNPs) binds to biotin which produces a visually detectable signal upon silver staining.](image)

7.4. Control experiments to identify the source of the signal

Control experiments were also done to identify the source of the signal as shown in Figure 7.2. It can be observed that absence of target RNA (chip A) and biotin labeled dNTPs (chip B) in
different chips doesn’t yield any signal as well as use of dNTPs with Klenow extension (Chip C). This concludes the source of the signal observed on chip D was as a result of RNA hybridization followed by RNase H digestion, Klenow extension with biotin-labeled dNTPs which upon binding with gold labeled streptavidin and silver staining afforded the desire signal (black spot). Blocking using StartingBlock blocking buffer reduces the background which is one of the challenges with silver staining experiments. Visual monitoring of the silver shell deposition on the glass surface allows one to monitor this step and cut it off when appropriate signal has been achieved. The pictures were captured using a CCD camera.

7.4.1 Experimental

Different probes designed from bird flu (BF) Avian flu (AF) lacZ and B anthracis (BA) were immobilized on four different chips for control experiments. The four chips were then incubated with StartingBlock blocking buffer for 20 min. On all the chips, similar RNAs were applied in 5x SSC buffer (20 µL, 0.75M NaCl, 75 mM sodium citrate, pH 7.0) and incubated at 37 °C for 15 min except for chip A. The unbound RNAs were washed twice with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, pH 7.4)). The chips were then incubated with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) at 37 °C for 15 minutes. The digested RNAs were then washed twice with PBS buffer followed by application of Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and biotin labeled dATP (1 µL, 0.4 mM, Invitrogen, Carlsbad, CA) on chips A and D while chip C had dNTPs applied to it. The chips were then incubated at 37 °C for 30 minutes followed by washing with PBS buffer. The chips were incubated with gold labeled streptavidin (4 µL) in gold conjugate dilution buffer (100 µL, KPL, Inc, Gaithersburg, Maryland) for 1 hour followed by washing with PBS buffer. Subsequently, 100 µL silver enhancing solution was applied until dark spots were observed. The chips were washed with a lot of water to stop further silver deposition and air dried. The pictures were taken using CCD camera with exposure time of 0.6s.
Figure 7.2: Control experiments using gold labeled streptavidin. Similar probes were printed on each chip as shown in the figure. On chip A, targets RNAs were not applied while on chip B, target RNAs were applied but biotin-labeled dNTPs were not added at Klenow step. Chip C had target RNAs applied but dNTPs were used at the Klenow step other than biotin-labeled dNTPS. Chip D had everything applied to it resulting in a positive signal.

7.5 Selectivity and simultaneous detection on the RNA microchip

One main desirable properties for the RNA microchip will be the ability to selectively and simultaneously detect multiple target mRNAs in a sample. This will not only save time but also enhance the quality of the experimental results. Selectivity is important to ensure false positives
resulting from cross hybridization from other mRNAs are avoided. With the emergence of several infectious agents, the RNA microchip will be of great help to identify the infectious target or targets as well as elucidate the strain or serotype.

7.5.1 Visual multi-pathogen detection RNA microchip

A microchip that allows for simultaneous detection of multiple pathogens may present a promising approach in detection of infectious diseases as well as epidemics in the field. For example influenza viruses, a negative stranded RNA viruses are notorious for recurrent epidemics in many species including humans. Bird flu and avian flu outbreaks have been in the news recently with new infections emerging across the globe (H7N9, 2013). These outbreaks pose an important diagnostic problem. In case of an outbreak, the RNA microchip can simultaneously use different markers to identify the pathogenic microorganism as well as the strain. The viability of the RNA microchip was tested using four different infectious targets with clinical importance i.e. bird flu (BF), avian flu (AF), B. anthracis (BA) and E. coli.

The chips were immobilized with four different probes, namely, BF, AF, lacZ and BA as shown on the left side (Figure 7.3). The RNAs applied on each chip as indicated at the table below each chip. As seen on Figure 7.3, only BA had a positive signal when only BA RNA was applied on Chip A confirming the selectivity of the method. Other chips show selectivity and simultaneous results for the pathogenic detection microchip. These results are an indication of the viability of the RNA microchip. KPL buffer is recommended for washing unbound AuNPs after conjugation. But this did not improve the signal thus PBS was retained for all washes except the final wash after silver staining. Use of one buffer systems also simplifies the procedure.

Rapid and direct detection visual RNA microchip could revolutionalize diagnostics of pathogens around the globe. By designing specific probes for each target, any target can be detected directly on the chip. In situations where different pathogens have asymptotic symptoms, specific probes targeting each strain can be designed and detected on the chip. The signal on the RNA microchip can be visualized with naked eyes thus it can be used even in areas
with limited resources or without electricity. The technology requires minimum equipments making it a good candidate for a point of care (POC) device.

7.5.2 Experimental

Four different probes BF, AF, lacZ and BA were spotted and immobilized on the chip. The chips were then incubated with StartingBlock blocking buffer for 20 min. Subsequently, the chips were flooded with different RNAs in 5x SSC buffer and incubated at 37 °C for 15 min. The unbound RNAs were washed twice with PBS buffer. The chips were then incubated with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) at 37 °C for 15 minutes. The digested RNAs were washed twice with PBS buffer followed by incubation with Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and biotin labeled dATP (1 µL, 0.4 mM, Invitrogen, Carlsbad, CA) at 37 °C for 30 minutes followed by washing with PBS buffer. The chips were incubated with gold labeled streptavidin (4 µL) in gold conjugate dilution buffer (100 µL, KPL, Inc, Gaithersburg, Maryland) for 1 hour followed by washing with PBS buffer. Consequently, 100 µL silver enhancing solution was applied until dark spots were observed. The chips were washed with a lot of water to stop further silver deposition and air dried. The pictures were taken using a CCD camera.
Figure 7.3: Selective and simultaneous detection of multiple pathogenic mRNAs on the RNA microchip. The chips were immobilized with the corresponding detecting probes; bird flu RNA (BF), Avian flu RNA (AF), lacZ RNA (lacZ), Bacillus anthracis RNA (BA). BA RNA was selectively detected by incorporating biotin labeled dATP into the RNA. Selective detection of A) BA or Simultaneous and selective detection of B) lacZ and BA RNAs and C) BF, AF and lacZ RNAs when the three were present in the sample, while BA probe was a negative control. D) Simultaneous and selective detection of all four RNAs.

7.6 HNC Biomarkers identification on the RNA microchip

Cancer detection is the most important step in cancer management and treatment with early detection leading to better treatment options and cure. Early detection of HNC has been shown to boost survival rates by more than 70%\textsuperscript{115} A microchip that allows for early detection of HNC biomarkers may present a promising approach for early detection of HNC. For this microchip to be effective in identifying biomarkers, selectivity is important which ensures false negatives are avoided.

The chips were immobilized with four different probes, namely, IGFBP1, IL8, VCAM1 and BA as negative control. As shown in Figure 7.4, selectivity was demonstrated on chip A by detection of IGFBP1 RNA when only its RNA was applied on the chip while VCAM1, IL8 and BA probes acted as negative controls. Visual observation of the silver shell deposition on the glass surface also allowed one to monitor this step and cut it off when appropriate signal was achieved. Similarly, IL8 was selectively detected on chip B while the other probes acted as negative control. As observed, there was no interference or cross hybridization from other mRNAs.
Selectivity and simultaneous detection is demonstrated in other chips. In this case, different RNAs were applied onto chip C and D as indicated at the bottom of the chip. The main advantage was that the probes and RNAs could be used for both chemiluminescence detection and AuNPs silver staining method. This is not the case with methods that require labeling of targets during RT-PCR or use of thiol modified probes. Each detection method would require preparation of method specific probes.

### 7.6.1 Experimental

Different HNC probes; IGFBP1, IL8, VCAM1 and BA were immobilized on the chip for selectivity and simultaneous detection studies. The chips were then incubated with StartingBlock blocking buffer for 20 min. Subsequently, the chips were flooded with different RNAs in 5x SSC buffer and incubated at 37 °C for 15 min. The unbound RNAs were washed twice with PBS buffer. The chips were then incubated with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) at 37 °C for 15 minutes. The digested RNAs were then washed twice with PBS buffer followed by Klenow incorporation step. Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1 x New England Biolabs, Ipswich, MA) and 1 µL of 0.4 mM; biotin labeled dNTPs; biotin 14-dATP (Invitrogen, Carlsbad, CA), biotin 14-dCTP (Invitrogen, Carlsbad, CA), biotin 11-dGTP (PerkinElmer, Waltham, MA) and biotin 11-dUPT (PerkinElmer, Waltham, MA). (1 µL, 0.4 mM, Invitrogen, Carlsbad, CA) was applied on the chips and incubated at 37 °C for 30 minutes followed by washing with PBS buffer (3 x). The chips were then flooded with streptavidin labeled AuNPs (4 µL) in gold conjugate dilution buffer (100 µL, KPL, Inc, Gaithersburg, Maryland) for 1 hour followed by washing with PBS buffer. Subsequently, 100 µL silver enhancing solution was applied until dark spots were observed. The chips were then washed with a lot of water to stop further silver deposition and air dried. The pictures were taken using CCD camera.
Figure 7.4: Selective and simultaneous detection of HNC biomarkers using streptavidin labeled AuNPs on the RNA microchip. The chips were immobilized with similar probes, three HNC probes i.e. insulin-like growth factor binding protein 1 (IGFBP1), interleukin 8 (IL8), vascular cell adhesion molecule 1 (VCAM1) and bacillus anthracis (BA) as control. IGFBP1 RNA was selectively detected by applying it on the chip A. The RNAs applied on the other chips are as indicated in the table below the chips.

7.7 Specificity of detection on the RNA microchip

7.7.1 Introduction

Variation in the mRNA sequence can affect gene expressions as well as how humans responds to pathogens, chemicals, drugs, vaccines and other agents. SNPs which represents the most abundant form of genomic variation occurs at about one out of a thousand nucleotides in human genome. Some pathogenic bacteria strains and virus serotypes i.e. DENV only differs by SNPs which causes the virus to elicit different symptoms upon infection. SNPs are therefore regarded as potent genetic markers and valuable indicators for clinical diagnosis. Thus discrimination of SNPs for such species is important to ensure proper pathogen detection,
disease diagnosis and management. SNPs also present a variable molecular marker for drug development, disease therapy and biomedical research. Study of SNPs is therefore thought to be a key tool in genotyping and personalized medicine. The miniaturization, high probe density, selectivity and sensitivity of the RNA microchip technology offers the potential for simultaneous genotyping of SNPs.

SNPs often introduce perturbations to oligonucleotides which in turn reduces the binding interaction and stability in duplex formation. Binding is possible but the interaction is less stable than that of a perfect match. As a consequence, perfectly matching strands usually generate a high signal than strands having a mutation.\textsuperscript{200, 207} The application of the RNA microchip for direct detection of SNPs requires demonstration and validation of the ability of the RNA chip to discriminate single base pair mismatches or related species. This will avoid false positives which could be detrimental in life threatening cases.\textsuperscript{205}

7.7.2 Bacillus anthracis detection on the RNA microchip

To test the destabilizing effect of mismatches on our RNA microchip and the ability to discriminate SNPs, different probes were designed from Bacillus Anthracis. There are two main regions where mismatches can be introduced in our chimera probes, either the methylated region which stabilizes both the probe and target RNA or the Klenow template region. Introduction of mismatches in these regions will help us better understand the behavior of our probes. The mismatches are as underlined in the probe sequences (Figure 7.5).

Specificity results indicate it is possible to distinguish between perfect and mismatches duplexes on the chip even at 37 °C and 500 fmol target RNA. Probe BA-1 had two mismatches in the 2'-O-Me RNA region while probe BA-3 had a mismatch in the Klenow template region. Probe BA-2, the perfect match had the highest signal compared with BA-3 although the difference between the two was only one mismatch in the Klenow guiding sequence. BA-1 with two mismatches in the 2'-O-Me RNA region had no detectable signal at these conditions indicating
the stability at this region is quite important especially because the region plays an important role in stabilizing the primer mRNA during RNase H digestion and polymerization.

### 7.7.3 Experimental

Different probes were designed from *B. anthracis* for specificity studies; BA-1, BA-2 and BA-3. After immobilization, the chip was incubated with StartingBlock (TBS, Pierce, Rockford, IL) blocking buffer for 20 min. The chip was then flooded with BA target RNA in 5x SSC buffer (20 µL, 0.75M NaCl, 75 mM sodium citrate, pH 7.0) followed by incubation at 37 °C for 15 min. The unbound RNAs were washed twice with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, pH 7.4). The chip was then incubated with RNase H (0.5 µL, 5000 U ml⁻¹) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) followed by incubation at 37 °C for 15 minutes. The digested RNAs were then washed twice with PBS buffer followed by incubation with Klenow large fragment (0.5 µL, 5000 U ml⁻¹) in Klenow buffer (20 µL, 1x New England Biolabs, MA) and biotin labeled dATP (1 µL, 0.4 mM, Invitrogen, Carlsbad, CA) at 37 °C for 30 minutes followed by washing with PBS buffer. Subsequently, the chip was incubated with gold labeled streptavidin (4 µL) in gold conjugate dilution buffer (100 µL, KPL, Inc, Gaithersburg, Maryland) followed by washing with PBS wash. Consequently, 100 µL silver enhancing solution (1:1, BB International, Cardiff, CF14 5DX, UK) was applied until dark spots were observed. The chip was washed with water to stop further silver deposition on the AuNPs. Upon drying the pictures were taken using CCD camera.
Figure 7.5: Schematic of the RNA microchip for the single nucleotide polymorphism (SNPs) studies. Different probes were immobilized on the chip. Mismatches were introduced at different sections of the probes as shown in probe A and C.

5′-AUCUUAGAAGCA-UUAUCUGAAGAUAAG-AAAAAAAA-3′

**BA1:** TAGAAATCTTCGT-AAUAGCCUUCUAGUC-TTTTTTTT-5′

**BA2:** TAGAAATCTTCGT-AAUAGACUUCUAAUCC-TTTTTTTATAG-5′

**BA3:** TAGAAATCTTCGT-AAUAGACUUCUAAUCC-TTTTATTTAG-5′

Figure 7.6: RNA detection with single nucleotide polymorphism (SNPs). The probe could be discriminated by performing hybridization, RNase H and Klenow step at 37 °C.

### 7.7.4 Dengue serotyping on the RNA microchip assisted by silver staining

Virus infections pose a significant threat to human, animal and agricultural health. Dengue fever, also known as breakbone fever, is an infectious tropical disease caused by the dengue virus (DENV). The fever is caused by any of the four related viruses transmitted by mosquitoes, (DENV-1, DENV-2, DENV-3 and DENV-4). The four serotypes are mildly asymptomatic
and often difficult to recognize in the early phase of infection.\textsuperscript{182} But secondary infections with a different serotypes is characterized by severe forms of DSS/DHS. Besides, the genomes of the four serotypes differ with a few nucleotides. The SNPs can therefore be a valuable indicator for clinical diagnosis of DENV which will aid in disease therapy. Currently, there is a lack of a molecular method that can differentiate all the four serotypes. The application of the easy to use and cost effective RNA microchip for DENV serotyping was therefore demonstrated on the RNA microchip.

Hybrid probes were designed from the naturally occurring DENV serotypes. The probes have two regions, 3'-NH\textsubscript{2}-2'O-Me-RNA-DNA-5'. The probes were modified with an amino group at the 3'-end which affords immobilization on the chip surface. The RNA portion is 2'-'O-methylated and the DNA region serves as a guiding template for polymerase extension.

The four chips were immobilized with all four DENV probes and subjected to different conditions for optimization of DENV-1 RNA serotyping (Figure 7.7). As shown in the figure, chip A (5 pmol RNA) had a strong signal. Also, DENV-2 had a signal resulting from cross-hybridization. This required more stringent conditions to eliminate DENV-2 signal, thus chip B RNA was reduced to 500 fmol but DENV-2 was still visible. But an increase in temperature to 65 \textdegree C for the hybridization, RNase H and Klenow step led to increased specificity and chip C is a clear demonstration of the high specificity afforded on the RNA chip. The DENV-1 (300 fmol) virus was clearly discriminated at 65 \textdegree C for hybridization, RNase H and Klenow polymerization. As shown on the chip, increase in temperature increased stringency on the RNA microchip. This has a tendency of forcing weaker duplexes into single strands. DENV-1 can be detected even at lower fmol level but the signal is weaker (chip D).

DENV-4 serotyping was easily achieved at elevated temperatures (55 \textdegree C) for RNA hybridization (5 pmol), RNase H and Klenow incorporation. The high concentration required is an indication the duplex formation was very strong compared to the other mutated probes thus the specificity of the RNA microchip is very high. While this concentration is high, it is important to
note that lower fmol sensitivity can be achieved as will be discussed in the sensitivity experiments.

### 7.7.5 Experimental

Different probes were designed from different section of DENV viral genomic RNA. The four DENV serotypes probes were spotted at 100 µM and incubated at 37 °C for 30 minutes. Afterward, the chips were incubated with StartingBlock (TBS, Pierce, Rockford, IL) blocking buffer for 20 min. The chip was then flooded with DENV-1 RNA in 5x SSC buffer and incubated at 65 °C for 15 min. The unbound RNA was washed twice with PBS. This was followed by incubation with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) at 65 °C for 15 minutes. The digested RNAs were then washed thrice with PBS buffer followed by incubation with Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and biotin labeled dNTPs (1 µL, 0.4 mM, Invitrogen, Carlsbad, CA) at 65 °C for 30 minutes followed by washing three times with PBS buffer. This was followed by incubation with gold labeled streptavidin (4 µL) in gold conjugate dilution buffer (100 µL, KPL, Inc, Gaithersburg, Maryland) followed by washing three times with PBS. Consequently, 100 µL silver enhancing solution (1:1, BB International, Cardiff, CF14 5DX, UK) was applied until dark spots were observed. The chip was washed with water to stop further silver deposition. Upon drying the pictures were taken using CCD camera.
Figure 7.7: DENV-1 SNPs discrimination using AuNPs. The probes were designed using different regions of viral RNA genomes. Underlined are SNPs which differ from DENV-1.

Figure 7.8: DENV-1 serotyping on the RNA microchip. Discrimination was achieved at 65 °C for RNA hybridization (300 fmol), RNase H and Klenow step.

Figure 7.9: DENV-4 serotyping on the RNA microchip using AuNPs. Discrimination was achieved at 55 °C for RNA hybridization (5 pmol), RNase H and Klenow step.
7.8 Sensitivity of detection on the RNA microchip

7.8.1 Introduction

Sensitivity is one of the most important parameters for a biological sensor. This is because only a small amount of the biological material can cause contamination especially for the pathogenic probes. Other times it is not easy to get the labile materials and therefore one should be able to work with the lowest available material to achieve reliable and conclusive result. Detection on the RNA microchip can be done using biotin labeled probes or through our specific hybrid probes which allows for the incorporation of biotin dNTPs. Generally, most of the reported methods use biotin labeled targets for AuNPS silver staining detection method.\textsuperscript{207} Our 3'-labeling approach is a departure from this and allows direct detection of RNAs.

7.8.2 Detection on the microchip using 3'-labeling technique

Development of a microchip for pathogenic identification and detection requires a high level of sensitivity since sometimes there is not enough starting material. Generally many nucleic acids techniques especially RNA detection methods require reverse transcription and PCR amplification. This not only requires additional time but also increases errors and artifacts. The 3'-labelling technique enables direct detection of RNAs without polymerase amplification.\textsuperscript{102-103} Development of a direct and sensitive RNA microchip that doesn’t require RNA enrichment or amplification and other processes thereof will only save time with improved results. Sensitivity was demonstrated by using of lacZ probes at different RNA concentrations on the chip with 0 fmol lacZ applied on chip A), 5 fmol on chip B), 15 fmol on chip C) and 50 fmol on chip D. The method is sensitive for detection even to the lower fmol (Figure 7.10).

7.8.3 Experimental

The chips were immobilized with lacZ probe for 30 minutes after which they were incubated with TBS blocking buffer for 20 min. The chips were then flooded with lacZ RNA at different concentrations in 5x SSC buffer and incubated at 37 °C for 15 min. The unbound RNA was washed twice with PBS buffer. The chips were then incubated with RNase H (0.5 µL, 5000 U
mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) followed by incubation at 37 °C for 15 minutes. The digested RNAs were then washed twice with PBS buffer followed by incubation with Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and biotin labeled dATP (1 µL, 0.4 mM, Invitrogen, Carlsbad, CA) at 37 °C for 30 minutes followed by washing with PBS buffer. The chips were then incubated with streptavidin labeled AuNPs (4 µL) in gold conjugate dilution buffer (100 µL, KPL, Inc, Gaithersburg, Maryland) followed by washing with PBS wash buffer. Finally, 100 µL silver enhancing solution was applied until dark spots were observed. The chips were washed with a lot of water to stop further silver deposition. Upon air drying the pictures were taken using a CCD camera.

![Image](image.png)

Figure 7.10: RNA microchip detection sensitivity using lacZ RNA; images A–D; 0, 5, 15, and 50 fmol respectively.

### 7.8.4 Detection on the microchip using biotin labeled RNA

It was also necessary to demonstrate the sensitivity of the assay using biotin labeled RNA which is commonly used method for RNA detection. Normally, the probes are usually labeled during RT-PCR or chemical synthesis. In our set-up lacZ RNA was chemically labeled with biotin (24.2). *E. coli* lacZ probe was immobilized on the chip surface and later hybridized with different concentrations of biotinylated 24.2 RNA (Figure 7.11). Detection sensitivity was achieved at the lower fmol level without amplification. This labeling generally compares well with our methodology but prior labeling of the target RNA is required before detection on the chip.
7.8.5 Experimental

The chips were immobilized with $\text{lacZ}$ probe for 30 minutes after which they were incubated with TBS blocking buffer for 20 min. The chips were then flooded with biotin labeled $\text{lacZ}$ RNA at different concentrations in 5x SSC buffer and incubated at 37 °C for 15 min. The un-bound RNA was washed twice with PBS buffer. The chips were then incubated with gold labeled streptavidin (4 µL) in gold conjugate dilution buffer (100 µL, KPL, Inc, Gaithersburg, Maryland) followed by washing with PBS buffer. Subsequently, 100 µL silver enhancing solution was applied until dark spots were observed. The chips were washed with a lot of water to stop further silver deposition. Upon air drying the pictures were taken using CCD camera.

Figure 7.10: Detection sensitivity with biotin labeled dNTPs. A) RNA detection sensitivity; images 1–4: 50, 5, 1 and 0 fmol of biotin labeled RNA, respectively.

7.9 $\text{lacZ}$ mRNA detection from $E. \text{coli}$ total RNA

7.9.1 Introduction

For this method to be applicable for pathogen identification and cancer detection, it was important to demonstrate sensitivity using biological samples. Detection of specific mRNAs from total RNA presents a number of challenges. For one, it is not easy to label specific RNAs in an RNA mixture. Also, presence of other RNAs could also hinder the sensitivity of the method. Generally, the desired mRNA region is fished out of the total RNA mixture by PCR primers during reverse transcription followed by polymerization, isolation and purification of the amplified DNA. DNA microarrays are afterward used to confirm PCR products. There is therefore a search for direct RNA detection method. The RNA microchip is devoid of pre-processing of RNAs and thus is rapid and can be used for direct measurement of transcription levels.
The wild type *E. coli* bacteria strain; K-12 MG1655 was selected as the target pathogen in evaluation of the RNA microchip for biological application. *lacZ* gene was induced in the wild type *E. coli* in the presence of IPTG. A negative control was achieved by using glucose to suppress *lacZ* gene expression. The *E. coli* total RNA was extracted using MasterPure RNA purification kit followed by direct detection on the RNA microchip.

The total RNA was directly detected on the chip as shown on Figure 7.12 (chip B). Also, specificity on the chip was confirmed by the lack of signal with glucose-suppressed total RNA (chip C). Chip A acted as negative control. The total RNA was treated with 100 mM NaOH with heating for 5 minutes at 92 °C before application on the chip for fragmentation purposes. As discussed earlier, the secondary structures and lengths of nucleic acids affect the rates of nucleic acids, leading to reduced hybridization efficiencies and false negatives, thus fragmentation of the total RNA before detection on the chip has been adapted for this research.\(^{163} 208\)

### 7.9.2 Experimental; preparation of *E. coli* total RNA

The wild type *E. coli* bacteria strain, K-12 MG1655 was donated by Scarab Genomics (Madison WI). *E. coli* cells (5 µL) were grown in duplicate overnight in 6 mL Luria Broth (LB, Sigma Aldrich, St. Louis, MO) buffer at 37 °C with shaking at 220 RPM. The bacterium was then added to a 1L capacity Erlenmeyer flask containing 300 mL of sterilized LB buffer and cultured for 2 hours at 37 °C. Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma Aldrich, St. Louis, MO) was then added to one flask and to the other D-(+) glucose (Sigma, St Louis, MO) was added to make a final concentration of 1 mM. The bacteria were cultured for 6 hours at 37 °C. The cells were harvested and the pellets stored at -80 °C for nucleic acid extraction. Total RNA of *E. coli* cells was extracted and purified according to MasterPure Complete RNA Purification Kit with some modifications (Epicenter Biotechnologies Madison, WI) and the RNA was dissolved in TE buffer [10mM Tris-HCl (pH 7.5), 1mM EDTA]. After purification, the purity and concentration of the total RNA were determined by measuring absorbance using UV-Visible spectrophotometer (Varian Inc., Sana Clara, CA).
7.9.3 Detection of lacZ mRNA from total RNA on the RNA microchip

The chips were spotted and immobilized with lacZ probe. RNA fragmentation was carried out by heating the total RNA (30 µg) in 100 mM NaOH for 5 minutes. The hydrolysis reaction was stopped by adding 1N acetic acid. The RNAs were then added to 5x SSC and hybridization was done for 30 minutes. The unbound RNAs were washed with PBS buffer followed by RNase H digestion. The other procedures were followed as indicated in Section 7.8.

Figure 7.12: Detection of lacZ mRNA from E. coli total RNA. Figure A) had no RNA applied, B) had IPTG-induced total RNA and C) had glucose-suppressed total RNA.

7.10 Rapid and direct lacZ RNA detection from NaOH treated E. coli.

7.10.1 Introduction

The method developed for direct detection of E. coli in chapter 5 was applied in AuNPs detection. As indicated earlier, RNA extraction from E. coli cells require a simple treatment with alkaline solution. NaOH treatment not only lysed the cells but also fragments the RNAs to enable faster hybridization. This quick RNA extraction step is simple and cost effective. The method reduces RNA handling time by several hours or days thus the RNA degradation process by RNases is reduced. The method is specific as shown (Figure 7.13; chip B) with absence of a signal in glucose suppressed total RNA. This simple method opens the application of the RNA microchip to regions where RNA purification kits are not available due to funding problems. The rapid and direct RNA detection shortens the RNA microchip detection method; culture growth and harvesting, extraction and detection on the chip can be accomplished within 24 hrs. This is a great improvement from the RNA purification procedures and cDNA microarray which require RT-PCR, labeling, purification and long hybridization times (16 hrs for hybridization only).
7.10.2 Experimental; *E. coli* growth, hydrolysis and detection on the microchip

*E. coli* was prepared as previously described in 7.9 but was harvested in small portions for the lysis and hydrolysis purposes. Other than using commercial purification kit, total RNA was directly extracted and fragmented using NaOH. The chip was spotted and immobilized with *lacZ* probe for 30 minutes. RNA fragmentation was carried out by heating the bacteria (1.5 mL) with 400 mM (6.5 µL) NaOH for 5 minutes at 92 °C. The samples were cooled down, centrifuged and the supernatant aliquoted. This was followed by addition of 1N acetic acid for neutralization. The RNA samples were added to 5x SSC and applied on the chip. Hybridization was done at 37 °C for 35 minutes followed by washing with PBS buffer (3 x). The other procedures are as outlined in Section 7.8.

Figure 7.13: Rapid detection of NaOH treated *E. coli* bacteria; A) RNA extracted from IPTG-induced *E. coli* while B) is RNA extracted from glucose-suppressed *E. coli*.

7.11 Rapid detection on the RNA microchip

7.11.1 Introduction

Rapid detection of pathogens using the RNA microchip would greatly benefit many fields especially in the outbreak of food borne diseases. Infections are generally caused by consumption of contaminated subjects and are accelerated by person to person contamination. Rapid detection will come in handy in identification of the source of contamination to prevent further damage. The device can as well be applied as a routine check in organic farms, meat and food processing industries as a preventive method to curb the potentially harmful *E. coli* among other food related pathogens. A rapid RNA detection microchip will therefore be a versatile tool in monitoring communicable diseases e.g. the recent flu outbreaks.
Here we present a simple and rapid detection of mRNA using RNA microchip. Since we use short oligonucleotides, hybridization rates are faster which have enabled us to develop a rapid method where detection can be accomplished in one hour. There are several steps that can be optimized sequentially to ensure rapid detection (Figure 7.14, Figure 7.15 and Table 7.3). As shown, the reaction can be performed in one hour to afford a strong signal for the rapid detection. Three different probes were immobilized on the surface, \( \text{lacZ} \) labeled DNA as a positive probe, \( \text{lacZ} \) probe which was our testing probe and BA acted as a negative control probe.

Rapid detection could also be possible by combining the RNA hybridization, RNase H digestion and Klenow incorporation steps. This way, one is able to save time and reagents. Figure 7.16b is a chip showing the results for the three combined steps for 15 minutes. This was accomplished by combining all the components with RNase H and Klenow buffer system (2:8). There was no need for 5x SSC in this set-up. This result is an indication of the ruggedness of the RNA microchip.

### 7.11.2 Experimental

Several conditions were tested on the chips (Table 3) the best conditions for chip 13 reported here (Figure 7.16). The chip was immobilized with three different probes; biotin labeled \( \text{lacZ} \) probe, \( \text{lacZ} \) probe and BA probe. After immobilization, the chip was flooded with \( \text{lacZ} \) RNA sample at different concentrations in 5x SSC buffer and incubated at 37 °C for 5 min. The unbound RNAs were washed twice with PBS buffer. Consequently, The chip was incubated with RNase H (0.5 µL, 5000 U mL/1) in RNase H buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) followed by incubation at 37 °C for 5 minutes. The digested RNAs were then washed twice with PBS buffer followed by incubation with Klenow large fragment (0.5 µL, 5000 U mL/1) in Klenow buffer (20 µL, 1x New England Biolabs® Inc, Ipswich, MA) and biotin labeled dATP (1 µL, 0.4 mM, Invitrogen, Carlsbad, CA) at 37 °C for 5 minutes followed by washing with PBS buffer. The chip was incubated with gold labeled streptavidin (4 µL) in gold conjugate dilution buffer (100 µL, KPL, Inc, Gaithersburg, Maryland) at 37 °C for 10 minutes followed by washing...
with PBS buffer. Subsequently, 100 µL silver enhancing solution was applied and incubated at 37 °C for 15 min upon which the dark spots were observed. The chip was washed with water to stop further silver deposition and air dried. The pictures were taken using CCD camera. Detection was achieved in less than 1 hour.

\[-3' - d(AGAAAGTGTC) - 5'(UACACCUAACCGCUA) - d(TTTTTTGGTTG) - 5'\]

- RNA hybridization

\[5' - AUGUGGAUUGCGUAUUAAACAA - 3'\]
\[-3' - d(AGAAAGTGTC) - (UACACCUAACCGCUATTTTTTGGTTG) - 5'\]

- RNase H digestion

\[5' - AUGUGGAUUGCGUA - 3'\]
\[-3' - d(AGAAAGTGTC) - (UACACCUAACCGCUATTTTTTGGTTG) - 5'\]

- Klenow extension with biotin dNTPs

\[5' - AUGUGGAUUGCGUAUUAAACAA - 3'\]
\[-3' - d(AGAAAGTGTC) - (UACACCUAACCGCUATTTTTTGGTTG) - 5'\]

1. Streptavidin Au NPs
2. Silver staining

Figure 7.14: Flowchart for the rapid detection microchip. There are several reactions that can be optimized, namely; hybridization, RNase H digestion, Klenow extension with biotin dATPs, AuNPs binding and thereafter silver staining.
Table 7.2: Rapid detection optimization summary. There are several steps that were optimized in a stepwise manner to develop a rapid RNA microchip. Silver staining was accomplished in 5-20 minutes and picture was taken in 0.6 s using CCD camera.

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Figure 7.15: Rapid detection condition optimization. A few chips showing the results obtained with different conditions for optimization. From chips 11, hybridization, RNase H digestion, Klenow extension with biotin dNTPs, AuNPs binding and silver staining were done at 37 °C with timings as shown in the table above.
Figure 7.16: Rapid detection of lacZ RNA on the RNA microchip. A) with biotin labeled lacZ probe (35.2) acting as a positive control while BA acted as negative control. The detection was achieved in less than one hour. All steps were done at 37 °C. B) rapid detection on the microchip by combining the first three steps; RNA hybridization, RNase H digestion and Klenow incorporation (15 minutes).

7.13 Summary

A versatile visual detection biological sensor for RNA detection has been developed. The RNA microchip is rapid, sensitive and easy to use. Selectivity and simultaneous detection was demonstrated with both pathogenic RNAs and Head and Cancer markers. The targets were selectively and simultaneously detected without cross hybridization. Specificity of the method was demonstrated by SNPs discrimination using DENV-1 virus RNA as well as BA RNA to probe other regions. Even though our probes are short, they offer high level of specificity on the microchip. By using our specific probes, we are able to detection of lacZ mRNA from E. coli total RNA with high specificity against glucose-suppressed total RNA. The method is sensitive enough (up to fmol level) to detect real RNAs without amplification.

Direct nucleic acids extraction using a new, simple and rapid NAOH protocol further simplifies the method and reduces the cost of production. Detection on the RNA microchip can be accomplished in an hour which is a departure from DNA microarray which takes up to 16 hours for hybridization alone not to mention the processes involved during reverse transcription and PCR.

The method is rapid and can be accomplished in under 1 hour. Further simplification of the procedure was accomplished by combining RNA hybridization, RNase H digestion and Klenow incorporation step with good results. By consolidating the three steps combined with manual spotting (Appendix 1), the method can be used anywhere in the world with minimum
requirements. Once, the hybrid probes are made, all what one will need is a heating apparatus for temperature controls as the signal can be visually detected. If further signal analysis is required, no problem, the signal is permanent in comparison with chemiluminescence detection and fluorescence detection which requires special instruments and decay within hours due to enzyme substrate limit and photobleaching respectively. It is amazing how this procedure is easy, versatile and allows fine tuning to fit personal needs. The aforementioned desirable characteristics can be furnished for development of a POC pathogen detection device.
8. CONCLUSIONS AND SIGNIFICANCE

RNA microchips with different signal transduction methods has been developed, characterized and validated. There are several parameters needed to ensure the success of a biological biosensor and application thereof. These include selectivity, specificity, sensitivity, rapidity and cost effectiveness. While it is for a biological sensor to fulfill all these parameters, a trade off is made with the best method at the prevailing circumstances being used. Characterization of the RNA chip was accomplished by use of different markers from cancer, pathogens and virus RNAs. Test with this broad array of targets ensures the 3'-labeling assay and the detection methods there sued can be applied to any kind of RNA from any source, as long as target specific probes are designed.

With chemiluminescence detection, selectivity was demonstrated by use of different head and neck cancer markers. IL8 and VCAM1 were selectively detected from, synthetic RNA mixtures, cancer cell lines and colon cancer samples. There was no cross hybridization observed. Simultaneous detection was also demonstrated by using four different probes. From the cancer cell lines, VCAM1 was detected in all samples while IL8 was detected in all except 1 while IGFBP1 not detected in any of the samples. Specificity of detection was demonstrated by the signal profile observed in each cancer samples. For example TU 686 had both VCAM1 and IL8 detected while LN 686 had only VCAM1. Detection of the markers in low amount of colon cancer without amplification is a measure of sensitivity, specificity and rapidity of the RNA microchip. This is an indication that the method can be used for gene expression profiling and cancer diagnosis. DENV virus SNPS discrimination shows how powerful method can be in genotyping. Even with 1 or more mutation differences, the serotypes detected individually on the chip. A new E. coli total RNA extraction protocol simplifies the procedure further making it a more rapid and cost effective method RNA detection, genotyping and gene expression profiling.
AuNPs-silver staining afforded visual detection on the RNA microchip is a very attractive method with high selectivity, specificity and sensitivity. The signal can be observed with naked eye therefore it can be anywhere in the world with minimum requirement. A simple NaOH total RNA extraction protocol was also specific on the chip resulting in a cost effective and rapid method for RNA detection. The method is fast as detection can be accomplished in ~ 1 hour. Combination of RNA hybridization, RNase H and Klenow further simplifies the method without signal interference. Reduction of the steps ensures minimal operation which is a good characteristic for a POC device. Selectivity and simultaneous detection was accomplished by use of both cancer biomarkers and pathogenic markers without cross hybridization. The method was highly specific as observed with Dengue and *Bacillus anthracis* SNPs discrimination. The method exhibited high sensitivity by detection of lacZ mRNA from *E. coli* total RNAs without amplification. Specificity was demonstrated by detection of lacZ mRNA from only IPTG induced RNA and not from glucose-suppressed total RNA. Direct detection of RNA using NaOH was simple, rapid and specific. The rapid and direct RNA detection shortens the RNA microchip detection method: culture growth and harvesting, extraction and detection on the chip can be accomplished within 24 hrs without enrichment processes. This is a great improvement from the RNA purification procedures and cDNA microarray which require RT-PCR, labeling, purification and long hybridization times which are normally 16 hrs.

These two detection techniques have been tested with many different targets i.e. cancer, pathogen and virus by just designing target specific probes. The methods have been well characterized with very desirable sensor characteristics. The methods can therefore find applications in any RNA studies including gene profiling, cancer detection, pathogen detection, infectious diseases detection, forensic and detection of bioterrorist agents.
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Figure A1. Manual arraying using MicroCASTer 8-Pin System. 3'-labeling technique generated a positive on the chip.