

DNA Logic-A novel approach to semiconductor based genetics

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Abstract

In the coming years, genetic test results will be increasingly used as indicators that influence medical decision-making. With chronic disease on the rise and the continuing global spread of infectious disease, novel instruments able to detect relevant mutations in a point-of-care setting are being developed to facilitate this increased demand in personalized health care. However, diagnosis for such demand often requires laboratory facilities and skilled personnel, meaning that diagnostic tests are restricted by time and access. This thesis presents a novel configuration for Ion sensitive Field Effect Transistors (ISFETs) to be used as a threshold detector during nucleic acid base pairs match. ISFET-based inverters are used as reaction threshold detectors to convey the chemical reaction level to a logic output once a threshold has been reached. Using this method, novel DNA logic functions are derived for nucleotides allowing local digital computations.

The thesis also presents business models that enable such technology to be utilised in point of care applications, and experiment as results and business models given for an HIV point of care example are proposed.

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GLOSSARY OF ABBREVIATIONS

A	Adenine
AIDS	Acquired immune deficiency syndrome
ART	Anti-retroviral treatment
bdNA	Branched DNA
C	Cytosine
CAGR	Compound annual growth rate
CCR5	Chemokine receptor type 5
CD4	Cluster of differentiation 4
cdNA	Complementary DNA
CHEMFET(s)	Chemical field effect transistor(s)
CLIA	Clinical laboratory improvement amendments
CMOS	Complementary metal oxide semiconductor
CMV	Cytomegalovirus
CNV	Copy number variation
CpG	C-phosphate-G
DNA	Deoxy nucleotide acid
dNTP(s)	Deoxynucleotides (A, T, G, and C nucleotides)

dsDNA	double-stranded DNA
DTC	Direct-to-consumer
E. Coli	<i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
G	Guanine
HapMap	Haplotype map
HGP	Human genome project
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
ISFET	Ion sensitive field effect transistors
ISFET(s)	Ion sensitive field effect transistor(s)
IVD	<i>in-vitro</i> diagnostic
MB	Mega base
MOSFET	Metal oxide semiconductor field effect transistor
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NAT(s)	Nucleic acid test(s)
PCR	Polymerase chain reaction
PG-ISFET	Programmable gate ion sensitive field effect transistor

PITC	Provider-Initiated HIV testing and counselling
POC	Point of care
RNA	Ribose nucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SNP(s)	Single nucleotide polymorphism(s)
ssDNA	Single-stranded DNA
T	Thymine
UNAID	The joint United Nations Programme on HIV/AIDS
WB	Western Blot
WHO	World Health Organisation

LIST OF COMPANIES

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CHAPTER 1 – INTRODUCTION

As we enter a new wave of technology inspired by lifestyle and healthcare, precise and efficient point of care testing is becoming increasingly important. The technology to accurately detect a gene sequence in real-time would provide end-users with personal medical care yet is unavailable outside a laboratory. This presents opportunities in areas where a real-time result can save time, money and lives [1].

Contemporary sequencing and genotyping technologies are expensive, time consuming and laboratory based. However, rapid progress in genome science puts on a new horizon for low-density genotyping in POC settings. A robust, low cost, and laboratory free genotyping platforms are essential features in POC settings, which allow low-density genotyping to be extremely cost efficient without using heavy laboratory equipment and skilled personal.

1.1 DNA LOGIC

For year scientists have been trying to implement digital computations in cells by creating *in-vivo* digital logic circuits using DNA binding Proteins, and gene expression. Now, using the ISFET method for detection of nucleic acid, we are able to achieve digital computation out of biological cells and their genetics. It is the intention of this research to propose a novel method of DNA Logic using silicon based transistors with chemical interactions to create so called DNA switches that may perform *in-vitro* computation. This particular work will be confined to switching based upon the marker for nucleotide insertions (SNPs) with the application being diagnostics. It is not the intention of this research to develop a point-of-care genetic

testing device but rather to add some intelligence to the sensors so that local processing and computation of SNPs can be realized in real-time.

1.2 CHEMICAL SENSOR

A chemical sensor is generally comprised of sensing element, transducer, and the readout circuit. The sensing element is the sensing membrane in the case of ISFET, which can be silicon nitride or other oxides. The evaluation of sensor performance is generally done by a table of sensor characters. Specificity and sensitivity are the two basic characters that chemical sensors are evaluated by the users and regulated by the authorities [2, 3]. Common complications of chemical sensors includes hysteresis, baseline drift, fineness and cross reactivity [4]. Scientists have been working to optimise these issues in which sensors deviate from the ideal. By merging and integrating chemical sensing with electronic signal processing circuitry, many undesirable characteristics of chemical sensing can be mitigated through drift compensation or intelligent processing on heterogeneous sensors to improve selectivity [5].

1.3 BIO SENSING

A biosensor is a chemical sensor that integrates a bio-component with a physicochemical component for the purpose of detecting an analyte [6]. The sensor uses a biological recognition layer to bind with the concentration of the target species to a transducer, as we can see in [7]. Various applications have been employed using biosensors in past twenty years, including environmental analysis, medical treatments, toxicology studies and military defences [8-11]. The two major challenges in developing a biosensor are stability and reproducibility. The sensor often lacks the

capacity for continuous monitoring, rapid response times, intelligence and portability. [11]

We can categorize biosensors into two groups by the nature of the biological recognition process. [12] The first group, biocatalytic sensors, uses enzymes, cells or tissues as immobilized components. The second group, affinity sensors, are based on the recognition process of antibodies, membrane receptors or nucleic acids. In certain transduction mode, biosensors can be made in the solid-state to provide chip-based sensing of biomolecules, cells, neurons and nucleic acids. [13]

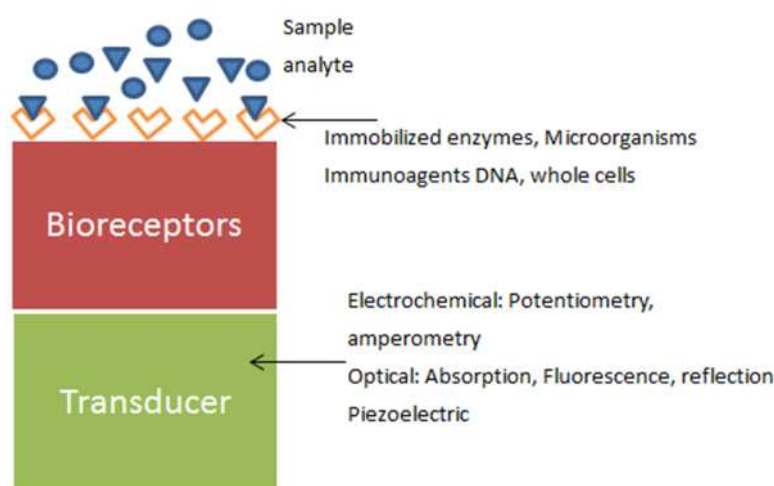


Figure 1-1 Biosensor architecture

1.4 ISFET PLATFORM HIV NUCLEIC ACID TEST

The key objectives are to implement HIV detection in a point of care setting and bring personalized medicine to the bedside in the management of infected patients with HIV. In addition to its timeliness in developing Point of Care (POC) testing which will be of immediate benefit to patients, the section will serve as a proof of principle for POC detection of other host genetic polymorphisms and the detection and

quantification of infectious organisms. The work will also be important in the development of lab-free diagnostics needed in the developing world and for other diseases.

1.5 RAPID HIV TESTING KIT: BUSINESS MODEL OVERVIEW

With healthcare budgets already overstretched and fewer careers – professional and non-professional – available to meet these increased needs, the forecasted additional demands are simply unsustainable using current practice. Factoring in the growing patient demands for personalized treatment, medicine and services, it results in huge cost pressures on healthcare. A new paradigm is required. Using the ISFET technology, we propose a novel business model where selling nucleic acid testing machines to point of care facilities and institutions is realised.

In summary the thesis will discuss the novel application of nucleic acid detection based upon a semiconductor approach using ISFETs. The concept of DNA logic is then invented as a means of implementing logical functions of genetic information. The application to the SNP detection as a point of care means for any application is then experimentally confirmed. In the final chapter, the application of the DNA Logic technique is applied to a novel HIV point of care detection system and experimental results are presented. The final chapter discusses the market potential of the technology and the business model followed by concluding remarks and future applications.

1.6 SOLID-STATE ELECTROCHEMICAL SENSING

Electrochemical sensing is a practice of detecting targeted species through electrons or by ion transfer across an electrolyte/electrode interface. Since the 1930s, electrochemical sensing has been widely used in practice. It is a very matured and well-characterized transduction method. [7]

Driven by commercial success in various applications, researches for more advance solid-state physical sensors have begun looking towards developments in fabricating chemical sensors on a semiconductor substrate. This will benefit the sensors by lowering manufacturing costs, standardizing wafer fabrication processes and integrating sensors with electronic circuitry. [14, 15]

There are clear advantages of utilising solid-state technologies in chemical sensing application. The approach leverages the great investment of solid-state sensor industry to meet the demand of better chemical sensors. The biological research works have consistently making new discoveries about the analytes and the corresponding ligands. The integration of the new ligands on solid-state sensors is possible due to the transduction nature of the ion or electron transfer of the targeted species and the solid-state substrate binding at the sensor surface. In addition, electrochemical detection is relatively more cost effective over other alternative solid-state transduction modes, which require lenses and fluorescent labels for detection. [4, 16]

In spite of many advantages with using electrochemical sensors, many of the undesirable characteristics mentioned in have not yet been overcome. Thus, till present day, researches to improve sensors performance has been an on-going assignment for many physicians and engineers.

1.7 SOLID-STATE ELECTROCHEMICAL MICROSENSOR TECHNOLOGIES

A solid-state micro-sensor is defined as a solid-state sensor embedded with sensing elements such that the physical dimensions are on the sub-millimetre scale and often as planar devices, fabricated using procedures from the microelectronics industry. Low cost, small dimensions, high reproducibility and integratability to electronic circuitry are several key advantages of solid-state electrochemical micro-sensors. [17] This section reviews various solid-state electrochemical micro-sensor technologies and their compatibility with the complementary metal oxide semiconductor (CMOS) fabrication process.

1.7.1 SENSOR MICROFABRICATION AND CMOS COMPATIBILITY

Sensor micro-fabrication is similar to semiconductor device fabrication. The main difference between them is the planar electrodes formed on the solid-state electrochemical sensors substrates. The substrates can be made of ceramic, glass, plastic and silicon to endure insertion into acids and bases, and to be resistive to various environmental conditions and physical deformation. By implementing thick-film lithography of conducting pastes, or by thin-film metal deposition processes, electrodes can be patterned onto substrates [16].

Using batch fabrication techniques and lithographic processing, sensor micro-fabrication is very low cost per sensor with high reproducibility. CMOS is the current industry standard in terms of the production technology for high performance digital and analogue integrated circuits. Its fabrication process is highly standardized and well characterized. CMOS-fabricated systems are currently the best method for the

realization of a minimum cost per sensor, minimum feature size and minimum process variation [18]. However, only a fraction of solid-state electrochemical sensors can be produced using generic CMOS process. The major limitations are due to restrictions on the process flexibility in commercial foundries.

1.7.2 CONDUCTIMETRIC MICROSENSORS

The conductimetric microsensor is based on the measurement of the conductivity of a sample to find out the concentration of charges in a solution. Therefore any particular species in the solution will affect the measurement. Thus this is a technique which exhibits low selectivity as we have mentioned in . With such a property, this sensor is often used as an online detector after separation of a mixture of ions by ion chromatography [19].

Chemiresistors and chemicapacitors are examples of conductimetric devices that serve as sensors for “electronic noses” and “electronic tongues”, where pattern recognition is more important than selectivity [20, 21].

1.7.3 AMPEROMETRIC AND VOLTAMMETRIC MICROSENSORS

A fixed potential is applied to a reference electrode, causing a redox reaction in the analyte, when implementing detection in amperometry. The reaction will trigger the current to flow between the electrode and a working electrode. Due to the possibility of causing the fixed potential to alter when current passes through the reference electrode, the three-electrode system is often used in practice to avoid such problems by adding a third electrode, known as a counter electrode.

Voltammetric is a technique using time-varying potential where current-voltage profiles are recorded and thus several species reacting at different applied potentials

can be determined simultaneously in a single experiment. As a result, voltammetric provides more information than amperometry and has less limitation [22].

1.7.4 POTENTIOMETRIC MICROSENSORS

Potentiometry is an electroanalytical method which is based on measurement of potential between two electrodes. Between the two electrodes there is only negligible current. The voltage is generated between an ion-selective electrode (ISE) and a reference electrode caused by a difference of concentration between the analyte solution and a reference solution. Potentiometric measurements enable selective detection of ions in the presence of a multitude of other substances. The reference electrode is normally a silver/silver chloride (Ag/AgCl) electrode used as reference. [23]

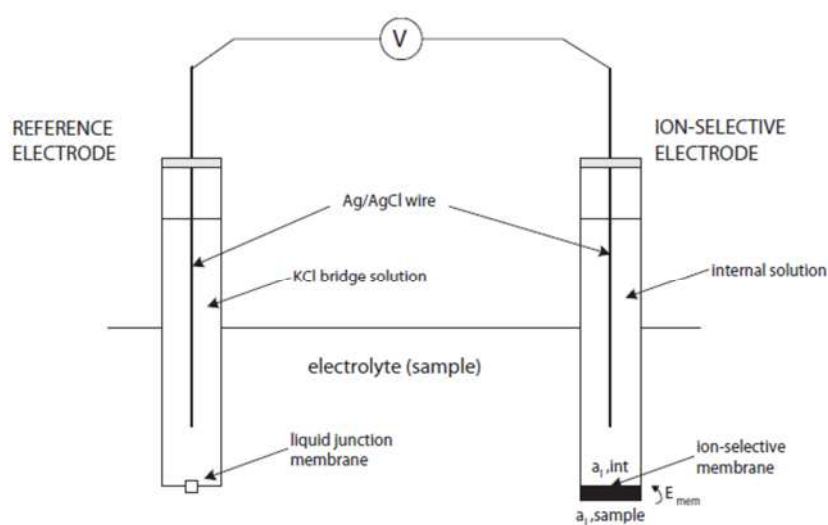


Figure 1-2 Electrochemical cell for potentiometric measurements with an ISE

At equilibrium, this potential is related to the ion activity a_i on either side of the membrane as defined by the Nernst equation:

Equation 1-1 Nernst equation of potentiometric measurement of an ion-selective electrode [24]

$$E_{mem} = \frac{RT}{z_i F} \ln \left(\frac{a_{i,sample}}{a_{i,int}} \right)$$

for the ion i of charge z_i , where R is the gas constant, T is absolute temperature and F is the Faraday constant.

Since the activity of the ion i in the internal solution is known, then

Equation 1-2 Ionic concentration dependence electrode potential

$$E_{mem} = \text{constant} + 2.303 \frac{RT}{z_i F} \log a_{i,sample}$$

thus giving a variation of $\frac{59.13}{z_i}$ mV in E_{mem} per decade variation in ion activity at a temperature of 298°K.

FET-based chemical sensors such as the ISFET, CHEMFET, and the Work function FET all fall into this class whereby the charge in solution, exposed to a floating gate, may be sensed via a stable reference electrode.

In this section, we have discussed various biosensors and with any chemical sensor priory mentioned, it is desirable to keep to minimum non-standard process steps. This is because such steps will increase in manufacturing complexity and thus will the cost per sensor.

Sensors we have discussed prior to this section are either not CMOS-process compatible or post-processing is needed either to deposit electrodes on the die or attach membranes to make the sensors selective to specific analytes.

Fabrication of ISFETs can be achieved using standard MOSFETs with the native passivation of the CMOS process. [25] This is even so when using ISFETs as a proton sensor that no extra processing steps are required to deposit the membrane and with an external reference electrode, making the devices selective to other ions requires deposition of other membranes. ISFETs are an attractive option for proton sensing because they can take full advantage of the merits which come with implementation in a standard CMOS process which include miniaturization, repeatability, minimal process variation, high yield and low cost of large volumes.

1.7.5 BENEFITS OF CHEMICAL SENSING WITH UNMODIFIED CMOS ISFETS

The ISFET has great potential to be commercialized due to its unique features in detecting genes through chemical reaction. Potential benefits of using ISFETs to build such systems include:

- Minimal or no post processing - CMOS ISFETs are inherently sensitive to hydrogen using the native passivation of the die [25]
- Low power processing - Can be operated in weak inversion in which current is governed by diffusion to give low power operation [26]
- Boltzmann relation to ionic current - Obeys a Boltzmann distribution in weak inversion which can be used to implement chemically-gated ion channels [18]
- Monolithic integration - Can be integrated with its MOSFET counterpart to make differential readouts as well as to apply well-known circuit techniques which are used in solid-state integrated circuits.

- Array processing - Can be grouped into arrays to provide redundancy and spatiotemporal mapping

With these in mind we proceed by describing the background of the applications of ISFET-based chemical sensor and operation of the ISFET.

CHAPTER 2 SEQUENCING AND GENOTYPING

Molecular diagnostics is becoming a necessary tool in modern clinic practice. The eruption of the knowledge comes not only from the gene sequences but also mutation-phenotype association and biomarker discoveries. Critical information such as drug adverse selection, viral load monitoring, and pathogen identification can be provided through diagnostic technologies in the molecular level for clinicians when treating patients. In more and more fields, molecular diagnostics are essential and the only tool to decide the treatment or prescription. These fields include infectious disease detection as well as individual variations. Examples are human leukocyte antigen typing (HLA) for transplant matching, mutation tests in oncology, and pharmacogenetic tests. These methods generally adapt the technologies that were originally developed in research laboratories. Generally, the clinicians in the first line facing patients are not trained for the skills of molecular diagnosis.

Demand for a rapid and simple nucleic acid test has been rising rapidly and the market in the United States has approximately a double digit growth from 2009 to 2010 [27]. The growth is partly due to the advance of molecular diagnostic technology as well as the availability of markers. Because of the short windows for effective action, in an area such as infectious disease control and management, rapid testing for nucleic acid has been needed in diagnosis as well as prognosis.

A classic example is the management of Human immunodeficiency virus (HIV) infection, where the traditional detection based on immunoassay requires long turn-around time and lacks critical information, such as virus strain. Nucleic acid tests have filled the gap and provided early detection and helps with therapeutic important decisions.

The field of nucleic acid test technology has been largely dominated with real-time Polymerase Chain Reaction (PCR) technologies which require a molecular laboratory setup in clinical setting or primary care environment. These methods are established in commercial molecular diagnosis kits, reagents, and instruments. The majority of the tests employ fluorescent signal and nucleic acid amplification methods.

A rising new era of personalized health care is demanding for rapid low-density genotyping in applications for quick diagnostics, drug efficacy with toxicity screening, and personal genetic tests. This made molecular medicine and medical diagnostic test characterised less by treating symptoms and more by looking into the fundamental causes of disease with real-time diagnostic tests. Therefore, more rapid, real-time and more specific diagnostic tests will make possible for earlier treatment of countless maladies.

Although the cost of the lab-based tests have been reduced dramatically and the tests become popular and essential in detection of many infectious disease, the gap is not being filled in to improve the turnaround speed and reduce the skill requirement. Further reduction of the cost will benefit users in applications which have been priced out and are not available. More detailed discussion follows in later chapters.

It is essential to include DNA sequencing in the discussion of molecular diagnosis. The sequencing provides the ultimate information of the genetic makeup of an organism, pathogen or host. Any nucleic acid based diagnosis can be considered as a reduction of the DNA sequencing technology. The basic principle and chemistry are similar between PCR and sequencing, which is enabled by the Watson-Crick base pairing nature of nucleic acid [16]. With sequencing technology, thousands to billions of nucleic acid sequences could be produced in one assay while one or few bases are

revealed by PCR or similar molecular diagnostic assays. The sequencing technology today has made a great progress but it still requires very specialised resources and discouraging cost to sequence a human genome. It is very difficult to use sequencing as a molecular diagnostic tool before the next major breakthrough in sequencing technology. As a solution, assays using relatively simple technologies, such as real-time PCR, are developed to fill the need.

With the speed of evolution of sequencing technologies, it should not be long before the first diagnostic application using sequencing. It is sensible that the enabling technology revolves around ultra-high density sequencing. At the moment, the major users of the current next-generation sequencing tools are research laboratories and gene-discovery companies for applications such as disease surveillance on dangerous mutations, flu virus genomes, mutation disco, linkage study of cystic fibrosis gene, and clinical lab for cancer/disease diagnostic or prognosis.

In October 1990, The Human Genome Project (HGP), formally began its strenuous thirteen years of international effort, concluded with a remarkable prominent achievement in biochemistry by completing the sequencing of 3 billion DNA subunits bases in the human genome [28, 29]. In early 2005, the materialisation of new DNA sequencing technologies has dramatically revolutionised the structure of the sequencing industry [30, 31]. This was a direct result of the arduous efforts made by the consortium, numerous genomics researchers and mostly importantly the emerging novel sequencing technologies. This later development allowed for a considerable reduction in the cost of the sequencing process in addition to supplying innovative and economical platforms for the next-generation of DNA sequencing. The scale of reduction of sequencing time and cost is exponential, which is comparable to Moor's law in semiconductor industry [26]. Without using Sanger's method [32], it is

possible now to sequence a whole human genome within days, which costs only tens of thousands US dollars [33]. This new generation of technologies and research has changed the landscape of DNA sequencing. The technology has become a push force that has established a positive feedback ecology between emerging applications [34, 35] and creation of new markets. As result, new research in DNA sequencing technologies will persistently grow[36] and continue to alter the spectrum of the DNA sequencing industry as innovative market opportunities arise.

Current state-of-art sequencing technology depends on synthesis of labelled nucleic acid or detection by synthesis. The target molecules could be DNA or RNA. Here we present some background knowledge about deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the first half of this chapter, followed by an overview of various sequencing applications by density throughput. Finally, we conclude this chapter with an overview of the current sequencing/genotyping technologies.

2.1 DEOXYRIBONUCLEIC ACID AND RIBONUCLEIC ACID

Living organisms are complex systems, which are made of cell or cells. It is estimated that as many as tens of thousands of different proteins are responsible for the normal cell functions [37]. In order to regulate and program such complicated networks of proteins, an enormous amount of information is required to be stored and processed correctly even in the most complex system. In this information system, it entails specific structures of proteins as well as the regulation instruction of proteins. The vast amount of information is coded and stored in the genome, which is made of nucleic acids [38].

The structure of a nucleic acid contains two main parts, ribose phosphate and nucleoside. The backbone of a nucleic acid is made of ribose sugar (RNA) or deoxy-

ribose (DNA) and phosphate connecting nucleic acids in a long chain. Each backbone ribose is attached to one of four different nucleoside bases. The order of the nucleoside in a long chain forms the basis of the sequence. The regulation and gene information are coded in the sequence. Genomic nucleic acid is mostly deoxyribonucleic acid (DNA) except in some virus genes, and are coded in ribonucleic acids (RNA). The famous double-helix structure of genomic DNA consists of two complimentary strands. Each strand consists of sequence of four nucleotide bases, adenine (A), cytosine (C), guanine (G) and thymine (T) as shown in Figure 2-1. In the structure of a double-helix, a nucleotide forms a Watson-Crick base-pairing relationship with another, such that A is complimentary to T and G to C. The double stranded structure is the basis for some crucial functions in life, such as replication of the genome, the process of copying itself to form new cells.

In the process of replication, the double-stranded structure is denatured with the help of replication machinery. Each strand then acts as a 'template', on which a new strand of nucleic acid grows by adding complementary nucleic acids to the template—adenine (A), which can only pair with thymine (T) and cytosine (C) can only pair with guanine (G). The process is known as 'chain extension' since the nucleic acid chain is extended by adding one nucleotide after another. As the chain extends, new double-stranded DNA is created consisting of a strand from the existing base template and another strand synthesised based on the template [38].

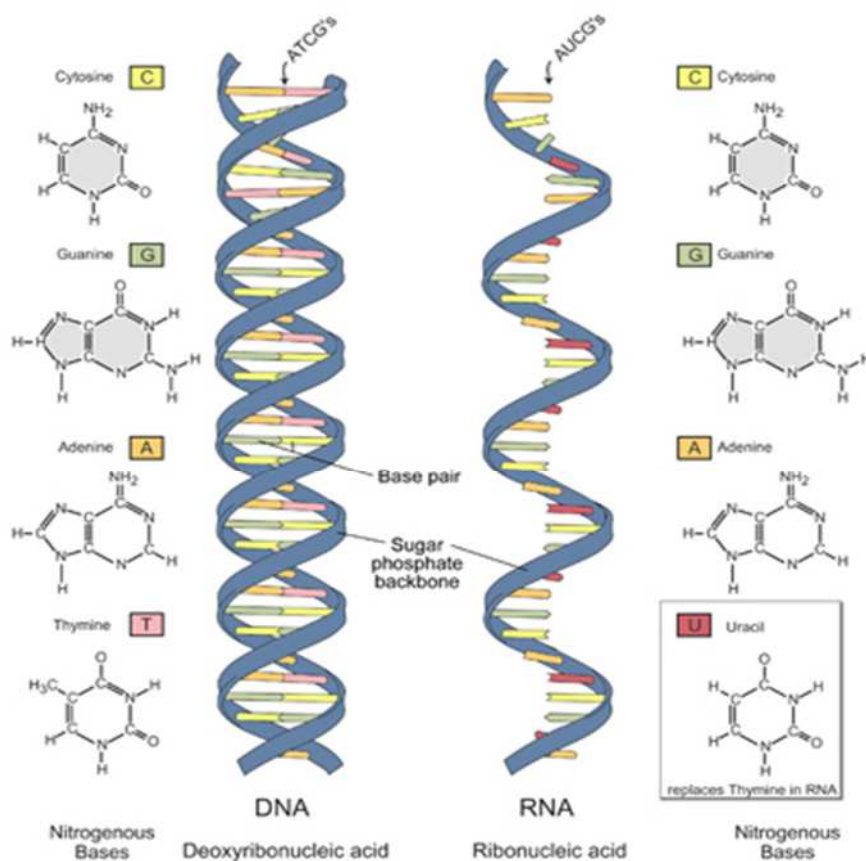


Image adapted from: National Human Genome Research Institute.

Figure 2-1: The structure of deoxyribonucleic acid and ribonucleic acid. Reproduced from National Human Genome Research Institute.

Unlike DNA, Ribonucleic acid (RNA) is usually a single-stranded molecule. There is hydroxyl group at ribose of RNA, which contributes to higher chemical activity, which makes RNA more susceptible to degradation. Another important feature of RNA is that thymine of the four nucleoside is replaced with uracil. (Figure 2-1) The uracil is paired with adenine in Watson-Crick base-pairing.

RNA plays an important role for the process of producing proteins. The messenger RNA, which is a transcription from genomic DNA, has the non-coding region of genomic DNA removed. As the result, the sequence of mRNA is more close to the sequence of protein following the corresponding codons. Study of the messenger

RNA reveals the snap-shot-of activity of the cells or tissues. The expression of the messenger RNA provides information as how cells are regulated and response to extracellular stimulation as well as the differentiation of tissues. It is also vital as a diagnostic tool such as the identification of cancer tissue or mechanism of the metastasis.

RNA is also the genomic material in retrovirus, such as influenza virus or HIV virus. The understanding and surveillance of the sequence of the RNA has been very important in containing the spread of diseases. On the occasion of a new influenza event, sequencing the RNA genome is the main methodology in the establishment of the nature of the virus.

2.2 SEQUENCING, MARKETS, AND APPLICATIONS

2.2.1 SEQUENCING

Sequencing has become an indispensable method for biological researches as well as applied service products. The emerging sequencing technologies developed on the shoulder of the capillary-based Sanger method have generated tremendous amount of the raw data. These raw data is not very useful without the equal development of bio-information innovations. The data generated from the new sequencing methods is generally highly fragmented genomic sequences. A generalised workflow of emerging sequencing technologies contains four steps. First, the nucleic acid (DNA or RNA) is purified and stabilised. The nucleic acid will be randomly fragmented to fit into the size of sequencing length and for some methods enriched by amplification in the second step. As a result of the fragmentation, 10^5 - 10^7 of sample sequence pieces are generated. Each sample is then subject to a sequencing step,

where the detection methods are involved. Two classes of detection methods would cover most of the current emerging technologies, sequencing by synthesis (pyrosequencing, Illumina/Solexa system, and Life Technology Solid system) and sequencing by direct reading the nucleoside sequence of a nucleic acid string (Pacific Biosciences, Helicos Biosciences and nanopore technologies) [39]. At the end of the sequencing step, a large number of the sequences of various lengths, which overlap each other, are assembled using bioinformatics methods. The degree of the overlapping between sequences generally refers to the coverage of how deep the sequencing is. A deep sequencing run can generate the number of nucleoside read 20 times more than the size of the sample. For example, the 20 times of human genome is 6×10^{10} nucleotide base output.

2.2.2 MARKET

Bioinformatics is applied to the large number of the information generated in a sequencing run. Genomic samples from different species can be resolved without tedious strain isolation in metagenomic studies. Similarly, deep sequencing is essential in fields such as epigenetic which plays an important role in cancer development, high genetic variation study such as human leukocyte antigen typing, and rare virus strain diagnosis in HIV therapy.

Consequently, the new sequencing methods are transforming the practices and enabling sequencing as a tool in clinical diagnostics, biotechnology, forensic biology, as well as basic biology. The advent of sequencing technologies and the emergence of novel applications have driven the market to grow immensely. The 454 Life Sciences sale figure for 2009 is \$143 million [40], \$80 million for Life Technology SOLid system in a CityGroup report [41]. The largest market share is occupied by Illumina,

which accounts for 60% of the total market in the same report. According to the consulting firm Scientia Advisors, the DNA sequencing market including system sales, reagents, and service, is expected to grow from \$1.2 billion in 2009 to more than \$3.6 billion in 2014 [42].

2.2.3 APPLICATION

The DNA sequencing market is divided into various segments by application: Life science research and drug discovery for further medical and bioscience developments. Due to the high cost associated with the current emerging sequencing methods and low throughput of capillary methods, the commercial applications are restricted at the moment.

Currently, major sequencing market sales are from biological research project founded by government or institutions or Pharma/biotech in the field such as drug discovery. The revenue has an estimated of \$920.1 million in 2010. Within 5 year, the markets are expected to increase to \$1.7 billion which is equivalent to a compound annual growth rate (CAGR) of 13% [43].

The sequencing tasks from research project tend to be gene discovery instead of gene testing. The genomic sequencing work is still active in more organisms or re-sequencing form individual variations.

Some limited applications are molecular diagnosis such as cystic fibrosis, where the variation is great. The interest is also increasing in oncology. As tumour is typically associated with multiple mutations and auto-mutations, identification of mutation components could be useful in making decisions for a specific therapy regime. It is one of the personalised medicine applications [44].

One high profile case that is trying to break away from the model is the direct to consumer (DTC) personal genome service. This type of model combines the technology providers, such as Illumina, and high street shops facing consumers, such as 23andme. The customer submits the sample such as saliva to the shop, which provides a list of pre-selected sequencing products. The sequencing factory is then commissioned to perform the test without knowing the identity of the customer. However, the type of service has been under scrutiny from regulatory bodies [45].

In a more narrowed field of sequencing, the interest has focused on some single nucleotide polymorphisms (SNPs). SNP is a type of common genome sequence variation in a population. The variation is typically a change of one nucleotide at a position while flanking regions are not changed. The application covers some common genetic disorder or variations such as drug adverse response. An example is the administration of Warfarin. US Food and Drug Administration (FDA) has advised taking a SNP test on VKORC1 and CYP2C9 polymorphism for drug dose decision. At the moment, high density sequencing, DNA microarray, and real-time PCR are three major technologies used in SNP assays.

It is expected that much more applications in personal genomics will be delivered in the near future. The applications cover SNP detection through complex genome wide screening or sequencing. Many applications in this field are yet to be established as a standard practice. However, the emerging applications carried most expectation for exponential market growth as predicted in the last market report. The emerging market value will be 10 times bigger in five years from now (Figure 2-2).

Other emerging applications include diagnostic sequencing, gene methylation analysis, and pathogen subtyping. Before sequencing is a standard routine clinically

diagnostic tool, cost and accessibility of the technologies would have to be cost effective.

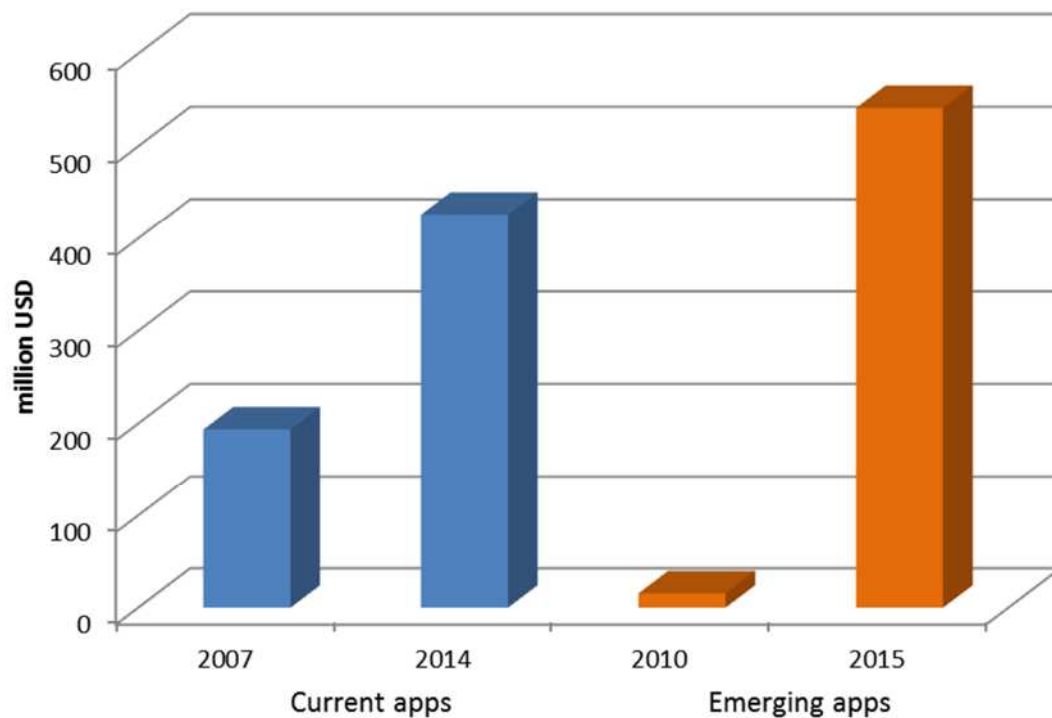


Figure 2-2: The projected grow of sequencing applications[43]. The emerging applications are expected to outgrowth the current commercial applications at a CAGR rate of more than 100% from 2010 to 2015.

High growth in the DNA sequencing market is foreseeable in the near future. Driven by technologies and market forces, the sequencing industry is shifting away from stagnant and declining grounds in sequencing discovery market. Advent of DNA sequencing as a premier genetic analysis tool used for existing applications such as de novo whole genome sequencing in infectious disease surveillance and entirely new applications like epigenetic tag sequencing are significant indication of this booming market.

2.2.4 HIGH-DENSITY SEQUENCING

The current sequencing technology development has been driven by the throughput of the sequence read per run. Part of the drive is due to the size and complexity of genomes. Part of the reason is the cost of the test. Due to constraints of the technologies available in preparing sequencing DNA, it is difficult to reduce the preparation steps. Fragmentation and genome wide amplification are essential for high quality sequencing data. Currently, there are few available proprietary methods, emulsion or bridge PCR, which limit the freedom of operation, let alone the preparation time incurred. In order to make the process cost-effective in terms of cost and time, a large throughput of sequencing data is required. This is achieved by using a higher density of the reactions and microfluidic cell structures. Cost per mega base read (USD/MB) is generally used to estimate the cost of the sequencing run. At the moment, the cost could be 2-40 dollar per MB, which is about one hundredth of the Sanger method used in HGP project (Table 2-1).

Because of the cost and technology complexity involved in high density sequencing, it is not cost effective to sequence target less than a few mega bases. On the other hand, a lower density or a small target would require different detection technology or special markets to keep the method market viable.

	USD/MB	No. run
Sanger	2742.9	340000
454	42.9	234
Illumina	2.1	40

Table 2-1: Cost and number of sequencing run of sequencing a human genome [39].

High-density sequencing application is defined as sequencing more than millions of base pairs per run. This includes full genome sequencing (Genome-wide de-novo sequencing/re-sequencing). The method is usually limited to very low sample numbers in exchange for an ultra-high base read. The cost of the sample preparation and sequencing reaction are spreading over the enormous size of genome. Even so, the reported retail cost for the new Illumina HiSeq2000 will cost 10,000 USD for one human genome wide sequencing announced by CEO J. Flatley in Goldman Sachs Healthcare Conference in 2010.

The capability of running deep sequencing makes high density sequencing a useful tool in monitoring the event of emerging infectious diseases. The method could detect conserved point mutations within samples along with the wild type. It is, therefore, cost effective in surveillance of dangerous mutations such as Influenza H1N1.

The main users in this market would remain research laboratories and biotechnology companies because of the high cost associated with the technologies. Current players in this market are 454 Life Sciences, Illumina Inc, and ABI Solid Sequencing. The average cost of sequencing systems in this market is over half a million USD.

2.2.5 LOW-MID DENSITY SEQUENCING

Sequencing is a powerful tool in understanding variations between individual as well as phenotype association. It is particular useful in the field of diagnosis, prognosis, or clinical study. Giga-bases reading is not required if the target of interest includes a few genes. Deep sequencing or coverage of the gene is desirable in finding out the variations, especially for highly variable regions.

Many researchers are interested in mutation discovery and linkage study such as the cystic fibrosis gene with larger sample number or cytochrome P450 variations with a number of samples. The application does not require the same number of base reading since the length of gene is much shorter than the genome.

The copy number of a sequence or a gene can vary and the variation is associated with a phenotype. The study is called the copy number variation (CNV). It has been associated with autism, schizophrenia, autoimmune, and cancer development [46-48]. The genes of interest for CNV study are established and knowledge of the variations is available. The needed CNV sequencing throughput is much less than the whole genomic discovery [49].

The works on gene variation or CNV form the basis for a diagnosis market which depends on sequencing. Diagnostic sequencing could be very specific to a known region of genome. Consequently, the sample preparation could target the sequence of interest. The fact that there is no need to randomise the fragmentation is crucial. When sequencing a known gene, a typical strategy contains amplification using specific primer sets. The primer sets are designed in such a way that they anneal to the conserved regions of the genome. The yield of the functional sequencing reactions will be much higher than the randomised method. It is also possible to automate the process without skilled users.

2.2.6 GENOTYPING

Gene variation between individuals or organism can be as small as one nucleotide. Single nucleotide variation (SNP) is known to associate with serious drug adverse effects or infection response [50, 51]. The international effort (SNP consortium) has been trying to discover novel SNPs, associate the SNPs to phenotypes, and build

haplotype of the variation (HapMap)[52, 53]. A few SNPs are associated with disease or drug response phenotype. The rest of the SNPs are called silent SNPs. A non-silent SNP could be found at crucial position of protein function or regulation of protein expression.

Consensus sequence, by known HLA-B57/5801-restricted epitope in Nef (amino acid position) and amino acid sequence			
KF9 (82–90)	HW10 (116–124)	YT9 (120–128)	GF14 (130–143)
KGALDLSHF	HTQGYFPDW	YFPDWQNYT	GPGIRYPLTFGWCF
.A.....	N.....		
.....	N.....		...V.H..C....
.....	N.....		
.....	N.....		...V....C....
.....	N.....		...V.....
SA.V.....	N.....		...V.....

Figure 2-3: Amino acid sequence variation in HLAB57/5801. The variation from consensus sequence is shown in the table. The amino acid sequence variation is corresponding to gene sequence mutation or SNP [54].

For SNP discovery, deep sequencing is a reliable method to find out the variation. The users of SNP discovery are mainly research institutes, biotechnology firms or pharmaceuticals. On the other end in the genotyping market, the mutation or variation is known. The task is to query the sample about its genotype. There are a few methods to detect SNP. Since the sequence in query is only one nucleotide, it is possible to use a PCR method or a DNA microarray to detect SNPs. Although these methods are strictly limited to pre-defined known variation, they can process much higher sample numbers. With a typical micro-titre plate assay, it is possible to run many PCR reactions using a standard 96-well format at a time. With PCR technology, the requirement for sample preparation is much less than any sequencing methods.

Therefore, the application of sequencing technology will be limited to highly variable regions, such that there are many SNP in a short region of sequence. It is very difficult to query multiple variations spreading over a short distance using PCR methods. An example is shown in Figure 2-3. In the case of GF14 epitope, the three variations are within six amino acids, which is equivalent to 18 nucleotides.

In such a field, direct sequencing is the best method to query the genotype of the sample. The low to mid density sequencing methods do not provide the whole picture of a genome. However, genetic variations are few and most of the genomic sequences are the same between individuals. It is sensible to target the variations that are relevant rather than sequence the whole genome. When the application is very specific, such as a particular drug response, low-mid density sequencing methods are the better tools that provide critical information.

2.3 CURRENT DNA SEQUENCING/GENOTYPING TECHNOLOGIES

2.3.1 INTRODUCTION

Innumerable technologies and resources have been promoted by the HGP project with profound impacts on biological researches, revolutionizing a wider spectrum of biomedical research, and clinical medicine.

HGP has relied heavily on Sanger sequencing, which is time consuming and extremely expensive (Table 2-1). The advanced DNA sequencing technologies have resolved such drawbacks. It brings down the sequencing cost dramatically. With the introduction of the HiSeq 2000 earlier 2010 [55], the cost of sequencing a full human genome had dropped to the \$10,000, which is helped by much higher throughput,

comparing with the price tag of \$48,000 a few years ago. In some cases, where the clinical relevance is obvious, the cost of sequencing a human genome can be reduced to \$9,500. The sequencing service is becoming affordable for mid-small research groups or individuals.

At the moment, the major DNA sequencing technology providers include Illumina (Solexa), Life Technology (ABI and Ion Torrent), Roche (454 Biosciences), Pacific Bioscience, and Helicos Biosciences. Companies developing the nanopore methods for sequencing [56-58] such as Oxford Nanopore Technologies [59, 60] are yet to produce commercial products in the market.

With the challenge of 1000 dollars per human genome, the most promising technologies are single molecule sequencing. Rather than reading the signal from thousands of pre-amplified molecules, single-molecule sequencing techniques can read the signal from a single molecule. The signal could be fluorescent labelled nucleotides, such as Pacific Biosciences, or direct reading using electrical signals, such as nanopore methods. However, single molecule detection is generally very sensitive to background variation. The cost of the sequencing assembly is also a challenging bottleneck.

Here is a short review about the evolution of the sequencing technology in detail.

2.3.2 1ST GENERATION SEQUENCING

The first generation sequencing is synonymous to the Sanger method. The method is based on the chain terminator reaction. The sequencing start from a purified double-stranded DNA. Sequencing reaction is divided into four ddNTPs. Newly synthesised DNA is labelled by strand (Figure 2-4) or by ddNTPs. The sequence from a specific

primer can be resolved by running on a high resolution denaturing gel [61]. For each Sanger run, the sample is split into four terminator reactions, (ddATP, ddGTP, ddCTP, and ddTTP). The terminator is a nucleotide analogue which is labelled with a fluorescence molecule. When a strand of DNA is incorporated with a terminator, the extension reaction is terminated due to the lack of active 3' hydroxyl group on the terminator. In each termination reaction, the extension from a primer will terminated at various positions. Once the terminator reactions are finished, each reaction is applied to gel electrophoresis. From the result of the gel read, a sequence can be established to around one thousand bases. The work flow is illustrated in Figure 2-4.

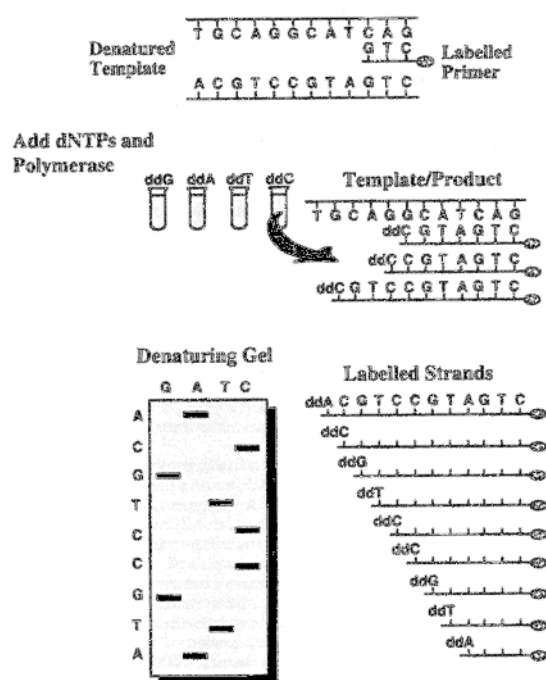


Figure 2-4: Work flow of Sanger sequencing method. Reproduced from Northwest Fisheries Science Center.

The method, however, requires large amounts of the fragment in separate reaction tubes. This is typically achieved by cloning technology. The genomic DNA has to be sheered into millions of fragments, which are transformed into bacteria. Bacteria grown in a single colony contains only one type of fragment. Each colony is grown

into larger amount in order to produce enough plasmid, which is purified for Sanger sequencing. The process is illustrated in Figure 2-5. The target DNA sequence has to be fragmented and cloned into a bacteria plasmid (bottom left of the graph). One bacterial dot grown on an agar plate (bottom right) represents one plasmid or one unique fragment of template [62].

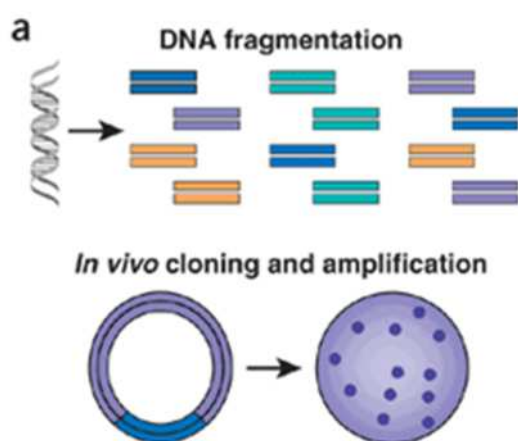


Figure 2-5: Cloning step for Sanger sequencing [62].

The cloning step has severely increased the complexity of the sequencing method. Millions of samples are going through growing bacteria, purification of the plasmid, sequencing reaction, and electrophoresis. It is not surprising that it has taken more than 10 years to finish a draft map of the human genomic sequence.

2.3.3 2ND GENERATION SEQUENCING

The next generation of sequencing methods have tackled the complexity issue in the preparation of the DNA sample. The complex cloning system is replaced by *in vitro* amplification methods. The challenge of creating amplified DNA fragments in a physically separated space was solved by performing emulsion PCR using micro beads. The reaction removes the need for cloning and purification altogether. The

method was pioneered by 454 Life Sciences, and is shown in Figure 2-6. In an emulsion PCR, micro particles and DNA fragments are mixed in a diluted solution therefore part of the beads contain only one copy of DNA fragment in the first step. Millions of bead in individual oil droplet go through PCR simultaneously in a tube. As the result, each template DNA is amplified without contaminating each other [63]. The genomic DNA is fragmented by sonication or sheering. After polishing both ends of double-stranded DNA fragments, an oligonucleotide adapter is added to the fragment. The adapter will guide the DNA template to anneal with a primer on a microbead. When the solution of microbead and DNA template is diluted, according the Poisson distribution, fraction of the beads will only have one DNA template per bead.

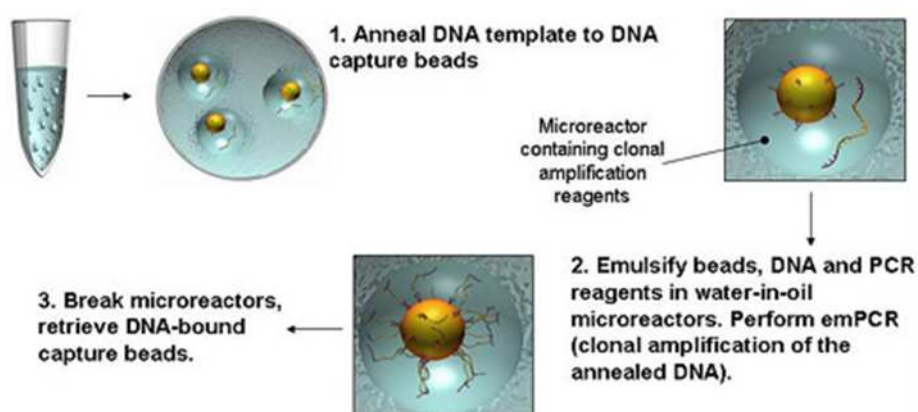


Figure 2-6: Work flow of emulsion PCR [63].

The ability of amplifying the genomic fragment in a single tube reduces the preparation time from weeks to a few hours. The cost saving is more than thousands fold as there is no need for growth media and colony management. As there is only one type of DNA template in each bead, the sequencing reaction at each bead represents a full sequencing of one Sanger's gel.

2.3.3.1 454 TECHNOLOGY

Life Science [30] published the first sequence map of genome in 2005 using a pyrosequencing method. The work-flow of the 454 sequencing system FLX contains the preparation of the DNA bead by emulsion PCR, loading the bead in the micro-wells, sequencing by synthesis in each well, and base-calling with sequence assembly. Nucleotide of A, T, G, and C flow through the bead sequentially. The reaction chemistry is based on the product from the DNA extension, pyrophosphate. Pyrophosphate is used as the energy source to drive light emission by luciferase as shown in Figure 2-7. The emission is proportional to the number of nucleotide extended, therefore multiple insertions of a single nucleotide can be resolved. The density of the 454 micro-well is about 400,000 per chip. However, the yield of read from the chip is about 50% due to multiple-bead in a well or empty bead without DNA template. The light emission is detected by optical detector through the optical fibre. The read throughput is restricted by the number of active beads per chip times the number of read length, which is about 400-800 bps.

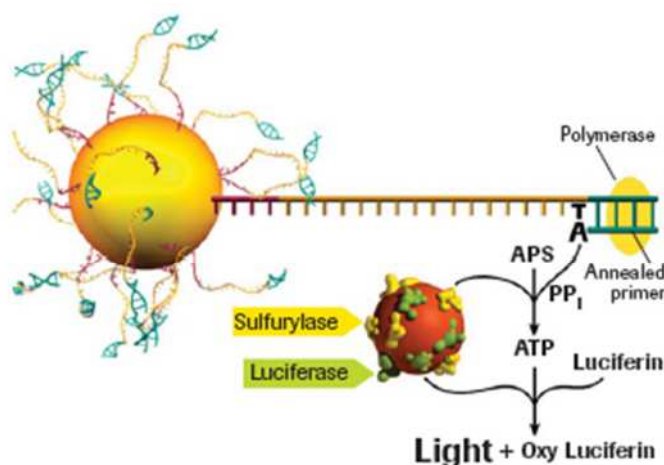


Figure 2-7: 454 pyrosequencing reaction mechanism. There are three types of microbead in 454's system. The largest one is the DNA beads. The enzymes and

polymerase is immobilised to other beads such that the assembly can go through many cycle of washing [64].

2.3.4 SOLEXA TECHNOLOGY (ILLUMINA)

The cumbersome step of cloning in Sanger sequencing is replaced by the polony method in Solexa Technology. The polony is defined as the DNA colony on a solid matrix in comparison to the bacterial colony on an agar plate. Solexa achieved the polony by performing bridge PCR on a micro slide as shown in Figure 2-8. The sequencing reaction is similar to Sanger method but the terminator could be activated. Each terminator (A, T, G, and C) carries a unique fluorescent label. Photos of four colours are taken and each colour represents an insertion of the corresponding nucleotide.

After the reading is done, the blocker at the 3' end of the inserted nucleotide is removed and the next round of terminators is introduced. Repeating the process builds the sequence of the target template.

The method can achieve very high density since the DNA cluster occupies a much smaller space (10-100 nm pitch distance) than a micro-well does. As the result, much larger data throughput, 100 million polonies per chip, is possible with the method.

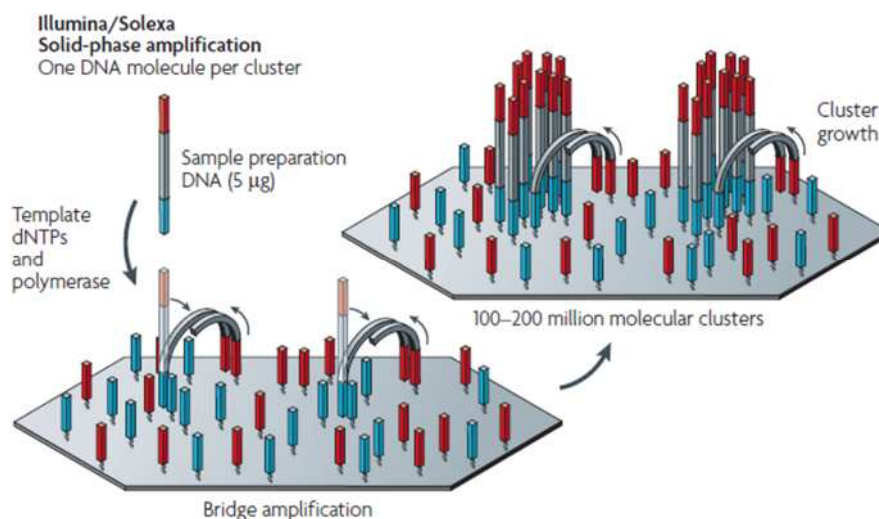


Figure 2-8: Solexa bridge PCR mechanism. Each fragment of DNA template is amplified and forms a small cluster on the surface of the chip, called polony [39].

2.3.5 HIGH DENSITY 3RD GENERATION SEQUENCING TECHNOLOGIES

Numerous sequencing technologies have emerged from companies such as Pacific Biosciences, Oxford Nanopore, Cracker Bio. However, these sequencing technologies either require optical labelled detectors with very high resolution/speed or the difficult to control pore detection at the current state-of-art setup. Many disadvantages then may be, but high hope has been that the technologies could one day remove the need for enzyme reactions used in pyrosequencing or amplification. It is still early in term of maturity of technology but single molecule detection has shown the potential for very long read (> 1,000 bases) and capability of reading the methylation of the nucleotide in CpG islands.

Oxford Nanopore Technologies and others are developing a new generation of DNA sequencing technology that does not require fluorescent labels or optical instrumentation. The method reads DNA bases directly. The measurement is possible

by passing a strand of DNA through a very small hole. Signals of different bases are differentiated and recorded in sequence as the strands of DNA moving through the nano pores.

2.3.6 LOW DENSITY GENE SPECIFIC GENOTYPING TECHNOLOGIES

The genotyping is the process of determining the genotype of an individual by the use of DNA micro arrays (Figure 2-10) or real-time PCR (Figure 2-9). Due to current technological limitations, the sequencing result from genotyping is partial even with the ultra-high density of DNA microarray. However, clinical relevant sequence information generally is very concentrated in short regions since the protein-encoded sequence only accounts for less than 2% of the total genome [65]. Sequencing by microarray or real-time PCR could be effective when the sequence variation is very well known and conserved. However, for *de novo* sequencing or sequence variation discovery, it is better to use high-density sequencing method or Sanger's method.

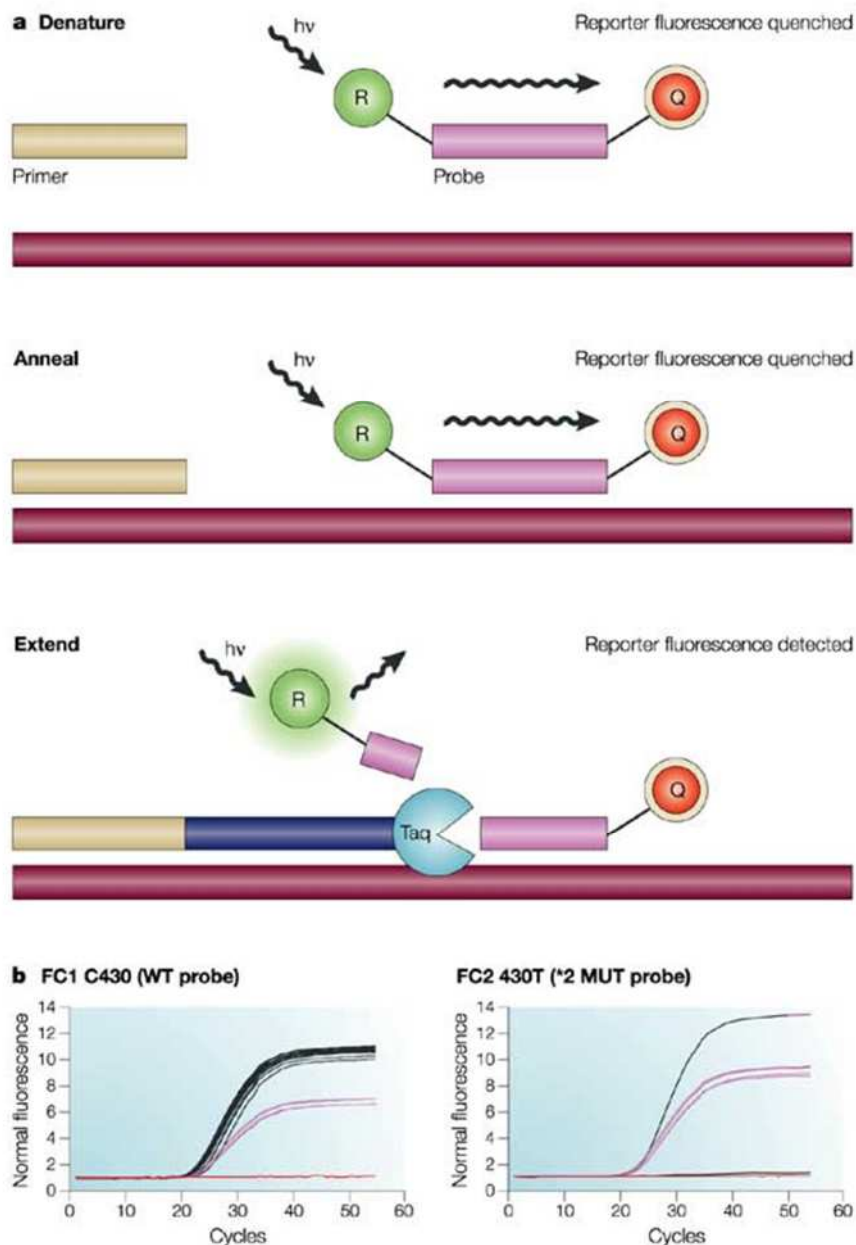


Figure 2-9: SNP detection using Tagman assay. The SNP in the graph is CYP2C9. In the bottom graphs, it contains several samples in each graph. The black lines represent the homozygotes while the pink lines in the middle represent heterozygotes [66].

Genotyping covers copy number variation (CNV), SNP, deletion and insertion of gene sequence. The new knowledge of epigenetic application will cover the methylation of the genome sequence [67]. It is also very useful in typing and identifying infectious diseases. Viruses for instance, or bacteria, can be detected

and genotyped for great clinical value [68]. Genotyping in this context may help in controlling the spreading of pathogens, by tracing the origin of outbreaks. This area is often referred to as molecular epidemiology or forensic microbiology.

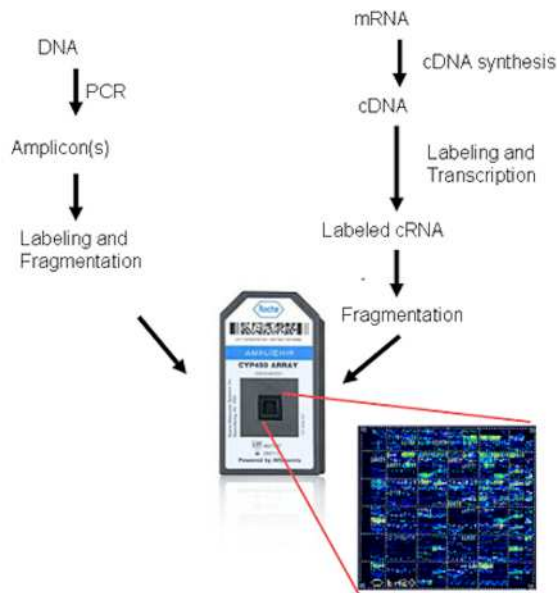


Figure 2-10: The DNA microarray [69]

Apart from pharmacogenetics applications in genotyping human beings, the technology is also crucial in individual identification. When testing for father-/motherhood for instance, scientists typically only need to look at 10 or 20 genomic regions (like single-nucleotide polymorphism (SNPs) to determine relationship or lack thereof. That is a tiny fraction of the human genome, which consists of three billion or so nucleotides.

Genotyping mainly focuses on molecular diagnostic, genome-wide SNP pattern discovery, direct-to-consumer (DTC) personal genetic tests such as 23andme and Navigenetics.

2.4 SUMMARY

DNA sequencing technologies continues to be currently dominated by the Sanger chain termination method. Motivation for faster, simpler technologies, which can sequence genomes within a day, are actively sought and many new techniques to rival the Sanger technique are emerging.

Nucleic acid tests (NAT) have a wide range of applications and markets. They can detect the presence of a few copies of cellular, bacterial, or viral genome and with a specificity that is capable of detecting a single base change in the nucleic acid sequence. The existing applications in the market include the detection of single-nucleotide polymorphisms (SNPs), human genomic variations, viral and bacterial detection and sub-typing. However, to date the current NAT systems require intensive capital investments, complex laboratory based machines, and trained laboratory technicians. We are starting to see a divergence in sequencing technologies, as each sequencing methods concentrates on having clearly defined advantages and potential applications that differ from all others.

In order to develop a point-of-care setting NAT device, a label free detection platform is inevitable. We propose a novel approach based on our core invention. This approach utilizes the fact that during the amplification of microbial nucleic acid by PCR, where there is an exponential release of protons resulting in a pH change, which can be detected using an Ion Sensitive Effect Transistors (ISFETs).

CHAPTER 3 – ISFET-BASED NUCLEIC ACID REACTION DETECTION

3.1 PROTON RELEASE MECHANISM

Nucleic acid detection by ISFET depends on the proton release from a nucleic acid reaction. The proton balance after the hydrolysis of a nucleic acid is dependent on the dissociation constants of acid and base species in the solution. The production of proton causes change of pH, which could be detected by ISFET. The Henderson–Hasselbalch equation derives pH from the acidity in biological and chemical systems as shown in Equation 3-1. [57] The equation is used to find the equilibrium pH in acid-base reactions and estimates the pH of a buffer solution. Equations are shown below:

Equation 3-1 Henderson Hasselbalch Equation

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

Equation 3-2 Equilibrium Constant

$$K_a = \frac{[A^-][H^+]}{[HA]}$$

where $pK_a = -\log(K_a)$, and K_a is the acid dissociation constant for the non-specific Bronsted acid-base reaction. In addition, in Equation 3-2, A^- represents the ionic form of the relevant acid, and $[HA]$ and $[H^+]$ are the molar concentration of the quantity within the solution.

According to the Henderson-Hasselbalch, the pH of a solution containing an acid-base conjugate pair can be calculated. Thus, the number of protons release is contingent to the acid-base equilibrium after the reaction. Essentially, when the protons generated pass the buffer capacity, the pH will drop to a new acid-based chemical equilibrium. [58]

Here are two example cases:

Case 1

The initial chemical equilibrium is established at pH_0 therefore

$$pH_0 = pK_a + \log \frac{[A^-]_0}{[HA]_0} \text{ and } K_a = \frac{[A^-]_0[H^+]_0}{[HA]_0} \quad (1)$$

Also, from the mass balance,

$$[HA]_{total} = [HA]_0 + [A^-]_0 \quad (2)$$

This is useful since the actual concentration at a given time is difficult to know. We know at least two things, (i) boundary condition (starting concentration) and (ii) one of the components in real time measurement. ($[H^+]$ in this case) We can work out the rest components by applying the mass balance and the equilibrium equations.

When one adds more $[A^-]$ salt, such as N_aA , the equilibrium balance is disrupted and new balance will be reached to maintain a constant K_a . At the new equilibrium, some of the $[A^-]$ will be removed by forming complex with $[H^+]$. Here we do not know the final $[A^-]$ after adding the known $[N_aA]_a$. We can measure the pH value. If the $pH = pH_1$, then

$$pH_1 = pK_a + \log \frac{[A^-]_1}{[HA]_1} \text{ and we can get the value of } \frac{[A^-]_1}{[HA]_1} \quad (3)$$

The final concentration of $[A^-]_1$ and $[HA]_1$ can be work out using mass balance, where

$$[HA]_{total} = [HA]_0 + [A_0] \quad (4)$$

$$[HA]_{total} + [NaA]_a = [HA]_1 + [A^-]_1 \quad (5)$$

Case 2

The same initial equilibrium was established. This time, we add more alkaline ($NaOH$) in with the pH meter recording the reading in real time. Adding the alkaline also disrupt the old equilibrium. When the new equilibrium reached, with the help of pH meter, you should know the pH value in real time. Let's assume it is pH_2 .

Again we can use the Henderson Hasselbalch equation to work out the ratio of $[A^-]_2$ and $[HA]_2$ since the K_a is a constant. That is to say,

$$\log \frac{[A^-]_2}{[HA]_2} = pH_2 - pK_a \quad (6)$$

From the mass balance, we know

$$[HA]_{total} = [HA]_2 + [A^-]_2 \quad (7)$$

In the end, you can find out the concentration of the $[HA]$ and $[A^-]$

Since the reaction is only depending on the nucleic acid reaction. It is plausible to extend a single base insertion to continuous multiple-base reactions, such as the case in DNA and RNA amplification. Because there is no required modification in the reaction mixture, the application is a truly label-free detection of nucleic acid.

3.2 DNA AMPLIFICATION BY POLYMERASE CHAIN REACTION (PCR)

The Polymerase Chain Reaction (PCR) was first introduced in 1984 for replication and amplification a fragment of double-stranded *dsDNA* from a given DNA template.

Typical components of a PCR include: [70]

1. Thermal cycling temperatures between 98-50°C
2. Nucleic acid template as few as 1 copy
3. Two oligonucleotides acting as sense and antisense primer and dNTPs
4. Potassium chloride for stabilization of *dsDNA* as well as ionic strength
5. Thermal stable *DNA* polymerase such as *Taq* polymerase
6. Magnesium ion for *Taq* polymerase activity.

PCR selectively amplifies a region of *dsDNA* to a typical 1×10^8 fold in 35 cycles of thermal cycling. The time needed for 35 cycles range from 10 minutes to 2 hours depending on the thermal mass of all materials in thermal cycling. When diffusion/activity is not considered, the amplification is exponential of 2^n , where n is the cycle number. In practice, the efficiency is less than one and therefore the fold of amplification will be less than 1.7^n . As the rule of nature, it is impossible to amplify anything indefinitely. The possible restrictions on PCR amplification include the increased concentration of pyrophosphate and decayed enzyme activity. [71]

3.2.1 INTRODUCTION TO NUCLEIC ACID LABEL-FREE DETECTION

We use PCR for two purposes, selective amplification for Klenow fragment extension and nucleotides detection in the integrated device. Klenow fragment is a *DNA* polymerase, which works at 37°C. The name comes from the Danish scientist, H. Klenow, who published the method of making the polymerase from *E. coli DNA* polymerase. The size of the Klenow fragment is about 70 *kDa* with dimension around 7nm x 8nm x 9nm Figure 3-1. It has one reactive $-SH$ group on the surface, which may be able to form a covalent bond with pure gold. [69]

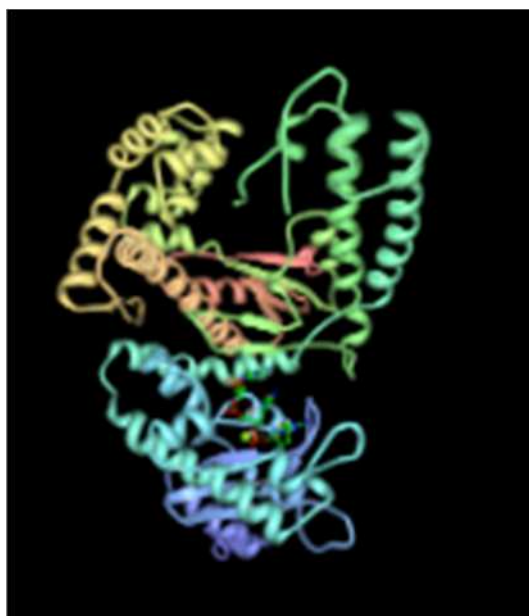


Figure 3-1 3-D structure of Klenow fragment.

To implement a Klenow fragment extension we must first use a pair of *ssDNA* primers to amplify both nucleotide variations in a targeted location. It is the detection *ssDNA* probe in the Klenow Fragment extension assay that differentiates and gives the signal if there is a match. An alternative method involves using a modified primer in the *ssDNA* primer set. The 3'-end of the modified primer matches with the targeted nucleotide and forms mis-pair with another nucleotide. *Taq*, like Klenow fragment,

which lacks of 3'-5' exonuclease activity, extends only the matched primer. Only the matched nucleotides are amplified exponentially when both primers match with the sample. The mismatched nucleotides are also amplified but the amplification is linear to the cycle numbers. [24, 72]

3.2.2 DNA POLYMERASE ACTIVITY:

The principle of nucleic acid detection relies on the function of DNA polymerases. DNA polymerases are responsible for matching DNA sequences with the right nucleotides and for catalysing the insertion of the nucleotides on a growing DNA primer. Below is a list of critical activities and observations that are important to the DNA Logic detection.

1. 3'OH extension from a *ssDNA* primer hybridized to a longer *ssDNA* template.
2. The activity is dependent on Mg^{2+} and use dNTP as the substrates. Mg^{2+} is known to affect the non-specific extension from a mis-matched 3'OH *ssDNA*.
3. The KF should only extend matched 3'OH primer-template duplex but we have seen non-specific extension from a mis-matched 3'OH *ssDNA*.
4. The pH tolerance is believed to be between *pH6* and *pH9*.
5. The stamina of KF is low (processivity) at around 100bps before it stops extending and dissociated from the *dsDNA*.

Taq DNA polymerase which has polymerisation activity extends the *ssDNA* primer from the 3'-OH. The enzyme is relatively stable at high temperature less than 100°C. There are varieties of *Taq* polymerase. Most of them are modified chemically or with antibody blockage to reduce the activity at room temperature. The blockage can be

removed via an activation step normally held at 95°C for a few minutes before the cycling programme. Each cycle comprises denaturation, annealing and extension. The cycle is repeated 30-40 times in a PCR reaction. Each of the three steps has a different temperature which is utilised to control the association of polymerases and dsDNA as well as the hybridisation of ssDNA. It is possible to merge the annealing and extension in one step, which reduces the total reaction time required to finish amplification. Below is a list of important observations and factors that are specific to a PCR reaction.

1. Extension from the 3'-end hydroxyl group of an *ssDNA* primer hybridized to a longer *ssDNA* template.
2. Magnesium ion is essential for activity. Typical concentration is between 1.5mM to 2mM on top of the Mg^{2+} -*dNTP* complex.
3. Higher magnesium ion concentration increases the activity but also increase likelihood of non-specific amplification.
4. The processivity is at hundreds bps and reaction speed is about 1 kbps per second.
5. We have seen not difference in amplification between pH8 and pH6.

1.1 RNA AMPLIFICATION

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is the reverse transcriptase where *RNA* is reverse transcribed to *cDNA* using a reverse transcriptase and primers. This is an important step allowing the performance of PCR since *DNA* polymerase can act only on DNA templates [73].

In the initial phase of the reaction reverse transcriptase (RT) converts the *RNA* to double stranded *DNA* as in RT-PCR Figure 3-2.

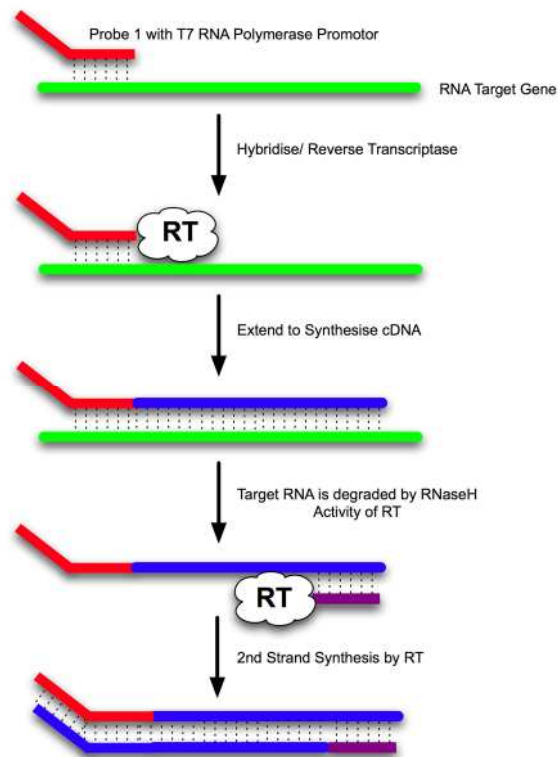


Figure 3-2 RT: Converts RNA to dsDNA

Subsequently the *cDNA* is cycled between *RNA* and *DNA* in an isothermal process Figure 3-3.

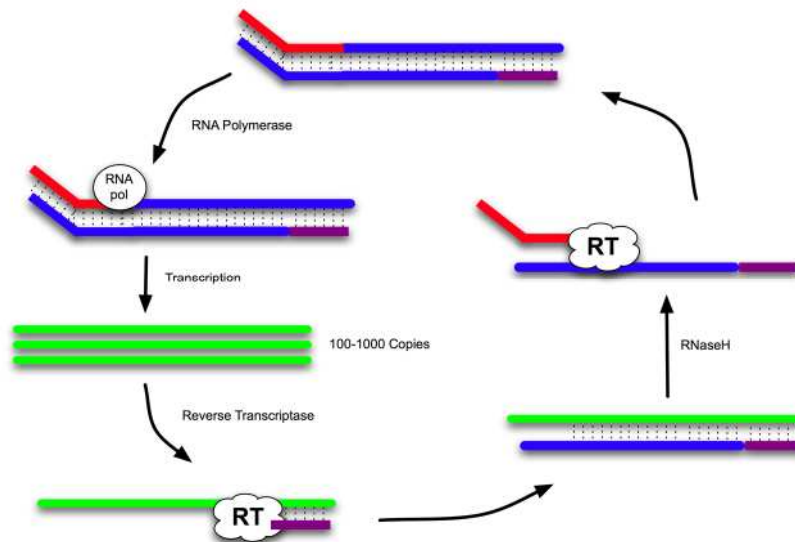


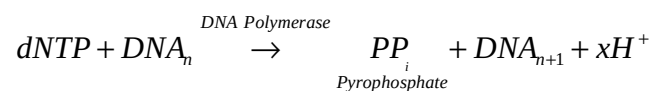
Figure 3-3 cDNA cycled between RNA and DNA in an isothermal process

The reactions above take place in a single process resulting in one million fold amplification in 60 minutes. [74]

1.2 FROM DNA TO PH DETECTION

Polymerase chain reaction (PCR) is the process of replication and amplification of a fragment *dsDNA* from a given *dsDNA* template. In the process, *dsDNA* is denatured into *ssDNA*. Once the melting process is completed, dNTP, DNA polymerase and magnesium ion will do the annealing and the extension to the melted *ssDNA* to form a new strain of identical *dsDNA*.

Equation 3-3 Protol reaction from nucleic acid extension



Klenow Fragment extension (Figure 3-4 and Figure 3-5), also called exponential amplification of nucleic acid, follows the same procedure as PCR but given a strain of targeted *ssDNA* primer. In other words, when the target primer matches the sample

fragment *ssDNA*, the fragment will extend while releasing protons and will not otherwise Equation 3-3.

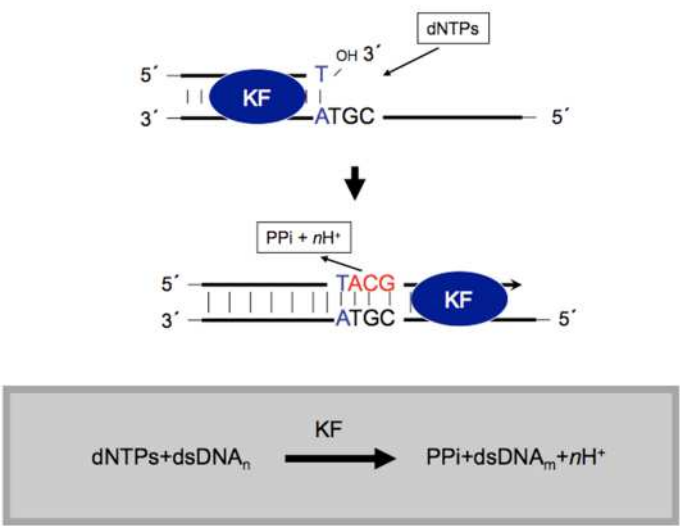


Figure 3-4 Klenow Fragment extension – matching – proton release

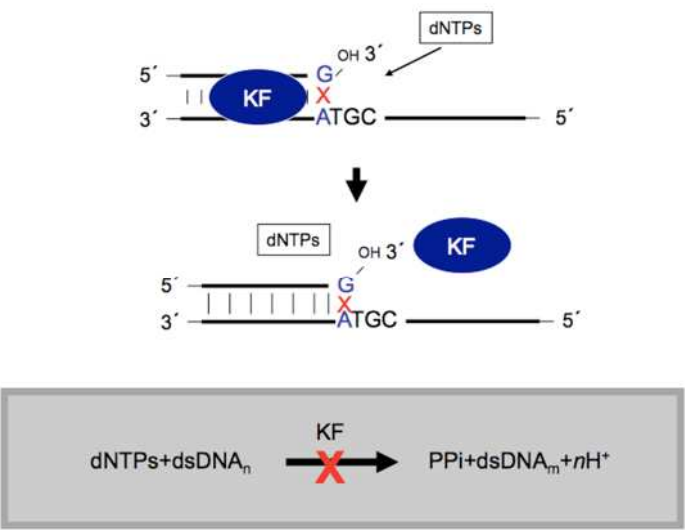


Figure 3-5 Klenow Fragment extension – mismatch with no proton release

1.3 ION SENSITIVE FIELD EFFECT TRANSISTORS REACTION MONITORING

1.3.1 CONCEPTS

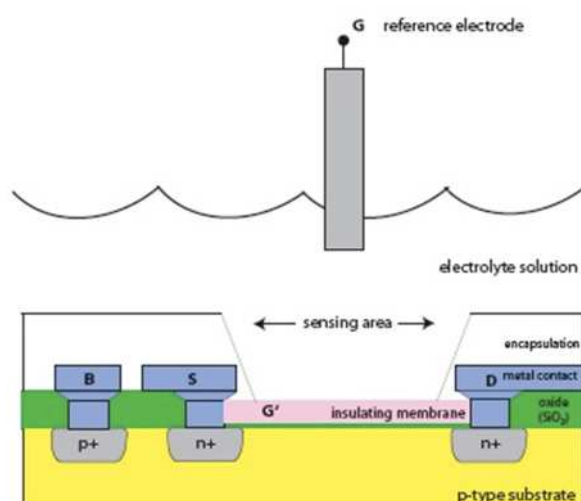


Figure 3-6 *pH*-ISFET scheme by Bergveld

The ISFET is a device that has gained much attention for use as a *pH* sensor. Being robust, cheap and small in size, it has rapidly become competitive with conventional ion selective glass electrodes. The device, which is well described in the literature [25, 75, 76], consists of a transistor similar to a traditional MOSFET but with the insulator surface exposed to an electrolyte solution to form an ion-sensitive area, and a remote gate provided by a reference electrode. The rest of the transistor is encapsulated to isolate the device from the electrolyte. The sensitivity is explained partly by the site binding theory and also by a fast dynamic electrochemical exchange component occurring at the surface of the ISFET [75]. The equilibrium between the binding and unbinding of charged ions at the gate surface becomes a function of analyte

concentration, which is measured as a change in the voltage drop across the electrolyte/insulator layers. This change in voltage is often described as a change in ISFET threshold voltage [25, 75] or alternatively as an intermediate, *pH*-dependent chemical potential between insulators surface and reference electrode [76] as illustrated schematically in Figure 3-7.

In the past, the ISFET has been prone to problems of hysteresis and drift. Therefore, there has been a reluctance to use it in critical biomedical applications. However, the method proposed in [77] made ISFET applicable. Changes between insertion and non-insertion of nucleotides are always compared such that the measurement of absolute *pH* values is not necessary. We expect the *pH* change from insertion reaction should be proportional to the number of insertions. Absolute measurement is not required when the relative difference between insertion and non-insertion is enough for monitoring. The use of the ISFET in this manner is considered as a novel method of applying the sensor for DNA analysis.

An ISFET *pH* detection mechanism for nucleotide incorporation creates a possibility of incorporating the detection method into existing cyclic array sequencing technologies such as pyrosequencing, allowing procedural simplification and cost reductions by eliminating the need for luminescence, fluorescence, and electrophoresis [77]. Thus, this particular method permits SNP detection outside mainstream laboratory facilities and allows identification by individuals such as clinicians and consumers in a point of care setting.

When chemical reactions cause discrete changes in chemical concentrations, these changes can be used as an indication for the presence of a reaction. For example, the sequencing of individual base pairs in a strand of DNA due to nucleotide

incorporation may cause a change in pH . Detecting this change in reaction serves as a positive marker for that nucleotide; therefore confirming its presence in that strand of DNA. The ISFET is based on a MOSFET with a remote gate (reference electrode) exposing with a chemically sensitive insulator to an electrolyte. Field-effects from ionic charge interaction at the insulator surface of an ISFET causes shifts in the ISFET $I_D - V_{GS}$ characteristic. The insulator in contact with the electrolyte is chosen according to its chemical properties and sensitivity to a particular ion. For the pH -ISFETs dealt in this thesis, use of silicon nitride and aluminium oxide membranes are common [25, 75, 76].

Any voltage applied to the reference electrode is coupled via the electrolyte to the insulator surface, where a pH -dependent charge from ions on this interface modulates the channel current, causing the observed shifts in the ISFET transfer characteristic, thus modulating its threshold voltage V_{th} .

Equation 3-4 Threshold voltage of ISFET [72]

$$V_{th(ISFET)} = (E_{ref} + \phi_{lj}) - (\phi_{eo} - x_{eo}) - \frac{Q_{ss} + Q_{sc}}{C_{OX}} - 2\phi_f + \frac{\phi_s}{q} = V_{th(MOS)} + V_{chem}$$

Equation 3-5 ISFET sensing and surface potential

$$V_{chem} = (E_{ref} + \phi_{lj}) - (\phi_{eo} - x_{eo}) - \frac{\phi_m}{q}$$

V_{chem} is a grouping of chemical potentials at the insulator surface of which ϕ_{eo} is the only pH -dependent term:

Equation 3-6

$$\phi_{eo} = 2.3U_T\alpha(pH_{pzc} - pH)$$

U_T is the thermal voltage kT/q , pH_{pzc} the pH required for zero charge on the insulator surface and α a dimensionless sensitivity parameter representing the ISFET's sensitivity S relative to the ideal Nernstian sensitivity:

Equation 3-7 Sensitivity of ISFET

$$\alpha = \frac{S}{S_{Nernst}}$$

The dependence of V_{chem} on pH is modelled using a combination of the site-binding theory and the Gouy–Chapman–Stern double-layer model [78], which can be reconciled in a behavioral macro-model representation such as that of Martinoia et al. as shown in Figure 3-7.

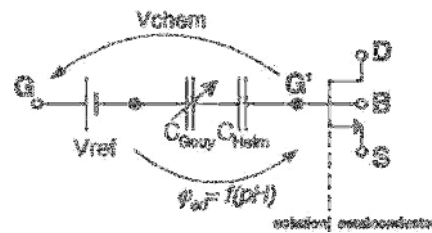


Figure 3-7 ISFET behavioural macro-model

A final simplification of this model is to group all chemical potentials independent of pH by substituting Equation 3-6 and Equation 3-7 into Equation 3-4 and Equation 3-5 giving:

Equation 3-8

$$V_{chem} = \gamma + 2.3\alpha U_T pH$$

where γ is a pH -independent grouping of chemical potentials. With α taken as constant to a first-order approximation, the threshold voltage of the ISFET is linearly proportional to pH , but is strongly temperature dependent due to scaling by the

thermal voltage. [76, 79] It is this change in pH representing in a change in transistor threshold voltage which gives rise to a change in the transistor's drain current. The device as with the normal MOSFET can operate in either strong or weak inversion.

1.3.2 WEAK-INVERSION ISFETS

The weak inversion region of a MOSFET is a region of operation of the transistor for which $V_{GS} < V_{th}$ and diffusion of charge carriers rather than drift across an electric field dominates the channel current. The large-signal equation for drain current in a MOSFET biased in the weak inversion region is given by:

Equation 3-9 Drain current in weak inversion region [74]

$$I_D = I_0 e^{\left(\frac{V_{GS}}{nU_T}\right)} \left(1 - e^{\left(\frac{-V_{DS}}{U_T}\right)}\right) = \left[2n\beta U_T^2 e^{\left(\frac{V_{GS}-V_{T0}}{nU_T}\right)} e^{\left(\frac{(n-1)V_{BS}}{nU_T}\right)}\right] \left(1 - e^{\left(\frac{-V_{DS}}{U_T}\right)}\right)$$

where $\beta = KW/L$, V_{T0} is the threshold voltage for $V_{BS} = 0$ and n is the sub-threshold slope factor and saturation is assumed for $V_{DS} > 4U_T$. It is suggested that this equation also holds for a weakly inverted ISFET, since all additional chemical phenomena are represented as the modulation of its threshold voltage by the potential V_{chem} , which is linearly proportional to pH . The significance of operating the ISFET in weak-inversion is not only the low current and voltages involved, but also that since the drain current of the ISFET is exponentially related to its threshold voltage which is proportional to pH , and pH is in turn logarithmically related to hydrogen ion concentration, a direct relation between drain current and hydrogen ion concentration will be apparent. [76, 79] A pH fluctuation, pH can be seen to scale the current as follows from Equation 3-4, Equation 3-5 and Equation 3-7:

$$\Delta V_{T0} = \Delta V_{chem} = 2.3\alpha U_T \Delta pH \quad (8)$$

From Equation 3-9, with $V_{BS} = 0$ and assuming saturation ($V_{DS} > 4U_T$):

$$I_D(V_{T0} + \Delta V_{T0}) = 2n\beta U_T^2 e^{\left(\frac{V_{GS}}{nU_T}\right)} e^{\left(\frac{-\Delta V_{T0}}{nU_T}\right)} = I_{DQ} e^{\left(\frac{-2.3\alpha U_T \Delta pH}{nU_T}\right)} \quad (9)$$

Finally substituting in the logarithmic dependence of pH on hydrogen ion concentration:

Equation 3-10 Definition of pH value

$$pH = -\log_{10}[H^+]$$

Equation 3-11 Drain current and proton concentration

$$I_D(V_{T0} + \Delta V_{T0}) = I_{DQ} e^{\left(\frac{-2.3\alpha U_T (-\ln \Delta[H^+]/2.3)}{nU_T}\right)} = I_{DQ} \Delta[H^+]^{\alpha/n}$$

This shows that the quiescent drain current I_{DQ} in a weak inversion ISFET that is scaled by a known and temperature independent power of a change in hydrogen ion concentration. This is a significant result, which could be manipulated into a more useful linear relationship. The index (α/n) of $[H^+]$ will always be between 0 and 1 since $0 < \alpha < 1$ and $n > 1$. To achieve direct proportionality between $[H^+]$ and drain current, ΔV_{TH} can be amplified by a gain A such that $\alpha A/n = 1$ before converting it to a current.

Alternatively, using a chemically tailored pH sensitivity such that $\alpha/n=1/x$ (where x is *real*), the drain current can be raised to a power x using simple trans-linear circuits. The latter method is presented herein because it is a large-signal approach and therefore operable over a wide pH range.

1.4 SIMULATION RESULTS

This simulation presents an output of an ISFET when there is a pH change. Here we assume a reverse transcription with RNA to form DNA such that DNA is the only input. The simulation does not include the boundary condition that the reagent will deplete in the end of the reaction. It only shows a general trend of the pH value trace.

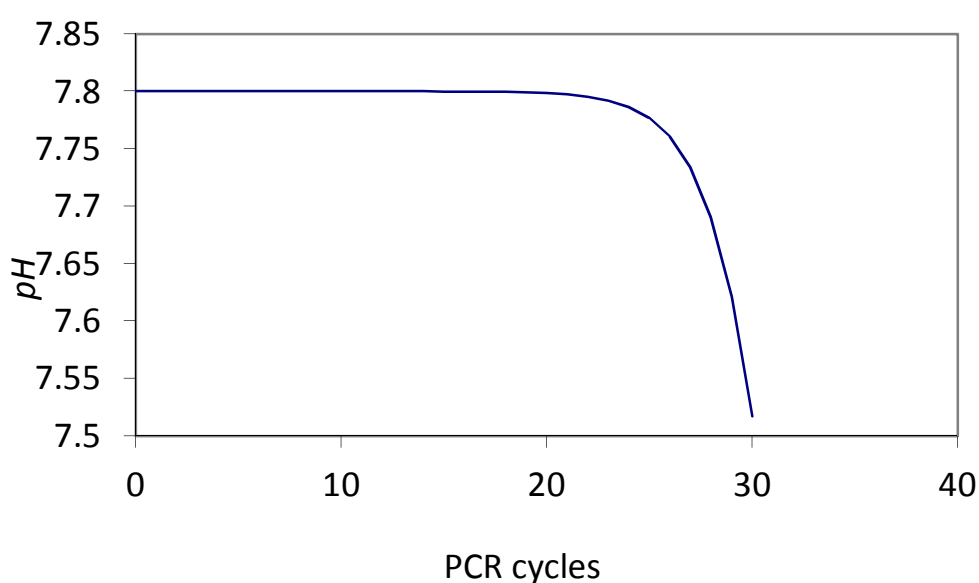


Figure 3-8 Simulation results - pH drop after reaction. The pH decreases rapidly when there is enough amplicon accumulated between 20 and 30 cycles.

The simulation demonstrated pH response in a PCR reaction. The condition used in the simulation is 0.1pM template concentration, 300 base-paired amplicon, and the amplification efficiency coefficient 0.7 in each doubling of PCR cycle. As shown in

The result above proves the theory that ISFETs can be switched on when protons are released from primers extension.

1.5 SUMMARY

In this chapter, we have demonstrated the theoretical basis for the chemistry as well as the ISFET responses. By monitoring the pH change of a nucleic acid reaction and comparing with a control experiment, it is possible to monitor the reaction. The integration of the reaction chemistry and ISFET sensor would enable a true lab-in-a-chip format of biosensor.

Building on the simple method of a single extension using ISFET, one can increase the density of the reaction drastically using standard CMOS process, which could be applied to ultra-high throughput sequencing. The leverage of the CMOS sensor technology also applies to POC, where critical information of pathogen infection could be identified by ISFET-dependent nucleic-acid amplification monitoring.

CHAPTER 4 – DNA LOGIC

4.1 INTRODUCTION

The feasibility of digital computation in cells has always attracted much interest. Attempts have been creating *in-vivo* digital logic circuits using DNA binding Proteins, and gene expression has gained much interest. [80]. Our method should permit numerous improvements for nucleic acid testing outside of mainstream laboratory facilities and allow identification by individuals such as clinicians and consumers in a point of care setting.

Similar to the process that electronic microprocessors to control electromechanical devices, biochemical reactions can be used as biochemical circuits to control discrete electrical events. Although the construction of these circuits has been explored theoretically since the birth of molecular biology, current practical implementations of reaction changes due matching biochemical events has led to the creation of chemical-logic circuits. This chapter proposes a novel method of DNA Logic, a subset of chemical-logic, using silicon based transistors with chemical interactions to create so called DNA switches that may perform *in-vitro* computation.

4.2 DNA LOGIC THEORY

The pH detection of CMOS ISFET is possible because of the *pH* sensing capability of the CMOS passivation layer, which happens to be silicon nitride. In order to create ISFETs in unmodified CMOS, the standard MOSFET from the technology is used with an extended floating gate to the passivation in order to sense ionic changes on the insulator. Because it is one and the same with the MOSFET, integration of

ISFET/MOSFET pairs is possible to create highly monolithic chemical sensing systems. In addition to this the decreasing feature size of the transistor according to Moore's law, allows implementation of miniaturized ISFETs, which can be combined to form large-scale arrays for parallel biochemical processing.

When chemical reactions cause discrete changes in chemical concentrations, these changes can be used as an indication for the presence of a reaction. For example, the sequencing of individual base pairs in a strand of DNA due to nucleotide incorporation may cause a change in pH [77]. Detecting this change in reaction serves as a positive marker for that nucleotide; therefore confirming its presence in that strand of DNA.

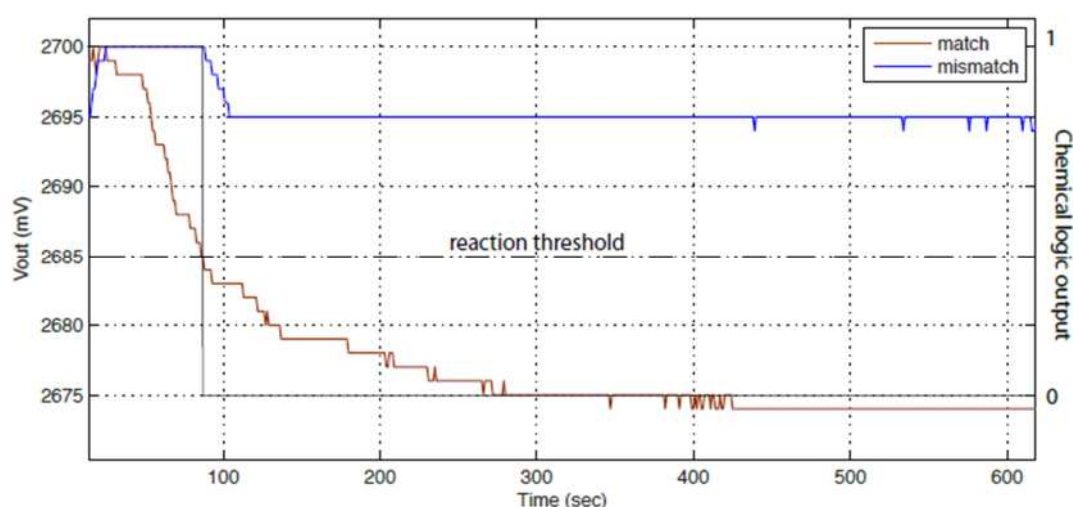


Figure 4-1 Reaction monitoring of a base pair match and mismatch

Figure 4-1 shows an example whereby we can see the reaction due to a matching nucleotide using the protocols described in [77]. The reaction is measured with an ISFET, whereby the decrease in source voltage indicates a decrease in pH . Assuming reaction took place at a pH of 7.8, and assuming near Nernstian sensitivity of 50mV, the change of 25mV results in a 0.5 pH change. By setting a threshold just above the

mismatch voltage, this reaction can be simplified as a chemical logic boolean 0-1 result which can confirm the presence of a match. An aggregation of all these results could then be used to sequence the genome using conventional combinatorial digital logic functions.

The operation of the ISFET as a means of implementing a “DNA logic” unit capable of calculating truth-table type outputs is an attractive idea for array-based algorithmic processing. The sooner the conversion to digital 0 or 1 signals, the less the effect of degradation from interconnects and sources of interference between the sensor chip and any off-chip interfacing.

To demonstrate how aptly ISFETs can be used for this type of operation, we present an adaptation of the standard CMOS inverter, Figure 4-2(a), which performs a pH thresholding operation when one of the MOSFETs is replaced by an ISFET, Figure 4-2(b). The circuit can be implemented in strong or weak inversion, but for low power operation, the analysis herein is restricted to weak inversion.

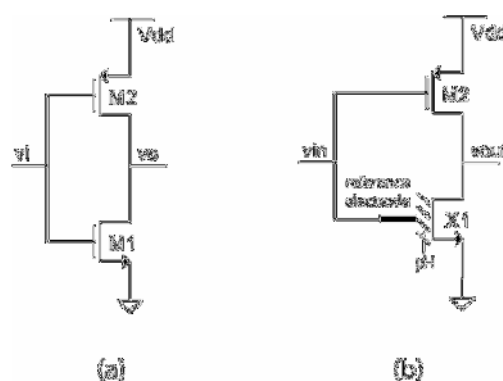


Figure 4-2(A) Standard CMOS inverter and (B) ISFET-based inverter OR “ pH thresholder”

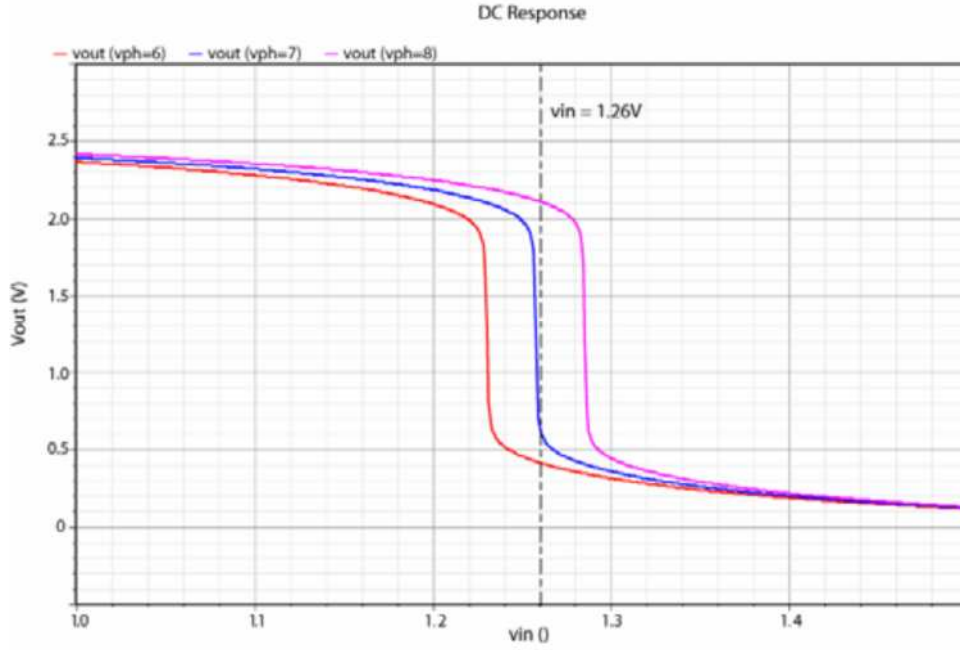


Figure 4-3 SIMULATED V_{out} VERSUS V_{in} AT pH 6, 7, 8

The standard CMOS inverter in weak inversion has the transfer characteristic shown Figure 4-3. At the switching threshold V_{inth} , M1 and M2 have equal drain currents giving:

Equation 4-1

$$V_{inth} = \frac{V_{DD} - |V_{Top}| + \ln\left(\frac{I_{sn}}{I_{sp}}\right)V_{Ton}}{1 + \ln\left(\frac{I_{sn}}{I_{sp}}\right)}$$

where the sub-threshold slope for NMOS and PMOS are assumed equal. We have already seen from a behavioural point of view that the ISFET can be considered to be a MOSFET with a chemically sensitive threshold voltage, where V_{chem} varies approximately 55mV/pH [74]. Therefore, if the NMOS in Figure 4-2(a) is replaced with by an n-channel ISFET as shown in Figure 4-2(b), the switching threshold will be given as [81]:

Equation 4-2

$$V_{inth} = \frac{V_{DD} - |V_{Top}| + \ln\left(\frac{I_{sn}}{I_{sp}}\right)[V_{Ton} + V_{chem}]}{1 + \ln\left(\frac{I_{sn}}{I_{sp}}\right)}$$

4.3 SIMULATION RESULTS

The simulated transfer characteristic of the ISFET-based inverter circuit Figure 4-2(b) in buffer solutions of pH 6, 7 and 8, is shown in Figure 4-3. The circuit was simulated in the UMC 0.25 μ m CMOS technology, $V_{dd} = 2.5V$, using a simplified version of the Martinoia macro-model [78] with perfectly linear pH sensitivity (constant α for all pH and temperature). Device dimensions were 50/50 for n-type ISFET X1 and 50/10 for p-type MOSFET M2.

pH	3	4	5	6	7	8	9	10
$V_{inth}(V)$	1.148	1.176	1.204	1.231	1.258	1.286	1.31	1.341

Table 4-1Effect of pH on switching threshold

The effect of pH on the switching threshold is shown in Table 4-1 and illustrated in Figure 4-3. A consequence of this characteristic is that if V_{in} is fixed, then switching will occur exclusively due to a change in pH . Moreover, the pH threshold at which this switching occurs can be set by our choice of V_i as shown in Figure 4-4. For illustration, it is shown that the output is high for $pH \geq 4$ when V_{in} is fixed at 1.18V and for $pH \geq 8$ when $V_{in} = 1.26V$.

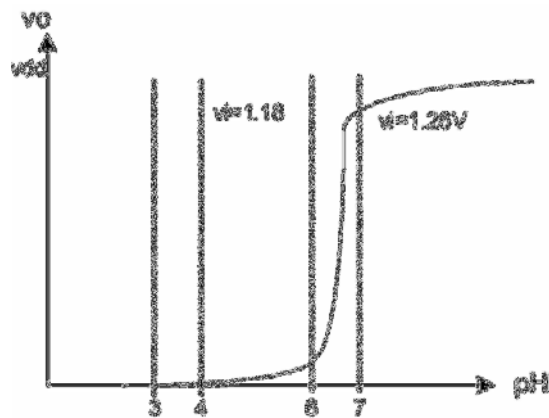


Figure 4-4 SIMULATED pH SWITCHING THRESHOLDS FOR DIFFERENT INPUT BIAS VOLTAGES

The significance of this simple circuit is that it is a type of pH comparator, not switched by a 0 or 1 on the gate input, but by a pH less than or greater than a chosen threshold. Simulation results have shown the uncertainty region around the threshold pH to be approximately ± 0.3 pH units. This is nevertheless a potentially useful technique for array-based thresholding operations where trends and not accuracy are the focus. Discrete pH changes caused by events such as primer extension for Single Nucleotide Polymorphism (SNP) detection extend “pH thresholding” to “DNA logic”, which has a practical utility in the direct transduction of a DNA input to a binary output as shown in Table 4-2. Figure 4-7 shows circuit simulations showing how the measured data from nucleotide incorporation would be converted to a digital output by the ISFET-based DNA logic “NOT” gate in Figure 4-2.

$pH < pH_{th}$	DNA Input	Output
0	mismatch	1
1	match	0

Table 4-2 Truth Table of DNA Logic “NOT” Gate

4.4 DNA LOGIC CHIP

4.4.1 MEASURED RESULTS

This section presents the measurements of the DNA logic chip. This unmodified CMOS ISFET chip was fabricated with AMS 0.35 μm technologies, containing four invertors in each corner for protons detection, Figure 4-6 and Figure 4-5 shows the layout of an inverter on the chip with p-type MOSFET and n-type ISFET, which is the same layout shown in Figure 4-2.

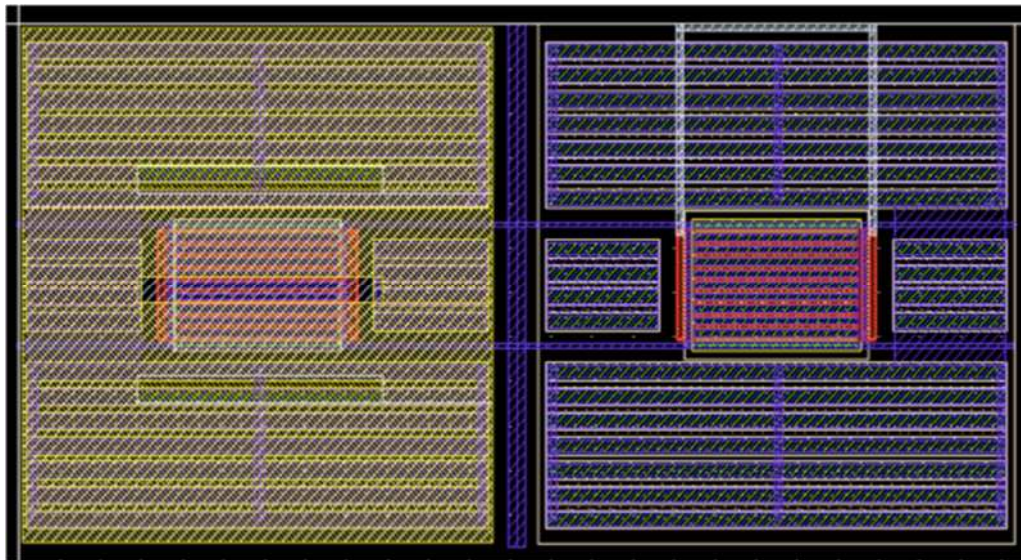


Figure 4-5 World first DNA Logic Inverter with p-type MOSFET and n-type ISFET[82]

Figure 4-7 shows the DNA logic inverter are pH sensitive, however the switching transient is about 1V. With the result, we can conclude an indication of a working inverter with an unmodified CMOS ISFET.

In addition, switching transient of 1V is an issue in practice. Normally in a point of care (POA) setting, the amount of sample input is often not enough to trigger the switching mechanism of an inverter. Thus, to resolve this problem, we propose a circuit solution by employing feedback to a programmable gate ion sensitive field

effect transistor's (PG-ISFET) electrical gate such that switching the transistor can be achieved for changes in pH as low as 0.1 units.

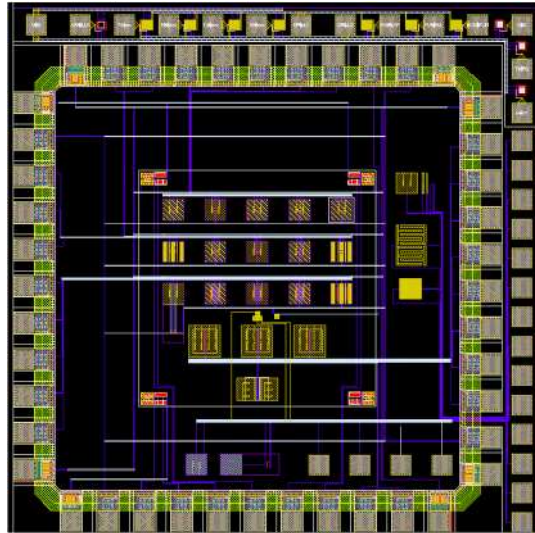


Figure 4-6 CMOS ISFET CHIP WITH FOUR ISFET INVERTERS

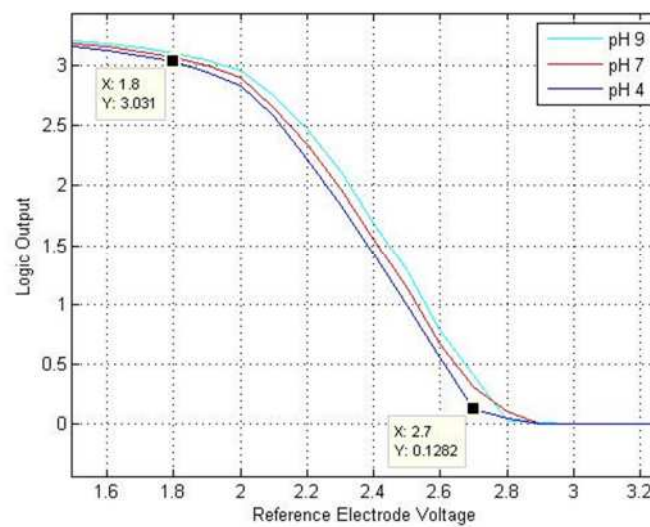


Figure 4-7 Data Results V_{out} versus V_{in} at pH 4, 7, 9

4.4.2 PG-ISFET BASED CHEMICAL LOGIC FOR REACTION MONITORING

This section presents the use of a Programmable Gate -Ion Sensitive Field Effect Transistors (PG-ISFET) to form chemical-logic gates for reaction monitoring. A PG-ISFET sensor is integrated with standard MOSFETs to form a chemical-inverter, which switches when a chemical reaction reaches a certain threshold. By employing positive feedback to the electrical gate, switching is achieved for changes in pH as low as 0.1 units. Using this, chemical-logic arrays can be synthesized to carry out highly parallel chemical processing functions for applications such as DNA sequencing.

Implemented in typical 0.35 μm technology, this implementation overcomes the limitations imposed on standard chemical logic inverters due to implementation in CMOS [82]. As a result, it can be used to digitize changes in reaction, which allows parallel combinational logic functions to be implemented, giving high throughput and instantaneous results from large-scale arrays.

In order for a chemical logic signal to be derived, a change in pH needs to be converted to a discrete value. In [82] a simple ISFET/MOSFET inverter shown in Figure 4-8 was proposed which could cause switching due to a change in pH . When implementing ISFETs in unmodified CMOS however, the voltage on the floating gate of the ISFET is divided down due to the passivation capacitance, which exists due to $S_{i3}N_4$ passivation [83]. The result is that a larger pH swing is needed to switch this type of inverter, which makes it extremely difficult for reactions, which only cause a change of 1 pH unit or less as in Figure 4-1.

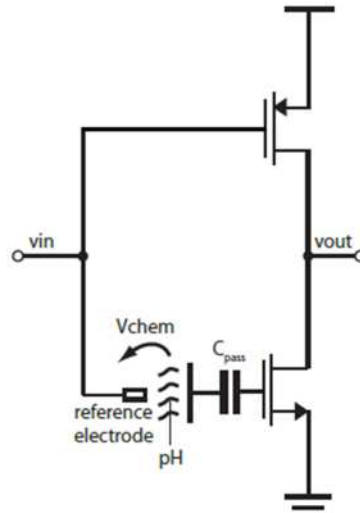


Figure 4-8 ISFET based chemical inverter

The PG-ISFET [84] can be used to resolve this issue by incorporating feedback to the programmable gate of the ISFET through a cascade of single stage amplifiers as is shown in Figure 4-9. As a result the switching of the chemical logic block occurs very rapidly even with small changes in pH . When the ISFET is biased with a stable reference electrode, typically $Ag/AgCl$, a potential, V'_G is established between the insulator of the device, which contains a pH dependence described by V_{chem} , and an offset due to the presence of trapped charge V_{tc} [83]:

Equation 4-3

$$V'_G = V_G - V_{tc} - V_{chem},$$

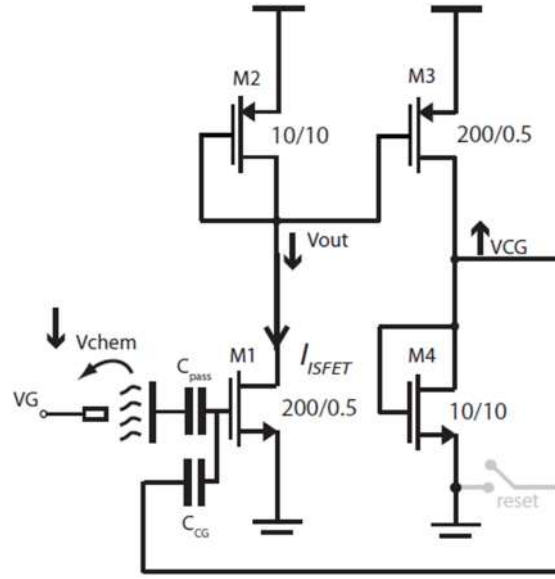


Figure 4-9 PG-ISFET chemical logic inverter

Equation 4-4

$$V_{chem} = \gamma + 2.3 \cdot a \cdot U_t pH$$

whereby U_t is the thermal voltage of the device, is a grouping of non-chemically related potentials and γ is a number ranging from 0-1, describing the reduction in sensitivity from the Nernstian response, typically 59mV/pH.

Equation 4-5

$$V_{FG} = \frac{V'_G \cdot C_{pass} + V_{CG} \cdot C_{CG} + V_S \cdot C_{GS} + V_D \cdot C_{GD}}{C_T}$$

The floating gate voltage of the PG-ISFET, V_{FG} , is established by a weighted sum of the voltages applied to the reference electrode, through V'_G , and the control gate, through V_{CG} , Equation 4-3[85]. Each weight is determined by the capacitance of its input normalized by the total capacitance of the floating gate. For V'_G this occurs

through the passivation capacitance, C_{pass} and for V_{CG} through the control gate capacitor C_{CG} . Connecting a diode between load and drain of the PG-ISFET and cascading the output to another single stage amplifier allows the output of the chemical-logic inverter to be described as:

Equation 4-6

$$V_{CG} = \frac{gm1 \cdot gm3}{gm2 \cdot gm4} V_{FG}$$

Considering now the case that pH decreases, this causes a decrease in V_{chem} , which causes an increase in V_{FG} , which causes an even larger increase in V_{CG} because of the two gain stages. By employing local feedback of this voltage to the electrical control gate of the PG-ISFET, a very rapid transition in V_{out} from 0-1 may be achieved for a very small change in pH if the device is biased, through the reference electrode, just below the switching threshold of the device. A reset switch has been added to ground the control gate prior to a reaction so that the inverter may switch.

The chemical logic inverter in Figure 4-9 was designed and simulated in a typical 0.35 μ m process using a Verilog-A model based on the measure results of the fabricated PG-ISFET. Figure 4-10 shows the output of the inverter with and without feedback when V'_G is increased ramped from 0-3.3V. What can be seen is that in the open loop case due to capacitive division through the passivation the inverter fails to switch whereas in the closed loop case it rapidly switches after a voltage of 693.3mV. Assuming the PG-ISFET in buffer solution is biased to be below this potential, thus any decrease in pH due to a chemical reaction will cause the inverter to switch. Figure 4-11 shows the results for a decrease in pH from 7.8 V with the reference electrode

biased at 1.47 V. What we can see is that the output switches by changes of 0.1 *pH* and lower satisfying the constraints for the reaction shown in Figure 4-1.

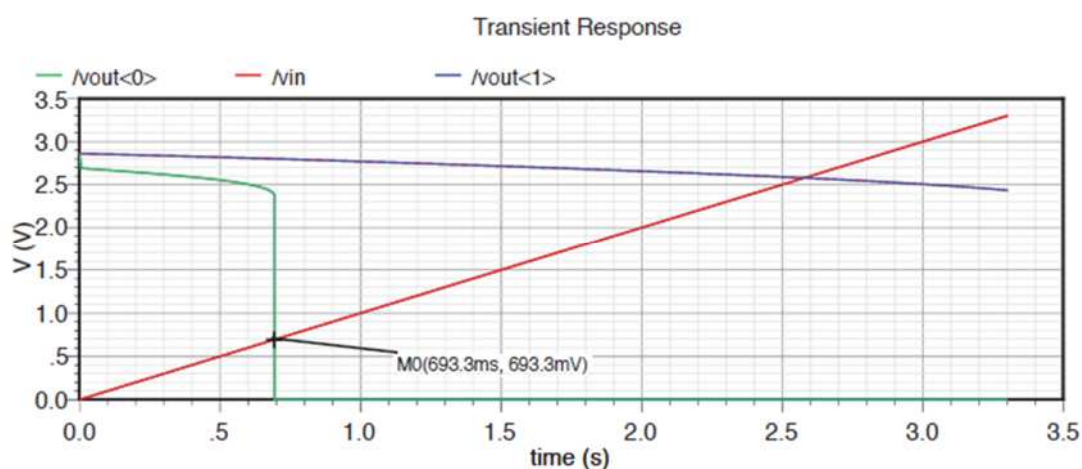


Figure 4-10 Open and closed loop simulated results

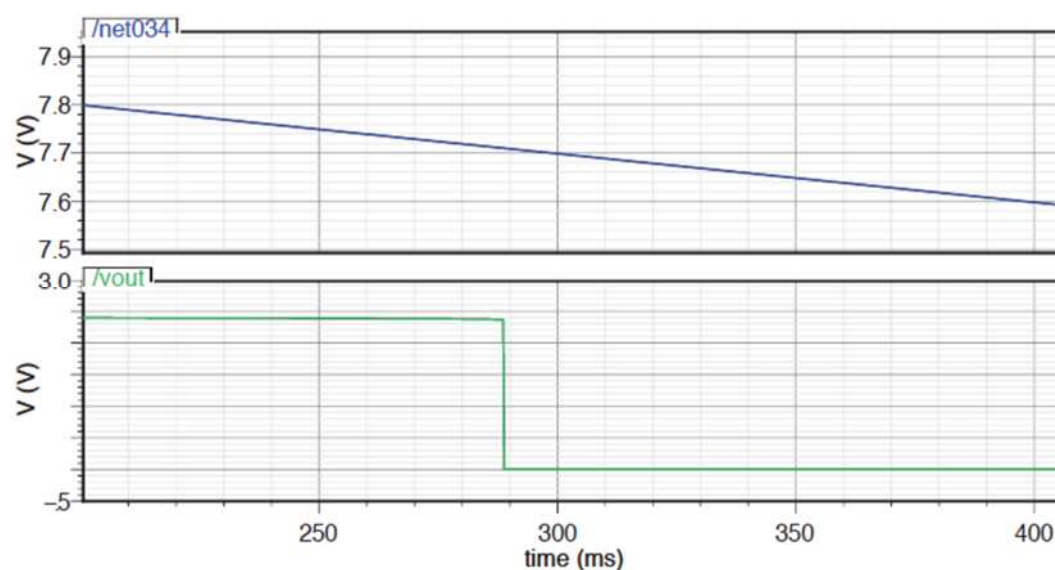


Figure 4-11 chemical logic simulation due to a change in *pH*

Here we presented a chemical-logic inverter based on the PG-ISFET. By employing local feedback the inverter is capable of switching for *pH* changes as low as 0.1 *pH*. This is useful for identifying chemical reactions such as those produced during base

pair incorporation of DNA. This then facilitates the use of standard CMOS combinational logic to sequence the genome in a parallel fashion.

4.5 DNA LOGIC APPLICATIONS

4.5.1 SINGLE NUCLEOTIDE POLYMORPHISM

This section presents the application of ISFET-based inverters for reaction threshold detectors to convey the chemical reaction level to a logic output once a threshold has been reached. Using this, DNA logic functions are derived for nucleotides allowing identification of multiple SNPs. This logic is extended to facilitate a combination of SNPs, deriving gates, which give a simple Yes/No answer to their presence. A DNA logic ‘NOR’ gate is derived for an application where two SNPs need to be absent in order to determine drug delivery.

The key aim is to achieve computation out of biological cells and their genetics. In this section we propose a novel method of DNA Logic using silicon based transistors with chemical interactions to create so called DNA switches that may perform *in-vitro* computation. This particular work will be confined to switching based upon the marker for nucleotide insertions, Single Nucleotide Polymorphisms, with the application being for diagnosis of disease.

SNPs are receiving considerable interest due to the potential they offer for disease diagnosis, screening and personalized drug therapy and medicine. A SNP is defined as a mutation of a single base affecting at least 1% of a defined population. Although they do not necessarily cause disease, their association with disease and with effects on the pharmacokinetics of many drugs provides information for diagnosis and pharmacological treatment options for many different diseases. There are currently

over 1.8 million identified SNPs. In this section we propose an extension to the method of applying ISFET technology to the detection of these SNPs, which could be applied to develop a cost-effective disposable device.

In prior sections, we have shown how the ISFET given a DNA input such as the presence or absence of a SNP will cause a change in pH which can be converted to a binary output using a simple adaptation of the “NOT” logic gate. Table 4-2

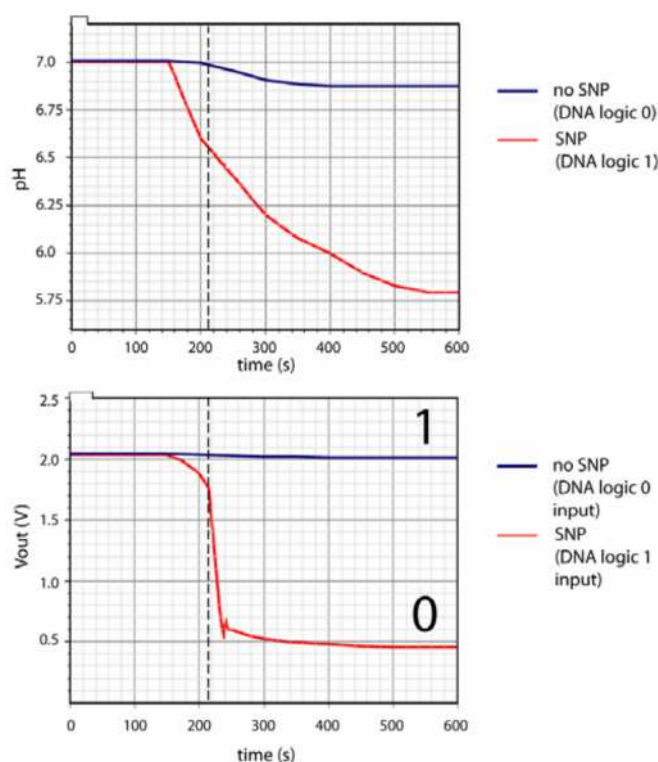


Figure 4-12(A) Measured data from pH response during nucleotide incorporation during primer extension when no SNP is present (logic 0) and when SNP is present (logic 1) and (b) simulated output from DNA logic inverter circuit

That principle can be extended to multiple DNA inputs to create other DNA logic circuits to perform simple logical operations, which provide a yes/no answer. Often the presence or absence of a combination multiple SNPs can give information about the risk of genetic predisposition to certain diseases such as heart disease or Type II

diabetes [86]. Furthermore, some SNPs are linked to drug metabolism and therefore can be used to predict whether a patient should take a high or low dose of certain prescription drugs. In the case of warfarin, a blood-thinning agent, a high dose in people with certain SNPs could cause excessive bleeding. Two SNPs in particular have been found to correspond with lower dose requirements, due to the fact that they indicate slower metabolism of the drug. These SNPs are on the “CYP2C9*2” and “CYP2C9*3” alleles, and a patient with either or both of these SNPs will require a lower dose of warfarin [87]. For illustration, a simplified “truth table” for warfarin dose as predicted by CYP2C9 genotype, is shown in Table 4-3.

CYP2C9*2	CYP2C9*3	Dose
Absent (0)	Absent (0)	High (1)
Absent (0)	Present (1)	Low (0)
Present (1)	Absent (0)	Low (0)
Present (1)	Present (1)	Low (0)

Table 4-3 Truth table of warfarin dose predicted from CYP2C9 genotype

Table 4-3 corresponds to a “NOR” relationship, which can be implemented using ISFET-based DNA Logic. The circuit of the DNA-NOR gate is shown in Fig. 7 where the classical MOSFET is replaced with ISFETs at the inputs, with appropriate DNA probes and biochemical reagents to detect nucleotide incorporation during primer extension [77]. The simulation results are shown in Figure 4-14, where the four vertical dash lines divide the simulation into four results corresponding to the truth table inputs shown in Table 4-4, where either SNP A or SNP B is present in a DNA

sample. This simulation clearly demonstrates that when a single base is detected on either ISFET, then the output will be zero. Only if neither ISFET detects a SNP will the result be a digital one.

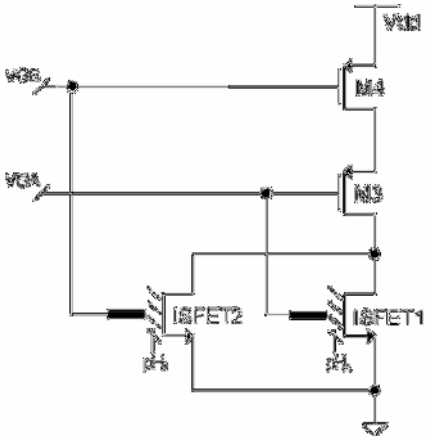


Figure 4-13 DNA Logic “NOR” Gate with ISFET

pH<pHthA (SNP A present)	pH<pHthB (SNP B present)	Vout
0	0	1
0	1	0
1	0	0
1	1	0

Table 4-4 Truth table of DNA Logic NOR Gate

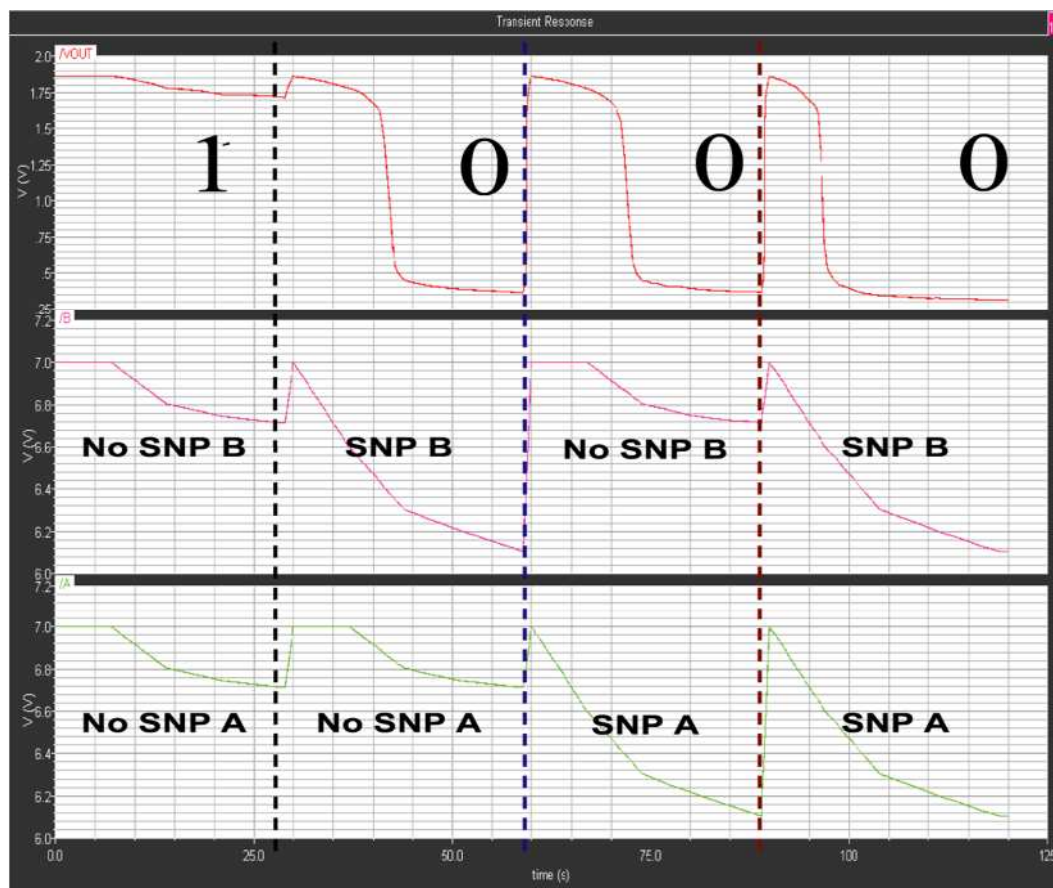


Figure 4-14 Simulated output response from DNA-NOR circuit for reactions in truth Table 4-3

In this section, we have demonstrated the feasibility of using DNA to switch on and off transistors to create a synthetic form of DNA logic. This will be useful in two applications, either to create DNA computation as an extension to the work already being pioneered within cellular computing or as described in this letter for point-of-care SNP logic on silicon chip based technologies with the advantages therefore of disposability, portability as well as leveraging on the economy of scales of semiconductor technologies.

4.6 SUMMARY

Here we have demonstrated the feasibility of DNA logic as a true DNA switch. The transistor is controlled by the presence or absence of a DNA target. By combining a few DNA logic switches, it is possible to perform genotyping in a POC setting. In the next chapter, we are presenting a business model for rapid viral detection using the ISFET detection platform.

CHAPTER 5 – ISFET PLATFORM HIV NUCLEIC ACID TEST

5.1 INTRODUCTION

Human immunodeficiency virus (HIV) is known to cause Acquired Immune Deficiency Syndrome (AIDS). The spread of the virus is considered by World Health Organisation (WHO) pandemic and has caused tens of millions of deaths worldwide since the first reported case. UN report in 2009 shows the number of global infection is about 30-33 million [88]. The trend of new infection peaked in 1996 as shown in Figure 5-1. It is clear that international effort between health regulators, pharmaceutical industry, charity organisations and biotechnology companies has paid off in reversing HIV spreading.

The effectiveness of the WHO in dealing with such a pandemic requires close collaboration in health policy, disease prevention, HIV diagnosis, therapy and patient care and support. One key area in combating the pandemic condition is awareness of the infection status for people living with HIV. The initial response of a person infected with HIV resembles a flu-like illness in the first four to eight weeks. Typical symptoms include fever, headache, diarrhoea, and fatigue. After an initial acute phase, the individual usually recovers but may live with HIV without knowing for 5-10 years before the AIDS illness starts. Without awareness of the infection, there is great risk that the disease will spread over the intermediate 10 years. It is therefore necessary and critical to implement early detection and constant surveillance of the epidemic.

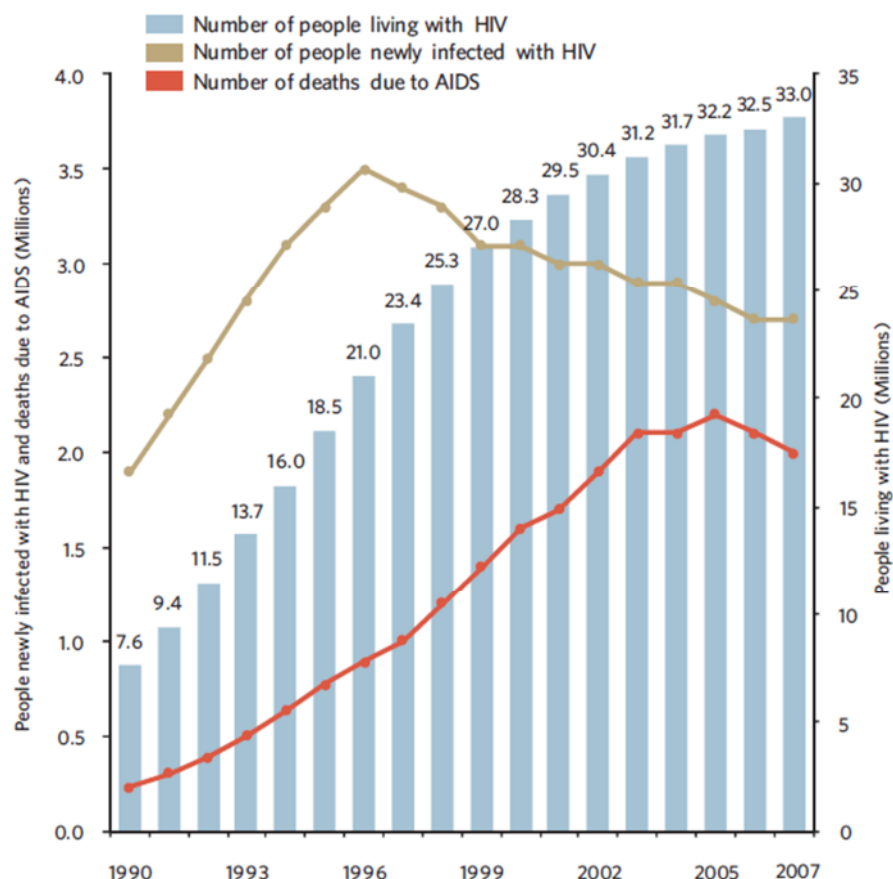


Figure 5-1: the statistics of HIV infection between 1990 and 2007 [88]. In recent year, 2.5- 3 millions are infected every year.

Awareness of people living with HIV has been one of the obstacles of managing the HIV infection. Fewer than 10 percent of people with HIV in Asia and Pacific and less than 25% in Sub-Saharan are aware of their sero-status [56, 89]. The undetected infection is not specific to developing countries. It is reported that 70 percent of those visiting sexually transmitted disease clinics left without known being a HIV carrier in developed countries [56, 90, 91]. There has been a new initiative, Provider-Initiated HIV Testing and Counselling (PITC), which encourages a more proactive approach in HIV testing. With the PICT, the HIV testing is initiated and provided by the primary health providers. In one report on immigrants in Belgium, the persistent stigma associated with the disease and fear for the outcome has been cited as the reason

stopping more uptake of HIV test [92]. In another report, the barrier includes long waiting times in getting a test, lack of supporting counselling, and privacy issues [93]. The new initiative is to improve testing coverage and therefore to enrol people living with HIV to care and supporting system along with existing voluntary counselling and testing route.

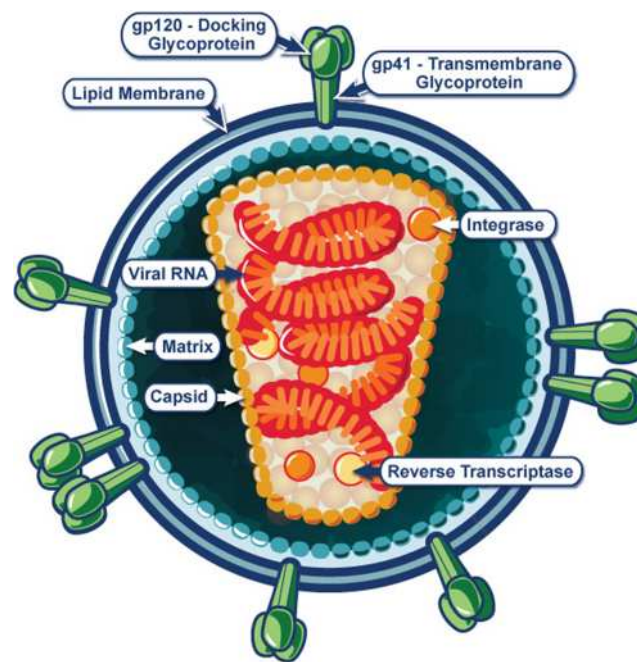


Figure 5-2: Structure of Human Immunodeficiency Virus. The size of the virus is about 100 nm in diameter [94].

To support both initiatives, it is crucial to provide a technology that enables testing and improves services. There are rapid HIV tests for diagnosis in field and laboratory-based detection methods. Each category covers different fields of HIV assays. The rapid tests are deployed as the front line transfusion/surveillance/diagnosis, while laboratory-based assays are more for confirmation tests and prognosis.

The rapid tests detect the presence of a human antibody, which is an immune response to HIV infection. The human antibody is produced after seroconversion, which is about few months after HIV exposure. As the result, there is a window of infection,

when the antibody test will fail in detecting the infection. On the other hand, laboratory-based methods detect the presence of virus, either protein or genetic, materials. Consequently, it is effective in diagnosis for acute HIV infection. One of the laboratory-based methods is to monitor the existence of viral genetic material, which also has applications in prognosis and care management such as viral load or drug resistance.

The structure of HIV has been studied extensively. The complete particle is also known as a virion, comprising a lipid-bilayer jacket and a bullet-shape capsid core as illustrated in Figure 5-2. HIV is a retrovirus in which the genetic material is RNA instead of DNA. In each virion, there are two identical copies of positive single-stranded RNA sequence and two copies of reverse transcriptase in the capsid. The capsid is made of two thousand copies of viral protein p24. Complementary DNA is produced by reverse transcriptase and integrated into the human genome by integrase once the virus enters a cell. On the outside, the virus jacket is a mixture viral protein gp120 and gp41 with the lipid bilayer originated from the human cell where the virus was produced. The number of surface protein, gp120/gp41 is about 70 copies per virion. For viral detection in the early course of HIV infection, viral RNA and p24 could be found in the circulation as early as 1 week after exposure. However the circulating p24 will disappear after the acute phase due to the infection cycle of HIV.

Two species of HIV infect humans: HIV-1 and HIV-2. Both viruses have a similar genetic makeup. The genome is composed of two copies of positive single-stranded RNA that codes for the virus's nine genes, enclosed by a conical capsid or viral coat. The single-stranded RNA requires enzymes for the development of the virion. One of these enzymes, reverse transcriptase (RT), transcribes single-stranded RNA into double-stranded DNA. The RNA genome consists of nine genes (*gag*, *pol*, *env*, *tat*,

rev, *nef*, *vif*, *vpr*, *vpu*, and *tev*), which in turn encodes 19 proteins. Three of these genes, *gag*, *pol*, and *env*, contain information needed to make the structural proteins for new virus particles. Genetic sequences within these genes are common to both HIV-1 and HIV-2, but not other viruses and so can provide a distinct recognition modality.

It is important to note that the gene sequences of HIV vary between regions and individuals due to the high error rate of the genome replication mechanism. The variation could be the result of a natural protection for HIV. Change of the genetic content result in the mutation in its protein. The chance of evading the host attack could be increased by mutations of the envelop proteins which are recognised by immune system. Since the structure of HIV is very simple, the odds of a tolerating mutation is much larger in HIV than in organisms such as yeast or *E. coli*. An example showing the degree of variations in three geographical populations is shown in Figure 5-3. It is therefore very important that the design of any HIV diagnostic kit takes into account the dynamic variation of the genes. It is also known that the dominant HIV strain in an individual living with HIV could change depending on the treatment regime and the discipline of exercising the regime [95]. It is usual that there are more than one strains of HIV co-infected a patient. The mutations or gene variations may or may not be associated with drug resistance. The HIV medications have to take these issues into account to deliver specific treatments. Therefore the effectiveness of the therapy is correlated with the surveillance and early diagnosis of the gene variations.

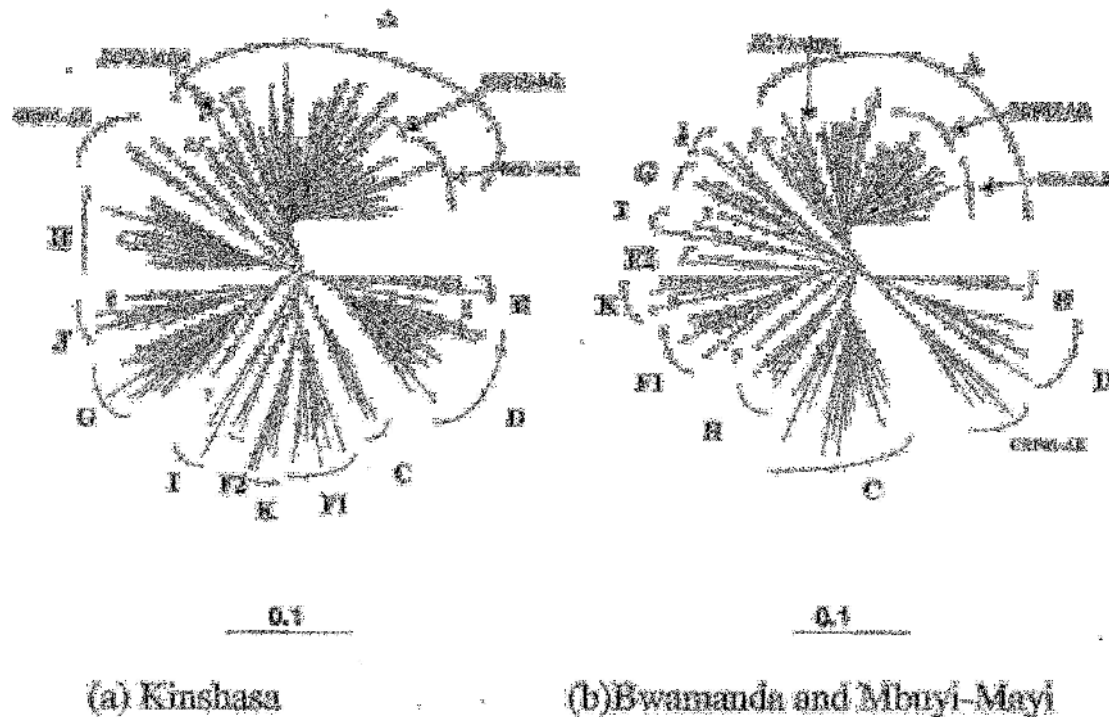


Figure 5-3: Phylogenetic tree of 197 HIV-1 samples from Kinshasa and from Bwamanda and Mbuyi-Mayi in Democratic Republic of Congo. The 0.1 bars at the bottom of the graph represent 10% variation [96].

Following infection with HIV, the virus binds to and enters a class of white cells known as T-lymphocytes. Only a subset of T-lymphocytes carrying cluster of differentiation 4 (CD4) is infected as the route of entry. The invasion of the HIV virus particle is initiated by the binding of CD4 receptor to the gp120 on the envelope of HIV particle, which initiates endocytosis processes [97].

The HV replication is illustrated in Figure 5-4. After a cell is infected, HIV multiplies within the CD_4^+ lymphocytes and is then released by killing the cell and releasing virus particles that then infect further CD_4^+ T-cells.

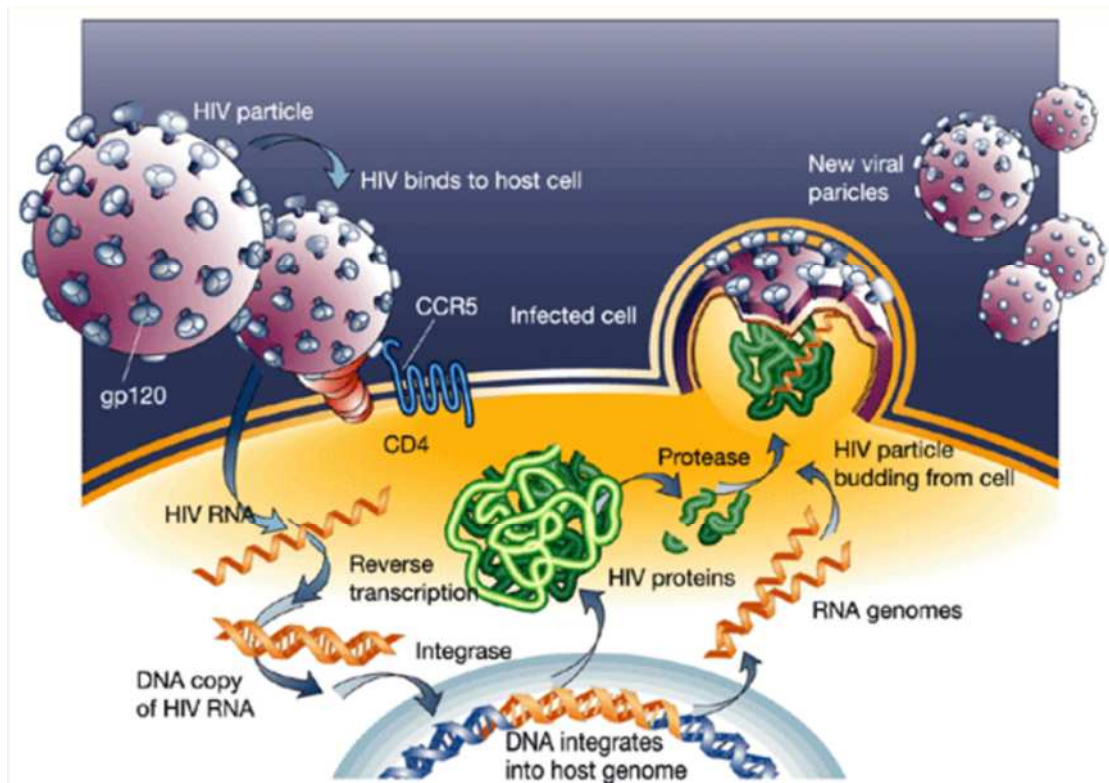


Figure 5-4: Primary HIV infection and replication. HIV particle associates with human lymphocyte via interaction between gp120, CD4, and CCR5 [98].

In a few weeks after viral exposure, the HIV replication process reaches a maximum as well the number of circulating virion. Billions of virus particles are produced each day in the acute phase and the individual is highly infectious at the stage. During this acute phase of the infection there are no overt symptoms although the body is producing antibodies against the virus, specifically against the gp120 protein. Also during the acute phase there is an increase in virus particles and a fall in T-cell numbers which we can see in the viral load and cell count in Figure 5-5.

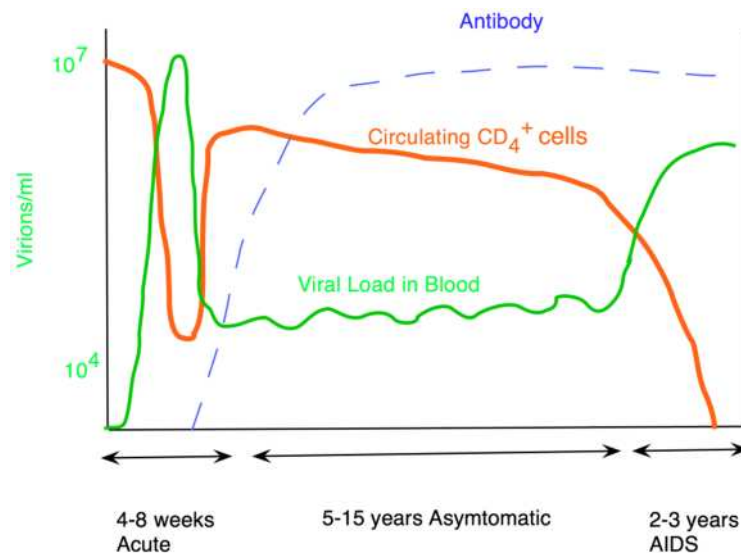


Figure 5-5: Viral load and immunological response of HIV infection.

Sometime in the 3-6 months following infection the individual ‘seroconverts’, the level of antibody reaches a plateau, virus numbers fall and CD_4^+ cell numbers recover. This asymptomatic state can last for years and it is usually during this period that individuals are diagnosed as being HIV positive.

It is not surprising that people living with HIV do not understand the viral conditions until the start of the AIDS illness due to the very long asymptomatic period. In more developed countries, late diagnosis contributes to a substantial proportion of newly infected HIV individuals [99, 100]. The late diagnosis has several implications in HIV management and care support. The choice of medication is limited in the AIDS stage and the health condition of the individual generally is too poor to support the therapy. Strategies to improve the early diagnosis are proposed to include routine HIV test in primary care condition such as the PITC scheme and providing HIV tests in non-medical environments where adequate counselling is available [101].

Studies show that early detection of the virus could radically prevent the spread of the disease. The majority of the peoples, who learned that thy are HIV positive, tends to

modify their behaviour to reduce the risk of HIV transmission [102, 103]. In addition, early detection of the virus also reduces morbidity and mortality and improves quality of life

5.2 HIV TESTING OVERVIEW

The testing can be classified as diagnosis and prognosis, in which diagnosis provides result for people newly living with HIV and prognosis offers the therapeutically effectiveness and medical support.

HIV diagnosis generally tests for the presence of antigen or antibody specific to HIV-1 and HIV-2. A reactive result indicates the presence or history of HIV infection. The standard WHO/UNAID protocol on HIV assays includes three strategies in calling the assay result as shown in Table 5-1. For screening of donated blood or organs for HIV, a one-step test is carried out. ELISA is usually used to in the strategy for its ability to process large quantities of samples with low cost. Reactive response or intermediate results will result the donated sample being discarded. When individuals are in clinical counselling for HIV, the diagnosis involves loops of testing using different methods and mechanisms depending on the prevalence of the case. In low prevalence situations such as early diagnosis when there is no symptom of illness, a supplement test is needed to confirm a positive reading. One of the confirmatory tests is Western Blot (WB), where the antibody specific to HIV proteins are being verified. WB requires more skilled intervention and is not designed for the first test.

Table A. UNAIDS and WHO recommendations for HIV testing strategies according to test objective and prevalence of infection in the sample population		
Objective of testing		Prevalences of infection
<i>Transfusion/transplant safety</i>		All prevalences
<i>Surveillance</i>		>10%
		≤10%
<i>Diagnosis</i>	Clinical signs/symptoms of HIV infection ¹	>30%
		≤30%
	Asymptomatic	>10%
		≤10%

Table 5-1: HIV testing strategies and test objective [104].

Another class of first-line HIV test is the rapid test, which produces result in less than 30 minutes. The rapid test can be performed in the field using whole blood, serum, urine, or even saliva samples. The rapid test could be used as a screening assay as well as diagnostic assay.

When the objective is medical support for people living with HIV, viral load and drug resistance are the focus. The viral load indicates the effectiveness of the medication. A viral load lower than 50 copies per ml of blood is considered as undetectable. Increaseing viral load is a rebound and sign of the failed therapy. Nucleic acid test is the standard methods in viral load assay. The test quantifies the amount of the viral RNA or pro-DNA using nucleic acid amplification technologies such as PCR. The type of assay is extremely sensitive and a much shorter window period compared with an antibody/antigen assay. However, specialised facility and training is required to ensure the test quality, which associates with much higher cost. The turn-around time is generally several hours before the result is available [105].

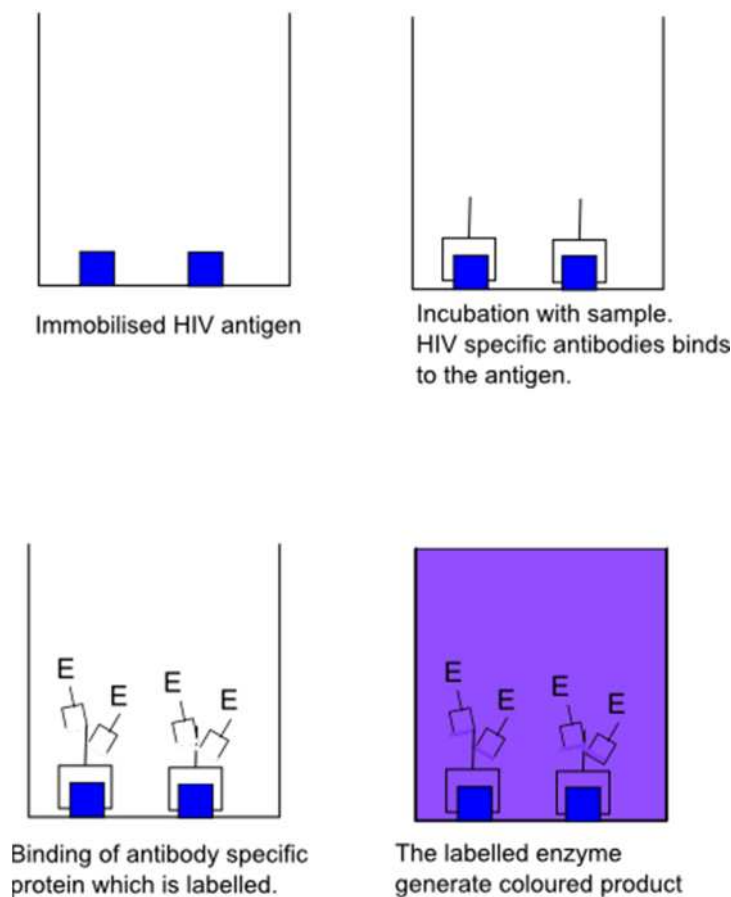


Figure 5-6: ELISA work flow example. In this example, the target antigen could be recombinant HIV fragments. The sample is applied to the immobilised antigen. If there is HIV-specific antibody, it will bind to the antigen. The detection of the antibody by immunoglobulin binder produces colour or light [75]. The graph is an illustration of a general work flow.

5.2.1 ANTIBODY AND ANTIGEN TESTS

These type of tests covers a broad range of products and assays. HIV antibody tests are highly sensitive and have advantages of being inexpensive and accurate. This makes it the most apposite test for routine viral detection. One of the first HIV test to be widely used is the enzyme-linked immunosorbent antibody test (ELISA, Figure 5-6). The type of assay could be automated in microliter plate format, which allows a really large volume of samples. When used in diagnosis, ELISA positive can be

inconclusive. Risk history of the individual, and clinical judgment on the visit should be included in the assessment. A second independent test is required as a confirmation test.

The emerging rapid tests circumvent the long turn-around time and provide alternatives to regions with limited resources and low sample numbers. The principle of detection of the rapid testing kit is the same as the ELISA. The rapid kit has the antigen and reagents packaged in a compact and machine free format. Typically recombinant peptides resembling the HIV proteins are immobilised on a membrane. In the presence of antibodies specific to HIV, the antibodies bind to the peptides on the membrane. These antibodies are only produced after seroconversion by the human body as the response to HIV invasion. The detection of the antibody-peptide complex is achieved by labelled immunoglobulin binder, such as protein, a from of bacteria. The immunoglobulin binder could be labelled with gold nano particles. When the binders are concentrated at the line/dot of the HIV antibody, a visible line or dot will appear. The detection cascade is sensitive and simple. Many of the tests have been granted CLIA waiver status in using whole blood samples (Table 5-2).

However, a clear disadvantage of antibody tests is the false negative result during the acute phase of infection. This is due to the gradual development of detectable HIV antibodies. The vast majority of people take approximately 30 days after infection for their antibodies to be noticeable.

Brand	Time	Viral type	Sample type	Format
OraQuick	20	HIV 1/ HIV 2	Blood, plasma or oral fluid	
Uni-Gold	10	HIV 1	Blood, serum, or plasma	
Clearview	15	HIV 1/ HIV 2	Blood, serum, or plasma samples	
Reveal HIV	20		Whole blood, serum, or plasma.	

Table 5-2: The short list of third generation HIV rapid tests.

5.2.2 NUCLEIC ACID TESTS

There is clinical correlation between the concentration of circulation virus and progress and risk of AIDS. When newly HIV positive, the supporting system will decide the therapy regime based on the individual living history, any medical complications, and the viral load in blood.

For people who have lived with HIV, a key indicator of anti-retroviral treatment (ART) failure is an increase in viral load. Currently the turnaround time for such tests is usually one week, and in the event that a patient on ART has an increased viral load, it is necessary for the patient to re-attend clinic, commonly four weeks from the time of the first appointment, and during this period it is likely that drug resistance

will have continued to develop, thus limiting the available options for changes in the drug regimen. The development of a lab-free HIV viral load test would serve to avoid such problems and reduce laboratory costs in a UK setting, as well as being a priority in global health to determine ART failure (instead of CD4 cell count/clinical symptom development) and to diagnose mother-to-child transmission (instead of antibodies which limits tests to children 18 months of age). The work proposed would validate the detection and quantification of both viral HIV RNA and proviral DNA, paving the way to develop diagnostics for other viruses including hepatitis B and C.

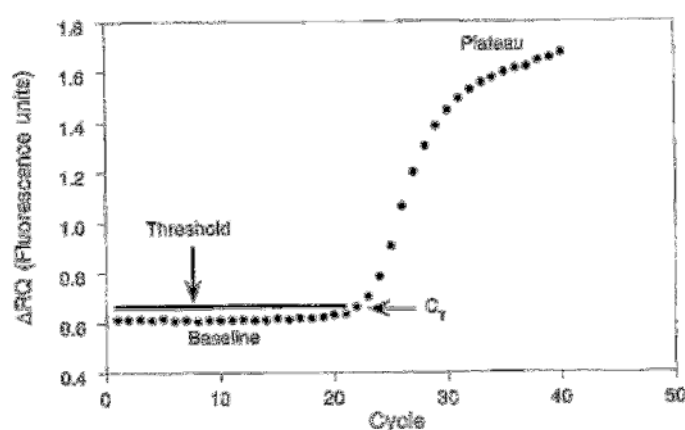


Figure 5-7: The fluorescence signal during a PCR. Threshold cycle number is usually used as the parameter in quantitating the amount of starting material [106].

Nucleic acid test (NAT) currently is the only established method for determine the viral concentration in the circulation. In the test, sample RNA or DNA, is amplified using polymerase mediated synthesis. The ratio of the product to the sample would reach 10^{8-9} at the end of the reaction. The speed of amplified nucleic acid accumulation is proportional to the amount of input sample. Quantification is done by comparing the accumulation against a known control. The amplification is hard to

match by ELISA while obtaining the specificity. Over the last twenty year, PCR has become the standard to quantify the small amount of nucleic acid which is otherwise undetectable [106-109]. The quantitative detection happens at the same time as the amplification. As the result, the method is also called real-time PCR. There are many techniques in obtaining the real-time quantification, which invariably require fluorescent intervention. One of the examples of the quantitative PCR is shown in Figure 5-7, where the technology is Sybr Green PCR.

The cost of a NAT assay is, however, considerably much more expensive compared with ELISA assays. If large sample number are to be processed using NAT, current products on the market would require substantial advanced instrumentation to be setup and automated. Although NAT is not being used as a primary assay for diagnosis, it could shorten the window period between infection and detectability of the disease. In order to enable NAT in a point of care environment, it will need new technology to circumvent the assay cost and machine complexity.

When using biological sample for NAT, the current protocol requires viral RNA is to be purified to high grade before amplification. It removes sample difference and inhibitors which affect polymerase. The following step is to convert RNA into cDNA by reverse transcriptase (RT) before being amplified by a second enzyme cassette.

PCR is the technology behind the Tagman assay [76], for example. Sets of short oligonucleotides specific to viral genome bind to the viral cDNA. The amplification is then followed using the hybridised oligonucleotides. Detection is by the hydrolysis of a labelled reporting oligonucleotide, which is specific to the amplified product. The release of the labelling probe is detected as fluorescence increases.

Amplification could also be achieved using T7 RNA polymerase/reverse transcriptase cassette in Gen-Prob products. After amplification, the resulting nucleic acid products are hybridized to specific oligonucleotides bound to the vessel wall, and are then made visible with a probe bound to an enzyme. The amount of virus in the sample can be quantified with sufficient accuracy to detect three-fold changes.

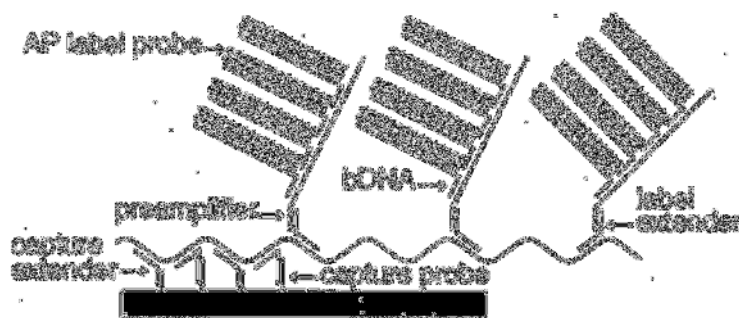


Figure 5-8: Branched DNA (bDNA) detection. The target nucleic acid is captured by capture probe, which is immobilised on the surface. The enzyme (Alkaline phosphatase) is conjugated to the bDNA [110].

There is another class of NAT which does not use polymerase to amplify the nucleic acid but use enzyme to amplify the signal. Branched DNA technology from Chiron uses densely packed enzyme on a recognition branched nucleic acid which associates to the target complex through hybridisation [110]. The target complex comprises capture/amplifier/preamplifier oligonucleotides as shown in Figure 5-8. Capture oligonucleotides are added to bind and immobilise the extracted RNA to the wall of the vessel. Amplifier and preamplifier oligonucleotides are added to bind at several locations with the RNA. Finally bDNA with enzyme conjugates bind to several locations to those oligonucleotides. The densely packed enzyme action causes a colour reaction, which allows quantification of the viral RNA. Monitoring the effects of antiretroviral therapy by serial measurements of plasma HIV-1 RNA with this test

has been validated for patients with viral loads greater than 25,000 copies per millilitre.

The NAT assay is effective in early diagnosis and essential in prognosis. There is a clear advantage for HIV detection and quick viral load results. However the performance of these methods mentioned, are not designed for a POC environment. With the advance of PCR technologies, improvement on polymerase performance, and integration with microfluidic structures, a new generation of NAT is coming to address the need. The first wave of the technology advance is the integration of the miniaturisation and traditional fluorescent PCR or hybridisation technologies. FDA approved assays and product such as Osmetech (eSensor[®] XT-8 Warfarin sensitivity) and Cepheid (Xpert[™] MRSA) are starting to test the acceptance in primary care environment. Despite the use of microfluidic structure and microchip, they require a large instrumental setup combining amplification and detection. As the result, these methods are classified as chip-in-a-lab devices.

5.3 ISFET NAT PLATFORM

We propose the ISFET NAT platform could provide an alternative to the current NAT methods based on the following:

1. Disposable sensor and analyser
2. The integration of detection of nucleic acid and amplification with CMOS is a true lab-in-a-chip technology.
3. It is versatile to form the platform into a high-density automatic unit or rapid result in a resource restricted environment.

Our objectives are to implement HIV detection in a point of care setting and to bring personalized medicine to the bedside in the management of infected patients with HIV. In addition to its timeliness in developing POC testing which will be of immediate benefit to patients, the section of this thesis will serve as a proof of principle for POC detection and quantification of other infectious organisms. The work will also be important in the development of a lab-free rapid NAT assay needed in the developing world and for HIV and other diseases.

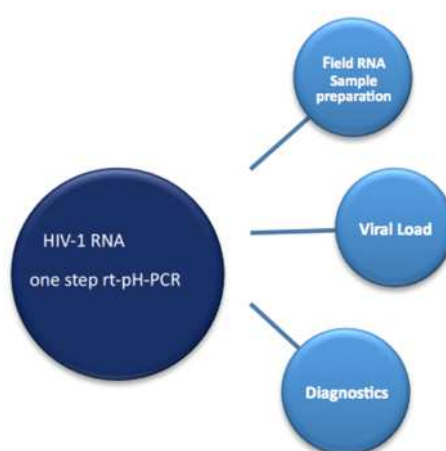


Figure 5-9: The goals and project of HIV-1 diagnosis. The projected project uses ISFET technology and one-step RT-PCR for viral amplification.

This work contributes to the proof-of-concept step which amplifies and detects the HIV virus in a simplified condition. The enabled technology platform will be incrementally developed to exploit its potential as a POC detection system for HIV-1 virus load testing and streamline the sample preparation (Figure 5-9).

The concept structure of the ISFET NAT platform is shown in Figure 5-10. It has been verified that the ISFET reads *pH* change on a nucleic acid chain extension that generates protons. The presence of a matching target sequence correlates with H^+

release as shown in the graph. The logic is established between the nucleic acid target and the ISFET readout. The method is highly scalable because

1. No camera or optical detector needed
2. Mass manufacturability powered by use of the standard CMOS process

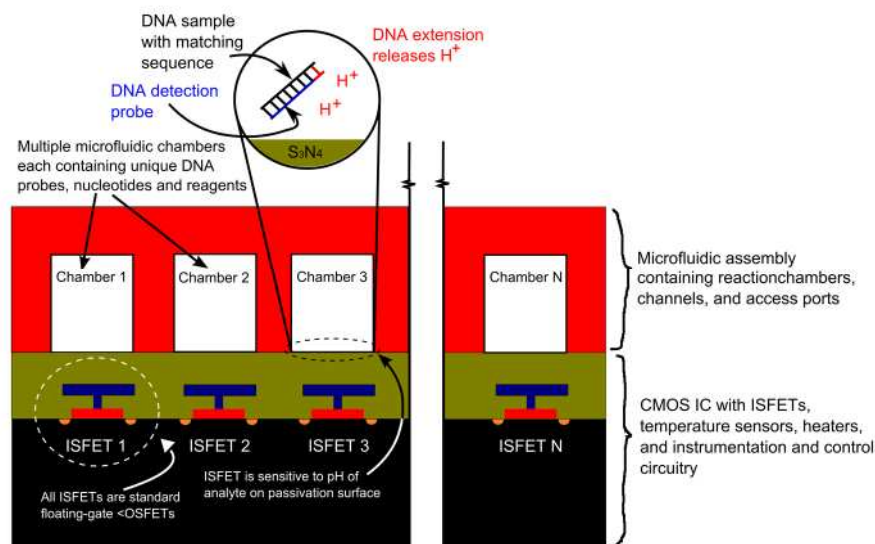


Figure 5-10: Device structure of POC ISFET device. Multiple sensors are integrated with corresponding fluidic structures. Detection of reaction is carried out in each discrete electrode.

5.4 LABEL-FREE HIV-1 DETECTION BY ISFET

A non-infectious strain of HIV-1 virus, 8E5 from SeraCare was used as the model sample. The virus is purified from cell culture and mixed with screened human serum with known concentration, 20,000 copies/mL. The virus has same sequence and structure as standard HIV-strain, HXB2[111], apart from one mutation at 3241 position which causes a frame shift to the polymerase [112, 113].

The development of the test has accomplished ISFET detection using a one step PCR divided into three stages,

1. Sample preparation
2. Validation of design and rt-PCR
3. pH-PCR of HIV in one step

The sequence of the primers used in the rt-PCR has been tested and optimised (Table 5-1). It is found that the extra primer (SID4) for reverse transcription step increases the yield significantly. The PCR primers (SID5/SID6) amplified the ssDNA initiated by SID4.

Name	Sequence 5' → 3'
SID4	CTGTAATAAACCCGAAAATTTTGAA
SID5	ACGGTTAGGGCCGCCTGTT
SID6	TTCCCCTGCACTGTACCCCC

Table 5-3: The sequence of oligonucleotides in HIV RT and PCR

Typical rt-PCR started with two steps PCR. In the first step, the reverse transcriptase (SuperScript III from Invitrogen) is mixed with SID4 and HIV RNA in proprietary buffer. The reaction is allowed to occur for 30 minutes, which produces cDNA. The reaction mixture is then heat-inactivated at 95°C for 5 minutes.

	SID05 100.0%	EcoRI
4101	CAGTAAAAAC AATACATACT GACAATGGCA GCAATTTTAC CGGTGCTACG GTTAGGGCCG CCTGTTGGTG GGCAGGAATC AAGCAGGAAT TTGGAATTCC	~~~~~
	GTCATTTTTG TTAIGTATGA CTGTTACCGT CGTTAAAGTG GCCACGATGC CAATCCCGGC GGACAACCAC CCGCCCTTAG TTCGTCCCTA AACCTTAAGG	
4201	CTACAATCCC CAAAGTCAAG GAGTAGTAGA ATCTATGAAT AAAGAATTAA AGAAAATTAT AGGACAGGTA AGAGATCAGG CTGAACATCT TAAGACAGCA	
	GATGTTAGGG GTTTCAGTTC CTCATCAICT TAGATACTTA TTCTTTAATT TCCTTTAATA TCCTGTCCAT TCCTAGTCC GACTTGTAGA ATTCTGTCT	
4301	GTACAATGG CAGTATTCAT CCACAATTTT AAAAGAAAAA GGGGGATTGG GGGGTACAGT GCAGGGGAAA GAATAGTAGA CATAATAGCA ACAGACATAC	
	CATGTTTACC GTCATAAGTA GGIGTTAAAA TTTTCTTTTC CCCCTTAACC CCCCATGTCA CGTCCCCTTT CTTATCATCT GTATTATCGT TGTCTGTATG	
	SID06 100.0%	
4401	AAACTAAAGA ATTACAAAAA CAAATTACAA AAATTCAAAA TTTTCGGGTT TATTACAGGG ACAGCAGAAA TCCACTTTGG AAAGGACCAG CAAAGCTCCT	
	TTTGATTTCT TAAIGTTTTT GTTAAATGTT TTTAAGTTTT AAAAGCCCAA ATAATGTCCC TGTCGTCTTT AGGTGAACC TTTCCTGGTC GTTTCGAGGA	
	SID04 100.0%	

Figure 5-11: Alignment of SID4/5/6 to the HXB2 sequence.

The cDNA from RT is then amplified using the PCR technique. Optimisation was done by varying the annealing temperature and concentration of primers. The amplification produced a greater *pH* decrease compared with a control reaction, as shown in Figure 5-12. In the control, one of the PCR primers was missing and there was no reaction happening in the control. A successful PCR requires both forward and reverse primers. The extension reaction only multiplied the template linearly by cycle number rather than exponential in a typical PCR. The missing primer was replaced by an equal amount of counter primer to compensate the buffer capacity variation.

The data shown in Figure 5-12 is the pH dependence on the PCR progress using cDNA. Each point was triplicated. The pH value at 5 cycles was used as the starting point, where the samples were cycled without substantial amplification. As the PCR progress, more amplicons are produced. As dNTPs converted to pyrophosphate in the PCR catalysed by polymerase, the pH decreased from 7.6 to 7.1. However, there is also a pH drop in the control between 5 and 35 cycles. This was likely caused by non-specific hydrolysis of dNTP, which was caused by thermal cycling.

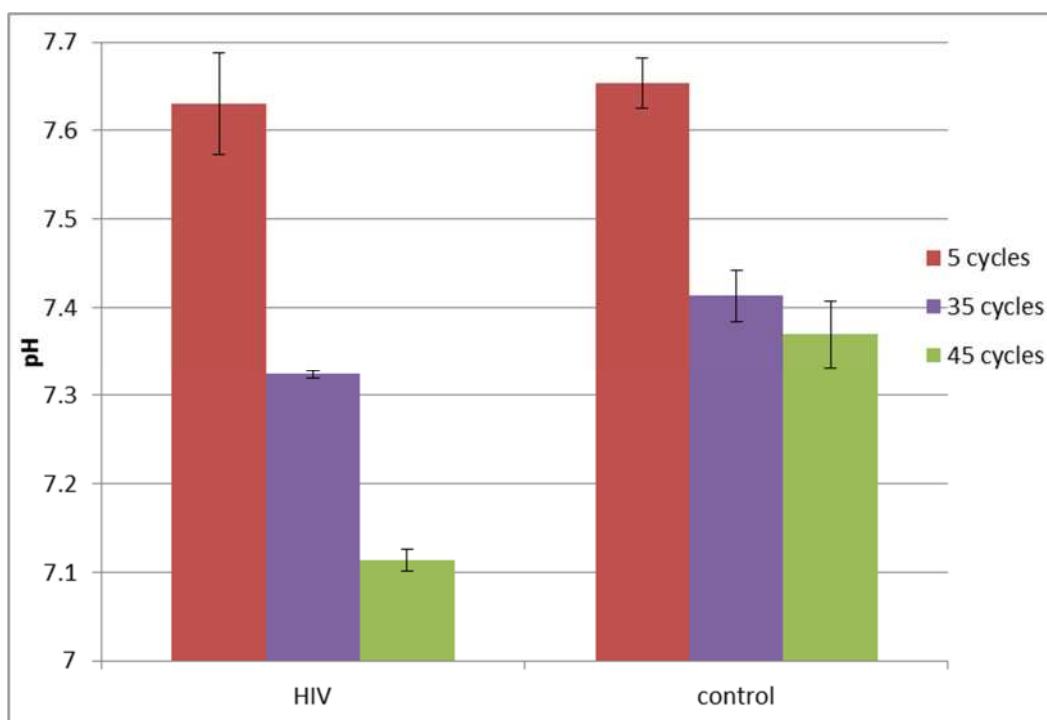


Figure 5-12: Amplification dependent pH change. Value of pH was measured at 5, 35, and 45 cycles of PCR using cDNA. The cDNA was prepared in a separate step by SuperScript III.

Integration of reverse transcription and PCR was not straightforward. Different enzymes are used in each reaction, in which the RT enzyme interferes with thermophile polymerase in PCR. Research work has been carried out trying DNA polymerases from various suppliers (Table 5-4). It is found that the combination of Invitrogen and Fermentas provided most consistent results. Crucial optimisation is done on mixing the right ratio of the RT enzyme and thermophile polymerase. A single reaction mixture is achieved to combined RT and PCR in a sealed chamber. The graph in Figure 5-13 shows a much bigger difference between the starting pH (cycle 5) and end pH (cycle 45) in the presence of HIV. The apparently lower starting pH in the control is the result of variation between assays. The reading of the signal is depending on the pH difference between two or more cycle numbers.

Company	Product
Invitrogen	AmpliGold
Fermentas	TrueStart
Promega	GoTaq
Clontech	Titanium Taq
KBiosciences	Klear Taq

Table 5-4 Enzyme vendors and products used in the assays

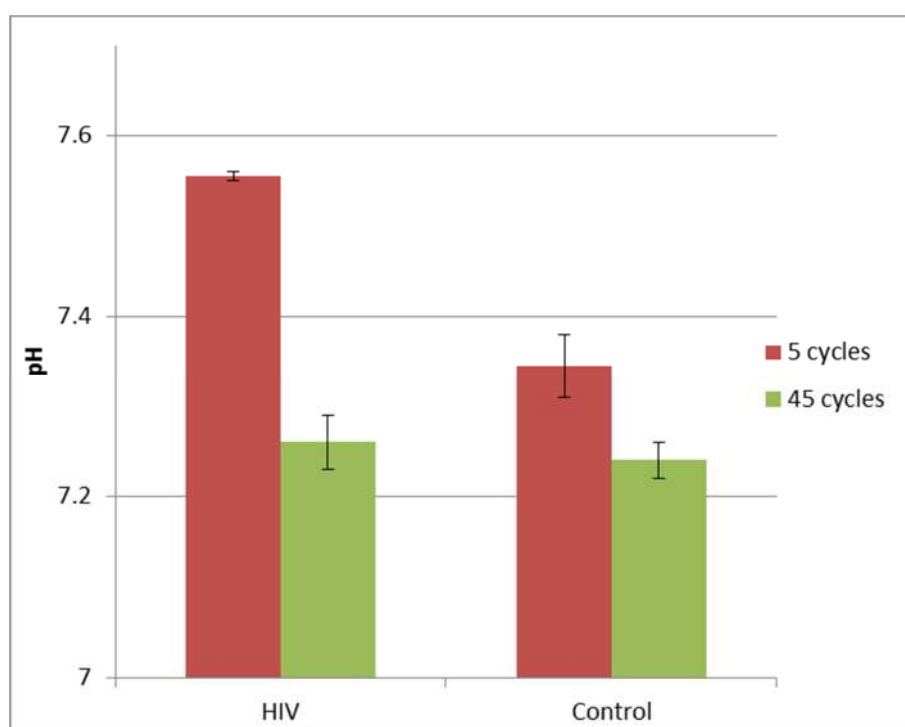


Figure 5-13: pH detection of HIV using one-step RT-PCR method

5.5 DNA LOGIC IN ISFET PLATFORM NAT HIV TESTS

Given the validated result in HIV detection using the ISFET, we designed an integrated DNA logic chip that combines the pH signal and decision making using transistor. For diagnosis, a device should help the user to reach a decision, such as the

type of drug or therapy care. Direct and concise instruction from the device is generally favoured without presenting raw signal or data such as the graph in Figure 5-13. Based on the concept, the DNA logic chip is to provide a logic operation on the ISFET signals from each target chamber shown in Figure 5-10. The result of the operation can offer simple binary result, Yes or No. It is essential to contain both negative and positive controls in any molecular diagnosis device. More control reactions are generally required to provide the noise level of non-ideal conditions. In HIV tests, the negative control is a reaction using buffer in the place of a template nucleic acid, which is called NTC control.. A synthetic target, RNA, is used as a positive control. A logic table in Table 5-5 reduces the assay result into a different infectious status or failed test. The confirmed positive requires both positive control and HIV sample being responsive while the buffer remains negative. A design of the trial chip is shown in Figure 5-15. Three reaction chambers and ISFETs are located at the corners of the square.

Buffer	RNA	HIV	Infected
0	0	0	x
0	0	1	x
0	1	0	NO
0	1	1	YES

Table 5-5 Logic Truth Table: Buffer = Negative control RNA = Positive Control

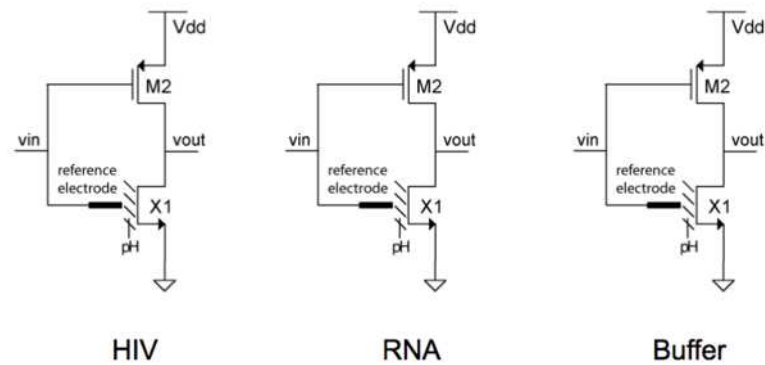


Figure 5-14: Three ISFET inverter pair circuit design of the logic table of three ISFET chambers

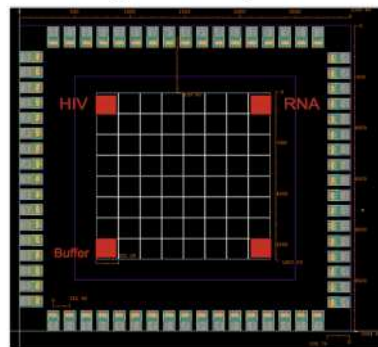


Figure 5-15: Floor plan of HIV logic chip, 1.5mm spacing between inverters

Logic gate design can also be applied to a sequence selectivity test. In the previous session, the principle of HIV detection using pH is shown. There is only one target sequence, which is part of the *pol* gene. However, it is normal to test multiple sites of HIV sequences to increase the selectivity. Conserved regions in the viral genome such as *pol* or *gag* are the frequent targets. Another conserved region is *vif* gene, which is used in the Roche detection kit along with *gag*. Here we present the logic gate design that accommodates two sequences such as *gag* and *vif* for the detection of HIV-1. The accuracy of testing is vitally important in order to access treatment, and knowledge of one's positive status can lead to behaviours to protect other people from infection.

<i>gag</i>	<i>vif</i>	<i>gag · vif</i>
0	0	0
1	0	0
0	1	0
1	1	1

Table 5-6: Truth table of genes, gag and vif

By having an “AND” gate, output 1 will only be given with both genes *gag* and *vif* are present in either blood or saliva samples. As a result, “AND” gate make feasible for more sensitive and more robust HIV-1 nucleic acid testing. This is an important step for such detection at home or at any POC setting without skilled personal and laboratory.

5.6 SUMMARY

HIV diagnosis is currently dominated by antibody-based assay. The kits described in the chapter provide detection with the risk of missing the early infection. The health authorities have managed HIV diagnostic policies based on the available detection technologies. The DNA logic and labelling free detection could improve the HIV management by increasing the sensitivity over the window period. We have demonstrated the proof-of-concept of the detection using ISFET. The detection and discrimination is directly correlated with the degree of amplification. By combining the HIV assay and DNA logic, it is possible to produce a single device that can indicate the early infection of HIV.

CHAPTER 6 – RAPID HIV TESTING: BUSINESS MODEL OVERVIEW

6.1 INTRODUCTION

Human Immunodeficiency Virus (HIV) is a compelling health issue that is currently plaguing the globe. This anxiety stems from the AIDS pandemic. First recognized in 1981, AIDS precipitated the death of more than 25 million people [114]. Present day, it is considered one of the most detrimental diseases recorded in history.

In spite of the constant advancement of antiretroviral treatments, AIDS is consistently still one of the most detrimental epidemics in the world. According to studies compiled by the Joint United Nations Programme on HIV/AIDS (UNAIDS), globally there are approximately 33.4 million people currently living with the disease. In 2010, it was surveyed that 2.7 million new cases of AIDS arise every year [115].

One of the key factors that contributed directly to this catastrophe was the insufficient amount of funds allotted for preventative measures and lack of access to antiretroviral treatment. In Kenya, antiretroviral therapy was offered to approximately 190,000 adults, but there is an estimated of 1.4 million existing HIV-infected patients. Only 12% of infected adults were receiving the treatment. In other words, more than half of infected patients are still not receiving antiretroviral therapy [116].

There has been a lot of debate regarding the amount of funds allotted for HIV/AIDS in the healthcare sector. This has been such a controversial matter because it has become an enormous burden on government budgets and resources. In 2006, \$8.9 billion was spent globally on HIV/AIDS activities. The spending only achieved 60

percent of the global need. Although the spending in 2008 was increased to \$13.7 billion, the fund was still 48 percent short of the actual global demand. Furthermore, UNAIDS estimated that the fund required for HIV/AIDS activities in 2010 will reach \$25.1 billion and forecasted that by 2015 the annual resource need will surpass \$54 billion [117] Figure 6-1.

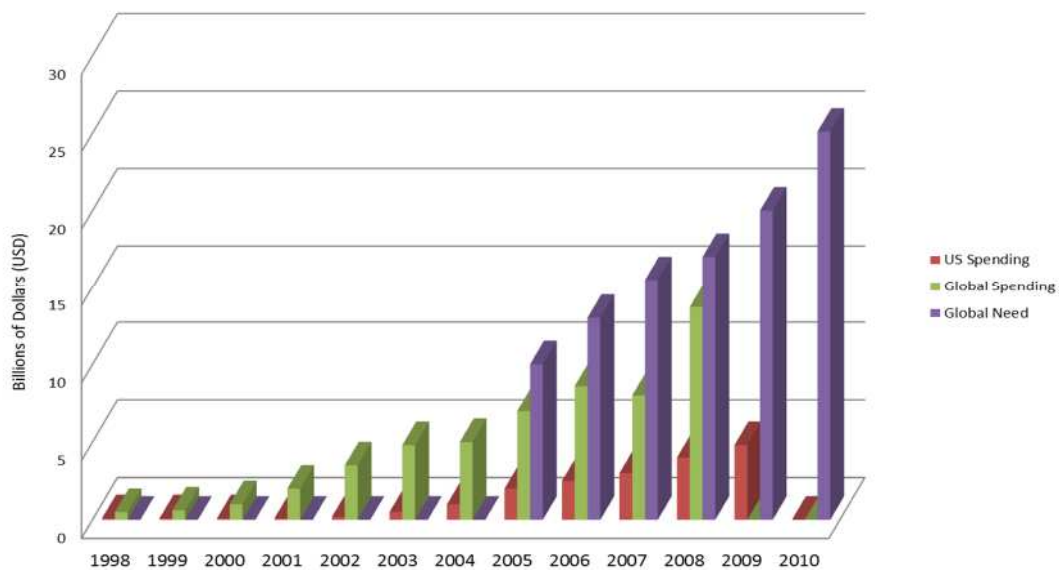


Figure 6-1 Global Spending on HIV/AIDS Activities [118]

Besides causing extreme grief and pain to loved ones, HIV and AIDS cause long-term permanent damages on the global economy and the obstruction of human capital growth. This is due to the impacts resulting from the increase in the proportion of young adults affected with HIV/AIDS. This pernicious disease has gravely crippled the taxable population and also reduced the amount of funding that was provided for public expenditures including education and health services [118].

Although governments and organizations have taken great care to implement preventative actions to impede the growing number of HIV/AIDS patients, the amount of victims that suffered from the contamination rose greatly regardless of

their immense efforts. Thus, vigorously expanding access to HIV counselling and testing shows the most potential to diminish the HIV/AIDS population. Implementing early HIV detection is without doubt a gateway into HIV prevention, treatment and care.

6.1.1 EARLY HIV DETECTION

Early HIV infection often causes no symptoms, making early detection extremely difficult. Present day, HIV testing programs rely heavily on HIV antibody tests because they are cheap and effective. However, HIV antibodies generally do not reach noticeable levels in blood for one to three months after infection. It may take the antibodies as long as six months to be produced in quantities large enough to show up in standard antibody tests [119].

In a 2006 UK report [120], it claimed that one quarter of an infected population visit and leaves the clinic not knowing they are HIV positive. This presents a particularly high risk, as subjects with acute HIV infection are most contagious because of high virus loads in their system. The reason causing false negative results is due to the intrinsic defect of any antibody-based tests within the window period before seroconversion. Thus, false negative results within this highly contagious period can pose a serious challenge in controlling such an epidemic. Hence, to determine whether a person has been recently infected, screening blood for the presence of HIV genes is consider one of the most effective method to avoid false negatives results. Direct screening of HIV genes is extremely critical in preventing transmission of HIV from recently infected individuals.

6.1.2 NUCLEIC ACID TESTING MARKET

Nucleic acid tests (NAT) is one of the fastest growing segment in the molecular diagnostics market. It is widely being used for improving IVD tests, tailoring personalized medicines, and discovering novel biomarkers. The growth of the NAT market is mainly driven by various applications such as viral load applications in HIV/hepatitis, detection of pathogens, and Cytomegalovirus (CMV) diagnostics. As a result, this thriving market is projected to reach 5.6 billion USD by 2015 [121].

NAT outperformed the traditional chemical reaction techniques in terms of sensitivity and sample to readout time, making NAT the most advanced diagnostic test for examining pathogens of interest and various genetic disorders. In addition, NAT is currently the only established method for determining the viral concentration in the circulation. This is particularly essential when it comes to therapeutic effectiveness and medical support. Although, in recent years, the advancement of NAT technologies has made the NAT more accessible, the adoption of NAT in the clinical laboratory field has been rather slow due to high cost and lacking in skilled lab personal [121].

Major players in NAT market include:

- Abbott Laboratories Inc. is a global company leading in the IVD business, specializing in immunoassays and blood screening. Various applications of medical tests and diagnostic instrument systems have been developed by Abbott. Abbott offers hospitals, laboratories, blood banks, and physician offices to diagnose and monitor diseases such as HIV, hepatitis, cancer, heart failure and metabolic disorders. Abbott also manufactures POC diagnostic products for

blood analysis and cardiac assays to provide immediate and precise diagnostics information [122].

- Applied Biosystems Inc. is a company that focuses on pharmaceutical, biotechnology and standardized testing researches. The company has an installed base of nearly 180,000 instrument systems in 100 countries. Applied Biosystems Inc. products have been widely used by pharmaceutical and biotechnology companies for enhancing discovering and developing new drugs [123, 124].
- Becton, Dickinson and Company, founded in 1897, is a medical technology company that manufactures, and sells medical systems and instruments. Its diagnostics department provides one-stop solution integration systems from safe collection and transport of diagnostics specimens to instrument systems and reagents for infectious diseases detection, healthcare-associated infections (HAI), and cancers. Its product lines include of safety-engineered blood collection products and systems, automated blood culturing systems, molecular testing systems for sexually transmitted diseases and microorganism identification and drug susceptibility systems [125].
- Myriad Genetic Laboratories, Inc. is most famous for its predictive medical products: [126]
 - I. BRAC*Analysis*: evaluates risk for hereditary breast and ovarian cancer
 - II. COLARIS: evaluates risk for hereditary colorectal and uterine cancer
 - III. COLARIS*AP*: evaluates risk for adenomatous polyposis colon cancer syndromes
 - IV. MELARIS: evaluates risk for hereditary melanoma

These predictive medical products patented by Myriad Genetic account over 40% of its revenue.

Myriad also offers the following personalized medicine tests: [126]

- I. TheraGuide: predicts toxicity to 5-FU-based chemotherapy
- II. OnDose: measures a patient's exposure to 5-FU chemotherapy
- III. PREZEON: assesses the status of the PTEN gene
- IV. PROLARIS: assesses the aggressiveness of prostate cancer

Myriad is one of the leading companies to make its profit through genome discoveries.

- Novartis AG is a Switzerland-based holding practicing research, development, manufacture and marketing of healthcare products. Novartis provides healthcare solutions include medicines, preventive vaccines, diagnostic tools, generic pharmaceuticals, and consumer health products. Operating in over 140 countries, its businesses are divided into four operating departments: [127]
 - Pharmaceuticals: discovery and developing new medicines
 - Vaccines and Diagnostics: developing human vaccines and blood-testing diagnostics
 - Sandoz: generic pharmaceuticals development
 - Consumer Health: manufactures and develops over-the-counter (OTC) medicines, animal health medicines, and contact lenses products.
- Roche's Diagnostics Division, in 2009 had a 20% share of the global IVD market, valued at an estimated 40 billion US dollars in annual sales. It is one of the top suppliers in the IVDs market. In addition, Roche's research products are widely used in hospitals and research labs for pathogens researches and discovery of new treatments [128].
- Siemens Healthcare Diagnostics, Inc. manufactures and provides clinical diagnostics and therapeutic systems. It has a wide range of product lines such as

integrated chemistry, immunoassay, routine chemistry, diabetes, urinalysis, blood gas monitoring, early detection, diagnosis, therapy, and care [129].

6.1.3 CHAPTER OBJECTIVES

The proposition of this chapter is to propose a business model using the ISFET chemical sensing technology as a platform to implement rapid HIV nucleic acid tests. In this Chapter, we started with a thorough analysis of the HIV testing market and a brief overview of current HIV testing products and manufacturers. Next we depicted a business opportunity using ISFET chemical sensing platform as a low cost, rapid, and robust HIV point of care detection device by constructing a business model with strategic comparative analyses and a revenue model.

6.2 HIV TESTS: MARKET OVERVIEW

6.2.1 INTRODUCTION

As we have mentioned in Chapter 4, HIV tests can be classified as diagnosis and prognosis, in which diagnosis provides result for people newly living with HIV and prognosis offers the therapeutically effectiveness and medical support.

There are three main objectives for HIV diagnosis tests:[130]

- I. Screening donated blood to reduce transmission of the virus through transfusions
- II. Surveillance of HIV prevalence
- III. Diagnosis of infection in individuals

The accuracy of HIV tests is measured by their sensitivity and selectivity. These operational parameters are determined by clinical condition of the patient, operator, environment, package design, and principle chemistry.

- I. Definition of sensitivity: null response in false negative. e.g. 2 false negative in 100 known positive samples, sensitivity is 98%
- II. Definition of selectivity: null result of false positive. e.g. If there are 4 false positive in 100 known non infected samples, selectivity is 96%

When the objective is to minimize false negative results, high sensitivity tests should be used. Otherwise if the objective is to minimize false-positives results, tests with high selectivity should be used. Present day, the sensitivity and selectivity requirement for a HIV test are 99% and 98% respectively. Due to cost-effectiveness and characteristics of various HIV tests, in practice, different types of tests are often used in combination to utilize limited resources [130].

Viral load and drug resistance are the focus when the objective is medical support for HIV patients. The viral load is a significant effectiveness indication of the HIV medication. Normally, viral load lower than 50 copies per ml of blood is considered as undetectable and an increase of the viral load during antiretroviral treatments is consider a rebound and sign of the failed therapy [131].

Nucleic acid test (NAT) is the standard methods in viral load assay. NAT is extremely sensitive and has a much shorter window period comparing with antibody/antigen assay. However, specialized facility and training is required to ensure the test quality, which associates with higher cost. The turn-around time is generally several hours to days before the test results are available [132].

The advancement of antiretroviral treatments has significantly improved therapies offered to HIV/AIDS infected individuals, which made HIV emerges as a persistent condition that needs efficient disease management rather than a life threatening disease. This not only prolong the lives of infected patients but also increased and encouraged people to have HIV tests when they find themselves exposed to HIV in a high risk situation. Thus, players in this sector have been motivated to develop more efficient, more robust, and more sensitive HIV testing products.

6.2.2 MARKET OVERVIEW

According to Global Industry Analysts, Inc, HIV screening tests market is projected to yield US\$1.3 billion revenues by the end of 2010 and is projected to exceed \$3.9 Billion by the year 2015. Although Africa, China, and India account for over 90% of world HIV population, according to the 2008 report, 60% of the worldwide HIV testing market is in United States and Europe. However, Asian countries such as China, India, and Southeast Asia are projected to encounter the fastest growth in demand for HIV tests in the next decade due to their economic growth and health education. This phenomenon is particular especially in China, booming market has pushed Chinese government to reform its health-care policies and increase government spending in the medical sector of its annual fiscal expenditure. These markets are very promising and offer enormous potential for manufactures with HIV/AIDS testing products [133].

By 2015, HIV viral load monitoring test market is projected to reach US\$1.7 billion due to the advancement of antiretroviral treatments. However only countries such as US and Europe, accounting for roughly 75% of the monitoring test market, can afford

and access such treatments. In addition, in US alone, HIV/AIDS confirmatory test market is projected to reach US\$32 million by 2015 [133].

The rapid test kits market is one of the fastest growing HIV testing markets. It is due to the nearly instant results as they claim to offer. Comparing with enzyme-linked immunosorbent assay (ELISA) testing kits, rapid test kits are easier to use with faster results.

6.2.3 CURRENT KEY PLAYERS IN HIV TESTING MARKET

Key players in HIV/AIDS testing market with significant presence (Table 6-1):

- Abbott Laboratories produces HIV medicine Kaletra and Aluvia. According to WHO, Kaletra/Aluvia is the most widely registered HIV medicine in the world, approved in 170 countries [122, 124].
- Adaltis Inc. is a company whose primary product for HIV tests is the Nexgen Four/Personal Lab instruments.
- Siemens Healthcare Diagnostics is one of the most advance HIV testing product manufactures. Its ADVIA Centaur HIV Ag/Ab Combo assay claims to aid in the early detection and diagnosis of HIV infection by simultaneously detecting the presence of both the p24 antigen and the HIV antibodies. The ability to detect both the antigen and the antibodies at the same time can increase the likelihood of early detection and treatment for HIV patients [129].
- Bio Mériex offers two test ranges, QUICKVUE and VIKIA. These rapid tests are designed to resolve the difficulties implementing diagnostic tests at a point-of-care (POA) setting. QUICKVUE and VIKIA tests cover a wide range of infectious diseases such as HIV, Hepatitis B, Influenza, RSV, Viral Gastroenteritis, and Chlamydia.

- Calypte Biomedical Corporation develops innovative *in-vitro* diagnostic testing solutions for HIV. The tests are known to be reliable and rapid non-invasive HIV diagnostic tests.
- Innogenetics NV. develops and markets diagnostic products in the area of infectious diseases include screening and confirmation tests for HIV, HCV, HTLV, and syphilis; genotyping tests for hepatitis B virus (HBV), human papillomavirus, and mycobacterium sp; and drug-resistance tests for TB and HBV.
- OraSure Technologies Inc. develops, manufactures, markets, and sells oral fluid diagnostic products. OraSure's HIV testing products include OraQuick ADVANCE HIV-1/2, a point-of-care test for antibodies to the HIV-1 and HIV-2 and OraSure, an oral fluid collection device for the detection of antibodies to HIV-1. Further, the company also sells immunoassay tests and reagents for western blot HIV-1 confirmatory test.
- Roche Diagnostics quantitative tests for HIV-1 provide health information to be used by physicians, helping them to determine viral load and monitor their patients' disease progression and response to therapy [128].
- COBAS AmpliPrep and COBAS TaqMan systems are the first fully automated real-time, continuous load IVD platform for infectious diseases detection such as HIV-1. It has features of sample-in and result-out capability. It is designed to increase laboratory efficiency and productivity with automated sample preparation, amplification and quantitation of RNA/DNA.

	Product name	Sample source	Time
HIV GENOTYPING	Bayer : TRUGENE® HIV-1 genotyping assay	Plasma RNA amplified by RT-PCR Detection by sequencing electrophoresis	Many hrs
	Celera Diagnostics/Applied Biosystems; ViroSeq HIV-1 genotyping system	Plasma RNA amplified by PCR Detection by capillary electrophoresis.	Many hrs
HIV VIRUS LOAD	Roche :Tagman HIV-1 Monitor Test v1.5	Plasma RNA amplified by real-time RT-PCR Detection by fluorescence (real-time PCR)	5-6 hrs
	Roche Amplicor HIV-1 Monitor Test v1.5	Plasma/Serum RNA by RT-PCR Detection by EIA	6-8 hrs
	Roche : Cobas Amplicor HIV-1 Monitor test v1.5	Plasma/serum RNA amplified by Cobas AmpliPrep RT-PCR Detection by EIA	6-8 hrs
	Gene-probe :APTIMA HIV-1 RNA	Plasma RNA amplified by TMA Detection by chemiluminescence (hybridization protection assay, HPA)	5-8 hrs
	Siemens Healthcare Diagnostics VERSANT HIV RNA 3.0 Assay (bDNA)	Plasma RNA amplified by RT-PCR Detection by chemiluminescence (bDNA) Covering HIV-1 subtypes 50copy/mL DOL	8 hrs
	Abbott:LCx HIV1 RNA quantification kit	Plasma RNA amplified by RT-PCR	5-8 hrs
	Abbott: Real-Time HIV-1	Plasma RNA amplified by RT-PCR rTth polymerase Detection by fluorescence (real-time PCR)	
	Organon Teknika: (Biomérieux) NucliSens EasyQ HIV-1	Plasma RNA amplified by NASBA Detection by Electro-chemiluminescence	2.5-3 hrs
	Primagen: Retina Rainbow (collapsed?)	Plasma RNA amplified by NASBA Detection by	1.5 hrs
HIV PROVIRAL DNA	Roche: Amplicor HIV-1 DNA test v1.5	Plasma DNA amplified by PCR Detection by EIA Case: 1-500µL infant whole blood with DNA sample from Rwanda to USA	7-8 hrs

Table 6-1 HIV assays and products

6.3 BUSINESS OPPORTUNITIES: ISFET PLATFORM HIV NAT

6.3.1 MARKET OPPORTUNITIES

Market provides revenue, produces profit, and sustains growth. Thus, it is significant to determine if there are demands for a new HIV test product. In section 6.2.2, a growth trend of the HIV testing market is foreseeable. Thus, there is a market for a more competitive and more cost-effective HIV testing product.

In chapter 1 we have discussed various sequencing markets, applications, and detection techniques. Here in this chapter, we focus on a novel low density genome typing methodology for HIV tests. Present day, HIV testing products such as HIV tests using NAT systems listed in Table 6-1 HIV assays and products, rapid tests such as OraQuick, or antibodies tests such as ELSA and Western bolt are either lab-based, require skilled lab personals, very costly or lack of sensitivity before seroconversion. Presently, in order to overcome shortcomings in each single HIV test, detecting HIV with combinations of different HIV tests as a cocktail solution is the only method to resolve the dilemmas.

6.3.2 BUSINESS OPPORTUNITIES: ISFET PLATFORM HIV NAT

As we have mentioned in section 6.2.1, the sensitivity and selectivity of a HIV test are essential. Generally, the sensitivity and selectivity requirement for a standard HIV test are 99% and 98% respectively. Thus, as identified in 6.1.2, NAT based tests are particularly appropriate for the task where NAT assays in the market have sensitivity better than 99% and selectivity better than 99%. However, section 6.1.2 also stated that NAT based tests are costly and difficult to implement.

As we have mentioned earlier, various chemical sensors have their unique properties, characteristics, and flaws. Therefore, there exists a market where users demand a low cost, sensitive, and rapid HIV tests. ISFET platform sensors are particularly suitable in the case for POC HIV screening tests. There are many comparative advantages using such technology in IVD market as it can reduce the costs by leveraging the semiconductor industry, while augmenting the efficiency and robustness of HIV tests, based on the core technology of NAT.

6.4 BUSINESS ANALYSIS

6.4.1 BUSINESS ANALYSIS OVERVIEW

Here we propose a business model, selling ISFET NAT machines and cartridges to clinics, governments, and charity foundations. STP analysis is shown in Table 6-3, listing all the potential buyers, market segmentation of this novel application using ISFET NAT. Furthermore, a SWOT analysis of the ISFET platform HIV testing is illustrated in Table 6-2 to analysis the strength, weakness, opportunities and threats of ISFET based NAT.

Strength	Weakness
Currently the only technology for rapid NAT detection from a purified and amplified saliva/blood sample within 15 minutes ⇒ rapid screening	FDA approval (Timing Consuming)
Scalable mass producible silicon-chip technology (CMOS) ⇒ Low cost	New testing technology ⇒ Cost to build credibility
Single assay: batch processing not required to achieve economics of scale	Ethical issues
Accurate – NAT detection ⇒ no “window period”	Confidential issues ⇒ Disclosure of personal information
Therapy support – Rapid NAT viral load test	Government regulated disease ⇒ difficult for self-testing market
Disposable cartridge component enhance hygiene and repeated revenue	
No skilled lab personal needed ⇒ self-test kit	
Reduce cost of therapy ⇒ early detection ⇒ early therapy ⇒ enhance disease control	
Opportunity	Threats
Epidemical World Wide – especially in Africa, China and South East Asia	Government regulations
Demands for accurate rapid HIV tests in regions lacking skilled personals and laboratory facilities	Liability for false negative
Effective ⇒ Excludes Window Period	

Table 6-2 SWOT ANALYSIS – ISFET PLATFORM HIV TESTING

Segmentation:

Rapid screening during window period
Niche market for rapid NAT detections
Cost-effective low density typing machine

Target Markets:

Government (Medical Centers, Hospitals, and Clinic office)
Machines and Cartridges
USAIDS
Worry wells – end consumers (unregulated regions)
Self-test kit in retails
Charity Foundations
Gates Foundation
William J. Clinton Foundation
Clinics for rapid detection

Position in the Market:

Clinic ISFET NAT Machine Vendor
HIV Testing Kit Supplier

Table 6-3 STP Analysis – HIV Testing Market

6.4.2 ESTIMATED REVENUE MODEL

Market Size: Clinics, Governments and Charity Foundations spending on HIV disease

ISFET NAT Machine Price: 5,000 USD

Estimate Cost of Machine: 2,000 USD

Cartridge Price: 5 USD/unit (Price of competitors from 1.5 USD to 5 USD)

Estimate Cost of Cartridges: 1 USD/unit

Forecast Machines Sales: 50 units/year

Forecast Cartridges Sales: 100,000 units/year

Forecast Revenue: 750,000 USD/year

Forecast Gross Margin: 550,000 USD/year

6.5 CONCLUSION

In this chapter, we proposed HIV diagnosis as the suitable application for DNA Logic. From the perspective of the global market of HIV testing, there is a growing demand for more cost-effect devices with better sensitivity. The impact of the new infection is significant on the life of individuals. The market and healthcare system need to narrow the non-responsive period of the current methods. DNA Logic differentiates from other NATs because the POC factor. In regions with less resources, HIV test in POC would have saved many infections and managed more treatment regimes that are more responsive. ISFET based NAT has all the ingredients to fill the need, such as right technology, proved manufacturability, lower cost, and strong market. HIV diagnosis is only one of DNA Logic applications. It has a great potential to be an important player in the diagnostic business.

CHAPTER 7 FUTURE WORK ON APPLICATIONS AND CONCLUSIONS

7.1 FUTURE WORK ON NAT APPLICATIONS

In previous chapters, we have shown the feasibility of using ISFET platform nucleic acid testing (NAT) to perform a HIV test. In addition, we also depicted and analyzed a business model selling ISFET NAT machines and cartridges for the detection of HIV/AIDS. However, as mentioned in the earlier chapter, the ISFTE technology is a platform technology. ISFET NAT not only is useful in HIV/AIDS application but it can also detect any genes of interest. In other words, this method is capable of detecting any nucleic acid sequence in any living organism. Thus, here we proposed some critical applications especially in infectious disease and pathogen detection could be benefited from POC NAT technologies such as ISFET platform. Two examples are given below, H1N1 and Chlamydia.

7.1.1 H1N1

In April 2009 H1N1, a new influenza virus is first detected in the United States causing illness in people. The novel strain is virulent and live threatening. Among the whole possible H1N1 infected individual in the US States, it is about 0.4% to be hospitalised as shown in Table 7-1. This epidemic is spreading rapidly around the world similar as regular seasonal influenza viruses. On June 11, 2009, WHO announced the emergence of H1N1 as a pandemic underway and raised the level of influenza pandemic alert from phase 4 (sustained human to human transmission) to phase 5 (widespread human infection).

2009 H1N1	Mid-Level Range	Estimated Range
Cases		
0-17 years	~20 million	~14 million to ~28 million
18-64 years	~35 million	~25 million to ~52 million
65 years and older	~6 million	~4 million to ~9 million
Cases Total	~61 million	~43 million to ~89 million
Hospitalisations		
0-17 years	~87,000	~62,000 to ~128,000
18-64 years	~160,000	~114,000 to ~235,000
65 years and older	~27,000	~19,000 to ~40,000
Hospitalisations Total	~274,000	~195,000 to ~403,000
Deaths		
0-17 years	~1,280	~910 to ~1,880
18-64 years	~9,570	~6,800 to ~14,040
65 years and older	~1,620	~1,160 to ~2,380
Deaths Total	~12,470	~8,870 to ~18,300

Table 7-1: H1N1 case and related deaths in US from April 2009 to April 2010[134]

Certain people are at high risk of serious complications, including severe illness and death. These people at high risk include old people from 65 years and older, young children less than five years old, pregnant women, and people with chronic medical conditions. Thus, once a case is confirmed, treatment should be initiated as early as possible. According to studies, therapies and treatments are most effective in the first 2 days of illness. However, the typical incubation period for influenza is 1-4 days. Therefore, early detection of the virus is essential to support effective treatments. New mutated influenza emerges every couple years and the emergence cycle of mutating influenza has shortened in the past few years due to missuses and overuse of antibiotics.

Early detection or self-diagnosis will prevent and stop virus spreading. The requirement for a fast development to accommodate the novel mutation eliminates the antibody-based detection based methods. Developing rapid ISFET NAT screening for H1N1 can help epidemic control and achieve early detection for early treatments to save life s from severe illness and death.

7.1.2 CHLAMYDIA

Chlamydia is a very common sexually transmitted disease (STD) caused by the bacterium, *Chlamydia trachomatis*. Symptoms of Chlamydia are often mild or absent, but serious irreversible complications, including infertility, can occur "silently" before a woman ever recognizes a problem.

Chlamydia is the most frequently reported bacterial sexually transmitted disease in the United States. In 2006, 1,030,911 Chlamydial infections were reported to the CDC, and this is a vast underestimate of the true number of infections; true figures are at least double this. A conservative estimate indicates that more than 90 million people a year get infected with Chlamydia. Chlamydia can be transmitted during vaginal, anal, or oral sex and it can also be passed from an infected mother to her baby during childbirth. Chlamydia can also be found in the throats of women and men having oral sex with an infected partner. In addition, women infected with Chlamydia are up to five times more likely to become infected with HIV, if exposed.

To help prevent the serious consequences of Chlamydia, screening at least annually for Chlamydia is recommended for all sexually active women age 25 years and younger. An annual screening test also is recommended for older women with risk factors for Chlamydia. All pregnant women should have a screening test for Chlamydia.

In pregnant women, there is some evidence that untreated Chlamydial infections can lead to premature delivery. Babies who are born to infected mothers can get Chlamydial infections in their eyes and respiratory tracts. Chlamydia is a leading cause of early infant pneumonia and conjunctivitis in new-borns.

There is a need for a rapid test for chlamydia but the current rapid tests are not sensitive in the low prevalence environment [135, 136]. There are no good antibody tests for Chlamydia due to their poor specificity and low sensitivity. The current gold standard is the laboratory nucleic acid test (NAT). These are exclusively PCR based, requiring expensive laboratory equipment. Thus, using the ISFET NAT, such concealed STD can be effectively identified and monitored at a very low cost.

7.2 CONCLUSIONS

It is only about half a decade since Watson and Crick published the X-ray structure of DNA and proposed the complementary double-strands DNA helix [32]. The knowledge of the chemical structure of DNA is the basis of modern genetics and molecular diagnostics. The advance of technical development has helped to accelerate the process. The first human genome draft mapping released in 2003 took 13 years to finish. Within a few years after the genome publication, the first low-cost sequencer from 454 Life Sciences was released, which achieved the whole human genome mapping in one week with cost only a fraction of the Human Genome Project. The speed of the technology is partly driven by the urge for knowledge of genetic information. Pharmacogenetics has been established to consolidate the correlation between gene variation and drug response. Molecular diagnostics has been utilising the genomic information of pathogens for more sensitive test. The DNA Logic

described in the thesis holds a key to unlock the genetic information accumulated so far and offer a unique solution for molecular diagnosis.

Nucleic acid testing is moving into a new phase. In this thesis we have depicted the transformation of the technology from a lab-based labelled test to an ISFET label-free NAT, allowing complex genetic/specific gene and genomic tests into back-offices or even on the store's counter. This rapid progress in genome science, stimulating vast potential of applications, has spurred observers to predict that medical biology will be the foremost science of the 21st century.

From ISFET platform NAT, DNA Logic was derived to make local intelligent computations. This not only allows users to see vast intelligent results but also enhances the performance and the robustness of the test.

Various applications can be derived from the ISFET platform NAT. One of the applications illustrated in this thesis is the detection of HIV/AIDS. We have made an unprecedented proof of science using an ISFET to detect HIV. This major breakthrough has proven the feasibility of using this platform to detect HIV in any POC setting. In conclusion, in this thesis we have shown vast applications using ISFET based NAT and the feasibility of using it as a HIV test. We have also depicted a business model showing the possibility of using such technology as a platform for pathogen detection.

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REFERENCES

1. Koch, W.H., *Technology platforms for pharmacogenomic diagnostic assays*. Nat. Rev. Drug Discov, 2004. **3**(9): p. 749-761.
2. Gardner, J.W., ed. *Microsensors: Principles and Applications*. 1994, Wiley.
3. D. Diamond, e., *A series of monographs on analytical chemistry and its applications*. Principles of Chemical and Biological Sensors, 1998. **150 of Chemical Analysis**.
4. Wilson, D., *Analog VLSI Architectures for Chemical Sensing Microsystems*. 1995, Georgia Institute of Technology.
5. Georgiou, P., *Chemical Bionics - A novel design approach using Ion Sensitive Field Effect Transistors*, in *Electrical and Electronic Engineering*. 2008, University of London, Imperial College of Science, Technology and Medicine.
6. *Compendium of Chemical Terminology* 2ed. 1997, International Union of Pure and Applied Chemistry.
7. Yang, G.-Z., ed. *Body Sensor Networks*. 2006, Springer.
8. Rogers, K., *Biosensors for environmental applications*. Biosensors and Bioelectronics, 1995. **10**(6-7): p. 533-541.
9. Wilkins, E., *Glucose monitoring: State of the art and future possibilities*. Medical Engineering & Physics, 1996. **18**: p. 273-288.
10. Bousse, L., *Whole cell biosensors*. Sensors and Actuators B: Chemical, 1996. **34**: p. 270-275.
11. Paddle, B., *Biosensors for chemical and biological agents of defence interest*. Biosensors and Bioelectronics, 1996. **11**(11): p. 1079-1113.
12. Wang, J., ed. *Analytical Electrochemistry*. 2 ed. 2000, Wiley.
13. Maes, H.E., et al., *Trends in microelectronics, optical detectors and biosensors*. Advanced Engineering Materials, 2001. **3**: p. 781-787.
14. Janata, J. and R.J. Huber, eds. *Solid State Chemical Sensors*. 1985, Academic Press, Inc.
15. Madou, M.J. and S.R. Morrison, *Chemical Sensing with Solid State Devices*. 1988: Academic Press, Inc.
16. Nam, H., et al., *Micropotentiometric sensors*. Proc. IEEE, 2003. **91**(6): p. 871-880.
17. Lambrechts, M. and W. Sansen, *Biosensors: Microelectrochemical Devices*. 1992: Taylor & Francis.
18. Shepherd, L., *Low-power computational interfacing with CMOS ISFETs*. 2008, Imperial College London.
19. Brett, C.M.A. and A.M.O. Brett, *Electroanalysis*. Vol. 64. 1998: Oxford Chemistry Primers, Oxford University Press.
20. Hines, E., E. Llobet, and J. Gardner, *Electronic noses: a review of signal processing techniques*. Proc. IEE Circuits, Devices and Systems, 1999. **146**: p. 297-310.

21. Koickal, T.J., et al., *Analog VLSI circuit implementation of an adaptive neuromorphic olfaction chip*. IEEE Trans. Circuits and Systems I - Fundamental Theory and Applications, 2007. **54**(1): p. 60-73.
22. Sansen, W., et al., *A smart sensor for the voltammetric measurement of oxygen or glucose concentrations*. Sensors and Actuators B: Chemical, 1990. **1**(1-6): p. 298-302.
23. Cane, C., *Multilayer isfet membranes for microsystems applications*. Sensors and actuators,, 1996. **35**(1-3): p. 136.
24. Saiki, R., *Enzymatic Amplification of β -globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sick Cell Anemia*. Science, 1985. **230**: p. 1350-1354.
25. Bausells, J., et al., *Ion-sensitive field-effect transistors fabricated in a commercial CMOS technology*. Sensors and Actuators B-Chemical, 1999. **57**(1-3): p. 56-62.
26. Shepherd, L.M. and C. Toumazou, *A biochemical translinear principle with weak inversion isfets*. Circuits and Systems I: Regular Papers, IEEE Transactions on [see also Circuits and Systems I: Fundamental Theory and Applications, IEEE Transactions on], 2005. **52**(12): p. 2614-2619.
27. *In vitro Diagnostics*. 2010 (June), Freedopnia Focus.
28. Venter, J.C., et al., *The sequence of the human genome*. Science, 2001. **291**(5507): p. 1304-51.
29. Human Genome Sequencing, C., *Finishing the euchromatic sequence of the human genome*. Nature, 2004. **431**(7011): p. 931-945.
30. Margulies, M., et al., *Genome sequencing in microfabricated high-density picolitre reactors*. Nature, 2005. **437**(7057): p. 376-80.
31. Bennett, S.T., et al., *Toward the 1,000 dollars human genome*. Pharmacogenomics, 2005. **6**(4): p. 373-82.
32. Watson, J.D. and F.H. Crick, *Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid*. Nature, 1953. **171**(4356): p. 737-8.
33. *Human genome at ten: The sequence explosion*. Nature. **464**(7289): p. 670-1.
34. Flusberg, B.A., et al., *Direct detection of DNA methylation during single-molecule, real-time sequencing*. Nat Methods. **7**(6): p. 461-5.
35. Eisen, J.A., *Environmental shotgun sequencing: its potential and challenges for studying the hidden world of microbes*. PLoS Biol, 2007. **5**(3): p. e82.
36. Astier, Y., O. Braha, and H. Bayley, *Toward single molecule DNA sequencing: direct identification of ribonucleoside and deoxyribonucleoside 5'-monophosphates by using an engineered protein nanopore equipped with a molecular adapter*. J Am Chem Soc, 2006. **128**(5): p. 1705-10.
37. Claverie, J.-M., *GENE NUMBER: What If There Are Only 30,000 Human Genes?* Science, 2001. **291**(5507): p. 1255-1257.
38. Alberts, B.J., A; Lewis, JJ; Raff, M; Roberts, K; Walter, P, *Molecular Biology of the Cell*. 4th ed. 2002, New York: Garland Science.
39. Metzker, M.L., *Sequencing technologies [mdash] the next generation*. Nat Rev Genet. **11**(1): p. 31-46.
40. Karow, J. *Roche's Sequencing Sales Decline 15 Percent in Q1*. 2010; Available from: <http://www.genomeweb.com/roches-sequencing-sales-decline-15-percent-q1>.

41. *Life Technology Citi Report*. 2010, Citygroup Global Markets Inc.
42. Balachander. *DNA Gene Sequencing Firm Plans IPO*. International Business Times 2010; Available from: <http://au.ibtimes.com/articles/43855/20100818/dna-pacbio-smrt-technology-pacific-biosciences-ipo-complete-genomics-illumina-life-technologies-ion.htm>.
43. *DNA Sequencing: Emerging Technologies and Applications*. 2010, BCC Research.
44. Maitra, A., et al., *Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray*. Mod Pathol, 2003. **16**(9): p. 902-12.
45. *Letter to 23andMe Personal Genome Service*, D.o.H.a.H. Services, Editor. 2010, Food and Drug Administration.
46. Ionita-Laza, I., et al., *Genetic association analysis of copy-number variation (CNV) in human disease pathogenesis*. Genomics, 2009. **93**(1): p. 22-26.
47. O'Donovan, M.C., G. Kirov, and M.J. Owen, *Phenotypic variations on the theme of CNVs*. Nat Genet, 2008. **40**(12): p. 1392-1393.
48. Braude, I., et al., *Large scale copy number variation (CNV) at 14q12 is associated with the presence of genomic abnormalities in neoplasia*. BMC Genomics, 2006. **7**(1): p. 138.
49. Moore, G., *Cramming More Components Onto Integrated Circuits*. Electronics Magazine, 1965. **38**(8): p. 114.
50. Yin, T. and T. Miyata, *Warfarin dose and the pharmacogenomics of CYP2C9 and VKORC1 -- Rationale and perspectives*. Thrombosis Research, 2007. **120**(1): p. 1-10.
51. Blanpain, C., et al., *CCR5 and HIV infection*. Receptors Channels, 2002. **8**(1): p. 19-31.
52. Thorisson, G.A. and L.D. Stein, *The SNP Consortium website: past, present and future*. Nucleic Acids Research, 2003. **31**(1): p. 124-127.
53. Sabeti, P.C., et al., *Genome-wide detection and characterization of positive selection in human populations*. Nature, 2007. **449**(7164): p. 913-918.
54. Navis, M., et al., *A nonprogressive clinical course in HIV-infected individuals expressing human leukocyte antigen B57/5801 is associated with preserved CD8+ T lymphocyte responsiveness to the HW9 epitope in Nef*. J Infect Dis, 2008. **197**(6): p. 871-9.
55. Sanger, F. and A.R. Coulson, *A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase*. J Mol Biol, 1975. **94**(3): p. 441-8.
56. WHO, *WHO/UNAID/UNICEF: Towards universal access: scaling up priority HIV/AIDS interventions in the health sector. Progress Report*. 2007.
57. Henderson, L.J., *Concerning the relationship between the strength of acids and their capacity to preserve neutrality*. (Abstract). Am. J. Physiol., 1908. **21**(4): p. 173-179.
58. Po, H. and N. Senozan, *Henderson-Hasselbalch Equation: Its History and Limitations*. J. Chem. Educ., 2001. **78**: p. 1499-1503.
59. Flatley, J., *Illumina Inc at Goldman Sachs Global Healthcare Conference* Goldman Sachs Global Healthcare Conference, 2010.
60. Khaja, R., et al., *Genome assembly comparison identifies structural variants in the human genome*. Nat Genet, 2006. **38**(12): p. 1413-8.

61. Parker, L.M., P; Waples, RS, *Application of DNA Technology to the Management of Pacific Salmon*. NOAA Technical Memorandum NMFS-NWFSC-17, 1993.
62. Shendure, J. and H. Ji, *Next-generation DNA sequencing*. Nat Biotechnol, 2008. **26**(10): p. 1135-45.
63. University, I.o.H.G.-N. *High throughput sequencing and genotyping*. 2010; Available from: <http://www.ncl.ac.uk/ihg/research/facilities/flx/empcr.htm>.
64. Sciences, L. *Sequencing Chemistry*. 2010; Available from: <http://454.com/products-solutions/how-it-works/sequencing-chemistry.asp>.
65. Elgar, G. and T. Vavouri, *Tuning in to the signals: noncoding sequence conservation in vertebrate genomes*. Trends Genet, 2008. **24**(7): p. 344-52.
66. Koch, W.H., *Technology platforms for pharmacogenomic diagnostic assays*. Nat Rev Drug Discov, 2004. **3**(9): p. 749-61.
67. Mungall, A.J., *Meeting review: Epigenetics in Development and Disease*. Comp Funct Genomics, 2002. **3**(3): p. 277-81.
68. Espy, M.J., et al., *Real-time PCR in clinical microbiology: applications for routine laboratory testing*. Clin Microbiol Rev, 2006. **19**(1): p. 165-256.
69. Klenow, H. and I. Henningsen, *Selective Elimination of the Exonuclease Activity of the Deoxyribonucleic Acid Polymerase from Escherichia coli B by Limited Proteolysis*. Proc Natl Acad Sci, 1970. **65**(1): p. 165-175.
70. S.Cheng, et al., *Effective amplification of long targets from cloned inserts and human genomic DNA*. Proc Natl Acad Sci., 1994. **91**(12): p. 5695-5699.
71. Rychlik, W., W. Spencer, and R. Rhoads, *Optimization of the annealing temperature for DNA amplification in vitro*. Nucl Acids Res, 1990. **18**(21): p. 6409-6412.
72. Saiki, R., *Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase*. Science, 1988. **239**: p. 487-491.
73. Joyce, C., *Quantitative RT-PCR. A review of current methodologies*. Methods Mol Biol, 2002. **193**: p. 83-92.
74. Bustin, S., *Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays*. Journal of Molecular Endocrinology, 2000. **25**: p. 169-193.
75. Bergveld, P., *Thirty years of ISFETOLOGY - What happened in the past 30 years and what may happen in the next 30 years*. Sensors and Actuators B-Chemical, 2003. **88**(1): p. 1-20.
76. Shepherd, L. and C. Toumazou, *Weak Inversion ISFETs for ultra-low power biochemical sensing and real-time analysis*. Sensors. and Actuators B: Chemical, 2005. **107**(1): p. 468-473.
77. Purushothaman, S., C. Toumazou, and C.P. Ou, *Protons and single nucleotide polymorphism detection: A simple use for the Ion Sensitive Field Effect Transistor*. Sensors and Actuators B: Chemical, 2006. **114**(2): p. 964-968.
78. Martinoia, S. and G. Massobrio, *A behavioral macromodel of the ISFET in SPICE*. Sensors and Actuators B-Chemical, 2000. **62**(3): p. 182-189.
79. Toumazou, C. and L. Shepherd, *Using transistors to linearise biochemistry*. Electronics Letters, 2007. **43**(2): p. 65-67.
80. Amos, M., ed. *Cellular Computing 2004*, Oxford University Press.

81. Shepherd, L. and C. Toumazou, *Towards an implantable ultra-low power biochemical signal processor for blood and tissue monitoring*. IEEE Int. Symp. on Circuits and Systems, 2005: p. 5226-5229.
82. Wong, W.J., et al., *Towards ISFET based DNA logic for rapid nucleic acid detection*. Sensors, 2009. IEEE International Symposium, 2009.
83. Georgiou, P. and C. Toumazou, *ISFET characteristics in CMOS and their application to weak inversion operation*. Sensors & Actuators: B. Chemical, 2009.
84. Georgiou, P. and C. Toumazou, *CMOS-based programmable gate ISFET*. Electronics Letters, 2008. **44**: p. 1289.
85. Shibata, T. and T. Ohmi, *A functional mos transistor featuring gatelevel weighted sum and threshold operations*. Electron Devices, IEEE Transactions, 1992. **39**(6): p. 1444-1455.
86. *deCODE diagnostics*. 2008; Available from: <http://www.decodediagnostics.com>.
87. Gage, B.F., et al., *Use of pharmacogenetics and clinical factors to predict the maintenance dose of warfarin*. Thrombosis and Haemostasis, 2004. **91**(1): p. 87-94.
88. *The Millennium Development Goals Report*. 2009, United Nations Department of Economic and Social Affairs. p. 32.
89. Hallacy, J. *UN agencies call for concerted effort to rapidly scale up access to HIV testing and counselling services in Asia and the Pacific*. 2007; Available from: http://www.searo.who.int/en/Section316/Section503/Section2358_13304.htm.
90. Van der Bij, A.K., et al., *Low HIV-testing rates and awareness of HIV infection among high-risk heterosexual STI clinic attendees in The Netherlands*. Eur J Public Health, 2008. **18**(4): p. 376-9.
91. Day, S., et al., *Improving uptake of HIV testing in patients with a confirmed STI*. Int J STD AIDS, 2004. **15**(9): p. 626-8.
92. Manirankunda, L., et al., *"It's better not to know": perceived barriers to HIV voluntary counseling and testing among sub-Saharan African migrants in Belgium*. AIDS Educ Prev, 2009. **21**(6): p. 582-93.
93. van Dyk, A. and P. van Dyk, *"To know or not to know": service-related barriers to voluntary HIV counseling and testing (VCT) in South Africa*. Curationis, 2003. **26**(1): p. 4-10.
94. *Structure of HIV*. 2009 March 31, 2009 [cited 2010; Available from: <http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/structure.aspx>.
95. Little, S.J., et al., *Antiretroviral-Drug Resistance among Patients Recently Infected with HIV*. New England Journal of Medicine, 2002. **347**(6): p. 385-394.
96. Vidal, N., et al., *Unprecedented Degree of Human Immunodeficiency Virus Type 1 (HIV-1) Group M Genetic Diversity in the Democratic Republic of Congo Suggests that the HIV-1 Pandemic Originated in Central Africa*. J. Virol., 2000. **74**(22): p. 10498-10507.
97. Kozak, S.L., et al., *Roles of CD4 and Coreceptors in Binding, Endocytosis, and Proteolysis of gp120 Envelope Glycoproteins Derived from Human Immunodeficiency Virus Type 1*. Journal of Biological Chemistry, 1999. **274**(33): p. 23499-23507.
98. Weiss, R.A., *Gulliver's travels in HIVland*. Nature, 2001. **410**(6831): p. 963-7.
99. Schwarcz, S., et al., *Late Diagnosis of HIV Infection: Trends, Prevalence, and Characteristics of Persons Whose HIV Diagnosis Occurred Within 12 Months of Developing AIDS*. JAIDS

- Journal of Acquired Immune Deficiency Syndromes, 2006. **43**(4): p. 491-494
10.1097/01.qai.0000243114.37035.de.
100. Girardi, E., et al., *Increasing Proportion of Late Diagnosis of HIV Infection Among Patients With AIDS in Italy Following Introduction of Combination Antiretroviral Therapy*. JAIDS Journal of Acquired Immune Deficiency Syndromes, 2000. **25**(1): p. 71-76.
 101. Girardi, E.M.D., C.A.P. Sabin, and A.d.A.M.D. Monforte, *Late Diagnosis of HIV Infection: Epidemiological Features, Consequences and Strategies to Encourage Earlier Testing*. JAIDS Journal of Acquired Immune Deficiency Syndromes, 2007. **46**: p. S3-S8
10.1097/01.qai.0000286597.57066.2b.
 102. Steward, W., et al., *Behavior Change Following Diagnosis with Acute/Early HIV Infection—A Move to Serosorting with Other HIV-Infected Individuals. The NIMH Multisite Acute HIV Infection Study: III*. AIDS and Behavior, 2009. **13**(6): p. 1054-1060.
 103. Johnson, W.D., et al., *Behavioral interventions to reduce risk for sexual transmission of HIV among men who have sex with men*. Cochrane Database Syst Rev, 2008(3): p. CD001230.
 104. *HIV Assays: Operational Characteristics: report 15 antigen/antibody ELISAs*. 2004, World Health Organization.
 105. *Sources and prices of selected medicines and diagnostics for people living with HIV/AIDS a joint UNICEF, UNAIDS, WHO, MSF project*. 2005, WHO.
 106. Heid, C.A., et al., *Real time quantitative PCR*. Genome Research, 1996. **6**(10): p. 986-994.
 107. Espy, M.J., et al., *Diagnosis of Herpes Simplex Virus Infections in the Clinical Laboratory by LightCycler PCR*. J. Clin. Microbiol., 2000. **38**(2): p. 795-799.
 108. Flexman, J., et al., *Differences between the quantitative antigenemia assay and the cobas amplicor monitor quantitative PCR assay for detecting CMV viraemia in bone marrow and solid organ transplant patients*. Journal of Medical Virology, 2001. **64**(3): p. 275-282.
 109. Holland, P.M., et al., *Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase*. Proc Natl Acad Sci U S A, 1991. **88**(16): p. 7276-80.
 110. Collins, M.L., et al., *A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml*. Nucleic Acids Res, 1997. **25**(15): p. 2979-84.
 111. Grate, L., *Potential SECIS elements in HIV-1 strain HXB2*. J Acquir Immune Defic Syndr Hum Retrovirol, 1998. **17**(5): p. 398-403.
 112. Quillent, C., et al., *Reversion of a polymerase-defective integrated HIV-1 genome*. AIDS Res Hum Retroviruses, 1993. **9**(10): p. 1031-7.
 113. Clavel, F., et al., *Genetic recombination of human immunodeficiency virus*. J Virol, 1989. **63**(3): p. 1455-9.
 114. *Report on the global HIV/AIDS epidemic 2008: executive summary*. 2008, Joint United Nations Programme on HIV/AIDS (UNAIDS).
 115. *UNAIDS Outlook Report 2010*. 2010, Joint United Nations Programme on HIV/AIDS (UNAIDS)
 116. *AIDS epidemic update: November 2009*. 2009, Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO).
 117. *What countries need: Investments needed for 2010 targets*. 2010, Joint United Nations Programme on HIV/AIDS (UNAIDS)

118. Bonnel, R., *HIV/AIDS and Economic Growth: A Global Perspective*. S.A.J. Economics, 2000. **68**(5).
119. *Skip CDC - HIV Testing Basics for Consumers*, S.a.K.C.P.H. Department, Editor. 2007.
120. *HIV and other Sexually Transmitted Infections in the United Kingdom: 2007*. 2007, Health Protection Agency.
121. *Nucleic Acid Testing - A Global Strategic Business Report*. 2010, Global Industry Analysts, Inc.
122. *About Abbott*. 2010; Available from: <http://www.abbott.com/>.
123. *Applied Biosystems Inc*. 2010; Available from: <http://www.appliedbiosystems.com>.
124. *Abbot Laboratories Worldwide Fast Facts and Statistics* Retrieved March 13, 2009, Abbott Laboratories.
125. *BD Company Description*. 2009; Available from: http://media.corporate-ir.net/media_files/irol/64/64106/reports/AR09/description.html.
126. *About Myriad*. 2010; Available from: <http://www.myriad.com/about/>.
127. *Welocome to Novartis*. 2010; Available from: <http://www.novartis.com/about-novartis/index.shtml>.
128. *Roche Diagnostics-USA*. 2010; Available from: <http://www.roche-diagnostics.us/about/index.html>.
129. *SIEMENS*. 2010; Available from: http://www.medical.siemens.com/webapp/wcs/stores/servlet/CategoryDisplay~q_catalogId~e_-111~a_categoryId~e_100005~a_catTree~e_100005~a_langId~e_-111~a_storeId~e_10001.htm.
130. *HIV testing methods*. 1997, UNAIDS.
131. Baggaley, D.R., *HIV for non-HIV specialists: Diagnosing the undiagnosed*. 2008, Medical Foundation for AIDS & Sexual Health (MedFASH).
132. Heid, C.A., et al., *Real time quantitative PCR*. Genome Research, 1996. **6**(10): p. 986-994.
133. *HIV/AIDS Testing*. 2008, Global Industry Analysts, Inc.
134. *Updated CDC Estimates of 2009 H1N1 Influenza Cases, Hospitalizations and Deaths in the United States, April 2009 – April 10, 2010*. 2010; Available from: http://www.cdc.gov/h1n1flu/estimates_2009_h1n1.htm.
135. Rani, R., et al., *Is there any role for rapid tests for Chlamydia trachomatis?* Int J STD AIDS, 2002. **13**(1): p. 22-4.
136. Herring, A., et al., *Evaluation of rapid diagnostic tests: chlamydia and gonorrhoea*. Nat Rev Microbiol, 2006. **4**(12 Suppl): p. S41-8.