

Acknowledgements

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I'd like to thank my family and my friends for their love and support over the years.

Abstract:

Electrochemical biosensors have long been used for measuring pH changes in complex biological samples. This research work involves investigating iridium sensors as a potential tool for detecting viral RNA by means of pH sensing during transcription mediated amplification (TMA). The iridium oxide film was formed on gold wire by electrochemical deposition. When complementary DNA/RNA bases are hybridized, they release a pyrophosphate molecule which is eventually hydrolyzed, contributing to the pH change during the TMA reaction which in turn is measured in voltage (mV) by the iridium sensor.

Firstly a standard method for bacteriophage MS2 RNA amplification method was established. A Real Time PCR assay for MS2 RNA using TaqMan probe chemistry was used to quantify the MS2 RNA and used as a gold standard for later project. We then used transcription mediated amplification (TMA) technique using reverse transcriptase and RNA polymerase enzymes for producing MS2 RNA amplicons and resulting protons generated during amplification were measured with iridium sensors. The iridium oxide sensor showed linear pH potential response and showed a near ideal Nernstian behaviour in the pH range 2 – 12 with a slope of -60mV/pH. Since TMA is an isothermal amplification technique the iridium sensors produced reproducible and reliable measurements. To increase the cation selective permeability and inhibit possible interferences the iridium oxide sensor was coated with 5% Nafion. The sensor showed the same linearity in voltage response against RNA concentrations in protein enriched samples (20 mg/ml albumin) compare to RNA in water.

The iridium oxide sensor was a rapid method of viral RNA detection as the detection time for MS2 RNA was 10 minutes. The lower limit of detection of the iridium sensor was 5ng of MS2

RNA which was comparable to the established real time PCR sensitivity. To further enhance the sensor's the sensitivity the iridium oxide sensor was prepared on a 3 mm × 3 mm glass slide which increased the slope for 5 ng of MS2 RNA from 3.25mV/min to 4.07mV/min resulting in an improved limit of detection.

Contents:

1. INTRODUCTION:	17
1.1.AN OVERVIEW OF CURRENT PATHOGEN DETECTION METHODS:	17
1.2.CURRENTLY AVAILABLE RNA QUANTIFICATION ASSAYS:	21
1.2.1. Real Time PCR:	21
1.2.1.1.Advantages of Real-Time PCR:	23
1.2.1.2.qPCR steps:	23
1.2.1.3.One-step qRT-PCR:	24
1.2.1.4.Overview of qPCR and qRT-PCR components:	24
1.2.1.4.1. DNA polymerase:	24
1.2.1.4.2. Reverse Transcriptase:	25
1.2.1.4.3. dNTPs:	25
1.2.1.4.4. Magnesium Concentration	25
1.2.1.4.5. Real-time PCR Primer Design:	26
1.2.2. Reaction Formats:	27
1.2.2.1.Use of dsDNA binding dyes:	27
1.2.2.2.Dual-Labelled Probes:	28
1.2.2.3.FRET Probe System:	30
1.2.2.4.Molecular Beacon Probes:	31
1.2.2.5.Amplifluor Assay:	32
1.2.3. An Overview of Commercially Available Real Time PCR Systems:	35
1.2.4. Nucleic Acid Sequence-Based Amplification (NASBA):	36
1.2.4.1. The Applications of Molecular Beacon in NASBA:	38

1.2.4.2. Advantages of NASBA:	39
1.2.5. Branched DNA method:	39
1.2.6. The Transcription Mediated Amplification Technique:	42
1.2.6.1 Advantages of TMA:	45
1.3. POTENTIONMETRY:	46
1.3.1. NUCLEIC ACID DETECTION BY pH SENSING:	48
1.3.1.1. pH Sensors:	49
1.3.2. Iridium Oxide Sensors:	51
1.4. MS2 BACTERIOPHAGE:	52
1.5. Aims of Research	54
CHAPTER 2: MATERIALS AND METHODS	55
2.1. PHAGE AND HOST STRAINS:	55
2.2. MS2 Bacteriophage:	55
2.3. PLASMIDS:	55
2.4. GENERAL CHEMICALS:	56
2.5. ENZYMES:	56
2.6. BUFFERS AND SOLUTIONS:	56
2.6.1. LB Media:	57
2.6.2. LB Agar Plates:	57
2.6.3. Kanamycin:	57
2.6.4. 10 × TAE Buffer:	57
2.6.5. SOB medium:	57
2.6.6. SOB Agar plates:	58
2.6.7. SOC Medium:	58
2.6.8. 1 M MES (pH 6.3):	58

2.6.9. 1M Potassium Acetate (pH 7.8):	58
2.7. REVITALIZING of MS2 PHAGE:	59
2.7.1. Double Layer Agar Technique:	59
2.8. MS2 RNA PURIFICATION:	60
2.9. ABSORPTION SPECTROSCOPY:	60
2.10. General Molecular Methods:	61
2.10.1. Reverse transcriptase Polymerase Chain Reaction (RT-PCR):	61
2.10.1.1. Reverse Transcription:	61
2.10.2. DNA amplification:	61
2.11. PREPARATION OF PLASMID DNA:	62
2.12. RESTRICTION DIGESTION OF PLASMID DNA:	63
2.13. AGAROSE GEL ELECTROPHORESIS:	63
2.14. GEL EXTRACTION OF MS2 RNA:	64
2.15. PRIMERS:	64
2.16. REAL TIME PCR:	65
2.16.1. Real Time Amplification Reagents:	65
2.17. HIGH RESOLUTION MELT:	66
2.18. ELECTROCHEMICAL METHODS:	67
2.18.1. Preparation of Iridium Oxide Sensors:	67
2.18.2. Preparing the Sensor Surface:	67
2.18.3. Iridium Oxide Deposition:	68
2.18.4. Nafion Coating:	69
2.18.5. Nafion-Polyphenol Coating:	70
2.18.6. Sensor Calibration:	70
2.18.7. Preparation of Iridium Sensor on Glass Slides:	71

2.18.8. Immobilizing of Oligonucleotides on the sensor surface:	72
2.19. TRANSCRIPTION MEDIATED AMPLIFICATION (TMA):	73
CHAPTER 3: RESULTS AND DISCUSSION	75
3.1. GEL ELECTROPHORESIS RESULTS:	75
3.2. ABSORBANCE MEASUREMENTS:	76
3.3. REAL- TIME RT-PCR:	77
3.3.1. Quantitation Data:	78
3.4. HIGH RESOLUTION MELT (HRM):	81
3.5. pH-POTENTIAL RESPONSE CURVE:	82
3.6. Application of the Iridium Oxide Sensor in TMA.	83
3.7. Dose Response Curve:	85
3.8. Sensor Response in Complex Samples:	88
3.8. Improvement of Iridium oxide sensor:	89
3.8.1. Coating of Iridium Sensors with Nafion:	89
3.9. Dose Response Curve with Nafion Coated Iridium Sensor:	91
3.10. Effects of TMA Components on Iridium oxide Sensor:	94
3.11. Dose Response Curve in Albumin:	95
3.12. Lower Limit of Detection in Albumin:	98
3.13. Detection of MS2 RNA using Nafion-Polyphenol Iridium oxide Sensors:	99
3.13.1. Nafion-Polyphenol Coating:	99
3.13.2. Dose Response Curve with Nafion-Polyphenol Coated Iridum oxide Sensor:	100
3.13.3. Limit of Detection with Nafion-Polyphenol coated Iridium oxide Sensor:	102
3.14. Effects on Sensitivity on with Larger Surface Area:	104
3.14.1. Iridium Sensor on a Glass Slide:	104

3.15. Iridium Sensor with Immobilized Oligos:	107
4. Conclusion and Future Challenges	111
Bibliography	113

List of Figures:

Figure 1.1: A Screen Shot of the Output from a Real-Time PCR Instrument.	22
Figure 1.2: As shown in the figure higher concentrations of SYBR Green can cause the dye jumping effect and can redistribute the dye molecules back into the dsDNA resulting in no signal. This effect can be eliminated by using LC Green. (Wittwer <i>et al.</i> , 2003; Herrmann <i>et al.</i> , 2006)	28
Figure 1.3: Dual Labeled Probe:	29
Figure 1.4: Schematic representation of FRET probe reaction.	31
Figure 1.5: Schematic representation of real time PCR with molecular beacons.	32
Figure 1.6: Schematic representation of Amplifluor assay.	34
Figure 1.7: A typical NASBA reaction.	37
Figure 1.8: A schematic representation of the combination of NASBA amplification with molecular beacon based detection.	38
Figure 1.9: Basic bDNA assay components.	41
Figure 1.10: A detailed overview of TMA cycling	43
Figure 1.11: A Schematic representation of HPA. The AE-labeled probe hybridizes to target RNA in solution. After washing of unhybridized probe the label protected hybridized probe is detected by chemiluminescence	45
Figure 1.12: Schematic presentation of the base incorporation reaction.	47
Figure 1.12: Schematic presentation of the base incorporation reaction.	52
Figure 2.1: The iridium oxide sensor as shown with iridium being deposited on to the gold surface.	68

Figure 2.2: The iridium sensor shown was prepared on a glass slide. The glass side was first plated with gold and then iridium oxide was deposited following Nafion coating.	70
Figure 2.3: The C6 amino linker to aid in surface immobilization using a dPEG cross-linker (amino-dPEG ₁₂ acid).	71
Figure 3.1: Agarose gel electrophoresis analysis of the purified MS2 RNA.	75
Figure 3.2: Plot showing raw fluorescence acquired at different cycles of PCR after the reverse transcription of MS2 RNA. The fluorescence generated in the plot by MS2 standards are shown in red whereas the yellow and green are the unknown concentrations of MS2 RNA purified after revitalization of the bacteriophage along with the NTC shown in purple.	77
Figure 3.3: Plot showing fluorescence generated by MS2 standards in red curves whereas the unknown MS2 RNA samples are shown in yellow and green curves during Real Time PCR.	78
Figure 3.4: Standard curve plotted by Rotor Gene 6000 with CT values of standards against the MS2 RNA concentration.	79
Table 3.1: Ct values of the known MS2 standards as well as the unknown MS2 RNA samples.	79
Figure 3.5: The change in fluorescence of MS2 RNA samples is shown when subjected to melt by decreasing the temperature after PCR amplification.	81
Figure 3.6: Plot showing change in voltage measured by iridium oxide sensors in different pH solutions.	82
Figure 3.7: A triplicate of 50 ng MS2 RNA was detected by using iridium oxide sensor during TMA reaction for 80 minutes.	83
Figure 3.8: A triplicate of 50 ng MS2 RNA was detected by using iridium oxide sensor during TMA reaction for 10 minutes.	84
Figure 3.9: Plot showing change in voltage (mV) for different concentrations of MS2 RNA.	86
Figure 10: The dose response curve for different concentration of MS2 RNA has been shown in the above plot.	87

Figure 3.11: Plot showing change in voltage of MS2 triplicates 50 ng in 20mg/ml albumin solution.	88
Figure 3.12: Plot showing triplicates of 100 ng purified MS2 RNA being detected by 3 different Iridium sensors coated with 1%, 5% and 10% w/v Nafion.	90
Figure 3.13: Plot showing the change in voltage for different concentration of MS2 RNA being detected by 5 % Nafion coated iridium sensor at regular intervals.	92
Figure 3.14: Dose response curve shown with 5% Nafion coated iridium oxide sensor at different concentrations of MS2 RNA.	93
Figure 3.15: Plot showing the change in voltage against time for MS2 RNA samples with different TMA components.	94
Figure 3.16: Plot showing change in voltage for different concentrations of MS2 RNA in 20mg/ml albumin using iridium oxide sensor coated with 5 % Nafion.	96
Figure 3.17: Dose response curve against different concentrations of MS2 RNA in 20 mg/ml of albumin.	97
Figure 3.18: Plot showing the slope against the lower concentrations of MS2 RNA in albumin.	98
Figure 3.19: Plot showing the voltage response curve for MS2 RNA with Nafion-polyphenol coated iridium oxidized sensor.	100
Figure 3.20: Dose response curve against different concentrations of MS2 RNA detected with Nafion-polyphenol coated iridium oxidized sensor.	101
Figure 3.21: Plot showing the lower limit of detection for MS2 RNA with Nafion-polyphenol coated iridium oxidized sensor during TMA.	103
Figure 3.22: Plot showing the change in voltage when various concentrations of MS2 RNA in 20 mg/ml albumin was detected with 5% Nafion coated Iridium oxide sensor on a 3mm×3mm glass slide.	105
Figure 3.23: Dose response curve against different concentrations of MS2 RNA using 5% Nafion coated iridium oxide sensor on a	106

3mm×3mm glass slide.

Figure 3.24: Plot showing voltage change with different concentrations of MS2 RNA in 20 mg/ml albumin using iridium oxide sensor with immobilized oligonucleotides. 108

Figure 3.25: Dose response curve for different concentrations of MS2 RNA using iridium oxide sensor with immobilized oligonucleotides. 109

Abbreviations:

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
Bp	Base pair
AEIROF	Anodically Electrodeposited Iridium Oxide Films
AMV	Avian Myeloblastosis Virus
b-DNA	branched DNA
C	Celsius
cfu	Colony Forming Units
Ct	Threshold Cycle
CSF	Cerebrospinal fluid
Ct	Threshold Cycle
cDNA	Complementary Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dsDNA	Double Stranded Deoxyribonucleic acid
DMSO	Dimethylsulphoxide
DTT	Ddithiothreitol

dATP	2'-Deoxyadonsine 5'-Triposphate
dGTP	2'-Deoxyguanosine 5'-Triphosphate
dCTP	2'- Deoxycytidine 5'- Triphosphate
dTTP	2'-Deoxythymidine 5'-Triphospahe
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EBV	Epstein Barr Virus
FRET	Förster resonance energy transfer
GSP	Gene-specific primers
HBV	Hepatitis B
HCV	Hepatitis C
HPA	Hybridization Protection Assay
HRM	High Resolution Melt
IPTG	Isopropyl -D-1-thiogalactopyranoside
IIF	Indirect immunofluorescence
IPA	Isopropyl alcohol
LED	Light-emitting diode
LB	Luria or Lenox broth
MB	Molecular beacon
Mes	2-(NMorpholino) ethanesulphonic acid
MOPS	4-Morpholinepropanesulphonic acid
ml	millilitre
mM	millimolar

min	Minute
MMLV	Moloney Murine Leukemia Virus Reverse Transcriptase
MTB	Mycobacterium tuberculosis
NASBA	Nucleic acid sequence based amplification
NBD	7-nitrobenz-2-oxa-1,3-diazol-4-yl
ng	Nanogram
nm	Nanometer
NTA	Nickel-chelated tri-acetate acid
NTC	No Template Control
NHS	N-hydroxysuccinimide
NTP	Nucleotide triphosphate
nM	nanomolar
PCR	Polymerase Chain Reaction
PMT	Photo Multiplier Tube.
PMMA	Poly(methylmethacrylate)
PMMA	Poly(methylmethacrylate)
Q	Quencher
qPCR	Quantitative PCR
R	Reporter
RNA	Ribonucleic acid
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SE	Standard Error
SNP	Single Nucleotide Polymorphism
ssDNA	Single stranded Deoxyribonucleic acid

TBE	Tris-Boric-EDTA
TMA	Transcription Mediated Amplification
Tm	Melting Temperature

1. INTRODUCTION:

1.1. AN OVERVIEW OF CURRENT PATHOGEN DETECTION METHODS:

A tremendous effort has been made in developing various technologies for pathogen detection. These advancements in technologies have enormously contributed to the medical and healthcare communities. Over the years scientists have developed a variety of assays ranging from rapid, point of care, serological methods to molecular technologies to detect microorganisms. All these methodologies have limitations as to their sensitivity, accuracy, robustness, early detection of infection and their performance.

The primary and by far the oldest method is by culturing the microorganism. The basic cell culture techniques involve in case of bacteria is by growing them in a predetermined culture medium and in case of virus is by isolation of cultured cells with antibiotics present in the appropriate culture media [1]. Nowadays a wide range of culture media are commercially available which can grow a number of bacteria and viruses. For cell culturing clinical samples like blood, sputum, cerebrospinal fluid (CSF), nasopharyngeal aspirates can be used [2]. For many reasons these clinical samples can be specific for specific infections e.g., for respiratory infections the commonly used specimen is sputum [3]. Once the specimen is collected suspected of viral infection, it is then inoculated with different cell lines in order to find the most suitable host for the infectious agent. The inoculated cell cultures in the presence of appropriate cell culture medium could take up to several weeks of incubation for the infectious agents to grow and can be

examined under the microscope for the abnormalities in the cells caused by the infection. Besides, using a range of cell cultures detection of more than one infectious agent is also possible. The only disadvantage of cell culture methods is that in many cases it takes from days to weeks [4] for the infectious agent to grow which poses further risks to the patient's health and delays the timely treatment which is required.

The time-consuming cell culture methods led researchers to look for a more rapid technology for pathogen detection. Meanwhile a wide range of serological methods were developed in order to address the rapid detection of parasites, bacterial and viral infections in human samples [5]. These *in vitro* serodiagnostic assays include radioimmunoassay, enzyme linked immunosorbent assay (ELISA), antigen-coated particle agglutination, indirect immunofluorescence (IIF) and dot blot assays [6].

In an effort the indirect immunofluorescence (IIF) methods were developed in which the antigen was detected by immunofluorescence with specific monoclonal antibodies. This approach did not require the isolation and proliferation of infectious agent in contrast to cell culture techniques [7]. As a rapid methodology it offered enhanced sensitivity and specificity along with shorter detection time but was labour intensive, time consuming and required a high level of expertise for the technician to examine the stained antigens.

Later the above approach was modified with the ELISA assay that did not require immunofluorescence staining of antigen. Instead it used a sandwich based assay involving binding of antibody, antigen and a detecting antibody [8]. Another rapid testing approach was based on immunochromatographic flow system, which required the migration of pathogen's antigen along the test strip. These rapid tests neither required a high level of expertise to perform

nor any expensive instruments as the examiner can visually differentiate between the positive and negative results. The methods are also not expensive.

Amongst the most commonly used immunological method is ELISA due its low cost, enhanced sensitivity and ease of use. ELISA assays can be employed in two different ways. One way is to detect the specific antibodies to the infectious agent in the human specimen. This technique is usually referred as indirect ELISA. The other technique in contrast to indirect ELISA, the direct ELISA, uses monoclonal antibodies to recognize the presence of specific antigens in the specimen [9]. ELISA assays unlike cell culture not only detect the presence of infection but also are able to quantitate the specific antigen or antibody present in the clinical samples. For instant, the Adenovirus ELISA assay can detect and quantitate the presence of specific IgA antibodies against adenovirus in serum or plasma.

Literally all these ELISA assays are automatable where busy laboratories can benefit from maximum throughput without any user intervention from sample labeling to results. The major drawback of indirect ELISA methods for pathogen detection is the inability to detect the infection in the window period [10]. This window period is the time period between the infection and the response of the host immune system. For instant the average window phase for HCV is approximately 66 days whereas for HBV and HIV the average seronegative window phase is 59 and 22 days respectively [11].

In early 1990, major advances were achieved in molecular methods. These molecular methods initially included the polymerase chain reaction (PCR) and relied on amplification of one copy of a gene to a billion fold [15]. The technique offered tremendous advantages to the medical and health community over the traditional cell culture and serological methods. With the polymerase

chain reaction (PCR) one can directly amplify the DNA of a pathogen present in the clinical sample [12]. In case of RNA viruses, the RNA is first reverse transcribed with the help of reverse transcriptase enzyme into cDNA and then amplified by PCR in the presence of DNA polymerase enzyme. The reverse transcriptase polymerase chain reaction (RT-PCR) requires a set of primers (forward and reverse primers) complementary to the target template, reverse transcriptase and DNA polymerase enzymes and a peltier based thermal system to facilitate the complimentary strands' rapid displacement and hybridization. With such significant advances, the researchers were able to develop specific tests for a variety of molecular diagnostic tests such as influenza, hepatitis, HIV, MTB, malaria and many more [13]. The PCR technology significantly reduced the window period of seronegative samples to 1 – 2 days. It is therefore important to know that all blood donations are now monitored by means of molecular based screening methods to avoid the transmission of blood borne diseases like hepatitis and HIV.

The polymerase chain reaction is benchmark of molecular techniques and has opened an opportunity for developing more advanced methods like Real Time PCR, NASBA, TMA and electrochemical detection methods. Consequently researchers have developed better amplification systems, more rapid molecular techniques and less laborious methods. Some of the key advanced technologies are detailed below.

1.2. CURRENTLY AVAILABLE RNA QUANTIFICATION

ASSAYS:

1.2.1. Real Time PCR:

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology [14]. PCR is used to amplify a single gene copy to a billion fold. The PCR product can then be quantified by gel electrophoresis and analysis of the gel image. Whereas in real-time quantitative PCR (qPCR), the amount of PCR product is measured at each cycle [15][52]. The real-time PCR determines the initial amount of target sequence by monitoring the reaction during its exponential phase. PCR theoretically amplifies DNA exponentially, doubling the number of molecules present with each amplification cycle [16]. The number of cycles and the amount of PCR end-product can theoretically be used to calculate the initial quantity of genetic material (by comparison with a known standard), but a number of factors complicate this calculation. The ethidium bromide staining typically used to quantify endpoint PCR products prevents further amplification, and is only semi quantitative. PCR may not be exponential for the first several cycles, and the reaction eventually plateaus, so the amount of DNA should be measured while the reaction is still in the exponential amplification phase, which can be difficult to determine in endpoint PCR. To address these factors, the technique of real-time quantitative PCR was developed.

In real-time PCR, the amount of DNA is measured after each cycle by the use of fluorescent labeled probes that are incorporated into the PCR product [17][51]. The increase in fluorescent signal is directly proportional to the number of PCR product molecules (amplicons) generated in the exponential phase of the reaction. Fluorescent reporters used include double-stranded DNA

(dsDNA)-binding dyes, or dye molecules attached to PCR primers or probes that are incorporated into the product during amplification.

The change in fluorescence over the course of the reaction is measured by the real time PCR instrument which combines thermal cycling with measurement of fluorescence intensity. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction [18] (Figure 1.1).

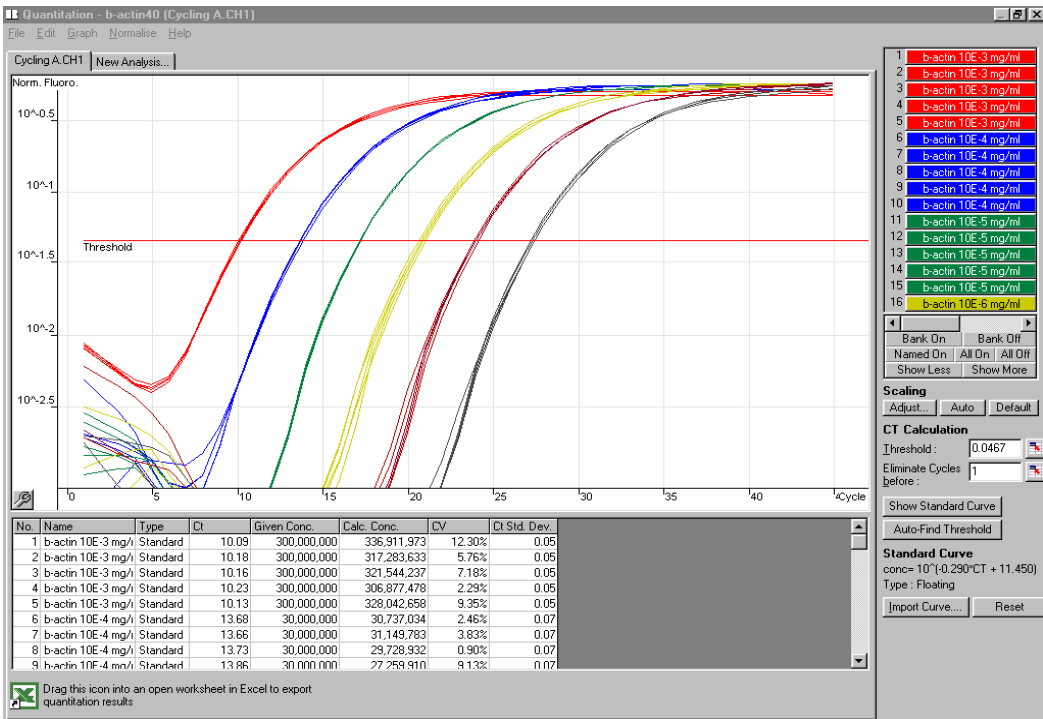


Figure 1.1: A Screen Shot of the Output from a Real-Time PCR Instrument.

1.2.1.1. Advantages of Real-Time PCR:

The advantages of real-time PCR include:

1. The ability to monitor the progress of the PCR reaction as it occurs in real time.
2. The ability to precisely measure the number of amplicons formed at each cycle.
3. A wider dynamic range of detection.
4. The combination of amplification and detection in a single tube, which eliminates post-PCR manipulations.

Over the past several years, real-time PCR has become the leading tool for the detection and quantification of DNA or RNA. Using this technique, one can achieve precise detection that is accurate within a factor of two, and a dynamic range of 6 to 8 orders of magnitude.

1.2.1.2. qPCR steps:

There are three major steps that make up a qPCR reaction. Reactions are generally run for 40 cycles.

1. Denaturation — The temperature should be appropriate to the polymerase chosen (usually 95°C). The denaturation time can be increased if template GC content is high.
2. Annealing — Use appropriate temperatures based on the calculated melting temperature (T_m) of the primers (5°C below the T_m of the primer).

3. Extension — At 70–72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in qPCR is small, this step is often combined with the annealing step using 60°C as the temperature.

1.2.1.3. One-step qRT-PCR:

One-step qRT-PCR combines the first-strand cDNA synthesis reaction and qPCR reaction in the same tube, simplifying reaction setup and reducing the possibility of contamination (Bustin, S.A. et al.). For one step qRT-PCR Gene-specific primers (GSP) are required. This is because using oligo (dT) or random primers will generate nonspecific products in the one-step procedure and reduce the amount of product of interest.

1.2.1.4. Overview of qPCR and qRT-PCR components:

This section provides an overview of the major reaction components and parameters involved in real-time PCR experiments.

1.2.1.4.1. DNA polymerase:

PCR performance is often related to the DNA polymerase, so enzyme selection is critical to success. One of the main factors affecting PCR specificity is the fact that *Taq* DNA polymerase has residual activity at low temperatures. Primers can anneal non-specifically to DNA, allowing the polymerase to synthesize non-specific product. The problem of non-specific products resulting from mis-priming can be minimized by using a “hot-start” enzyme. Using a hot-start

enzyme ensures that no active *Taq* is present during reaction setup and the initial DNA denaturation step.

1.2.1.4.2. Reverse Transcriptase:

The reverse transcriptase (RT) enzyme is as important to the success of qRT-PCR as the DNA polymerase. It is important to choose an RT that not only provides high yields of full-length cDNA but also has good activity at high temperatures. Currently, Moloney Murine Leukemia Virus (MMLV) and Avian Myeloblastosis Virus (AMV) are the most chosen RT enzymes for performing RT-PCR. High-temperature performance is also very important for tackling RNA with secondary structure or when working with target specific primers. In one-step qRT-PCR, an RT that retains its activity at higher temperatures allows you to use a GSP with a high melting temperature (T_m), increasing specificity and reducing background.

1.2.1.4.3. dNTPs:

It is recommended that both the dNTPs and the *Taq* DNA polymerase be purchased from the same vendor, as it is not uncommon to see shifts of one full threshold cycle (C_t) in experiments that employ these items from separate vendors.

1.2.1.4.4. Magnesium Concentration

In qPCR, magnesium chloride or magnesium sulfate is typically used at a final concentration of 3 mM. This concentration works well for most targets; however, the optimal magnesium concentration may vary between 3 and 6 mM.

1.2.1.4.5. Real-time PCR Primer Design:

Good primer design is one of the most important parameters in real-time PCR. When designing gene-specific real-time PCR primers, it should be kept in mind that the amplicon length should be approximately 80–250 bp, since longer products do not amplify as efficiently. Optimal results may require a titration of primer concentrations between 50 and 500 nM. A final concentration of 200 nM for each primer is often effective for most reactions.

In general, primers should be 18–24 nucleotides in length. This provides for practical annealing temperatures. Primers should be specific for the target sequence and be free of internal secondary structure. Primers should avoid stretches of polybase sequences (e.g., poly (dG)) or repeating motifs, as these can hybridize inappropriately to the template. Primer pairs should have compatible melting temperatures (within 5°C) and contain approximately 50% GC content. High GC content results in the formation of stable imperfect hybrids, while high AT content depresses the T_m of perfectly matched hybrids. If possible, the 3' end of the primer should be rich in GC bases to enhance annealing of the end that will be extended. The sequences should be analyzed to avoid complementarity and prevent hybridization between primers (primer-dimers).

For qRT-PCR, design primers that anneal to exons on both sides of an intron or span an exon/exon boundary of the mRNA to allow differentiation between amplification of cDNA and potential contaminating genomic DNA by melting curve analysis. To confirm the specificity of your primers, a BLAST search may be performed against public databases to be sure that your primers only recognize the target of interest.

1.2.2. Reaction Formats:

1.2.2.1. Use of dsDNA binding dyes:

This approach relies on fluorescent dyes which have the ability to differentiate double stranded DNA from single stranded DNA i.e., these dyes only bind to dsDNA and cause a change in fluorescent signal intensity. Using such dye, the increase in fluorescence intensity during amplification is directly related to the increased number of DNA copies. There are numerous dsDNA binding dyes commercially available including SYBR Green and LC Green [19]. The major drawback with using these dyes is that they bind to every dsDNA molecule present in the reaction hence decreasing the specificity of the assay as well as producing inaccurate quantification of the DNA or RNA. Besides if used in higher concentrations, they can inhibit the PCR which can result in 'dye jumping' during amplicon melting which in turn decreases the sensitivity of heteroduplex detection.

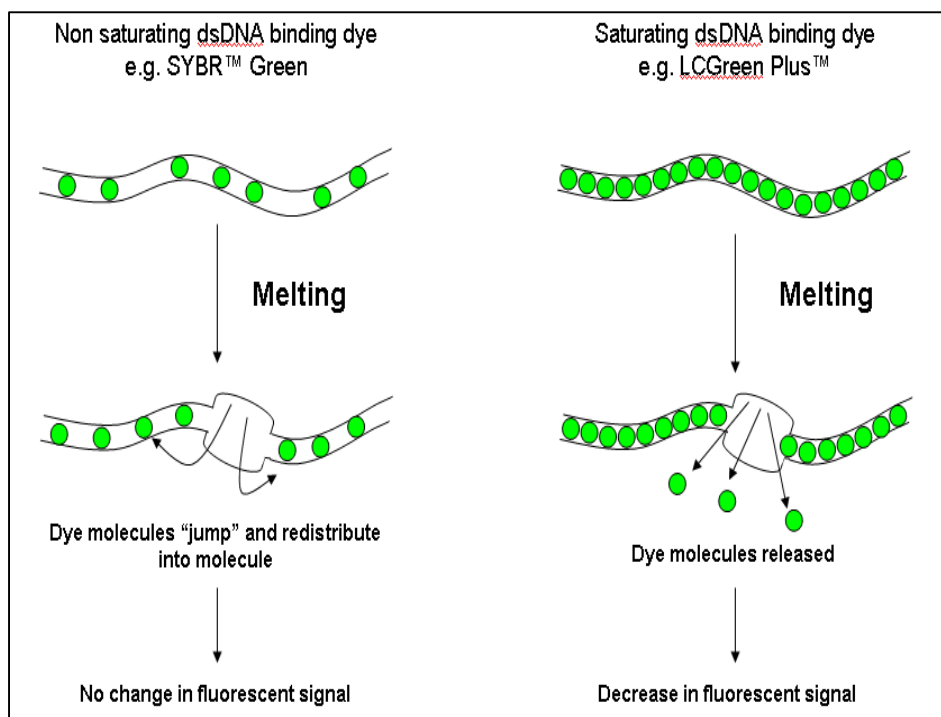


Figure 1.2: As shown in the figure higher concentrations of SYBR Green can cause the dye jumping effect and can redistribute the dye molecules back into the dsDNA resulting in no signal. This effect can be eliminated by using LC Green [20].

1.2.2.2. Dual-Labelled Probes:

This system uses short oligonucleotides of about 13-24 bp long, attached to a fluorescent reporter at the 5'-end and a quencher at the 3'-end in order to keep the quencher in close proximity to the reporter. These dual labelled probes, when intact, means the reporter does not produce any fluorescence. In real time PCR the exonuclease activity of the Taq polymerase during cDNA strand formation cleaves the dual labelled probe. When the probe is degraded and the reporter is not quenched, an increase in fluorescence is detected [8].

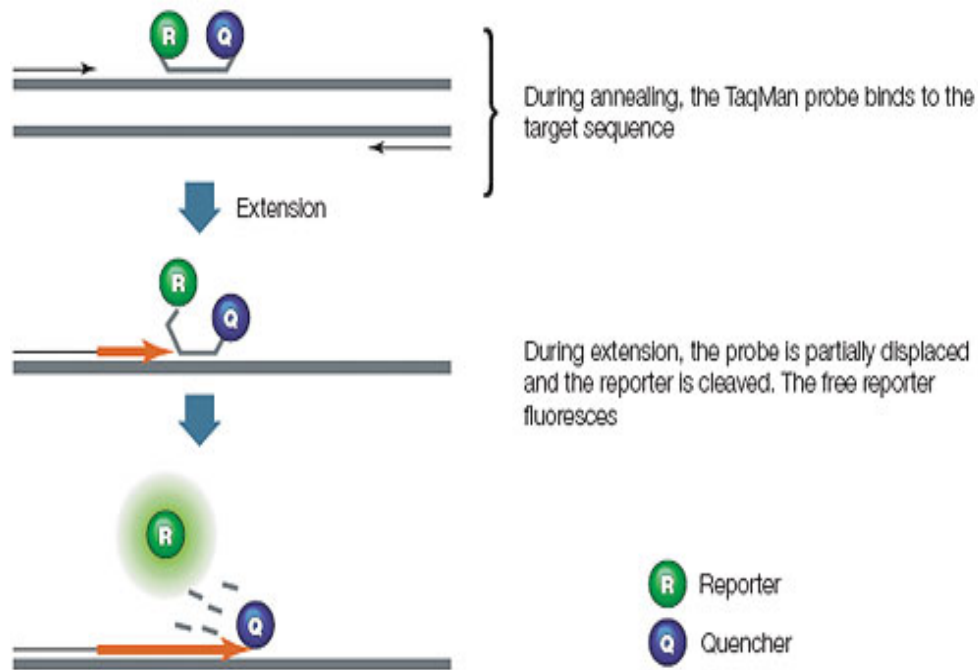


Figure 1.3: Dual Labeled Probe: The 5'-nuclease activity of the DNA polymerase used in the polymerase chain reaction (PCR) degrades the dual labeled probes during the extension step, which releases the reporter fluorophore (R) from a quencher (Q). Fluorescence emitted when excited by an external light source ($h\nu$) at each PCR cycle is proportional to the amount of product formed.

The increase of fluorescence in each amplification step is detected by the real time instrument due to the cleavage of the probes [21]. Since the reporter is degraded it is not possible to do the melt curve analysis. Dual labeled probes, also named as TaqmanTM probes, are sequence specific to the gene of interest and only bind to complimentary regions. The second reason is that the dual labeled probe for each amplified copy releases only one molecule of fluorescent dye. Due to increased specificity of the dual labeled probes this detection method works well for low copy number templates.

1.2.2.3. FRET Probe System:

Another approach in real time PCR is the use of Förster resonance energy transfer (FRET) probes. The approach uses the transfer of energy from one fluorescent dye to another [22]. The mechanism involves the use of two different sequence specific oligonucleotides labeled with fluorescent dyes. The upstream probe has a donor molecule on the 3'-end and the downstream probe has an acceptor molecule on the 5' end. The probes hybridize adjacently to each other on the target sequence which brings the donor and acceptor fluorescent dyes in close proximity. This results in the transfer of energy from the donor to acceptor fluorophore, which emits at a different wavelength. Either the decrease in the fluorescence of the donor or the increase in the fluorescence of the acceptor can then be detected. Just like TaqmanTM probes FRET probes can not be used for melt curve analysis. They are extremely useful for genotyping, single nucleotide polymorphism (SNP) detection and other mutation detections.

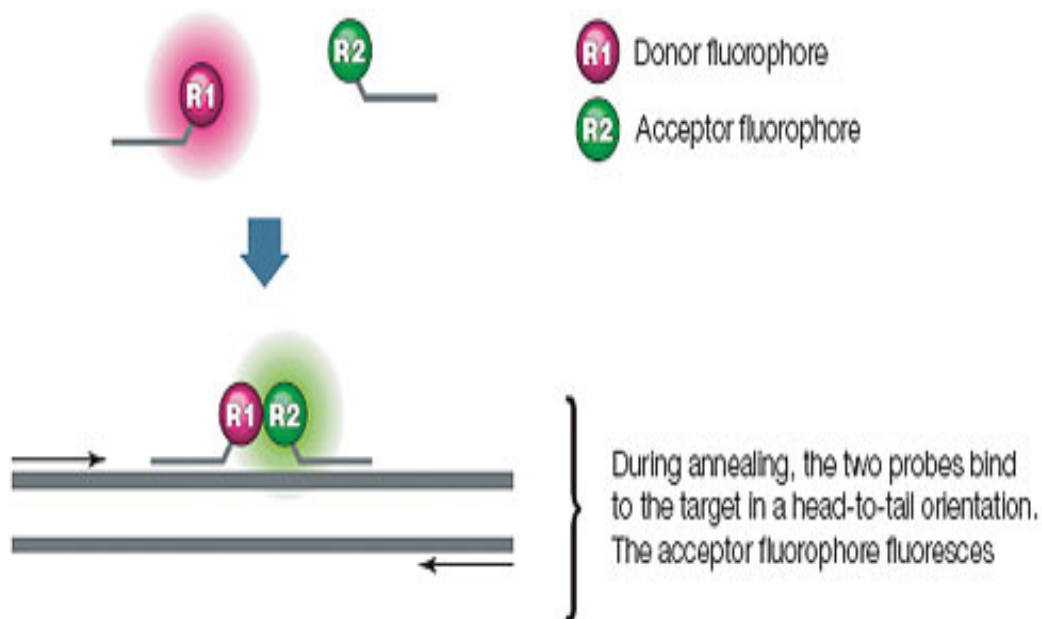


Figure 1.4: Schematic representation of FRET probe reaction. During the elongation period the 5' nuclease activity of the Taq polymerase releases the reporter fluorophore (R1) from the acceptor fluorophore and the fluorescence is detected by the FAM channel.

1.2.2.4. Molecular Beacon Probes:

The use of molecular beacon (MB) probes is relatively new in comparison with TaqmanTM and FRET probes. The probe is designed in a hairpin structure with a reporter fluorescent dye on one end and a quencher molecule on the other end [23]. The hairpin structure causes the MB probe to fold when not hybridized which brings the reporter and quencher molecule in close proximity resulting in zero fluorescence being emitted. However, when the MB hybridizes to the target gene, the hairpin structure is broken and the reporter dye is no longer in close proximity of the quencher. The increase in fluorescence caused by this is detected by the real-time instrument. Using MB probes one can do a melt curve analysis.

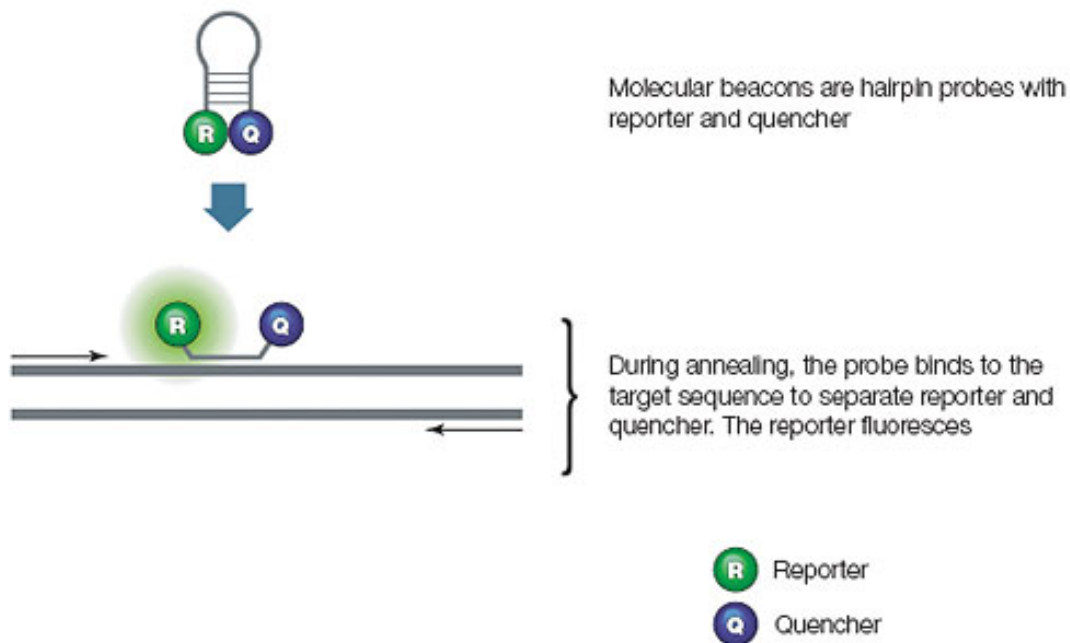


Figure 1.5: Schematic representation of real time PCR with molecular beacons. The molecular beacon has two dyes on each end, a reporter R and a quencher Q. In the hairpin loop structure, the quencher forms a non fluorescent complex with the reporter. When hybridized with the target sequence, quencher has no quenching effect on the reporter fluorophore and it emits fluorescence.

1.2.2.5. Amplifluor Assay:

This method employs a set of target-specific primers and one universal primer called the UniPrimer. The reverse primer is labeled with an 18-nucleotide long sequence called the Z-sequence that extends from the 5' end of one target-specific primer (called the Z primer); this same sequence forms the 3' end of the UniPrimer. The UniPrimer is designed in a hairpin structure just like the molecular beacon (MB) probe with a reporter and a quencher attached to the

5' and 3' ends respectively. In this hairpin structure because the reporter and quencher dyes are in close proximity, no fluorescence is emitted. In the first amplification round, the Z primer hybridizes to the template and is extended with the help of Taq polymerase (Figure 6). In the second amplification round, the other target-specific primer hybridizes to the template formed in the first round of amplification, and is used to synthesize a new target template that contains a sequence complementary to the Z-sequence. This template from the second amplification round can then serve as the template for the UniPrimer. During the third amplification round, the extended UniPrimer serves as a template for the next amplification cycle. In the fourth and last amplification round, extension of the template through the UniPrimer causes the hairpin structure to break and release the reporter from the quencher. This results in the emission of fluorescence which can be detected in a real time PCR instrument.

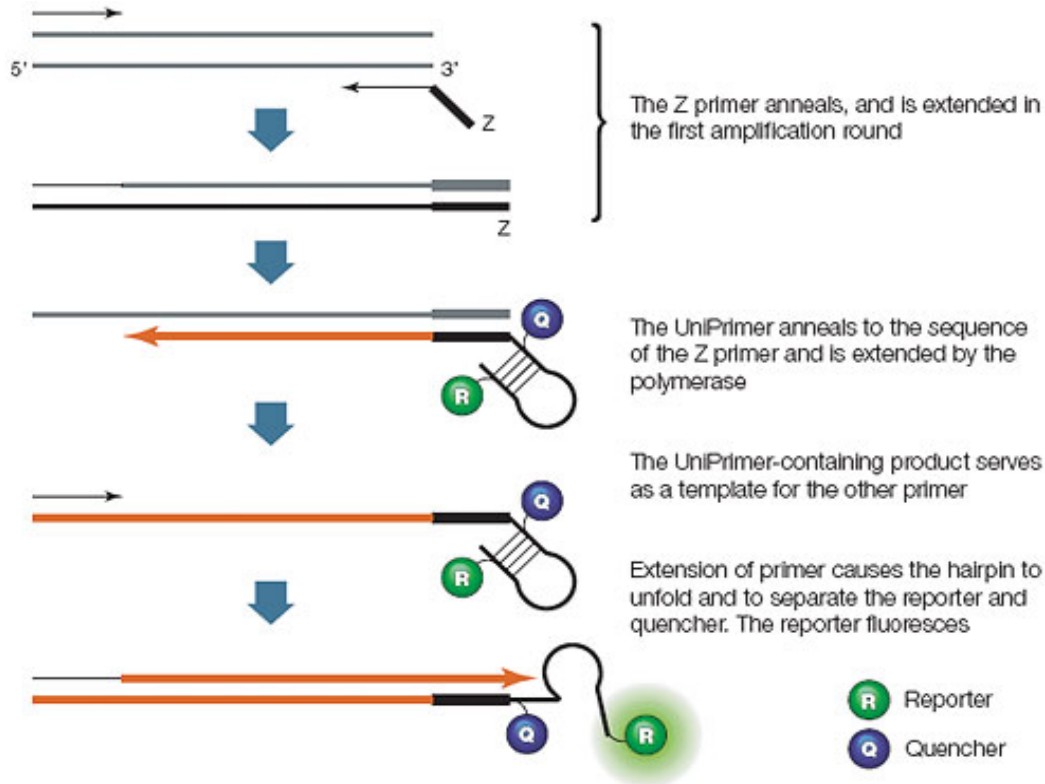


Figure 1.6: Schematic representation of Amplifluor assay. As shown in the figure in the first cycle of amplification, the reverse primer containing 18 bp long sequence (tag) hybridized and extended along the template. Similarly in the second cycle, the forward primer extends through the special sequence tag, forming a complementary sequence to the tag. In the third round, the UniPrimer hybridizes to this complementary sequence via the special sequence tag. The hairpin structure of the UniPrimer ensures that the quencher suppresses the fluorescence of the reporter. In the final step the hairpin structure is broken and the reporter from the quencher is released.

1.2.3. An Overview of Commercially Available Real Time PCR Systems:

Along with the rapidly evolving real time PCR chemistries, a continuing effort has also been made in developing better real time PCR instruments to provide accurate thermal conditions, larger multiplexing and higher throughput. A number of vendors have commercialized real time PCR with most commonly known are Rotor Gene 6000 from Qiagen, ABI 7900 HT from Applied Biosystems, Light Cycler from Roche, Smart Cycler from Cepheid, and ICycler from BioRad.

For clinical diagnostic applications the Rotor Gene and the Light Cycler are mostly used across the laboratories mainly due to their superior temperature accuracy during the thermal cycling. The Light Cycler however does not offer higher throughput and the specific glass capillaries used for samples are very expensive.

The Rotor Gene 6000 from Qiagen (formally Corbett Research, Australia) has four separate light-emitting diode (LED) light sources that excite at 470, 530, 585 and 625 nm. The excitation is detected using six filters at 510, 555, 610, 660, 580 and 610 nm and a photomultiplier (PMT). This instrument uses a centrifugal design for the sample rack in contrast to a blocked based system. This design facilitates the heating and cooling accuracy of the sample chamber which results in sample-to-sample variation of <0.01 °C.

1.2.4. Nucleic Acid Sequence-Based Amplification (NASBA):

Nucleic acid sequence based amplification (NASBA) is another approach for RNA detection and quantification. This proprietary technique has been used by Seimens Medical Diagnostic Solutions to develop viral load monitoring assays for Hepatitis C and HIV. NASBA is a complex and a time consuming method of amplification. Due to its complexity it poses more risk of cross contamination. Besides, this technique as compared to the previous methods is a one step isothermal process for amplifying RNA. It has proven to be successful in detection of both viral and bacterial RNA in clinical specimens [24].

The contents of a typical NASBA reaction are reverse transcriptase enzyme AMV, T7 RNA polymerase, RNase H and a set of forward and reverse primers complementary to the target sequence [24]. The amplification is more than 1000 fold in about 100 minutes. Since NASBA is an isothermal process, the technique can also be used for the amplification of ssRNA where the denaturation of dsDNA does not occur. The NASBA reaction does not show false positives caused by genomic dsDNA, as in the case of RT-PCR.

The steps of NASBA reaction are shown below in Figure 1.7. The amplification process consists of a repeating process of primer annealing attached with the T7 promoter sequence, forming a double-stranded DNA with a T7-promoter, and the transcription of multiple antisense copies of the target sequences (amplicons) by the T7-RNA-polymerase. Initially, a DNA intermediate is generated. Thereby, the first primer bind to the RNA or ssDNA input template, both of them (RNA or ssDNA) processable by the later used enzyme (AMV-polymerase). This first primer which consists of a target specific sequence has an additional T7-RNA-polymerase-promoter overhang, is elongated by the avian myeloblastosis virus reverse transcriptase (AMV-RT-

polymerase) and forms an RNA–DNA duplex. This duplex is degraded by the RNaseH function of the AMV-RT and thus enables binding of the second primer to the remaining ssDNA strand. This latter primer is then elongated by the AMV-RT, resulting in a double-stranded DNA intermediate containing the T7-RNA-polymerase-promoter sequence. This promoter sequence is required and used for the following *in vitro* transcription, resulting in RNA transcripts, which are in antisense orientation to the original input template and in turn can serve as new templates to restart the amplification process.

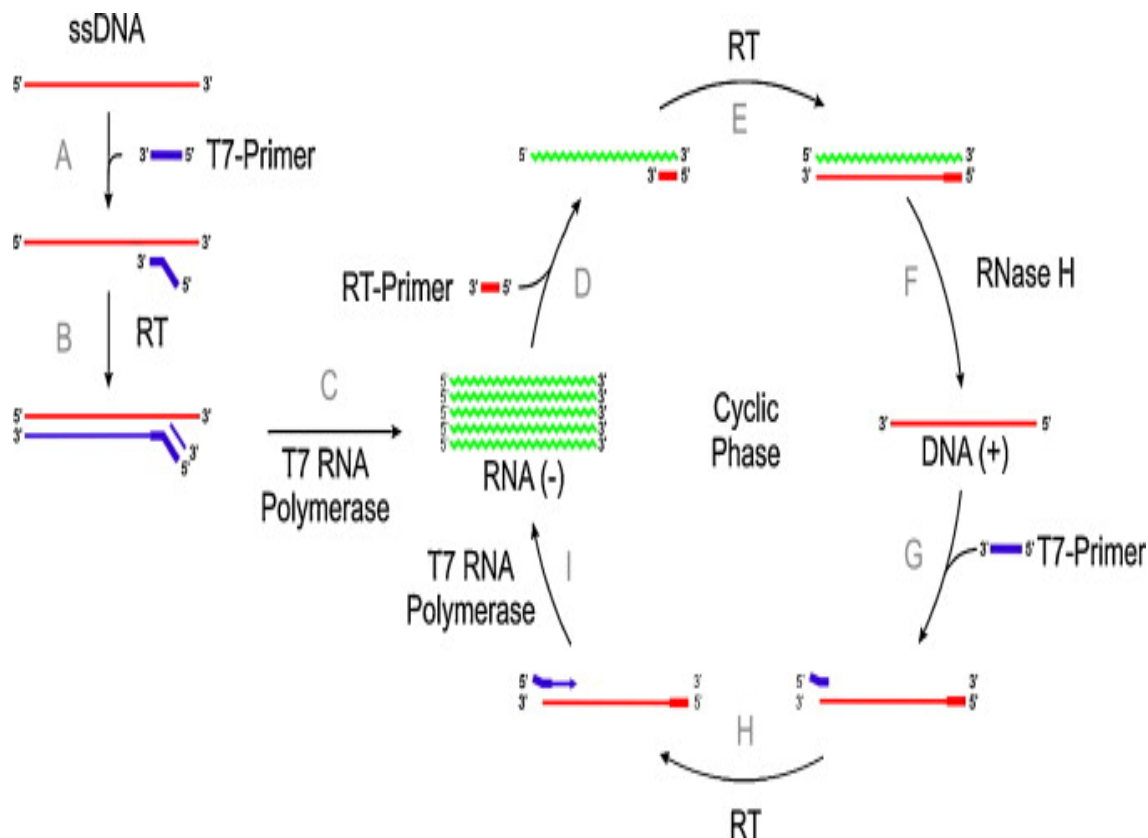


Figure1.7: A typical NASBA reaction. Single strand DNA is detected by a strand specific primer with a T7-RNA-polymerase-promoter overhang. Amplification is performed in an isothermal reaction and is driven by T7-RNA-polymerase *in vitro* transcription.

1.2.4.1. The Applications of Molecular Beacon in NASBA:

Researchers have also attempted to combine NASBA amplification with a real-time detection system by using molecular beacons. As previously described these MB probes have hairpin structures with a fluorescent label reporter at the 5' and a quencher at the 3' end. The sequence of the molecular beacon is complementary to its target sequence and it forms a stable hybrid with its amplified target RNA, when present in a NASBA amplification reaction [57].

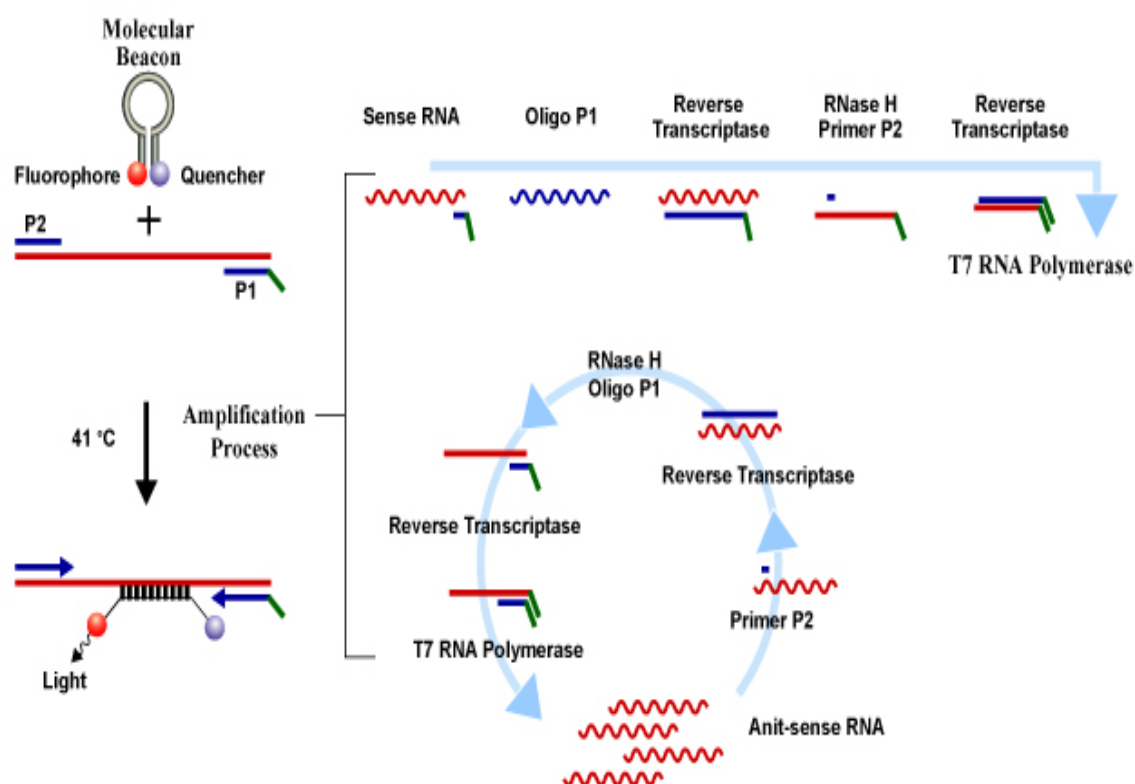


Figure 1.8: A schematic representation of the combination of NASBA amplification with molecular beacon based detection.

In the above approach which includes the binding of primer P1 to the target RNA and with the help of reverse transcriptase enzyme (AMV) the P1 forms a DNA/RNA hybrid which is later

hydrolyzed by the RNase H activity of the AMV. In the next step the P2 primer binds to the newly formed DNA strand and is extended by the AMV enzyme to yield a double-stranded DNA. Since the P1 primer is tagged with the T7 RNA polymerase promoter sequence, it generates multiple copies of RNA using a DNA template. The new copies of DNA are generated using RNA. The process is same as that followed for sense strand where P2 will bind first.

1.2.4.2. Advantages of NASBA:

Apart from being a labour intensive and time consuming amplification method, NASBA offers a wide range of advantages. Since it is an isothermal amplification method, there is no need to have an expensive thermal cycling system or a real time PCR instrument. Besides it helps in better RT-PCR reaction as it offers faster amplification kinetics. The technique has shown promising results in gene expression studies and in particular, in the detection of DNA viruses by detecting mRNA expression. It can also detect the human mRNA sequences without the risk of artifacts from DNA contamination.

1.2.5. Branched DNA method:

Another major advancement in the field of molecular amplification methods is the branched DNA (bDNA) technique [25]. bDNA has been used for quantification of bacterial and viral DNA. The technique involves a series of sequence specific hybridization reactions in a microwell plate and then chemiluminescent detection using enzyme labeled hybridized probes. The target DNA binds to the surface of a microwell plate by a number of probes that are complementary to both the capture probe and the target. Another series of probes is then added, designed to be complementary to the target and which also binds synthetic branched DNA molecules. Each

bDNA molecule has 15 arms, each arm able to bind to 3 alkaline phosphatase labeled probes. Thus, 45 alkaline phosphatase molecules can bind to each bDNA (Figure 1.9).

The branched DNA (bDNA) technology is not as sensitive as NASBA or Real Time PCR [25]. The bDNA procedure is similar to an enzyme immunoassay, and the sensitivity is limited by non-specific background. In order to reduce the non-specific hybridizations, two new bases, iso-cytosine (iso-C) and iso-guanosine (iso-G), were synthesized and utilized in the development of new probes. These bases form strong hybrids with each other, but, as they are not naturally occurring bases, they do not hybridize with natural guanosine and cytosine. In this manner, nonspecific hybridization between the bDNA amplifier and the capture probes has been reduced by substituting some cytosine and guanosine bases with iso-C and iso-G.

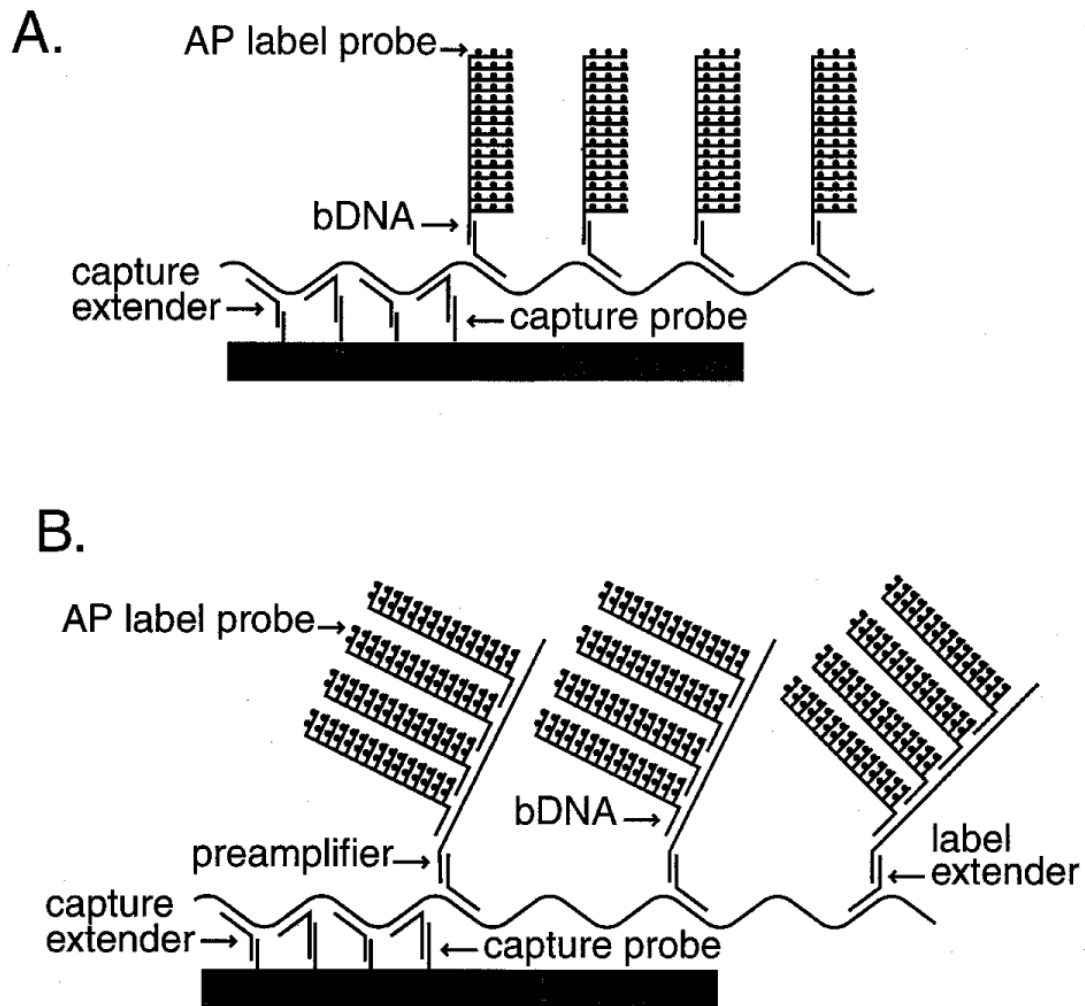


Figure 1.9: Basic bDNA assay components. (A) First generation assay; (B) second and third generation assays. The preamplifier (heavy lines) is unique to the second and third generation assays.

1.2.6. The Transcription Mediated Amplification Technique:

Recently the researchers at Gen Probe, USA have developed another isothermal amplification method called transcription mediated amplification (TMA). This method produces billions of RNA amplicons from a single target gene in 90 -120 minutes [26]. Since the amplification reaction is performed at the same temperature, there is again no need for thermal cycler. TMA can be used with any type of nucleic acid target including rRNA, mRNA or DNA. The TMA is performed in three stages. The first step consists of sample purification which then follows the amplification of target DNA or RNA and finally the detection is done by chemiluminescence method.

The sample preparation steps required the isolation of nucleic acid. This can be performed by several methods. There are a number of commercial kits available for RNA isolation. Alternatively this can be performed by mechanical means such as sonication. Once nucleic acid is released the samples are heated at 95°C to denature the target nucleic acid and to inactivate infectious agents. The lysate contains free nucleic acid that serves as a template for amplification.

Once the RNA is extracted the method follows the amplification step. The reagents required for TMA reactions are reverse transcriptase usually MMLV enzyme, RNA polymerase, dNTPs, NTPs, a set of primers complementary to the target sequence with the forward primer tagged with the T7 promoter sequence. The reaction begins with the forward primer with T7 promoter being hybridized to the RNA template and extended with the help of reverse transcriptase. This results in the formation of DNA/RNA hybrid. This hybrid is then degraded by the RNase H activity of the reverse transcriptase. At this point the other primer binds to the newly formed DNA strand and elongates with the help of reverse transcriptase to form a double stranded DNA. The RNA

polymerase then recognises the promoter sequence in the DNA and starts replicating the RNA amplicon. The process then continues by the using the newly formed RNA amplicon to re-enter the TMA cycle to produce more RNA amplicons. Since each of the DNA templates can make 100-1000 copies of RNA amplicon, this expansion can result in the production of 10 billion amplicons in less than one hour from a single target molecule.

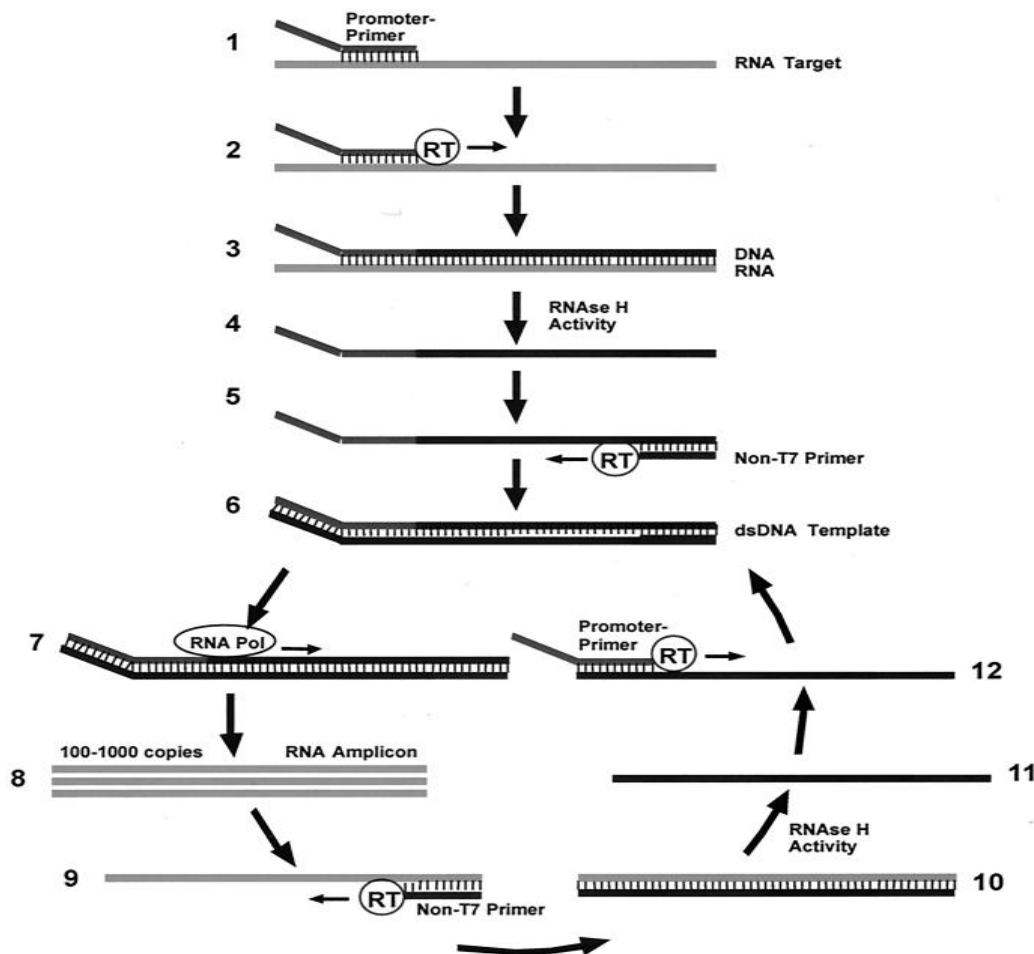


Figure 1.10: A detailed overview of TMA cycling is shown above. (i) A promoter-primer binds to the RNA target and is extended via the DNA polymerase activity of the reverse transcriptase (RT) (steps 1 and 2). This results in a RNA/DNA hybrid duplex, with the new DNA strand being complementary to the virion RNA target (step 3). (ii) RNase H activity of the reverse transcriptase specifically digests the RNA strand of the RNA/DNA hybrid, leaving only the

cDNA (step 4). (iii) Following the RNA degradation, the non-T7 primer (i.e., the primer without the T7 promoter) base pairs to its complementary sequence on the newly created DNA strand, and the reverse transcriptase catalyzes the synthesis of another new DNA strand using the cDNA as a template (step 5). The product of this reaction is a DNA intermediate that contains the bacteriophage T7 RNA polymerase promoter sequence in double-stranded form (step 6). (iv) The bacteriophage T7 RNA polymerase now recognizes the promoter sequence on the DNA intermediate and transcribes multiple copies of the RNA amplicon (steps 7 and 8). The RNA amplicon strands are opposite in polarity from the original RNA target and contain a region complementary to the probe used for amplicon detection. (v) The RNA strands created in (iv) are able to base pair to the non-T7 primer (step 9). Subsequently, the reverse transcriptase catalyzes the formation of a cDNA strand by extending the 3' end of the non-T7 primer, and again an RNA/DNA hybrid duplex is formed (step 10) as in step 3 and the TMA cycling continues [27].

For the detection of RNA amplicons produced at the end of the TMA reaction several methods can be used. The most commonly used method is the Hybridization Protection Assay (HPA) [27]. Alternatively in this thesis research work we describe an electrochemical detection method being coupled with TMA. The HPA detection method is initiated by adding acridinium ester-labeled DNA probes which specifically bind to the target amplicon (Figure 1.11). The final step of the assay is to measure the chemiluminescent signal in a luminometer after the addition of detection reagents. This is a homogenous assay format which requires no wash steps which could potentially spread contamination throughout the laboratory.

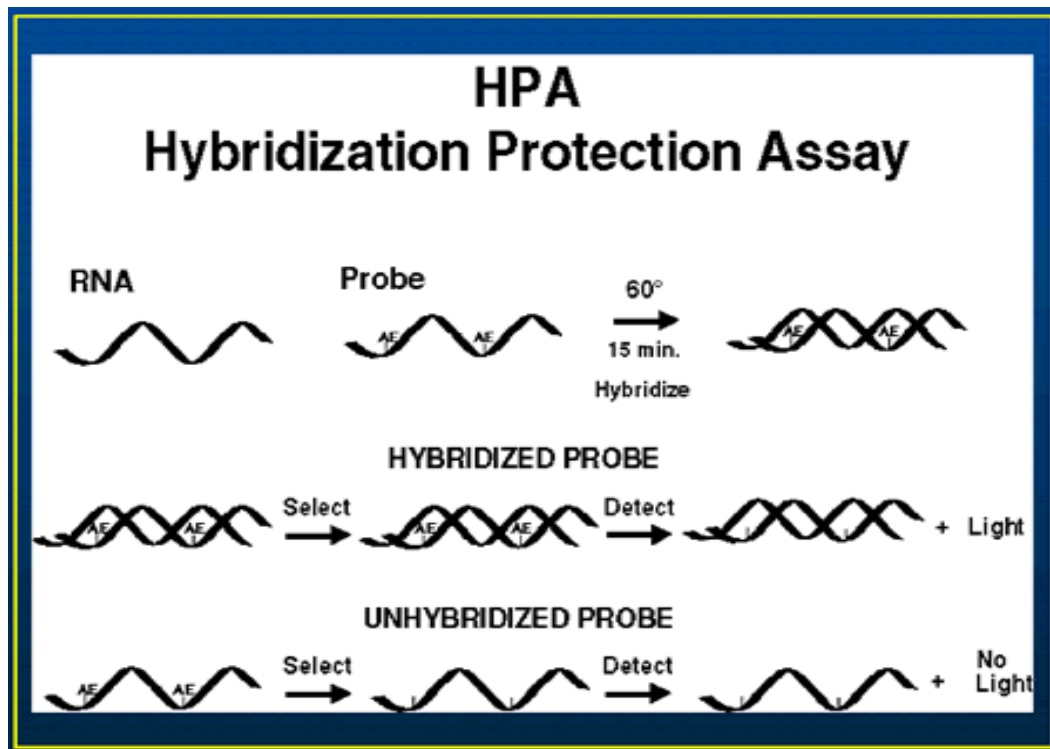


Figure 1.11: A Schematic representation of HPA. The AE-labeled probe hybridizes to target RNA in solution. After washing of unhybridized probe the label protected hybridized probe is detected by chemiluminescence.

1.2.6.1 Advantages of TMA:

Transcription mediated amplification or TMA has many technological advantages over the previously discussed technologies and molecular methods. Since it is an isothermal procedure, a number of detection methods can be readily coupled with the process including sensor devices. Since the amplification is done on the same temperature, the cost of buying expensive thermal cycling machines can be saved. Since the kinetics of TMA is very fast it produces billions of RNA amplicons in less than an hour. Also the end product formed by the TMA is an RNA not a DNA which is a temperature sensitive molecule and hence cannot contaminate laboratory where

as other amplification methods which produce DNA product can pose risk for laboratory contamination.

1.2.6.2. Reasons for Choosing TMA to Combine with Chemical Sensors:

TMA is a very sensitive technique and does not require RNA purification prior to amplification. It is an isothermal technique and generates protons in 5 stages during the amplification. Every time a complementary DNA strand is formed, protons are released which can be easily detected. Since the temperature remains the same during the amplification, temperature sensitive pH devices can have a great potential to produce more accurate results.

The rapid production of RNA amplicons also makes this a viable technique to employ pH sensing detection methods during the amplification cycles. This could potentially reduce the time for signal detection providing faster detection.

1.3. POTENTIOMETRY:

The principle of potentiometry is to measure the potential generated between an ion-selective electrode (ISE) and a reference electrode caused by a difference of concentration between the sample (analyte) solution and a reference solution.

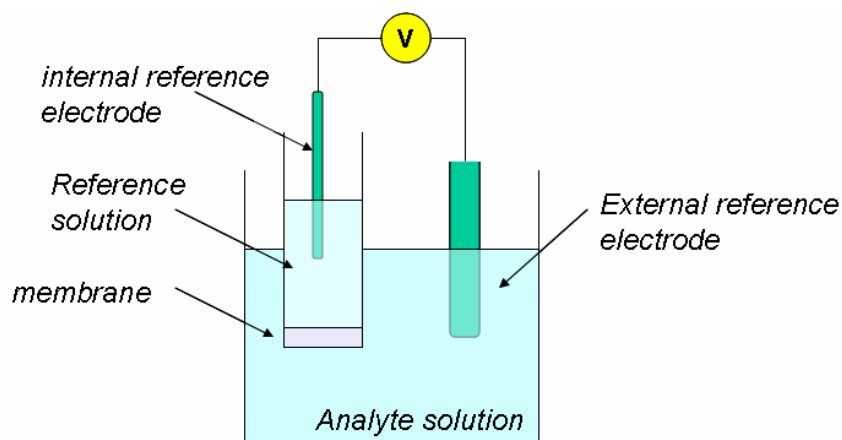


Figure 1.1.1a: An electrochemical cell for potentiometric measurements with an ISE.

The set up is shown in Fig. and consists of an ISE which has a selectively- permeable membrane separating the sample from a reference solution of a constant activity (concentration) inside the electrode. The membrane is usually nonporous, water insoluble and mechanically stable and is designed to yield a potential arising primarily from the ion of interest via a selective process e.g., ion exchange at the membrane-solution interface. The binding process generates a phase boundary potential at the membrane-sample interface and another at membrane-internal solution interface on its inner surface, giving rise to a membrane potential which reflects the differences in ion distribution on either side. At equilibrium, this potential is related to the ion activity a_i on either side of the membrane as defined by Nernst equation:

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

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

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

Thus giving a variation of $59/z_i$ mV in E_{mem} per decade variation in ion activity at a temperature of 298K.

The membrane potential is measure as the potential difference between the ISE and the reference electrode. The reference electrode is commonly a silver/silver chloride (Ag/AgCl) electrode which being a metal electrode in contact with a sparingly soluble salt of its own cation, provides a stable voltage reference.

1.3.1. NUCLEIC ACID DETECTION BY pH SENSING:

Nucleic acid testing by pH sensing has been a challenge for scientists due to the complexity of the procedures. The theory behind the phenomenon requires single stranded DNA/RNA to incorporate to the dNTPs being added in the reaction in the presence of polymerase enzyme.

Upon addition of a dNTP the following reaction proceeds,



So for each base incorporation with the target DNA/RNA, a pyrophosphate molecule is released as shown in the figure 1.12.

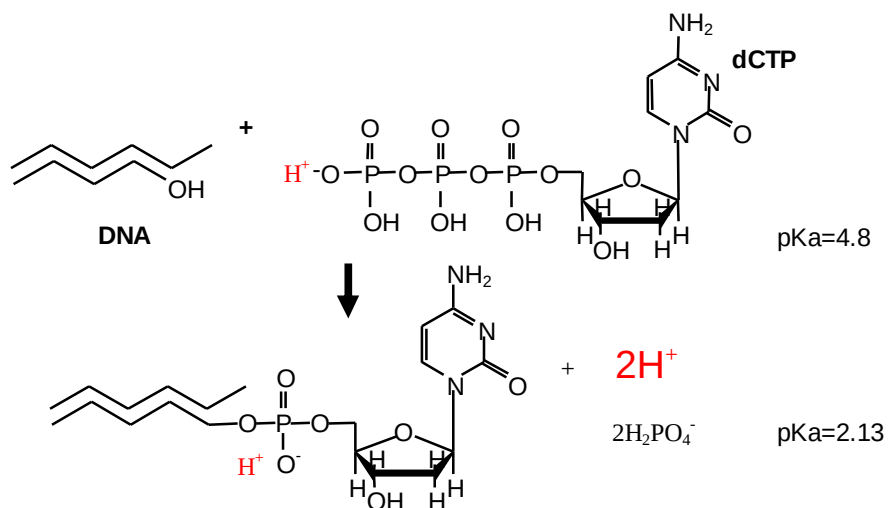


Figure 1.12: Schematic presentation of the base incorporation reaction.

This pyrophosphate ion is unstable in aqueous solution and is hydrolysed into 2 phosphates resulting in a change of pH of the reaction. The change in pH can be monitored by means of pH sensing devices.

1.3.1.1. pH Sensors:

The value of pH has long been one of the most important parameters of liquid samples and plays an integral part in agricultural industry, biology, pharmaceutical industry, environmental protection and many more [28]. The measurement of hydrogen concentration (pH) also indicates the chemical properties of materials and for the management of chemical reactions. So far numerous methods have been introduced for the determination of pH, most of which utilize

potentiometric electrodes [29]. Among them, the glass electrodes are widely used to measure the pH in various samples due to their stability, accurate performance, high reliability, dynamic pH range, and convenience of use [30]. These glass electrodes have a pH-sensitive thin glass membrane which is liable to be broken and hence restrict their use in methods requiring stirring along with other factors such as acid and alkaline errors and temperature sensitive nature [31].

Similarly since miniature pH electrodes are frequently required for many medical applications such as measurement of the pH of stomach fluid [32]. At the moment, the combined electrode which is integrated with glass electrode, reference electrode and temperature compensated electrode is used. Although this type of combined pH sensor has become compact, it has a limitation in miniaturization of the electrode part for the conventional liquid membrane type electrode using electrolyte solution [33]. In order to downsize the pH sensing part, the whole part (indicator electrode and reference electrode) needs to be an all-solid-state electrode.

As a result researchers are now more focused in developing non-glass pH electrodes. There is a lot of literature available on non-glass membrane pH sensor electrodes, which show low cation interference and resist high temperatures. Unfortunately this kind of electrode often shows significant redox sensitivity. The pH-response of these electrodes can be explained just by simple ion-exchange mechanisms, but often includes pH-dependent redox equilibrium. Up to now, the common non-glass pH electrodes have consisted of two main types, the polymer membrane pH electrode and metal-metal oxide pH electrodes [34]. The solid-state pH sensors based on metal oxides have gained considerable attention in terms of development of potential alternatives to the glass electrode for miniaturized systems, because metal oxides are mechanically robust, less sensitive to cation interference. So far various metal oxides have been attempted to be pH sensing electrodes, such as MnO_2 , PbO_2 , WO_3 , Co_3O_4 , TiO_2 , PtO_2 , RuO_2 , RhO_2 , OsO_2 and IrO_2

[35][36][37]. Examples of such electrodes are the zeroth class (electron exchanging or redox type) and the second and third classes (metal/metal oxide or oxides). Researchers have reported that the fabrication of anodically electrodeposited iridium oxide film pH microelectrodes in a tapered glass micropipette filled with a low melting point alloy with the linear slope of pH 0 – 12 [38]. A planar thick film electrode for the potential measurement of pH based on ruthenium dioxide hydrate held in a polymer matrix was also introduced [39]. The electrode had a near- Nernstian (-59.15 mV/pH) behavior in the pH range 2 – 10. Though metal-metal oxide electrodes have several advantages such as long lifetime and mechanical strength, which can overcome the disadvantages of the pH electrodes, they are still easy to disturb by redox reactions.

1.3.2. Iridium Oxide Sensors:

Iridium oxide sensors have been recently a focus for many scientists to use as a potential metal-metal oxide pH sensor. Some researchers have described the anodic electrochemical deposition of iridium oxide films “Anodically Electrodeposited Iridium Oxide Films” (AEIROF) which involves a procedure based on an alkaline iridium (IV) oxalate electrodeposition solution [40][53]. Anodic decomposition of such an oxalate complex resulted in a concomitant deposition of iridium oxide at the surface of the anode. Several publications have previously reported the pH sensing characteristics of such electrodeposited iridium oxide film (AEIROF) on sputtered platinum substrates [41][55]. The electrodeposition of IrO₂ is an easy and cost effective way of fabrication and offers the potential for using cheaper substrates than expensive base metals such as iridium itself.

In order to improve the fabrication of IrO₂ researchers have also attempted to modify the original electrodeposition solution prepared by Yamanaka *et al.* [42]. Unfortunately this modified

electrodeposition solution required larger amounts of expensive iridium salts and was sensitive to air. The resulting pH sensors had a limited lifetime and showed a severe break in the calibration plots at pH 6. Similarly some researchers have attempted deposition of AEIROF on SnO₂ and platinum substrates using K₃IrCl₆ and IrCl₃ respectively in the electrodeposition solutions instead of IrCl₄ as the iridium source [43][58][56]. But these efforts did not produce any effective advantages than using the original electrodeposition solution mentioned above.

Recently the deposition of iridium oxide on electronic conductors i.e., stainless steel, and used that as a pH sensor [44][54]. But most work has focused on characterizing AEIROF on platinum substrates and used that as a pH sensor [45]. This prototype showed a number of applications in monitoring pH in physiological studies. Although having several attempts to produce a successful pH sensor, there have been many issues which need to be addressed including shorter time for preparing electrodeposition solutions and cost effective substrates for AEIROF.

Applying the Nernst equation to an iridium oxide chemical equation gives:

$$2 \text{IrO}_2 + 2\text{H}^+ (\text{aq}) + 2\text{e}^- = \text{Ir}_2\text{O}_3 + \text{H}_2\text{O}$$

$$E = E^\circ + \frac{2.303 RT}{F} \log[\text{H}^+] = E^\circ - 59.15 \text{ pH}$$

There is a 59.15 mV difference for every power of ten change in ionic activity. Thus, there is an expected -59.15 mV slope for every change in pH.

1.4. MS2 BACTERIOPHAGE:

MS2 is an icosahedral male-specific bacteriophage of *Escherichia coli* belonging to the family *Leviviridae*. The MS2 phage has a very small genome comprising of 3,569 bases of single-stranded RNA. The single stranded RNA encodes four proteins including assembly, lysis, coat, and RNA replicase [β chain] proteins (Figure 1.13). The MS2 bacteriophage is non-infectious

phage to human, plants and animals. Due to its small size and simple structure it has been used as a surrogate in place of other infectious RNA viruses such as Hepatitis, EBV and other viruses. Besides much research has used the phage in studies ranging from clinical testing to environmental biology due to its non-infectious nature, it is easy to culture and easy to detect. Recently researchers have used the MS2 phage for insertion of a known quantity of a small region of HCV genome in the assembly protein. This known quantity of inserted HCV genome stays stable for very long periods and can be used as quantitative standards for a HCV viral load monitoring diagnostic assay.

Similarly other researchers in recent studies have used MS2 RNA as an internal control for real time PCR in multiplex assay [46]. If in a multiplex assay the target RNA is not amplified and the internal control is amplified then the sample is considered as true negative. Whereas if the sample and the internal control both are negative then it is indicated that the reaction did not occur due to possible PCR inhibition therefore eliminating the risk of false negative results.

In order to develop a rapid molecular detection method for pathogens, this research used MS2 Phage mainly due to its simple structure, non-infectiousness, easy culturing and small size. Additionally there are strict regulations for using pathogens for the testing of medical devices or molecular methods. The pathogens are normally kept in isolated environments to eliminate the risk of spreading. Consequently surrogates or viral models like MS2 phage are mostly used for testing new molecular detection assay design for pathogen detection.

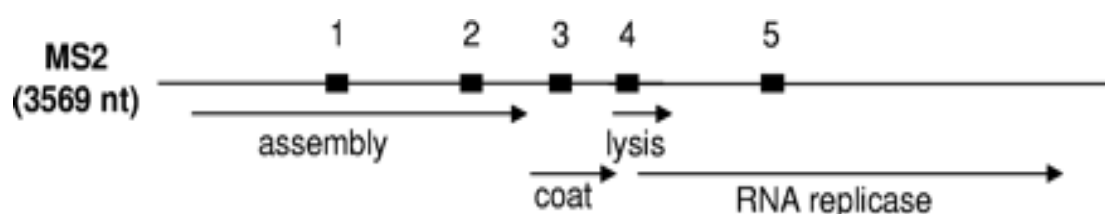


Figure 1.13: Linear map of the MS2 genome. The approximate locations and sizes of the amplicon products are indicated by thick bars superimposed on the linear map.

1.5. AIMS OF RESEARCH:

The aims of this work involved investigation of solid-state pH sensor for the detection of RNA during a robust RNA amplification method and to design a rapid point of care device for quantification of viral RNA in complex samples. To achieve this, real-time PCR was established as a gold standard for viral RNA quantification and for subsequent method comparison. For solid-state pH sensor, an iridium oxide sensor with a near ideal Nernstian behaviour of 60mV/pH was prepared for measuring pH change. The sensor was investigated with a number of polymers to serve as sensor surface coat for enhancing the selectivity of species diffusing through the polymer to reach the sensor surface and to reduce any possible interferences. Nafion proved to be the ideal candidate and was later used for sensor surface coating. The sensor was later used for RNA detection during transcription mediated amplification (TMA) method which generates protons at 5 stages in each amplification cycle. TMA is an isothermal amplification method and did not effect the performance of iridium sensor. The RNA model, bacteriophage MS2 investigated in this work, served as a surrogate for RNA viruses due to its non-infectiousness and small size. MS2 RNA was then amplified using TMA and voltage measurements were taken at regular interval. The sensitivity and performance of the iridium oxide sensor for detection of MS2 RNA during TMA will be compared to the gold standard i.e, real-time PCR method and the potential for using this iridium oxide sensor as a rapid point of care device will be exploited.

CHAPTER 2: MATERIALS AND METHODS

2.1. PHAGE AND HOST STRAINS:

2.1.1 *E.coli* Strains:

The *E.coli* strains used in the work described in this thesis were DH5 α XL10-Gold from Stratagene (California, USA) and BL21(DE3) from Novagen (Wisconsin, USA).

E.coli strain XL10-Gold containing the F-plasmid was also used as a host for the growth of phage MS2 by the double-agar-layer plaque technique.

2.2. MS2 Bacteriophage:

The phage used in this research was Bacteriophage MS2 DSM13767 and it was purchased from DSMZ (Braunschweig, Germany).

2.3. PLASMIDS:

The plasmid used in this work were, pET24a from Novagen (Wisconsin, USA) and pBluescriptSK(+) from Stratagene (California, USA) and F plasmid.

2.4. GENERAL CHEMICALS:

Agarose was purchased from Biometra Ltd (Maidstone, UK), yeast extract, peptone from casein (Tryptone), and agar were from Merck (Darmstadt, Germany). dNTPs used were from Roche (Basel, Switzerland). IPTG was from Apollo Scientific (Cheshire, UK). PBS buffer was from Fisher Scientific UK (Loughborough, UK), Betaine ((carboxymethyl) trimethylammonium inner salt, Oxynurine) was from Aldrich (Poole, UK). Rubidium Chloride, antibiotics, Sodium Chloride, EDTA, DMSO, and other chemicals of high purity were obtained from Sigma (Poole, UK). Poly(methyl methacrylate) (PMMA) substrate (1 mm thickness), 2-(N-morpholino) ethanesulfonic acid (Mes), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), phosphate, and Tris buffer solutions were obtained from Sigma (UK).

2.5. ENZYMES:

The restriction enzymes Acc I, Apa I, Bam HI, Cla I, Dra I, Eco RI, Dpn I were purchased from Helena Biosciences. DNA *Taq* polymerase, reverse transcriptase and RNA polymerase were purchased from New England Biolabs.

2.6. BUFFERS AND SOLUTIONS:

Water was purified by a combination of reverse osmosis, ion-exchange and carbon filtration and stock solutions were prepared as follows,

2.6.1. LB Media:

10 g Tryptone

10 g NaCl

5 g Yeast Extract

Dissolve in 950 ml of water. Adjust the pH to 7 with 5M NaOH (~ 0.2 ml). Then adjust the volume to 1 l and autoclave.

2.6.2. LB Agar Plates:

The LB agar plates were prepared with the same recipe as above with an addition of 15 g of Agar before autoclaving.

2.6.3. Kanamycin:

The concentration of Stock was 30 mg/l in deionised water.

2.6.4. 10 × TAE Buffer:

48.4 g Tris Base

10.9 g Glacial Acetic Acid

3.72 g EDTA

1 l of purified H₂O

2.6.5. SOB medium:

20 g Tryptone

5 g Yeast Extract

0.5 g NaCl

10 ml of 250 mM KCl

Add the above to 950 ml of water. Adjust the pH to 7.0 with 5M NaOH (~0.2 ml). Adjust the volume to 1 l. Autoclave the medium. Just before use, 5 ml of filtered MgSO_4 solution was also added.

2.6.6. SOB Agar plates:

The SOB agar plates were prepared with the same recipe as above with an addition of 15 g of Agar before autoclaving.

2.6.7. SOC Medium:

The recipe for the SOC medium is same as SOB medium except that it contains 20 ml of filtered 1 M glucose added once the SOB medium has been autoclaved.

2.6.8. 1 M MES (pH 6.3):

For 100 ml add 19.52 gm of MES. Adjust the pH 6.3 with 10 mM HCl.

2.6.9. 1M Potassium Acetate (pH 7.8):

For 100 ml add 9.815 g of Potassium Acetate and then adjust the pH to 7.8 with 5 M NaCl.

2.7. REVITALIZING of MS2 PHAGE:

MS2 phage was supplied as cell-free lysates in complex medium from DSMZ, Germany. The bacterial host cells and debris were eliminated by centrifugation and filtration (45 µm pore size). The phage suspensions were delivered in a high titre solution in a range of $1 \times 10^8 - 1 \times 10^{11}$ pfu/ml. The medium used for the phage suspension was described below.

2.7.1. Double Layer Agar Technique:

A host medium containing 1 ml of *E. coli* XL-Gold ultracompetent cells ($\geq 5 \times 10^9$ cfu/µg pUC18 DNA), the following was prepared,

Peptone = 5 g

Meat Extract = 3 g

Agar = 15 g

Distilled H₂O = 1 l

The pH was then adjusted to 7.

Similarly a phage buffer with the following composition was prepared,

Na₂HPO₄ = 7g

KH₂PO₄ anhydrous = 3 g

NaCl = 5g

0.1M MgSO₄ = 10 ml

0.1 CaCl₂ = 10 ml

H₂O to 1000 ml

The host medium was then poured onto an agar plate. From the ampoule containing the MS2 phage, the filter paper (containing the phage suspension) was placed in the middle of the host plate.

Then 0.1 ml of the following MS phage buffer was added on the filter paper and this plate including the phage and the host was incubated at 36 °C overnight. The lysis of the host cells lead to a clear zone and phage caused turbid plaques around the filter paper. After overnight incubation the filter paper was removed and the medium around the filter paper was collected as a phage stock suspensions. This was then stored at 2-9 °C temperature for up to 6 months.

2.8. MS2 RNA PURIFICATION:

The MS2 RNA was purified from the phage stock suspension using a Qiagen mini RNA viral kit from Qiagen, Germany. The purified RNA was stored at -20 °C freezer.

2.9. ABSORPTION SPECTROSCOPY:

The purified RNA was quantified by measuring the absorbance at 260 nm. The extinction coefficient used for RNA was 0.025 ml cm⁻¹ µg⁻¹ (or 40 ng cm µl⁻¹). The absorbance measurements were performed with a UV-Vis spectrophotometer ND-1000 (NanoDrop) using a 1 µl sample for each measurement.

2.10. General Molecular Methods:

2.10.1. Reverse transcriptase Polymerase Chain Reaction (RT-PCR):

2.10.1.1. Reverse Transcription:

The MS2 RNA was converted into cDNA using SuperScriptTMIII Reverse Transcriptase (Invitrogen). A total of 50 µl of the reverse transcription reaction was run for each round of the selection as follows. Ethanol precipitated RNA was suspended in 24 µl water and 1 µl of 10 mM dNTP mix and 1 µl of 400 µM reverse primer (GGCCACGTGTTTTGATCGA) were added and incubated at 65 °C for 5 min to denature the secondary structures present in the RNA that could inhibit reverse transcription reaction. The mixture was briefly chilled on ice before adding 8 µl of the supplied 5×RT-PCR buffer (250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM MgCl₂) and 2 µl of DTT (100 mM). This mixture was then preheated to 50 °C and 4 µl of reverse transcriptase (200 U/µl) was added and incubated for 1 hour. Finally, the temperature was raised and maintained at 70 °C for 15 min to deactivate the enzyme and stop the reverse transcription reaction. The resulting cDNA was then subjected to DNA amplification.

2.10.2. DNA amplification:

After the reverse transcription the cDNA was amplified using DNA *Taq* polymerase (Invitrogen). For each round, a total volume of 2 ml of the PCR reaction mix was employed as follows. The 50 µl of cDNA from the reverse transcription reaction was transferred to a 1.8 ml DNA-LoBind Eppendorf tube containing 1670 µl of water and incubated on ice. It was then followed by addition of 200 µl of 10×PCR reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl); 60 µl

of 50 mM MgCl_2 ; 10 μl of 25 mM dNTP mix; 1 μl of forward primer (GTCGCGGTAATTGGCGC) and 1 μl of reverse primer (GGCCACGTGTTTTGATCGA) both at the same concentration of 400 μM and mixed well.

A 100 μl trial reaction was carried out before progressing to a large-scale amplification. 1 μl of recombinant *Taq* DNA polymerase (5 U/ μl) (Invitrogen) was added to 100 μl aliquot of the PCR mix. The amplification was then run with the following parameters: denaturing at 94 °C for 30 sec; annealing at 45 °C for 30 sec; and extension 72 °C for 1 min. An extra 3 min of denaturation at the beginning and another 5 min of extension at the end of the amplification were applied. The samples were analysed by agarose gel electrophoresis as described before to determine if DNA amplification has occurred. This precaution guarded against under or over amplification of DNA.

After the optimum cycles for the amplification had been found, 19 μl of the recombinant *Taq* DNA polymerase was added to the remaining 1.9 ml and mixed well. This PCR mix was then split into 100 μl volumes in 96-well plate format and the amplification was then performed at the previously optimised conditions.

DNA obtained from the amplification was precipitated and then suspended in 50 μl of the water.

2.11. PREPARATION OF PLASMID DNA:

A single colony was picked and inoculated in 10 ml of LB medium containing kanamycin at concentration of 100 $\mu\text{g/ml}$. The cell culture was grown overnight at 37° C and with shaking at 200 rpm. The overnight cell culture was then centrifuged and the plasmid DNA was prepared using QIAprep Miniprep (plasmid purification) kit from Qiagen.

The purification of plasmid DNA using the kit was performed with an Eppendorf micro-centrifuge. The micro-centrifuge was run at maximum speed (13,200 rpm) unless indicated. The overnight culture was centrifuged for 1 min in 1.5 ml Eppendorf tubes. The following procedure was applied for an amount of cell pellet from a volume of overnight culture up to 5 ml: The pellet was first suspended in 250 µl P1 buffer (suspending buffer) containing Dnase I. 250 µl of alkaline P2 buffer (containing NaOH and SDS for cell-lysis) was added to the tube and gently inverted 4-6 times. The mixture was then neutralised with N3 buffer (containing acetic acid) and the whole mixture was mixed gently before being centrifuged for 15 min. The supernatant was then applied to a QIAprep spin column and centrifuged for 1 min. DNA bound to the column was then washed with PE buffer (containing 80% ethanol). The plasmid DNA was eluted from the column with 50 µl sterilised dH₂O.

2.12. RESTRICTION DIGESTION OF PLASMID DNA:

The restriction endonuclease cleavage was carried out in order to digest the reverse transcribed MS2 DNA from the pET24a vector by using appropriate restriction enzymes. Typically, 1 µg of plasmid DNA can be digested with 1 unit of enzyme in a recommended buffer in the Qiagen mini prep kit at 37 °C for 1 hr.

2.13. AGAROSE GEL ELECTROPHORESIS:

Agarose gel electrophoreses was carried out in order to confirm that the MS2 DNA is inserted in the pET24a vector. For that purpose a 10% w/v agarose gel (for 100ml, 1 g Agarose was added in 100 ml of 0.5 × TAE buffer) was prepared. The slurry was heated in a microwave for 1 minute for

the agarose to melt and cooled to 60°C in a water bath. Then 4.5 ul of ethidium bromide (10 mg/ml) was added. The solution was then poured into the gel plate and left to set for 30 minutes. DNA samples were mixed with 5× DNA loading buffer before being applied to wells. Electrophoresis was then performed at a constant voltage for 120 V for around 2 hrs. The gel was the visualized using

2.14. GEL EXTRACTION OF MS2 RNA:

The MS2 DNA containing plasmid was then was excised from the gel with a clean, sharp scalpel and extracted from the gel using QIAquick Gel Extraction Kit from Qiagen.

2.15. PRIMERS:

The primers for MS2 were designed using the Vector NTI software (Invitrogen) and were obtained from MWG Biotech.

The primers and probe obtained for MS2 RNA responsible for MS2 assembly protein, are as follows,

MS 2 Assembly Protein:

632 Forward primer 5'-GTCGCGGTAATTGGCGC-3'

708 Reverse primer 5'-GGCCACGTGTTTTGATCGA-3'

650 Probe FAM-5'-AGGCGCTCCGCTACCTTGCCCT-3'-BHQ

The degree of nucleotide sequence homology was checked by using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), which searches EMBL, GenBank and DDBJ databases.

2.16. REAL TIME PCR:

The Real Time PCR was performed on Rotor Gene 6000 from Corbett Research, Australia. The instrument has 6 excitation channels and due to its centrifugal design for tube-rack and accurate heating and cooling system it is very sensitive.

2.16.1. Real Time Amplification Reagents:

For the real time PCR Qiagen's QuantiTect Probe Kit was used. The kit contains a readymade mastermix, however the following concentration of primers, probe and magnesium were used after optimization.

MS2 Sense Primer = 2 μ l (200 nM)

MS2 Antisense Primer = 4 μ l (400 nM)

MS Probe = 2 μ l (200 nM)

Mastermix = 25 μ l

MgCl₂ = 2 μ l (4 mM)

Using this kit a slightly different PCR protocol was used which was specifically optimized for MS2 RNA amplification. The following protocol was used:

RNA Reverse Transcription = 30 minutes at 50 °C.

DNA polymerase activation = 10 minutes at 95 °C.

Then 40 cycles of 10 s at 95 °C, 15 s at 61 °C, 10 s at 72 °C.

The final step was measuring the high resolution melting curve of dsDNA with a ramp from 72 °C to 95 °C at 0.2 °C/s. The fluorescence was measured at 72 °C. An excitation wavelength of 470 nm and a detection wavelength of 510nm were used for the taqman probe labeled with FAM at 5' end.

2.17. HIGH RESOLUTION MELT:

For HRM a dye called SYTO 9 (Invitrogen) was used. This dye is a recommended dye for genotyping through HRM as it saturates the dsDNA and does not inhibit PCR unlike SYBR Green if used in large concentration. The cycling protocol used for HRM analysis is the same as shown in section (2.16.1).

The melt temperatures were measured using a ramp of 0.25 °C between 80 and 90 °C.

2.18. ELECTROCHEMICAL METHODS:

2.18.1. Preparation of Iridium Oxide Sensors:

The iridium oxide sensors were prepared by cutting 2 gold wires of about 4 cm long. Then a mixture containing equal amounts of Silver Epoxy Soft and Hard pastes were prepared. One of the wires was then coiled around the other one and the mixture of Silver epoxy soft and hard paste was then applied. This was then left for 15-30 minutes incubation in an oven at 60 °C.

Meanwhile 0.5 ml of Robnor resins hardner (Sigma) and 1 ml of Robnor resins soft (Sigma) were gently mixed in an eppendorf tube. After the incubation, the wires with epoxy paste were placed in a 200ul pipette tip and the tip was filled with the resin mixture to the top. The bottom end was then prevented from leaking by putting on some blue tack. The tip was then incubated for 1 hr at 60 °C.

2.18.2. Preparing the Sensor Surface:

After the incubation, the blue tack was taken off from the bottom. At this stage the Robnor resin solution was set in the tip along with the gold wire surfaced with epoxy paste. The tip surface was then cut with a sharp blade and carefully polished with Micropolish-II (Aluminium oxide paste from Sigma, UK) 0.05 micron for 8 minutes on a smooth surface. After that the sensor was sonicated in water for 7 minutes.

2.18.3. Iridium Oxide Deposition:

The Iridium oxide deposition solution was prepared by dissolving 0.15 g of $\text{IrCl}_4 \cdot \text{H}_2\text{O}$ in 100 ml of water and stirring for 30 minutes. Then 1 ml of aqueous hydrogen peroxide (30 % wt.) was added and the solution was stirred for approximately 10 minutes. After that 0.5 g of oxalic acid dihydrate was added to the solution and stirred again for 10 minutes. Finally to adjust the pH of the solution to 10.5, anhydrous potassium carbonate was added. This resulting solution was left for 2 days to stabilize. The colour of the solution changed from yellow to light-violet due to iridium being oxidized, indicating it was suitable for deposition. After stabilization, the solution was stored in a dark bottle at 4 °C in a refrigerator until use. The deposition solution could be used for up to 2 months after its preparation.

All electrochemical experiments were performed with a potentiostat/galvanostat. A DRIREF-450 (WPI) Ag/AgCl reference electrode, 450 μm diameter, was used for pH measurement inside microchannels.

The gold electrode was then placed into the Iridium deposition solution for 500 seconds and held at a constant current of 4.9×10^{-7} mAmp. Immediately after deposition, the IrO_x sensors were rinsed with distilled water and dried under a stream of nitrogen.

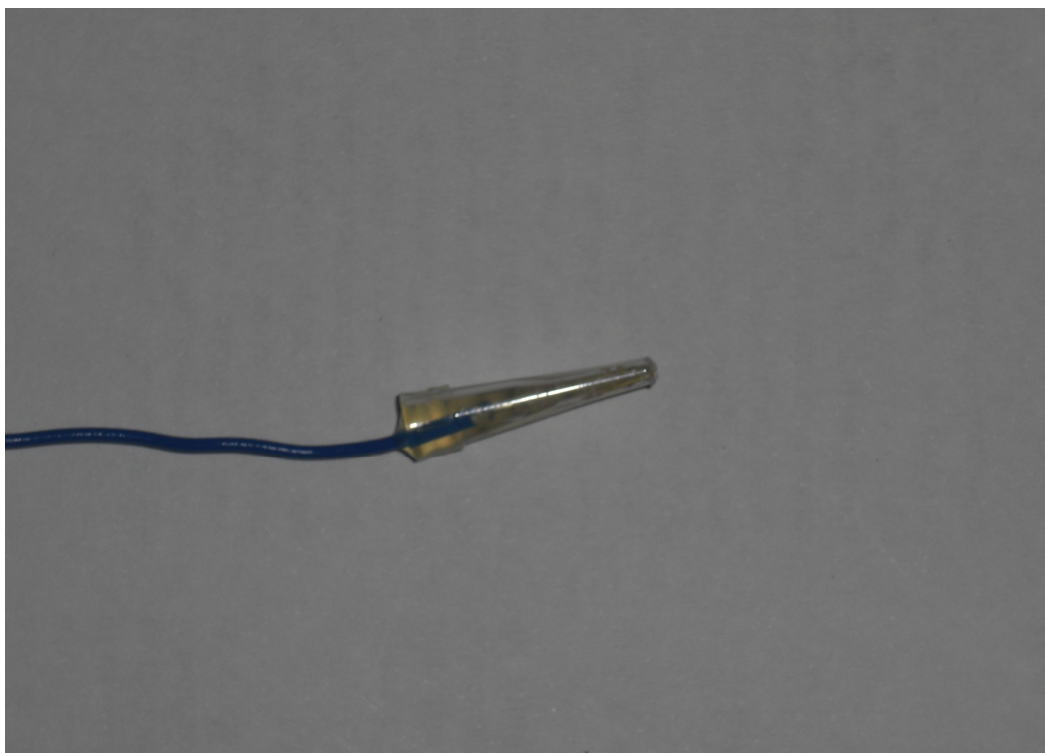


Figure 2.1: The iridium oxide sensor as shown with iridium being deposited on to the gold surface.

2.18.4. Nafion Coating:

Some of the Iridium oxide microelectrodes were coated with proton-exchanging Nafion resin by dipping them into 1%, 5% or 10% Nafion solutions and subsequently air-drying them at 80 °C. The pH microelectrodes were ready for use after they were soaked in a universal buffer of pH 7.0 for 2 days, which is essential to avoid potential drift [32][38]. When not in use, the microelectrodes were stored in universal buffer solution (pH 7.0) at room temperature in the dark.

2.18.5. Nafion-Polyphenol Coating:

A mixture of equal amounts of 5 % Nafion and 50 mM Phenol was prepared. The iridium oxide sensor was dipped into this solution and the polymer was formed by electropolymerization with a fixed current of 0.95 V for 30 minutes. The freshly formed polymer on the sensor surface was then dried under a stream of nitrogen.

2.18.6. Sensor Calibration:

The iridium oxide sensors were then calibrated in pH 4, pH 7 and pH 10 standard solutions. The following readings were obtained,

pH 4 = - 483 mV

pH 7 = - 275 mV

pH 10 = -61 mV

The sensors were routinely calibrated against the above standard solutions before using everytime.

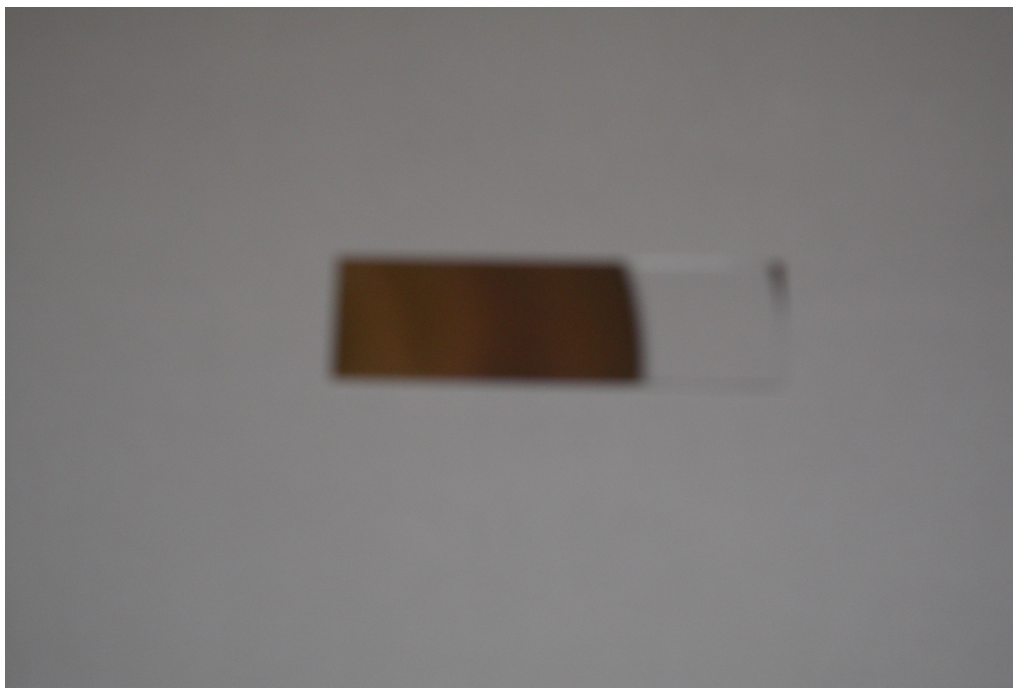


Figure 2.2: The iridium sensor shown was prepared on a glass slide. The glass side was first plated with gold and then iridium oxide was deposited following Nafion coating.

2.18.7. Preparation of Iridium Sensor on Glass Slides:

First, the slides are degreased by rinsing in acetone within an ultra-sonic bath for 15 minutes, then rinsing in isopropyl alcohol, then deionised water, followed by drying with nitrogen gas.

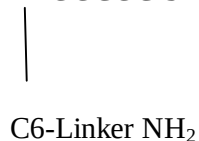
Then the slides were loaded into an evaporator and 20 nm titanium / 70nm of gold were respectively electron-gun evaporated onto them under high-vacuum (10^{-7} mbar)

Once the gold was sputtered onto the glass slides, Iridium oxide was deposited on the gold surface with the same protocol as described in section (2.18.3).

2.18.8. Immobilizing of Oligonucleotides on the sensor surface:

The reverse and forward primers were modified with thymine at 3' end was linked with C6 linker

Forward primer 5'-GTCGCGGTAATTGGCGC-3'



Reverse primer 5'-GGCCACGTGTTTTGATCGA-3'

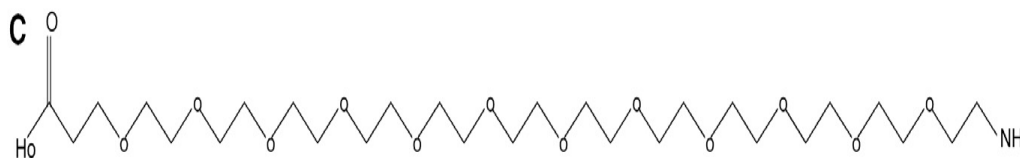
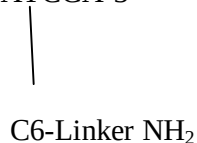


Figure 2.3: The C6 amino linker to aid in surface immobilization using a dPEG cross-linker (amino-dPEG₁₂ acid).

The poly(methyl methacrylate) PMMA when coated on iridium oxide glass slide produced surface-confined carboxylate groups by UV activation under a UV light (500 W) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) chemistry. The iridium oxide glass slides now coated with PMMA were then rinsed thoroughly with 2% isopropyl alcohol (IPA) and then with double-distilled water (ddH₂O), followed by drying under nitrogen gas. PMMA slides were then cross-linked with a dPEG spacer (amino dPEG₁₂ acid) obtained from Sigma (UK).

The steps in the EDC chemistry as mentioned above included the incubation of the surface with 100 mM Mes containing 10 mM EDC / 5 mM NHS for 30 min, followed by 100 μ M of the dPEG₁₂ for at least 5 h at room temperature, after which the slides were rinsed thoroughly in purified water and dried with compressed air. Therefore, in this step the amino group of the dPEG₁₂ was attached to the PMMA coated sensor surface, leaving the acid-functional group of the cross-linker available for attachment of the reverse and forward primers, which contained an amino group. Then the oligonucleotides were dissolved in 100 mM Mes (pH 5.5) containing 10 mM EDC/5 mM NHS to a final concentration of 100 nM, spotted onto the substrate by micropipetting 0.2 μ l of the appropriate solution onto the iridium sensor, and incubated for 2 h at room temperature.

2.19. TRANSCRIPTION MEDIATED AMPLIFICATION (TMA):

Transcription mediated amplification was performed using the following reagents,

1. T7 RNA Polymerase from New England Biolabs, UK.
2. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) from NEB, UK.
3. dNTPs, NTPs from Fermentas, UK.
4. DNase/ RNase free water from Sigma, UK.
5. Oligonucleotides from Eurofins, Germany.

Two reagent mixes were prepared for TMA experiments which are as follows,

a. Amplification Mix:

T7 promoter attached MS2 Sense Primer = 8 μ l (400 nM)

MS2 antisense primer = 8 μ l (400 nM)

dNTPs = 16 μ l (300 nM)

NTPs = 16 μ l (300 nM)

DNase/RNase free water = 22 μ l

b. Enzyme Mix:

M-MLV :4 μ l (200 units)

RNA Polymerase : 4 μ l (200 units)

1 $\times$ RNA buffer : 12 μ l

In the Enzyme Mix, the RNA buffer was firstly replaced with 1 $\times$ M-MLV buffer and then subsequently with water in order to observe the buffering effects which will be discussed later.

The TMA reaction was started by adding 10 μ l of purified MS2 RNA in 70 μ l of Amplification mix at a temperature of 62 $^{\circ}$ C for 15 minutes. The reaction was performed on 2 hot plates using Eppendorf 2 ml tubes. The total reaction volume was 100 μ l.

After the first incubation 20 μ l of Enzyme Mix was added and incubated for between 10 and 90 minutes at 42 $^{\circ}$ C.

The measurement of pH was performed using the Iridium oxide sensors at between 1 and 10 minutes intervals.

CHAPTER 3: RESULTS AND DISCUSSION:

3.1. GEL ELECTROPHORESIS RESULTS:

The MS2 RNA was purified after virus revitalization and ran on a 3% Agarose gel. The purified RNA showed visible bands in the following gel.

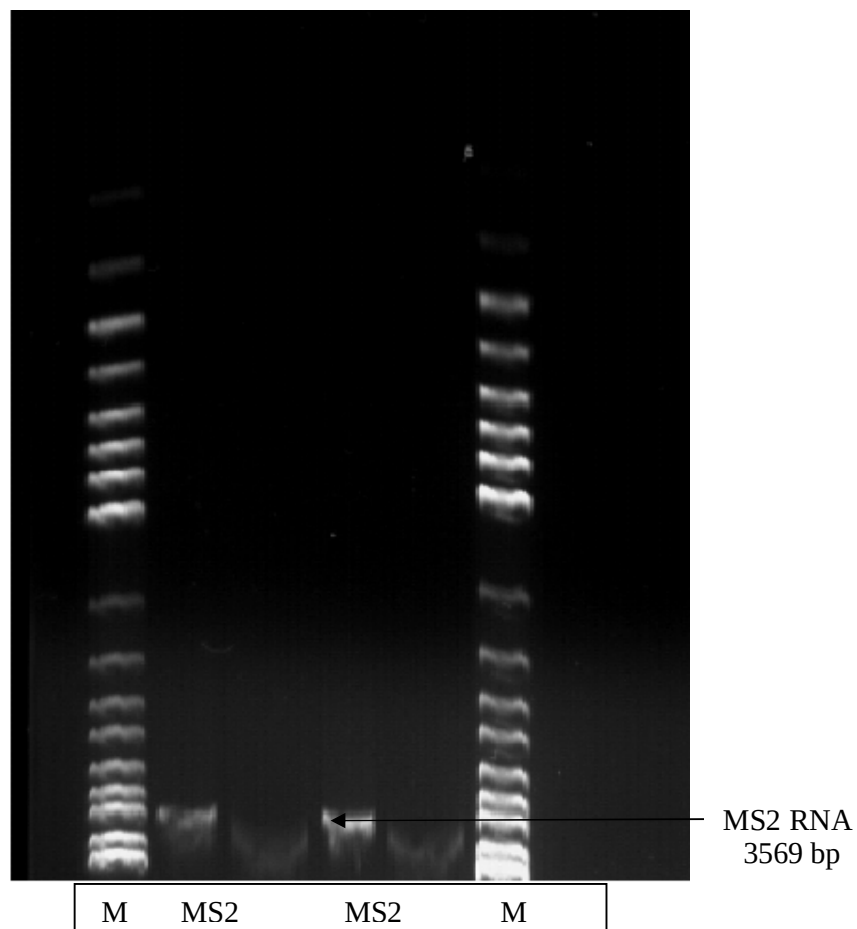


Figure 3.1: Agarose gel electrophoresis analysis of the purified MS2 RNA. The gel was 3% Agarose in TBE buffer and the RNA was visualised by ethidium bromide and UV light.

3.2. ABSORBANCE MEASUREMENTS:

The MS2 RNA was quantified by measuring the A260/A280 ratio and the following concentration was calculated,

Using Beer's law $A = \epsilon C l$

$$A = 0.08$$

$$\epsilon = 25 \mu\text{g}/\mu\text{l}/\text{cm}$$

$$l = 1\text{cm}$$

$$C = A / \epsilon l = A \times 40 \mu\text{g}/\text{ml} = 0.08 \times 40 \mu\text{g}/\text{ml} = 3.2 \mu\text{g}/\text{ml}$$

Since the dilution factor was 125

$$\text{MS2 RNA Concentration} = 125 \times 3.2 \mu\text{g}/\text{ml} = 400 \mu\text{g}/\text{ml}$$

3.2.1. MS2 RNA Concentrations:

All the concentrations of MS2 RNA shown in the following work are in nanograms (ng) in microlitres (μl).

3.3. REAL TIME RT-PCR:

The real time pcr experiments were performed in order to quantify the MS2 RNA with known concentrations of MS2 standards. The MS2 standards were prepared by inserting the nucleotide sequence of MS2 assembly protein into pET24 a vector.

Since we used Sybr green dye for quantification, we only had to calculate the data from the Green Channel. The plot below is showing the raw data collected in the Green channel. As we can see once the samples are amplified enough to be detected, they cross the threshold cycle and start to show fluorescence.

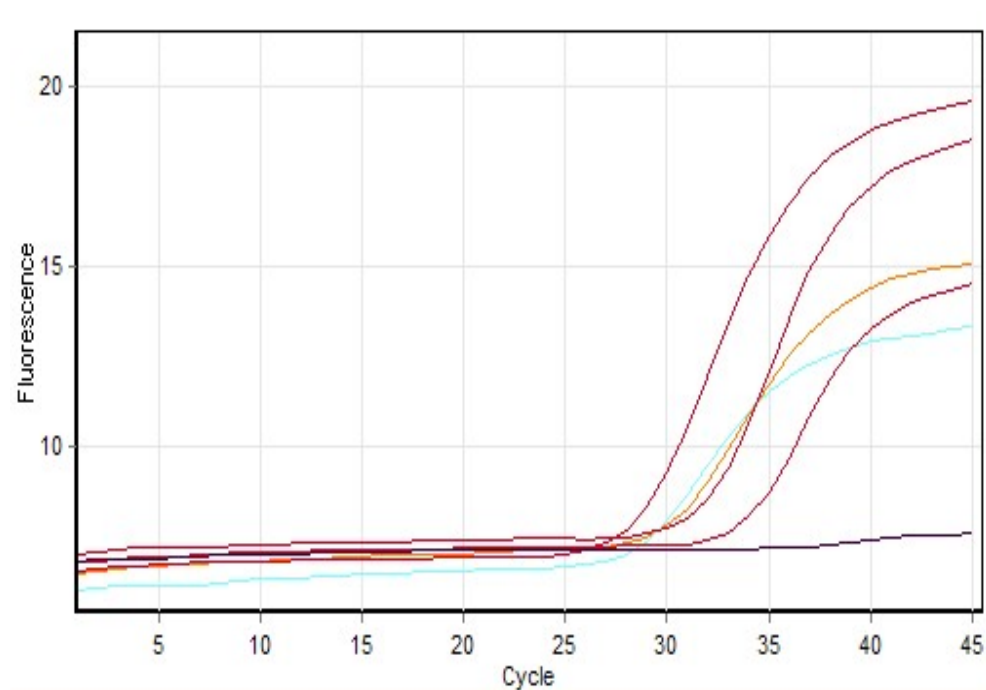


Figure 3.2: Plot showing raw fluorescence acquired at different cycles of PCR after the reverse transcription of MS2 RNA. The fluorescence generated in the plot by MS2 standards are shown in red whereas the yellow and green are the unknown concentrations of MS2 RNA purified after revitalization of the bacteriophage along with the NTC shown in purple.

3.3.1. Quantitation Data:

The raw fluorescence acquired by the real time instrument can then be converted into the quantitation data at the end of the reaction. By doing so one can then calculate the threshold (Ct) values for each of the know MS2 standards along with known MS2 RNA samples.

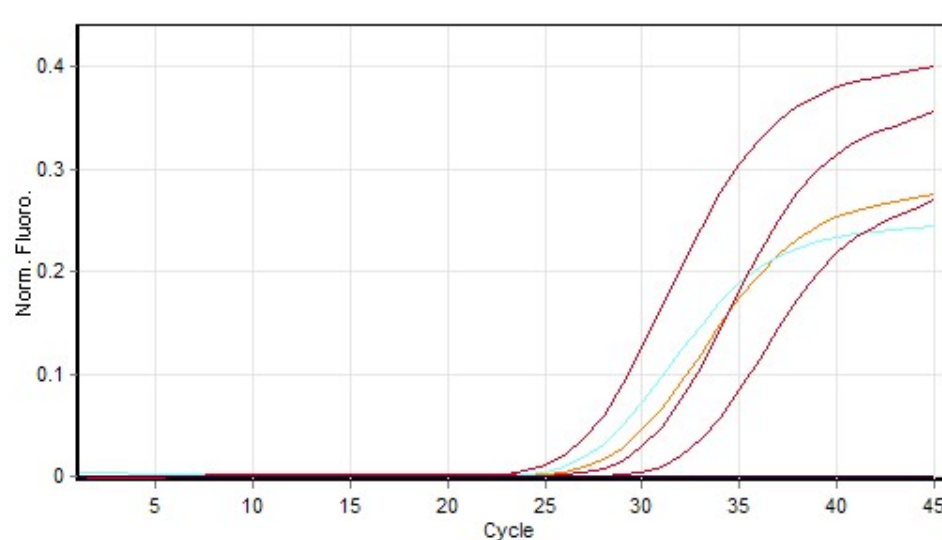


Figure 3.3: Plot Showing fluorescence generated by MS2 standards in red curves whereas the unknown MS2 RNA samples are shown in yellow and green curves during Real Time PCR. The threshold Ct value of all the above samples shown in figure 3.3 were then calculated and a standard curve was plotted as shown in figure 3.4.

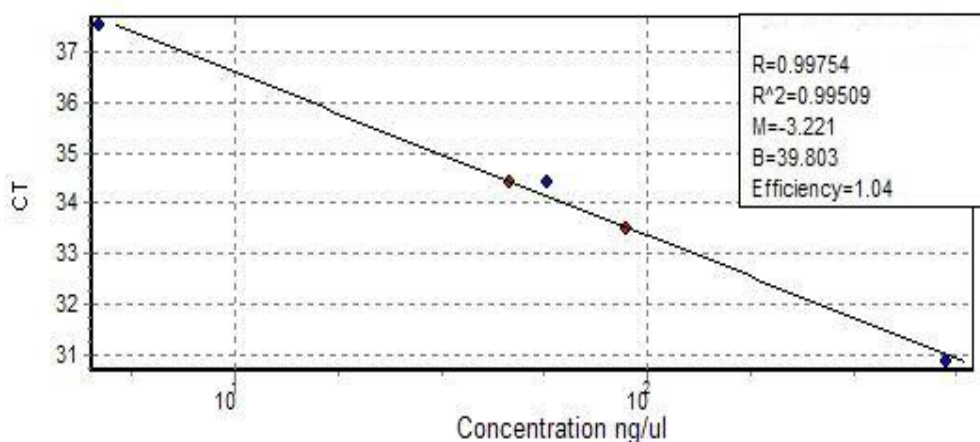


Fig 3.4: Standard Curve plotted by Rotor Gene 6000 with Ct values of standards against the MS2 RNA concentration.

The rotor gene software then by using the Ct values plotted the standard curve for the known MS2 RNA concentrations.

No.	Color	Name	Type	Ct	Given Conc. (ng/ul)	Calc Conc. (ng/ul)	% Var
1	Yellow	MS 2 RNA	Unknown	34.43		46.5	
2	Green	MS 2 RNA	Unknown	33.52		89.4	
3	Red	MS2 standard 3	Standard	37.51	4.7	5.1	9.50%
4	Red	MS2 standard 2	Standard	34.42	57	47.1	17.40%
5	Red	MS2 standard 1	Standard	30.88	532.7	589.4	10.60%
6	Purple	Neg. Ctrl.	NTC	NEG (NTC)			

Table 3.1: This table shows the Ct values of the known MS2 standards as well as the unknown MS2 RNA samples.

The concentration values for the unknown samples were calculated by interpolation of the standard curve. As we see in the above table the concentration for purified MS2 RNA were calculated as 46.5 ng/ul and 89.4 ng/ul.

The experiment was very reliable as we can see from the above table that % variation for the standards used ranged from 9.5% to 10.6% which is acceptable [47]. The experiment could further be improved by using dual-labeled probes since Sybr green does not specifically bind with the target gene and hence can produce non-specific fluorescence. For this we optimise the protocol of Qiagen Quantiprobe kit by using different concentrations of salt. It has been suggested in the previous studies that by using more than 3 standard we can further increase the accuracy of the standard curve as done in many studies in the past [48].

By quantifying our MS2 RNA we made our samples ready for the iridium oxide sensor based detection and establish a method for quantifying the RNA content of unknown samples.

3.4. HIGH RESOLUTION MELT (HRM):

Using HRM with SYTO 9 dye, the Rotor gene was used to further characterize samples based on sequence length, GC content and DNA sequence complementarity. HRM when performed after reverse transcription and DNA amplification for wild type MS2 RNA samples yielded the following results,

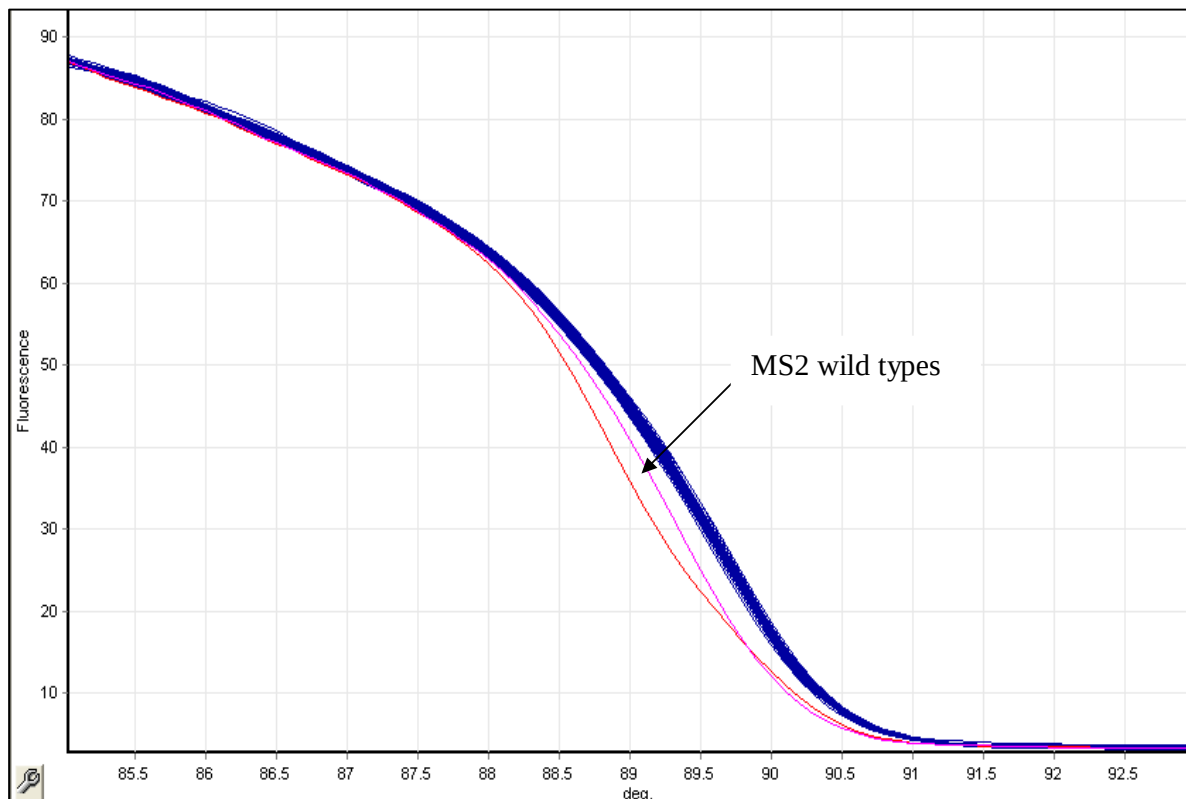


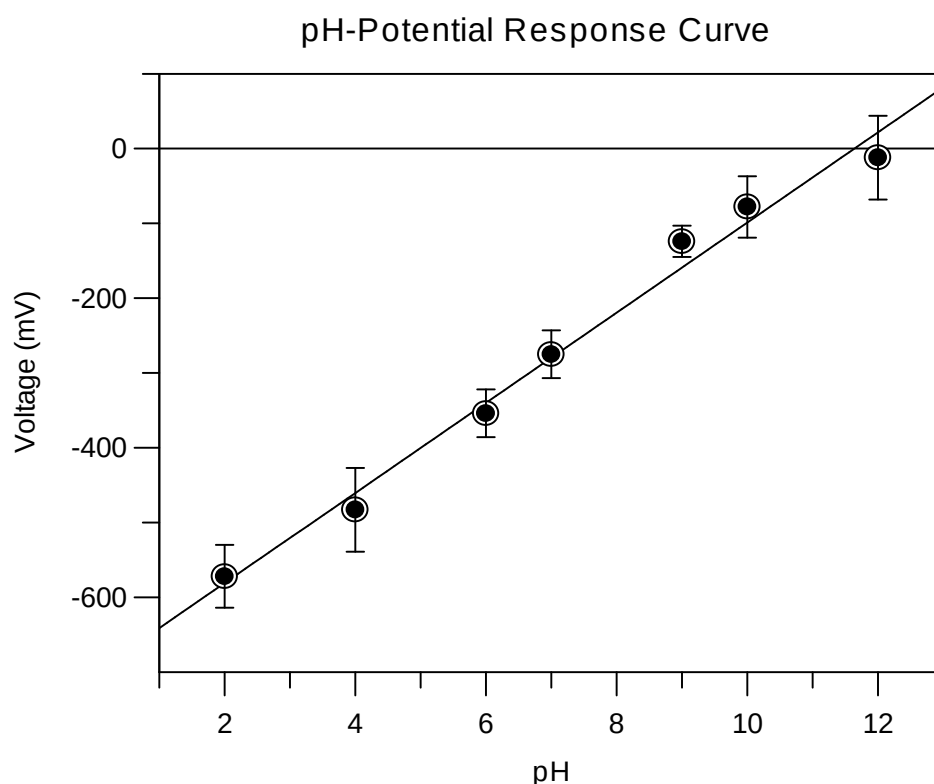
Figure 3.5: The change in fluorescence of MS2 RNA samples is shown when subjected to melt by decreasing the temperature after PCR amplification.

As can be seen, after amplification, the samples were melted and the fluorescence diminished since the dye only intercalates with dsDNAs but not ssDNAs. The samples were initially analyzed by the shift on the temperature axis. Since we know the length of the wild type MS2 RNA which is 3659 bp as seen in the plot in a cluster of blue lines, we found out that 2 of the samples shown in red did not show the same melting behaviour possibly due to a shorter length in the sequence

by some sort of deletion in the sequence. With these results we were able to use the wild type MS2 RNA for our further experiments.

3.5. pH-POTENTIAL RESPONSE CURVE:

The pH-potential response curve for iridium oxide sensor was measured in pH 2- 12 buffer solutions and the following behaviour was seen.



Parameter	Value	Std. Error
a (intercept)	-701.7451	24.6780
b (gradient)	60.2843	3.1487

Figure 3.6: Plot showing change in voltage measured by Iridium oxide sensors in different pH solutions in triplicate.

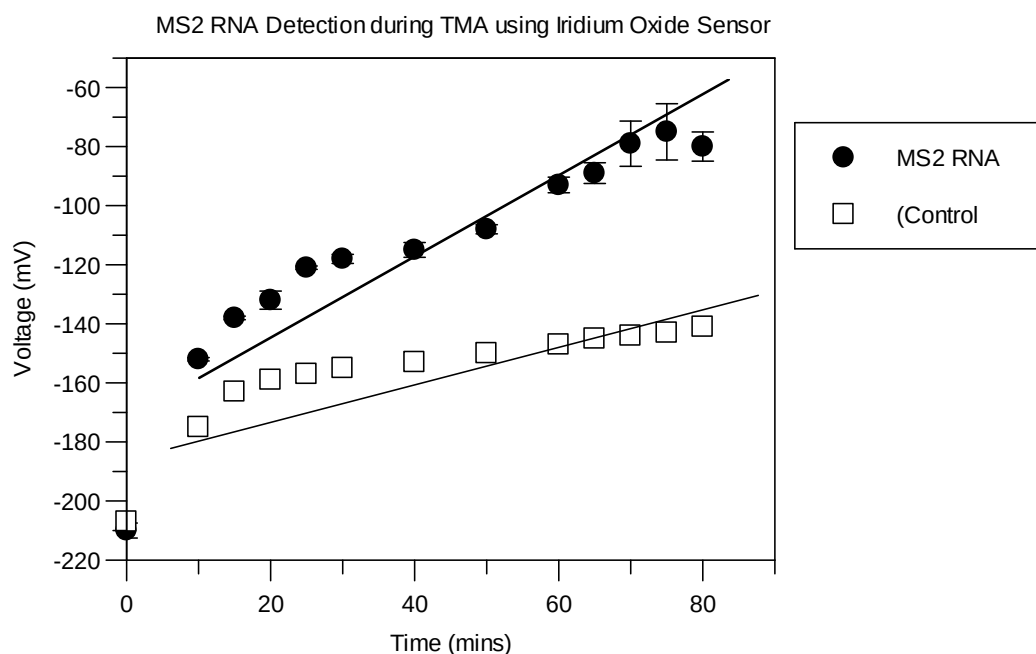
The above pH response curve represents a slope of -60 mV/pH and a correlation coefficient is 0.99. The voltage response of the iridium oxide sensor to the changing pH is linear and is near

ideal Nernstian behaviour in the pH range 2-12. The linear response also indicates that the anions present in the pH solutions did not have any adverse effect on the pH response. The standard error of 3 further gives a 95% confidence level. The pH-response curve was also observed regularly and it was noticed that iridium sensors show a drift of 3mV/pH in the calibration curve which may be due to the redox interference of the storage Tris buffer.

3.6. Application of the Iridium Oxide Sensor in TMA.

(a) Effect of Run time:

A series of TMA experiment were performed in order to determine the run time where detectable number of RNA copies and protons can be generated.



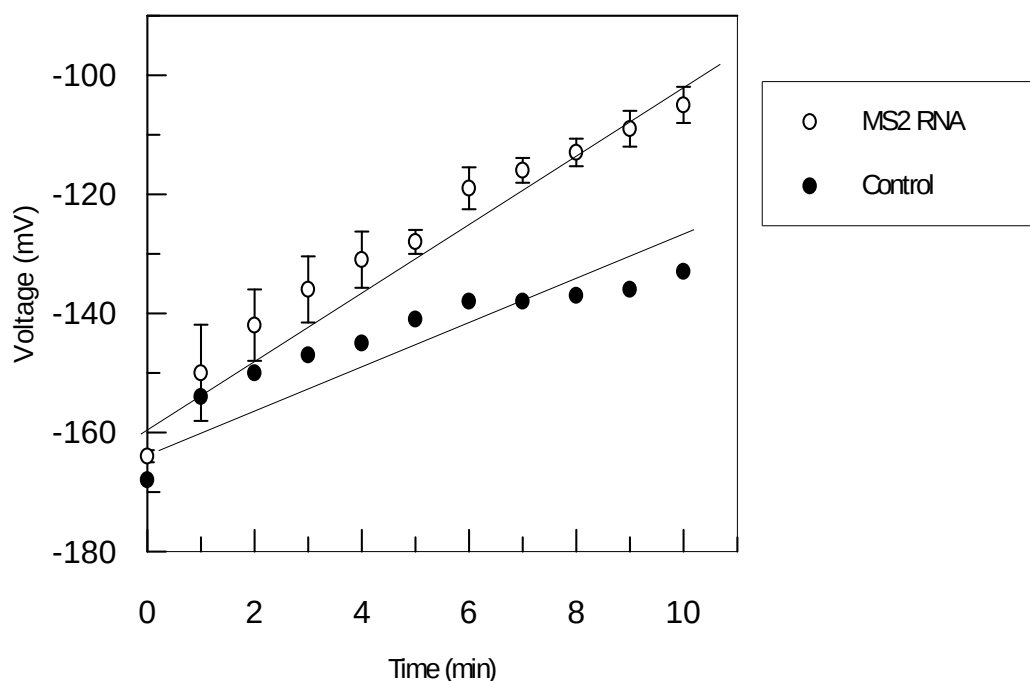
Parameter	Value	Std Error
a (intercept)	-168.899	8.0634
b (gradient)	1.2698	0.1649

Figure 3.7: A triplicate of 50 ng MS2 RNA was detected by using iridium oxide sensor during TMA reaction for 80 minutes.

As seen above in fig. 3.7 that significant change of around -60 mV in voltage was detected by the iridium oxide sensor in the first 10 mins of the TMA reaction. This voltage corresponds to 1 pH

unit change in the reaction according to the pH response curve. The rate of the change in MS2 RNA amplification decreases as the time progressed due to dNTPs and NTPs being used up during the reaction and resulting in producing less number of protons or enzyme activity may also be changing as a function of pH. This relates to the decrease in the change in voltage.

As most of the change in potential occurred in the first 10 minutes the measurements were repeated with greater time resolution.



Parameter	Value	Std Error
a (intercept)	-155.636	2.191
b (gradient)	5.4364	0.3703

Figure 3.8: A triplicate of 50 ng MS2 RNA was detected by using iridium oxide sensor during TMA reaction for 10 minutes.

The above plot illustrates that as soon as the enzyme mix is added, the reaction shows a significant change of around -12 mV even in the very first minute and then show a steady change in voltage in the next 9 minutes. This is may be due to the different buffer pH between the enzyme mix and the RNA solution. The slop for MS2 RNA sample was 5.4 mV/min whereas the slope for the control was 2.82 mV/min. Ideally the slope for the control should be zero but here in the case the slope is significantly higher corresponding to background pH drift. This was investigated later in this work. It was therefore decided to carry out the further study in a run time of 10 minutes.

3.7. Dose Response Curve:

The following change in voltage was observed while using the iridium sensors during TMA reaction with different concentrations of MS2 RNA. The reaction contained concentrations of purified MS2 RNA along with the other components of TMA in 0.2 ml tubes.

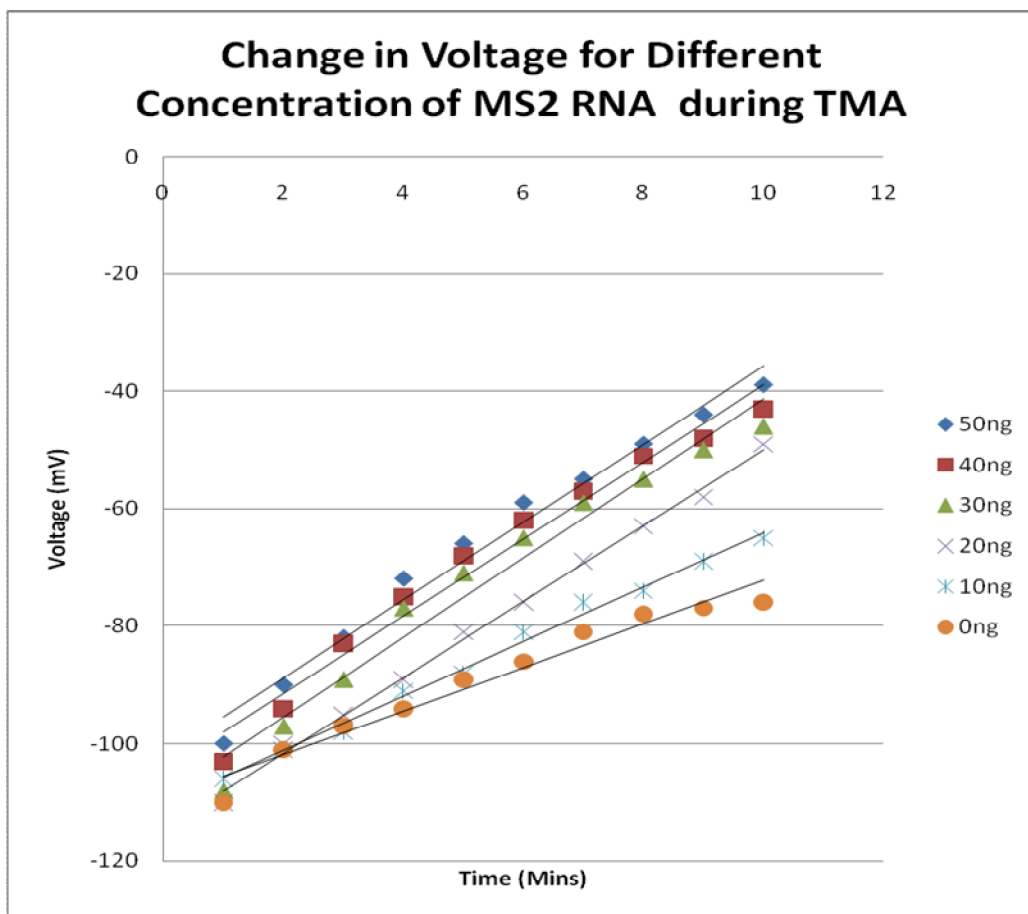
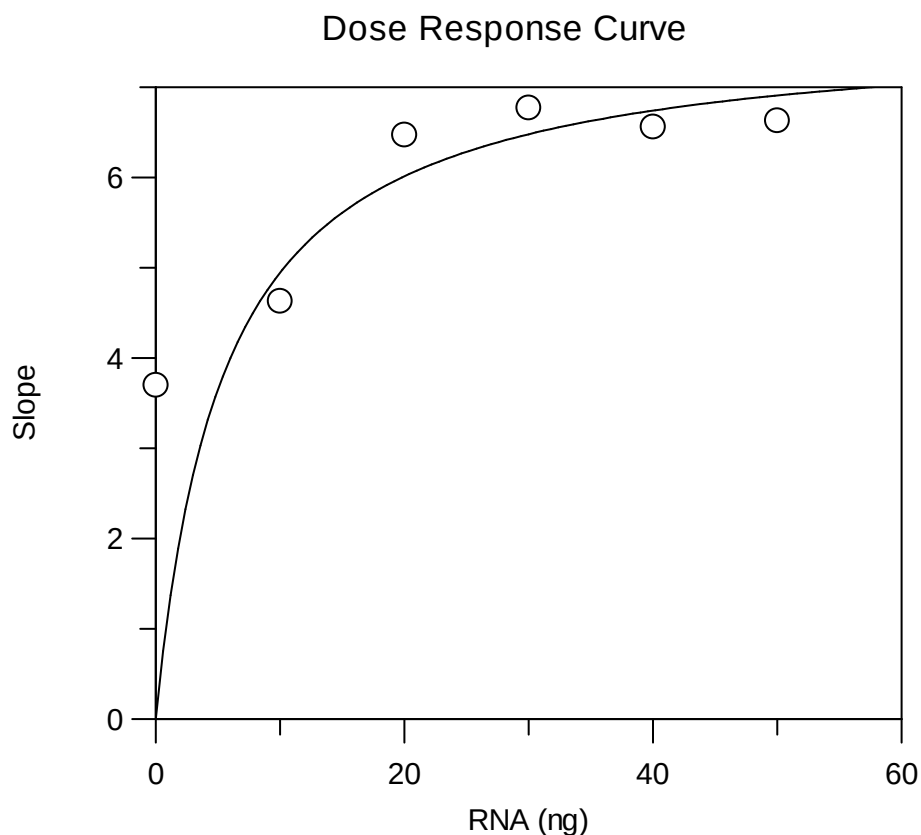


Figure 3.9: Plot showing change in voltage (mV) for different concentrations of MS2 RNA.



Parameter	Value	Std Error
a (intercept)	7.6691	2.3296
b (gradient)	5.5174	8.7705

Figure 3.10: The dose response curve for different concentration of MS2 RNA has been shown in the above plot.

As can be seen there is a difference in the slopes of various concentration of MS2 RNA, but a background signal is seen in the control which contains no RNA. The slope for 50 ng and 10 ng MS2 sample was 5.9 mV/min and 4.6 mV/min respectively whereas the slope for the control was 3.7 mV/min. These values do show the qualitative results by giving signal change for positive samples but do not show the quantitative response for increasing concentrations. In order to improve the discrimination between the control and sample responses and to investigate the

background signal control in the control and samples, a series of experiments were carried out which are described below.

3.8. Sensor Response in Complex Samples:

In order to see the effect of proteins on the response of iridium sensor, the purified MS2 RNA was spiked into PBS containing 20 mg/ml of albumin and the response to MS2 observed with the sensor on a series of three replicates of MS2 RNA as shown in figure 3.11.

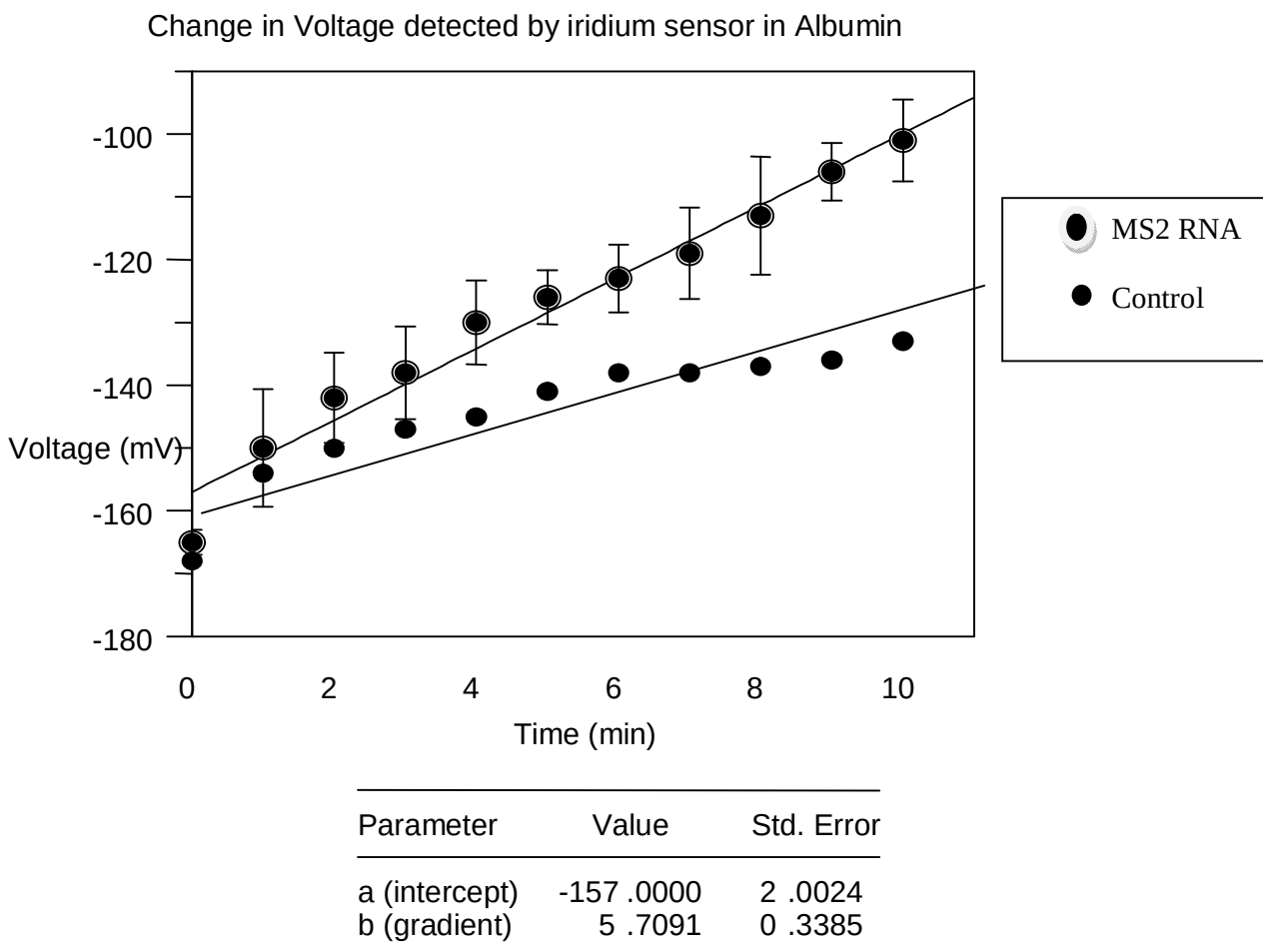


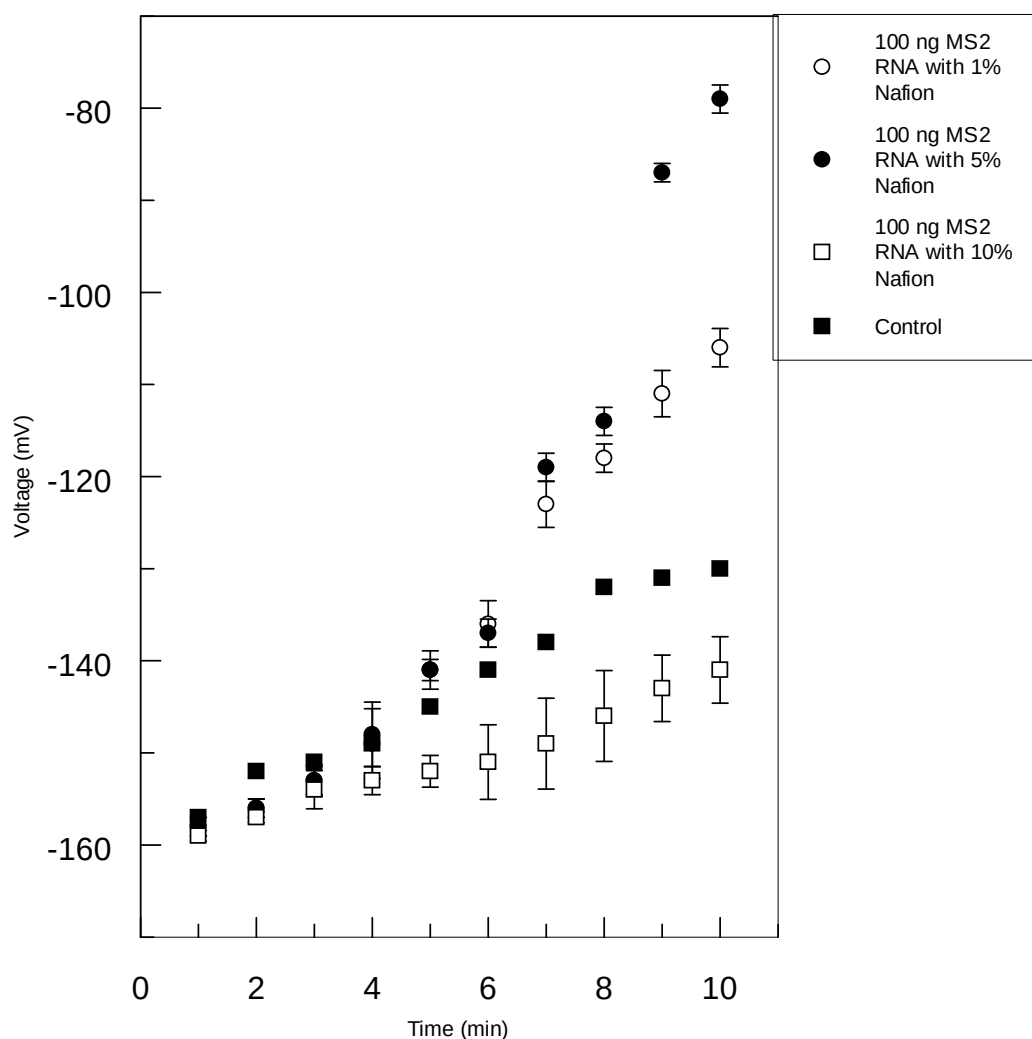
Figure 3.11. Plot showing change in voltage of MS2 triplicates 50 ng in 20mg/ml albumin solution.

As can be seen above the detection ability of the sensor was minimally affected while using a high concentration of Albumin. The slope for MS2 RNA without albumin was 5.43 mV/min whereas with 20 mg/ml the slope for the same concentration of MS2 RNA is 5.7 mV/min. Similarly the slope for the control with zero albumin was 2.87mV/min and with 20 mg/ml the slope was 2.81 mV/min. The standard error in both experiments was 0.37 and 0.34. The high level of protein concentration therefore did not seem to interfere with the sensor. However in the presence of albumin the standard deviations was slightly increased from 3.5 to 6.35 as can be seen by observing the error bars in figure 3.11. In order to produce reproducible results, linearity and lower the sample to sample variation the iridium sensors were coated a with layer of nafion.

3.8. Improvement of Iridium oxide sensor:

3.8.1. Coating of Iridium Sensors with Nafion:

Nafion is negatively charged polymer which exclude anions and attracts cations. It has been used as a coating film in a number of metal-oxide pH electrodes [49]. Since a high background signal was seen with iridium oxide sensor with no coating on it, it was decided to use Nafion as a selective membrane for detecting protons during TMA reaction and to stop any possible interferences. Firstly the sensor surface was coated with different percentage of Nafion concentrations (1%, 5% and 10%. w/v).



	Slope	Std Error
MS2 RNA detected with 1% Nafion coated iridium sensor	6.3091	0.3754
MS2 RNA detected with 5% Nafion coated iridium sensor	8.9697	1.0046
MS2 RNA detected with 10% Nafion coated iridium sensor	1.897	0.1075
Control with No Nafion coated iridium sensor	3.1636	0.159

Figure 3.12. Plot showing triplicates of 100 ng purified MS2 RNA being detected by 3 different Iridium sensors coated with 1%, 5% and 10% w/v Nafion.

As seen from the above plot the 5% Nafion coating showed us the highest average slope of 8.96 mV/min compared to 6.3 mV/min and 1.89 mV/min for 1% and 10% Nafion coating respectively. The slope for the control is 3.16 mV/min which is even higher than the slope for the MS2 RNA detected with 10% Nafion coated iridium oxide sensor which clearly indicate that by coating higher percentage of Nafion on iridium oxide sensor restrict the number of protons penetrating through the Nafion layer towards the sensor surface.

3.9. Dose Response Curve with Nafion Coated Iridium Sensor:

Since 5% Nafion gave the largest slope for the triplicates of MS2 RNA in the above reaction, we plotted a voltage response curve for purified MS2 RNA in water using 5% Nafion coated Iridium oxide sensor during TMA reaction.

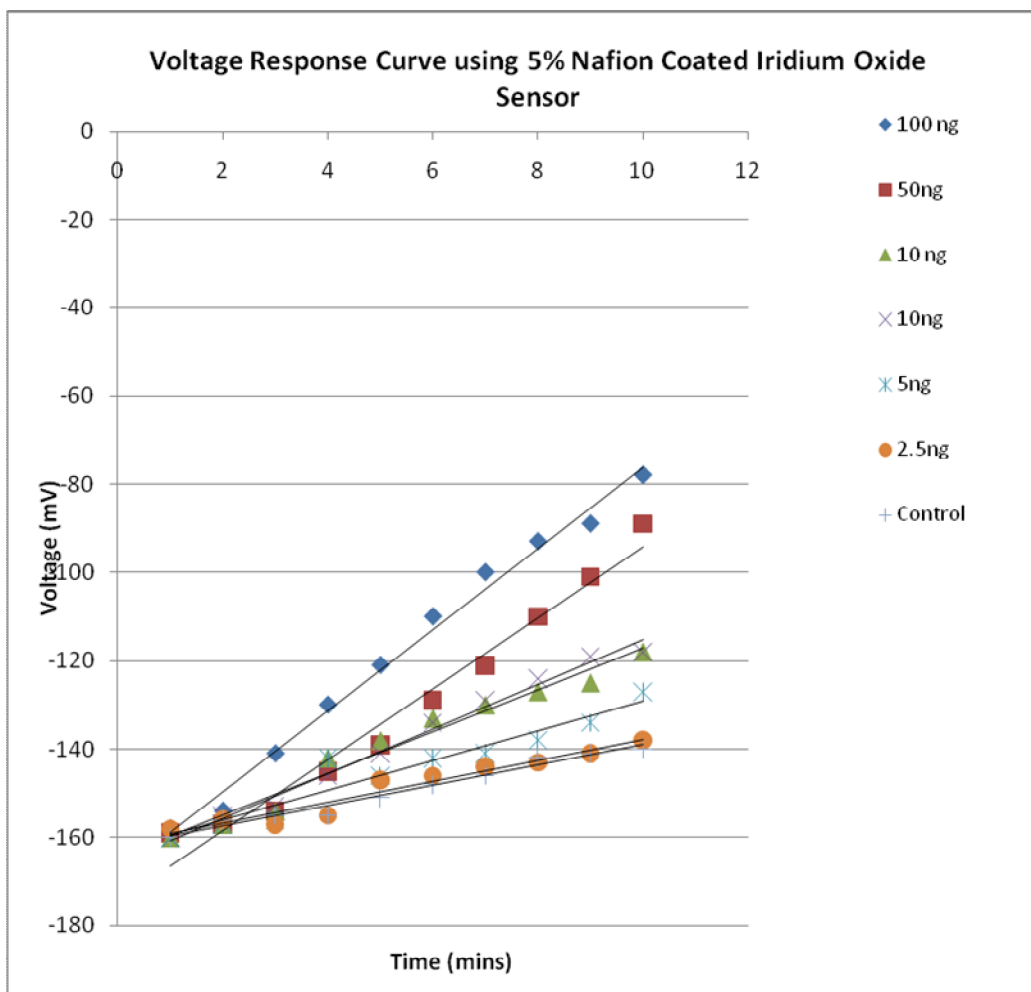
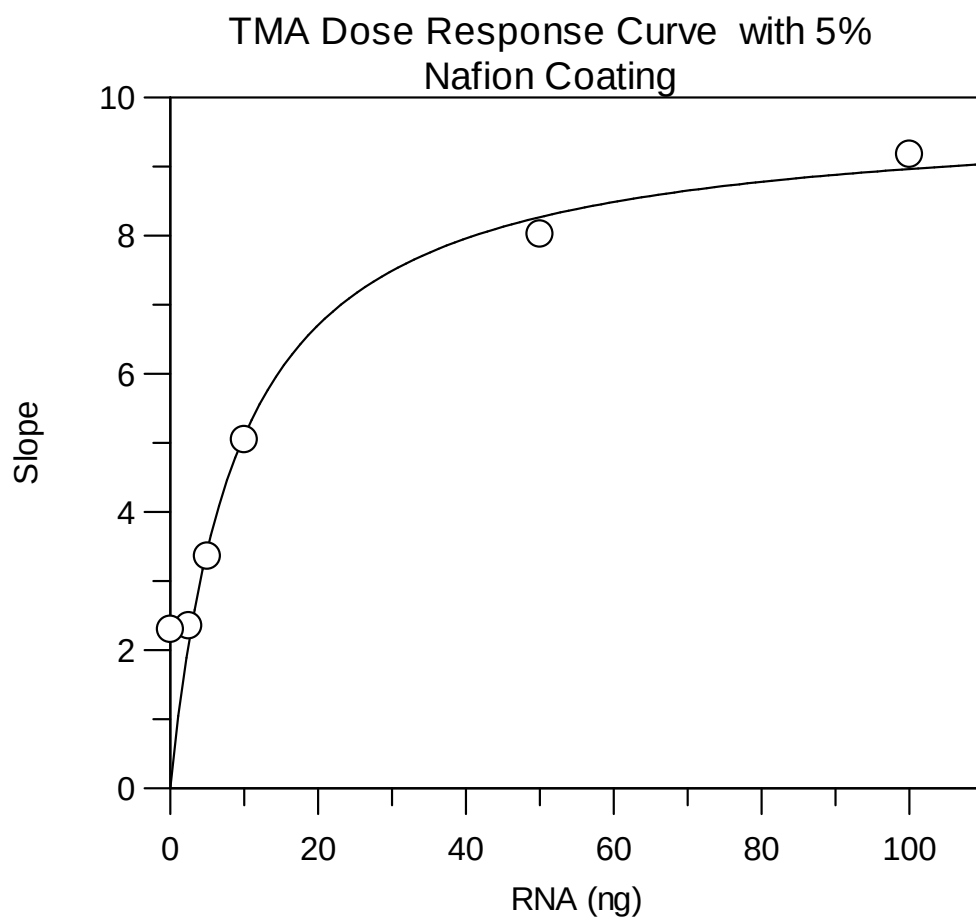


Figure 3.13: Plot showing the change in voltage for different concentration of MS2 RNA being detected by 5 % Nafion coated iridium sensor at regular intervals.

The above plot shows a quantitative change in voltage with varying concentrations of MS2 RNA. The slope for the 100 ng MS2 RNA is 9.18 mV/min whereas for 3.36 mV/min. However since the slope for 2.5 ng and the control are 2.35mV/min and 2.30 mV/min respectively, it is difficult to differentiate between the background signal the RNA detection.



Parameter	Value	Std Error
a (intercept)	9.7872	1.2396
b (gradient)	9.2079	4.024

Figure 3.14: Dose response curve shown with 5% Nafion coated iridium oxide sensor at different concentrations of MS2 RNA.

3.10. Effects of TMA Components on Iridium oxide Sensor:

Although with 5% Nafion coating we managed to decrease the background, however we further wanted to investigate the real cause of background signal in the control. For this purpose we carried out a series of experiments see the effects of different components of TMA such NTPs and enzymes on Sensor's detection capability during the reaction. Therefore 3 different controls were used, each missing one of the components suspected of causing the background signal. In the following reaction 20mg/ml albumin was used instead of purified MS RNA in water.

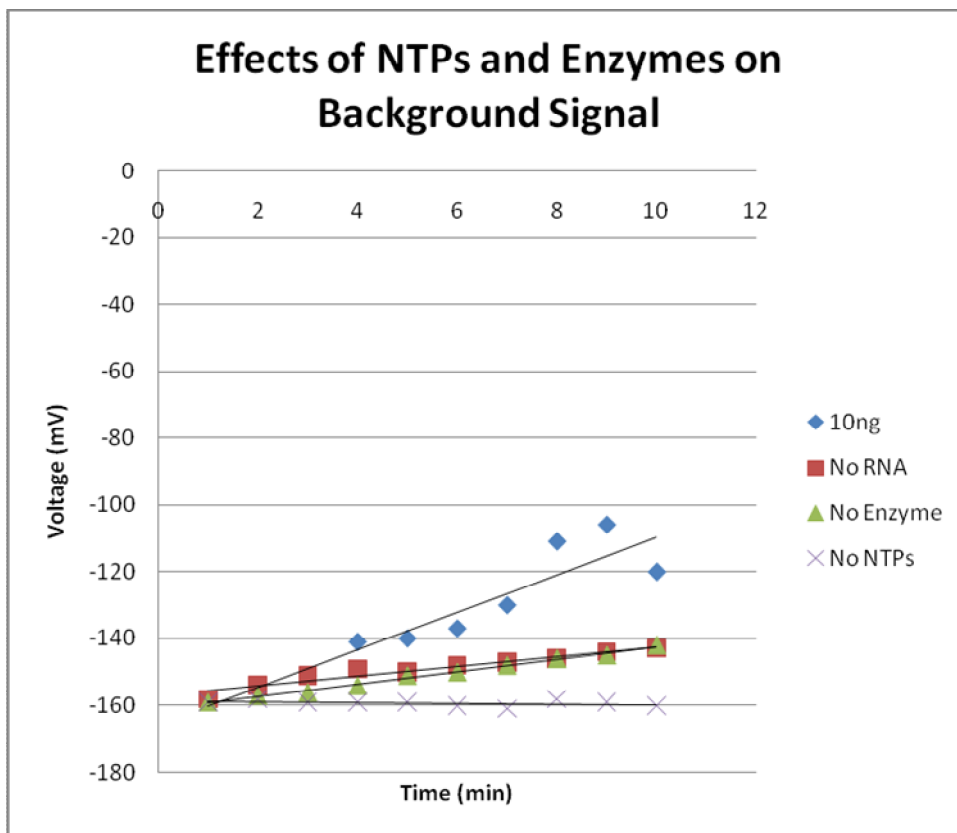


Figure 3.15: Plot showing the change in voltage against time for MS2 RNA samples with different TMA components.

As can be seen from the plot the sample containing 10ng of MS2 RNA with all the TMA components has a slope of 5.59 mV/min whereas the sample containing all the TMA components but no RNA showed a slope of 1.44 mV/min. Similarly the samples containing 10 ng MS2 RNA with all TMA components without enzymes (RNA polymerase and MMLV) and without NTPs showed slopes of 1.44 mV/min and 0.11 mV/min respectively. It was clear at this point that the background signal was mainly contributed by the NTPs whereas the enzymes do not have any effect in causing the signal noise. This interference was thought to be caused by the degradation of NTPs and producing protons during the TMA reaction. In order to minimize this, NTPs must be structurally modified to resist the release of protons. Similarly another approach is to use a more selective polymer membrane on the sensor surface to restrict the diffusion of undesired species.

3.11. Dose Response Curve in Albumin:

The iridium sensors with Nafion coating showed promising results for detecting purified MS2 RNA in water but in reality the sensor should show the same performance in complex samples as well. In order to detect the MS2 RNA in more complex environment, the water was replaced with a PBS buffer with 20 mg/ml albumin dissolved in it. This protein rich environment was then spiked with known concentrations of MS2 RNA and then detected with iridium oxide sensor.

A voltage response was observed for MS2 RNA in 20 mg/ml albumin using 5 % Nafion coated Iridium sensor to investigate if this high level of protein could have any effects on the newly prepared sensor. Please note the control used in the further experiments was without the NTPs in order to minimize the background signal.

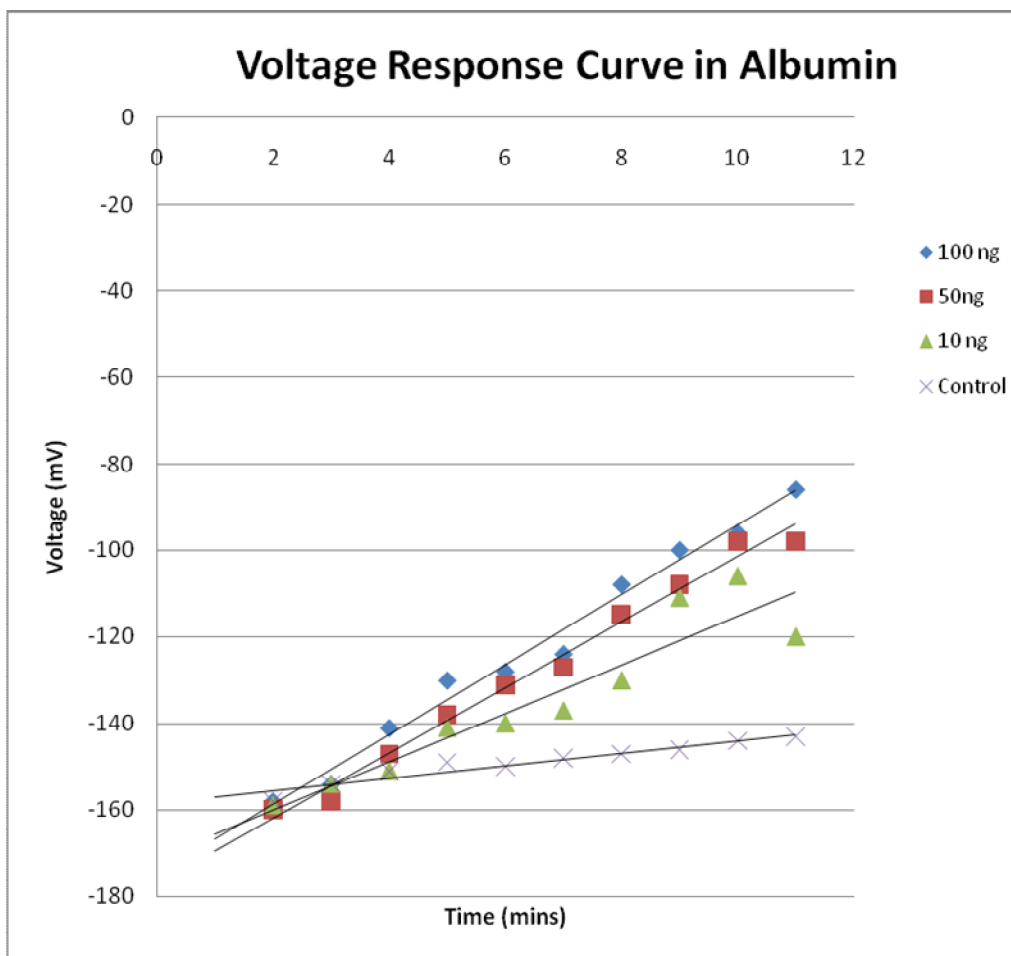
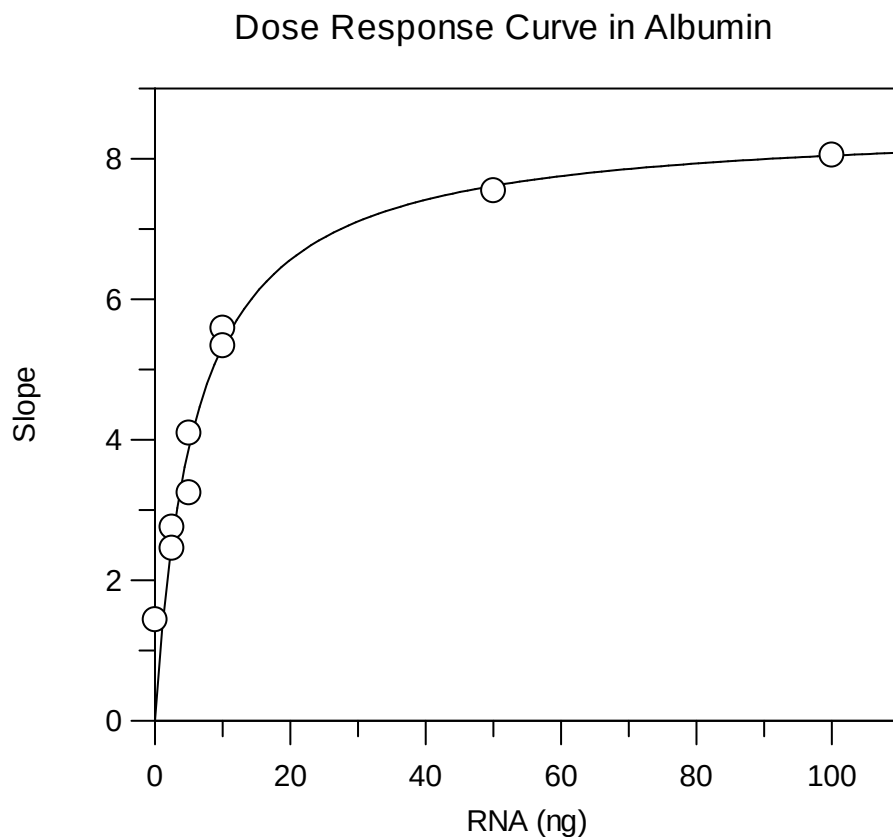


Figure 3.16: Plot showing change in voltage for different concentrations of MS2 RNA in 20mg/ml albumin using iridium oxide sensor coated with 5 % Nafion.

The above plot indicates that albumin seem to slightly suppress the voltage signal of samples with higher concentrations for MS2 RNA. For instant, the slope for the 100 ng MS2 RNA sample in purified water was 8.9 mV/min whereas when used MS2 RNA sample in 20 mg/ml albumin in PBS buffer the slope is 8.05 mV/min. Similarly for 50 ng the slope for MS2 RNA in water is 8.02 mV/min whereas in albumin the slope is 7.55 mV/min. Also the albumin did not effect the linearity of the plots with standard deviation of ≤ 2 mV/min.

This indication that iridium oxide sensor can be used MS2 RNA detection in more complex environment lead the work to plot a dose response curve for different concentrations of the bacteriophage .



Parameter	Value	Std Error
a (intercept)	8.5254	0.5541
b (gradient)	5.9957	1.184

Figure 3.17: Dose response curve against different concentrations of MS2 RNA in 20 mg/ml of albumin.

The dose response curve showed a standard error of 1.18 which gives us a 95% confidence level that the iridium sensor can detect MS2 RNA in protein rich environment ranging from 10 ng to 100 ng MS2 RNA concentrations.

3.12. Lower Limit of Detection in Albumin:

With a confidence level of detecting 10ng MS2 RNA in protein rich samples, further investigations were performed to determine the lower limit of detection for the iridium sensor with 5 % Nafion coating. The lower limit of detection is 3 standard errors above the control. The MS2 RNA samples in albumin but at concentrations lower than 10 ng in this study.

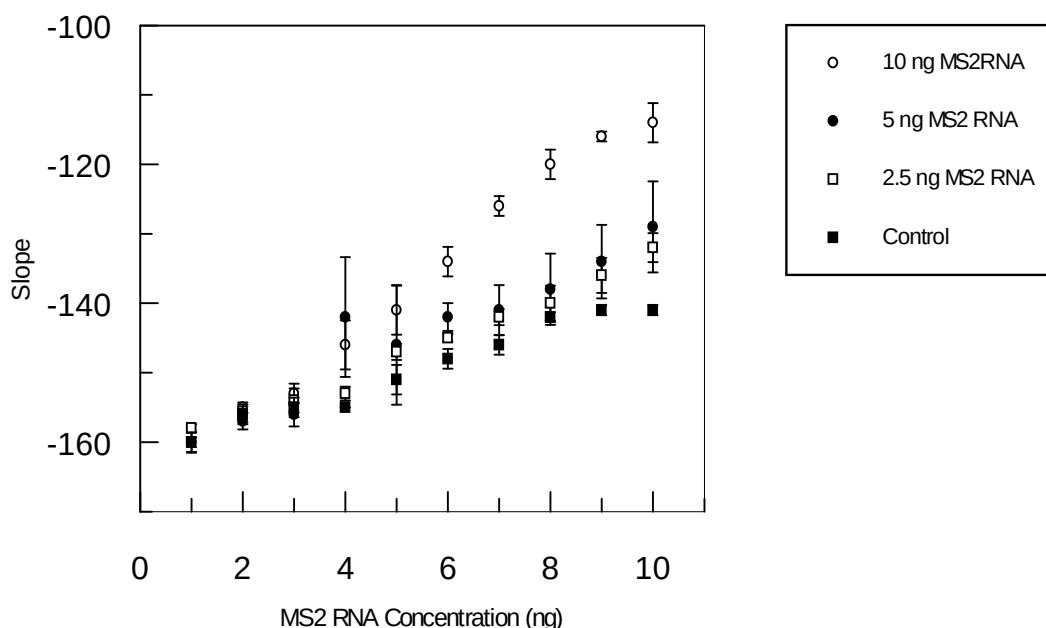


Figure 3.18: Plot showing the slope against the lower concentrations of MS2 RNA in albumin.

As it can be seen in the above plot that 10 ng and 5ng of MS2 RNA in albumin are detectable concentrations with a slope of 5.57 mV/min and 3.3 mV.min respectively with the error bars not over lapping the control's error bars. Whereas the slope of 2.5 ng and the control are 2.9 mV/min and 2.24 mV/min respectively and with the standard errors of 0.12 for 2.5 ng and 0.14 ng for

control it was established that 2.5 ng MS2 RNA concentration cannot be a detectable amount by iridium oxide sensor or it was simply the background signal.

3.13. Detection of MS2 RNA using Nafion-Polyphenol Iridium oxide Sensors:

3.13.1. Nafion-Polyphenol Coating:

Polyphenol is a size exclusion polymer and has been used in the past as coating film for pH electrode sensors [50]. Since in this study, the detectable species were the protons generated during TMA amplification there was also a need for cation attractive film. To achieve this, a mixture of polyphenol (50 mM) and Nafion (5 %) was created. This complex polymer was supposed to enhance the selectivity and permeability of the diffusing molecules to the electrode's surface surface during TMA reaction.

3.13.2. Dose Response Curve with Nafion-Polyphenol Coated Iridium oxide

Sensor:

The iridium sensor was coated with Nafion-Phenol oxidation to increase the sensitivity of the sensor and the following dose response curve was observed.

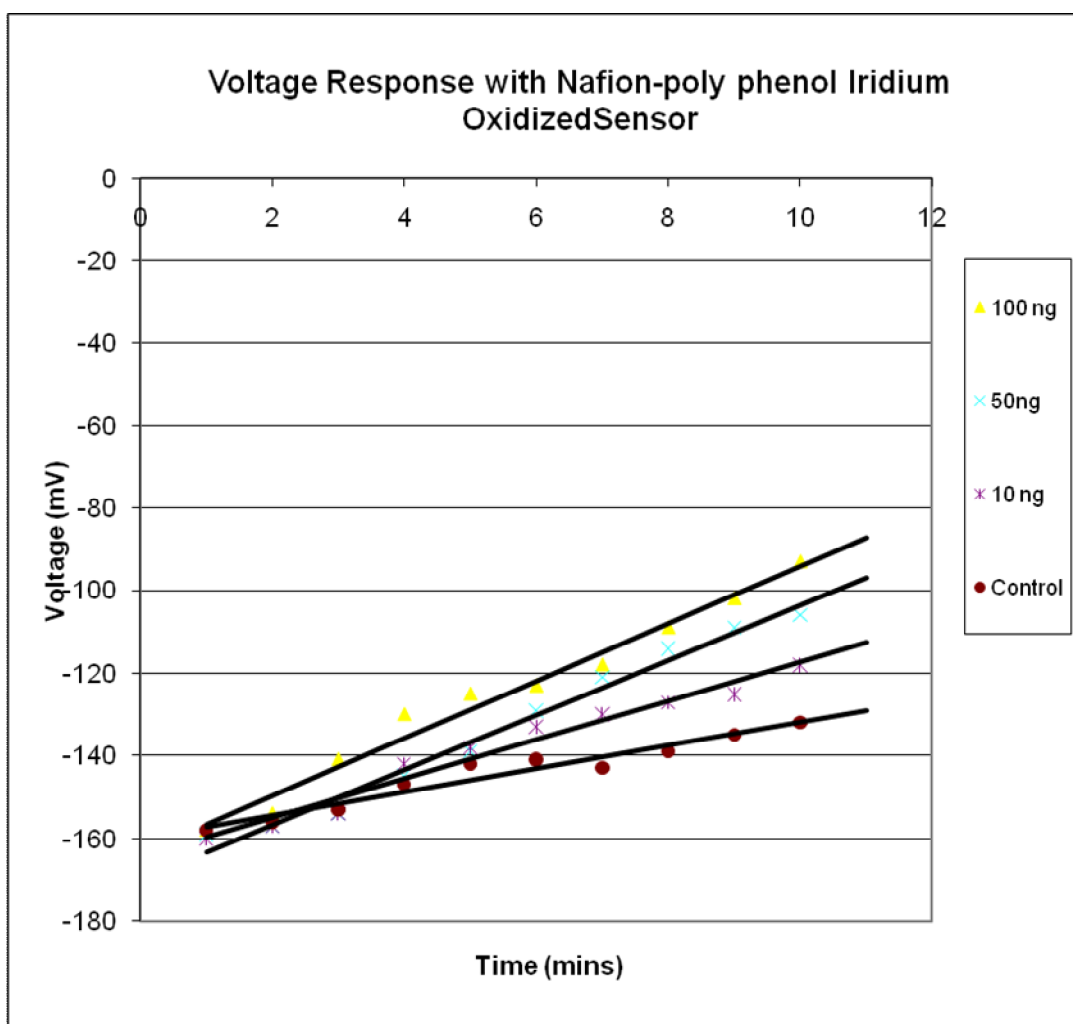
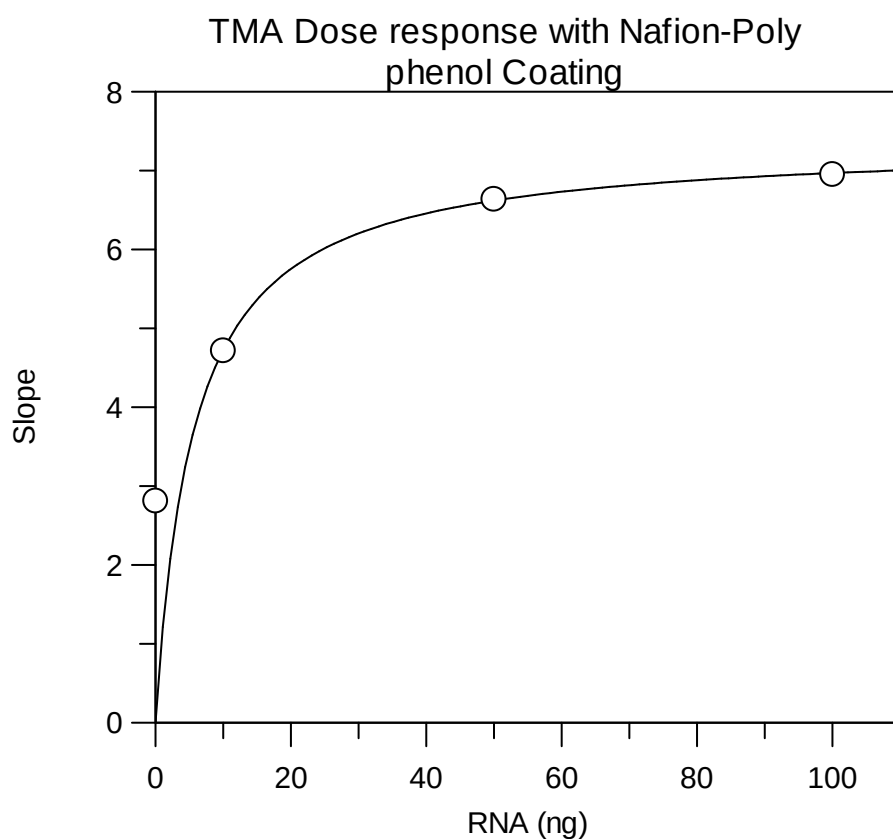


Figure 3.19: Plot showing the voltage response curve for MS2 RNA with Nafion-Polyphenol coated iridium oxidized sensor.

The response curve to same concentrations of MS2 RNA using Nafion-polyphenol coated sensors showed lower slopes when compared to the slopes measured using only Nafion coated iridium sensors. For instant the slopes for 100 ng and 50 ng of MS2 RNA were 8.05 mV/min and 7.55 mV/min respectively when measured with Nafion coated iridium sensor whereas with Nafion-polyphenol coated iridium sensor the slopes for 100 ng and 50 ng decreased down to 6.5 mV/min and 6.63 mV/min respectively.



Parameter	Value	Std Error
a (intercept)	7.3565	2.0771
b (gradient)	5.5882	9.1061

Figure 3.20: Dose response curve against different concentrations of MS2 RNA detected with Nafion-poly phenol coated iridium oxidized sensor.

When a dose response curve for MS2 RNA detection with Nafion-polyphenol coated iridium oxide sensor was studied, it was found that the standard error was enormously increased to 9.1 as compare to Nafion coated iridium sensor which was 1.18. With such standard error it was unable to establish the 95% confidence limit to detect the MS2 RNA concentrations ranging from 10 ng to 100 ng.

3.13.3. Limit of Detection with Nafion-Polyphenol coated Iridium oxide

Sensor:

In order to establish the limit of detection for detecting lower concentration of MS2 RNA using Nafion-Polyphenol iridium oxide sensor the following study was carried out in which 2.5 ng, 5 ng and 10 ng of MS RNA were used.

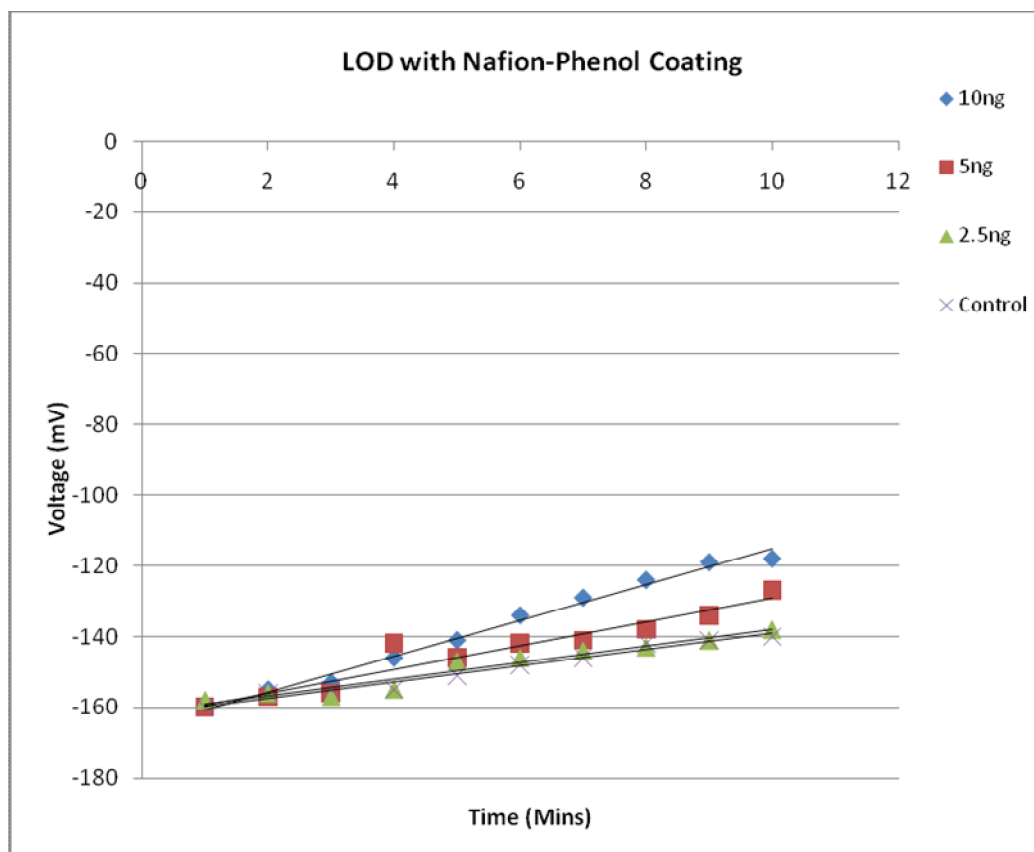


Figure 3.21: Plot showing the lower limit of detection for MS2 RNA with Nafion-poly phenol coated iridium oxidized sensor during TMA.

As seen in the below plot the lower limit of detection has decreased significantly for concentrations lower than 10 ng MS2 RNA when Nafion-polyphenol iridium oxide sensor was used to detect MS2 RNA during TMA. The slope for 10 ng of MS2 RNA with Nafion coated iridium oxide sensor was 5.57 mV/min and a similar slope of 5.01 mV/min was observed with Nafion-polyphenol coated iridium sensor but the response time was delayed since there is no change in the voltage for the first 2 minutes. Whereas for 5 ng MS2 RNA the slope was decreased from 3.2 mV/min to 2.35 mV/min which fell within the 3 standard error above the slope of the controls' slope

of 2.5. It was therefore established that the lower limit of detection of iridium sensors having Nafion-polyphenol coating with 95% confidence level was 10 ng.

The above observations however suggest that coating the iridium oxide sensor with Nafion-polyphenol restricted the permeability of protons to the surface of iridium oxide sensor. This also effectively decreased the response time of the sensor as well since the protons had to diffuse through a more complex polymer than Nafion to reach the surface of the electrode.

3.14. Effects on Sensitivity on with Larger Surface Area:

3.14.1. Iridium Sensor on a Glass Slide:

In order to design a point of care hand held device applicable for viral detection we made an iridium oxide sensor with 5 % (w/v) Nafion coating on 3 mm × 3 mm glass slide. The voltage for different concentrations were measured as shown in the figure 3.22.

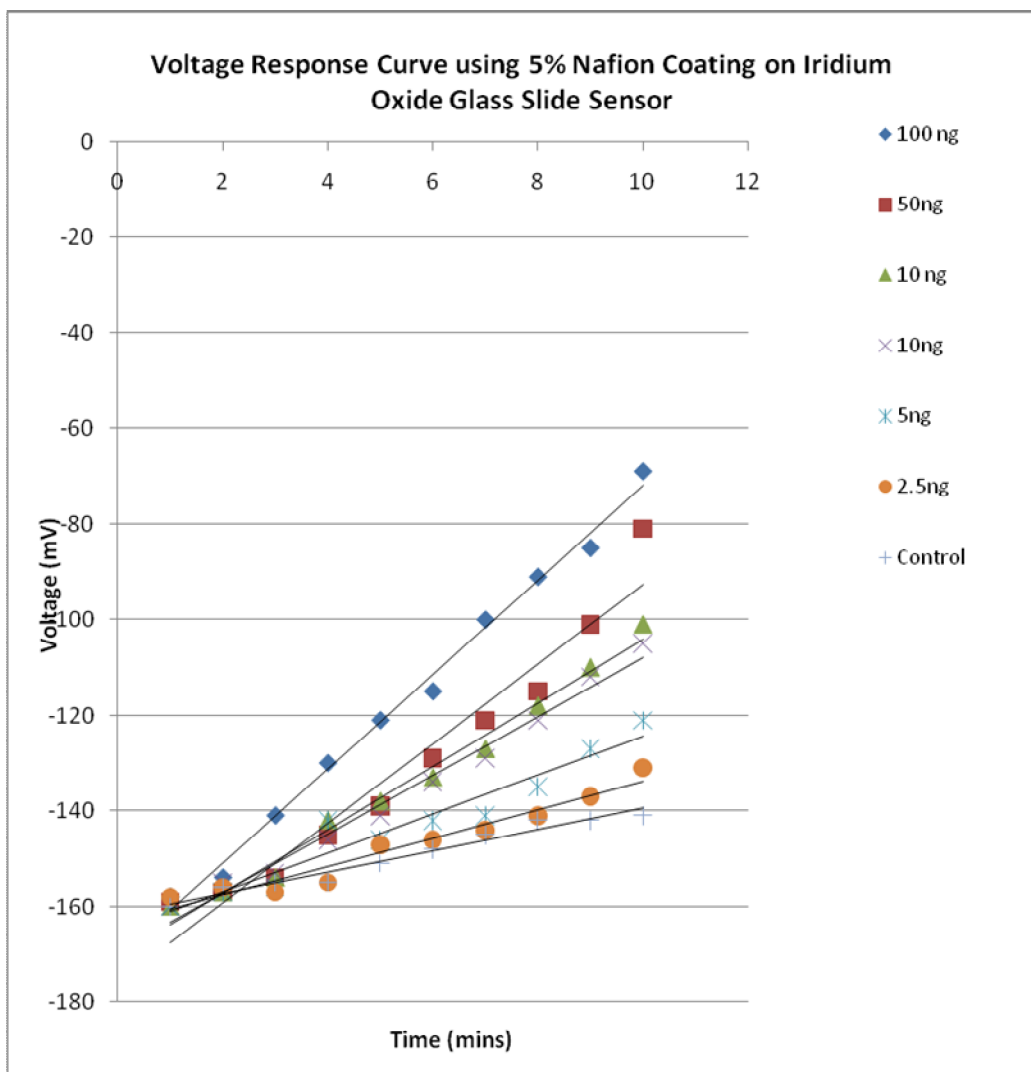
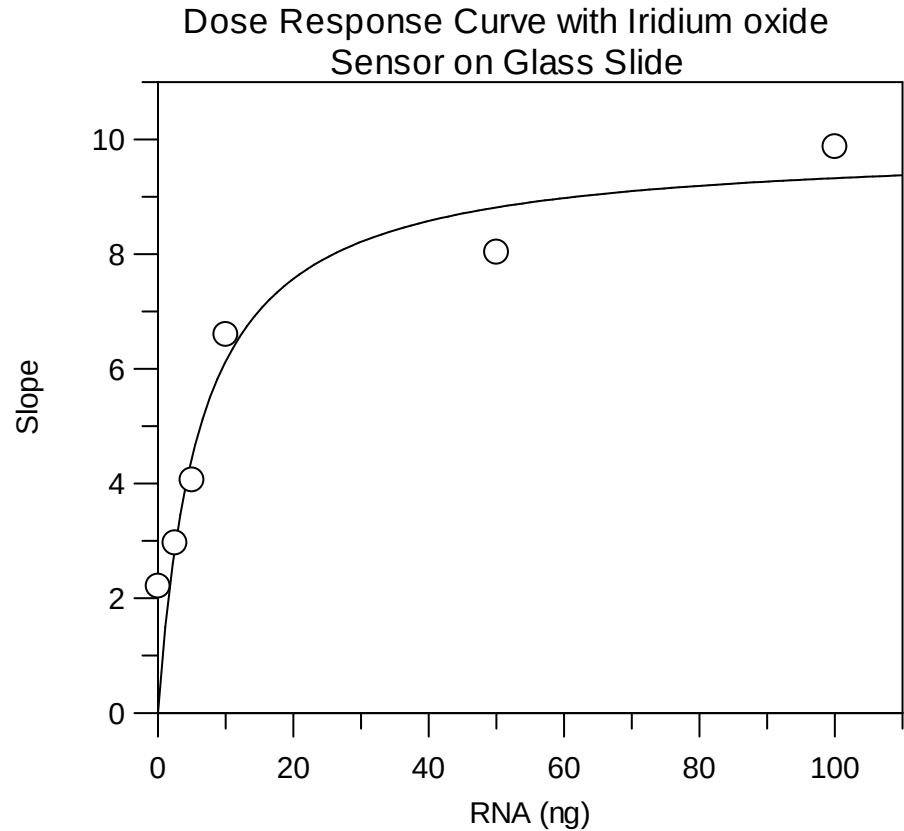


Figure 3.22: Plot showing the change in voltage when various concentrations of MS2 RNA in 20 mg/ml Albumin was detected with 5% Nafion coated Iridium oxide sensor on a 3mm×3mm glass slide.

The above plot shows a significant increase in the slopes of 10 ng and 5 ng as compare to the slopes shown by the previous iridium oxide sensor. The slopes for 10ng and 5 ng from the above plot are 6.5 mV/min and 4.08 mV/min whereas the slopes for 10 ng and 5 ng for 5.57 mV/min and 3.25 mV/min. The increase in slopes is possibly due to larger detection area exposed to the

cations. This means we can further reduce the detection time from 10 minutes to 5 minutes as the signals produced in 5 minutes can easily be distinguishable to the negative control.



Parameter	Value	Std Error
a (intercept)	9.897	1.1654
b (gradient)	6.1541	2.6813

Figure 3.23: Dose response curve against different concentrations of MS2 RNA using 5% Nafion coated Iridium oxide sensor on a 3mm × 3mm glass slide.

The dose response curve for MS2 RNA concentrations by iridium oxide sensor on a glass slide revealed a standard error of 2.68 which is higher than 1.184 the standard error produced by the original iridium sensor made in the plastic pipette tip. The standard error is higher in this case

may be due to the less number of samples being used. Even though the slope for 5 ng of MS2 RNA 4.08mV/min is above the 3 standard errors above the control's slope of 2.22 mV/min and therefore gives us a 95% confidence level of detection of MS2 RNA within the range of 5 ng to 100 ng.

3.15. Iridium Sensor with Immobilized Oligos:

In a further attempt towards the aim of designing a point of care device for viral detection, the reverse and forward primers were immobilized on Iridium oxide sensor, the dose response curve with MS2 RNA was plotted and observations were noticed.

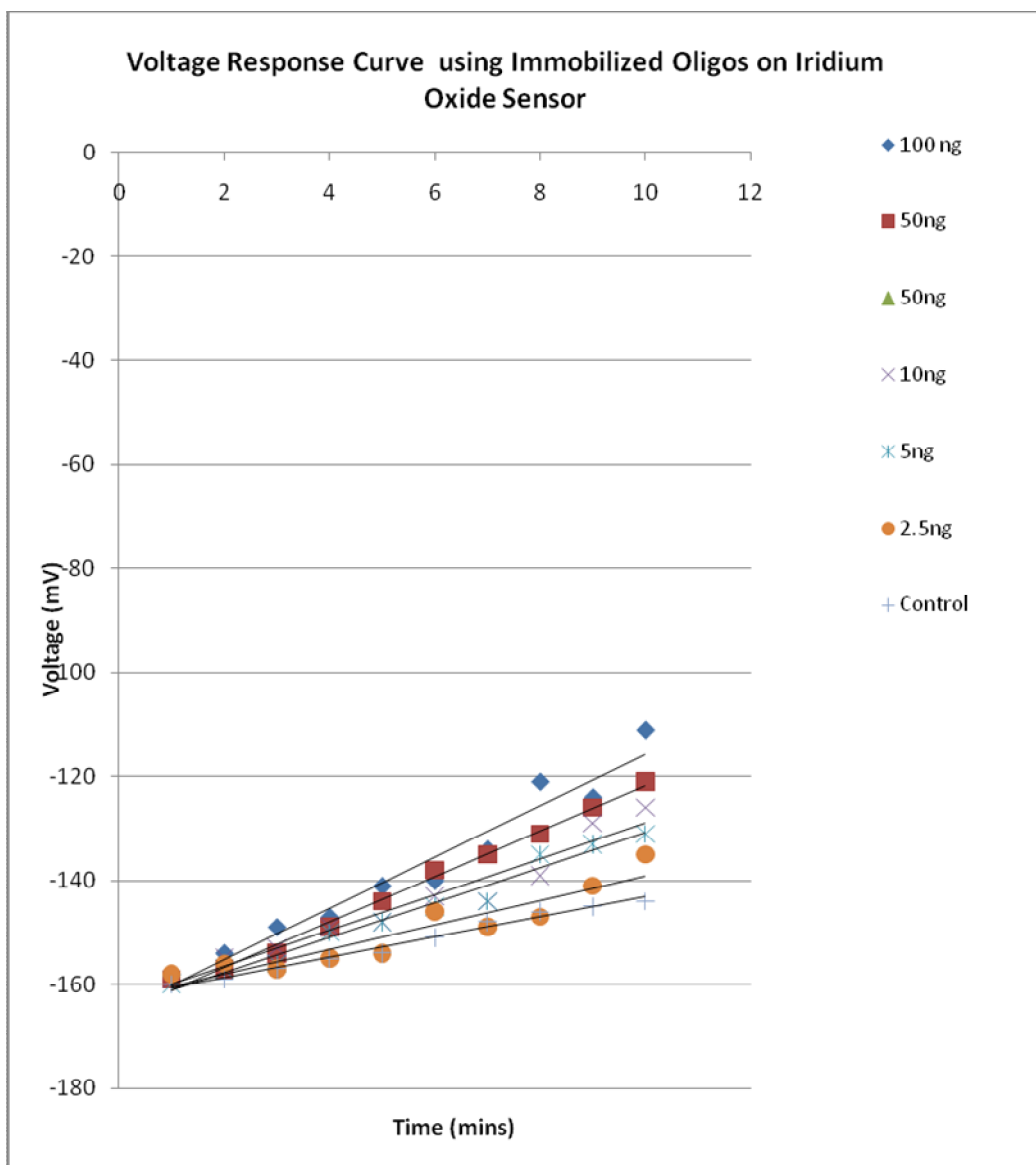
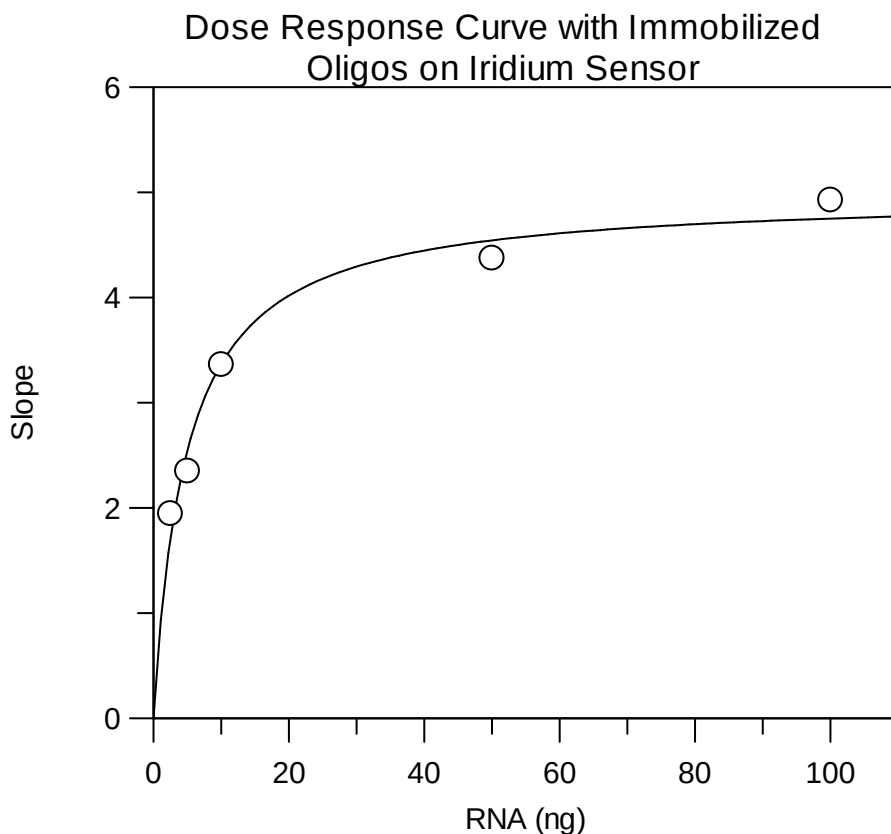


Figure 3.24: Plot showing voltage change with different concentrations of MS2 RNA in 20 mg/ml albumin using iridium oxide sensor with immobilized Oligonucleotides.

As seen from the above plot the immobilization of oligonucleotides did not produce better results. The slopes were significantly decreased for all concentrations of MS2 RNA. For instant the slopes for 10 ng and 5ng MS2 RNA were decreased from 6.60 mV/min and 4.07 mV/min to

3.34 mV/min and 3.36 mV/min. These slopes were found to be in the 3 standard errors of the control's slope of 2.25 mV/min.



Parameter	Value	Std Error
a (intercept)	4.977	0.1981
b (gradient)	4.7876	0.741

Figure 3.25: Dose response curve for different concentrations of MS2 RNA using iridium oxide sensor with immobilized oligos.

The dose response curve however shows a standard error of 0.741, much lower than the standard error of 2.68 for sensor without immobilized oligos due to less number of replicates being used. The replicates used in this experiment were 2 for each concentration whereas the replicates for

previous experiments were 3 – 5. Since the the slopes for 10 ng and 5 ng MS2 RNA were within the 3 standard error of the control, it was unable to establish a 95% confidence level for detecting even 10 ng of MS2 RNA with this sensor.

CHAPTER 4: CONCLUSION AND FUTURE CHALLENGES:

The overall aim of this work was to design a metal-oxide pH sensor which can be used for rapid detection of viral RNA during a RNA amplification method such as TMA. The iridium oxide sensor prepared in this work showed satisfactory results in comparison to the established real time PCR technology. TMA generated a series of protons during RNA amplification enabling the metal-oxide pH sensor to monitor the specific RNA by sensing the change in the pH in the sample. The iridium sensor showed a linear voltage response to the pH change and exhibited a near ideal Nernstian behaviour in the pH range 2 – 12 with a slope of -60 mV/pH. The iridium oxide sensor proved to be very sensitive which was able to detect 5 ng of MS2 RNA equally comparable to real time PCR. The detection time for MS2 RNA by iridium sensor was very rapid since a significant detectable change in signal was monitored in the first 10 minutes of the reaction. This detection time is far less than the current available technologies where the minimum detection time is around 2 hours.

The Nafion coating of the iridium-oxide sensor surface enhanced the selective permeability of the protons generated during TMA though the polymer and any possible interferences. The cause of background noise was found to be due to one of the components of TMA i.e, NTPs. The iridium sensor performed equally well when subjected to detect MS2 RNA in a protein samples thereby showing a potential sensor for clinical samples. The limit of detection remained the same i.e, 5 ng in albumin samples with a standard error of 1.18. The voltage response was further enhanced by designing the iridium sensor on a glass slide. The iridium oxide sensor on a glass slide showed better voltage response and slopes giving a possibility of reducing the detection time to 5 minutes.

Besides this methodology produced reproducible results and proved to be very cost effective and did not require any expensive instruments while the iridium sensors themselves were also cheap to prepare.

The iridium oxide sensor showed some great potential to be used a detection device for RNA viruses. The sensor when incorporated with an amplification technique such as TMA is very sensitive and specific methodology for detecting viral RNA. The method can be further explored by employing certain other membranes for coating iridium oxide sensor surface such as asolecitin or poly (N-vinylamide) to further reduce the interference and background caused by undesirable species.

Similarly the performance of the sensor should be analyzed with more complex samples such as blood or serum and should be validated with many other viruses like HCV, HSV and HIV. Since TMA can be used to amplify the complementary RNA of a DNA sample, the methodology is not limited to RNA detection and hence can be used to detect DNA viruses i.e, HBV and bacteria as well.

In order to develop a point of care diagnostic device for viral detection, more attention is required on the immobilization of oligonucleotides or perhaps the RT-PCR enzyme on the surface of the sensor. A further need of exploiting new linkage chemistries for the interaction between the iridium oxide sensor and the enzyme can help in the development of this device or slight modifications of the enzyme structure without comprising on the enzyme's functionality.

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