# Procedural Optimization of the Quartz Crystal Microbalance for rapid detection of *Escherichia coli O157:H7*


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# Declaration -

This thesis is an account of my research undertaken during the period January 2006 to November 2006 for the award of a Master Degree in Forensic Science at the University of Western Australia. Except as otherwise indicated in the text, the work described is my own. All contributions made by other individuals have been duly acknowledged.

This thesis has never been submitted to another University or similar institution for the award of any degree.

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# - Abbreviations -

| | |
|---|---|
| $^{o}C$ | Degree celcius |
| hr | hour(s) |
| kbp | kilo ($10^3$) basepairs |
| Mbp | Mega ($10^6$) basepairs |
| min | minute(s) |
| ml | Millilitres; $10^{-3}$ litres |
| µl | Microlitres; $10^{-6}$ litres |
| 16-MHDA | 16-mercaptohexadecanoic acid |
| AC | Alternating Current |
| APTES | (γ-aminopropyl)triethoxysilane |
| BARC | Bead Array Counter |
| BSA | Bovine Serum Albumin |
| BTCC | Biological Threat Characterization Center |
| CANARY | Cellular Analysis and Notification of Antigen Risks and Yields |
| CDC | Centers for Disease Control and Prevention |
| CFU | Colony Forming Units |
| CHEF | Contour-clamped Homogeneous Electric Field |
| CMV | Cytomegalovirus |
| CT-SMAC | Cefixime Tellurite – Sorbitol MacConkey agar |
| CymMV | *Cymbidium mosaic virus* |
| DHS | Department of Homeland Security |
| DNA | Deoxyribonucleic Acid |

| | |
|---|---|
| EBV | Epstein Barr Virus |
| E. coli | Escherichia coli |
| EDC | N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide |
| EEC | EHEC Enrichment broth |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FBI | Federal Bureau of Investigation |
| FDA | Food and Drug Administration |
| FET | Field Effect Transistor |
| Gb3 | Globotriaosylceramide |
| GMI | Giant Magnetoimpedance |
| GMR | Giant Magnetoresistance |
| GTRI | Georgia Tech Research Institute |
| HIMEC | Human Intestinal Microvascular Endothelial Cells |
| HIV | Human Immunodeficiency Virus |
| HSV-1 | Herpes Simplex Virus Type 1 |
| HSV-2 | Herpes Simplex Virus Type 2 |
| HUS | Haemolytic Uremic Syndrome |
| IDA | Interdigitated Array |
| IMFET | Immuno Field Effect Transistor |
| ISFET | Ion-Selective Field Effect Transistor |
| LAPS | Light-addressable Potentiometric Sensor |
| LPS | Lipopolysaccharide |
| NBACC | National Biodefense Analysis and Countermeasures Center |
| NBFAC | National Bioforensic Analysis Center |
| NCBI | National Center for Biotechnology Information |

| | |
|---|---|
| NHS | N-Hydroxy-succinimide |
| ORSV | *Odontoglossum ringspot virus* |
| PBS | Phosphate Buffered Saline |
| PEI | Poly(ethylenimine) |
| PCR | Polymerase Chain Reaction |
| PFGE | Pulsed Field Gel Electrophoresis |
| PNPP | *P*-nitrophenyl Phosphate |
| QCM | Quartz Crystal Microbalance |
| RBC | Red Blood Cells |
| RNA | Ribonucleic acid |
| RT | Room Temperature |
| SAW | Surface Acoustic Wave |
| SEB | Staphylococcal enterotoxin B |
| SMAC | Sorbitol MacConkey agar |
| SPR | Surface Plasma Resonance |
| SQUID | Superconducting Quantum Interference Device |
| SWGMGF | Scientific Working Group on Microbial Genetics and Forensics |
| TCSMAC | Tellurite-Cefixime Sorbitol-MacConkey agar |
| TSB | Triptocase Soy Broth |
| TSM | Thickness Shear Mode |
| TNF | Tumour Necrosis Factor |
| USDA FSIS | United States Department of Agriculture Food Safety and Inspection Service |
| VT1 | Verotoxin 1 |
| VT2 | Verotoxin 2 |
| VZV | Varicella-Zooster Virus |

| | |
|---|---|
| WHO | World Health Organization |

# - Abstract -

The applications of biosensors are rapidly expanding with the increased emphasis placed on the use of technology in the evaluation of food safety and also in military use. The United States food industry carried out 144.3 million microbiological tests in 1999 (Alocilja and Radke, 2003). These numbers are expected to rise with the recently implemented regulatory measures for food safety in the United States. In fact, similar trends in food safety are occurring on a global scale. Furthermore, with the recognition and establishment of Microbial Forensics as a new field of forensics, the interest in biosensor development for the detection of microbes will thrive. Moreover, the recent spate of biocrimes, notably the anthrax scares, has called for newer and improved techniques for the sensitive, rapid and reproducible detection of microbes. Biosensors have the capability to fill this role as an efficient device for microbial detection.

There is a wide range of biosensors available for different purposes. In addition, their versatility allows for their overlap in many fields. The quartz crystal microbalance (QCM) is a biosensor that is cost-efficient, sensitive, field-deployable with the ability to perform automated, real-time assays within minutes. The QCM is a mass sensitive device that works on the principle where a change in mass deposited on the crystal is inversely proportional to the change in the resonant frequency of the crystal. Therefore, frequency decreases with increasing mass deposited. The QCM has been used in several studies as a biosensor for the detection of a number of viral and bacterial species.

*Escherichia coli O157:H7* is a leading cause of kidney failure in children. The most common mode of *E. coli O157:H7* transmission is through the consumption of contaminated food products. Current detection techniques for the detection of *E. coli O157:H7* takes time (two days or more) and foods are generally consumed or sold by the time the testing regime is completed. Thus, a rapid technique for the detection of *E. coli O157:H7* will prove critical in the containment of the spread of infection.

This study describes the optimization of the QCM for the rapid detection of *E. coli O157:H7*. Several variables in the quartz crystal preparation processes were tested to obtain the optimal outcome in the minimal time and cost required. Six factors were examined: (1) optimal antibody incubation duration, (2) optimal antibody incubation concentration, (3) optimal antigen incubation duration, (4) optimal storage conditions for antibody coated quartz crystals, (5) regeneration and (6) reusability of quartz crystals.

There was a close relationship between antibody incubation duration and antibody incubation concentration. High antibody incubation concentration required a shorter antibody incubation duration. Conversely, low antibody incubation concentration required a longer antibody incubation duration. Furthermore, regardless of antibody incubation concentration, a distinct pattern in the rate of antibody binding with time was observed.

One hour antigen incubation at ambient room temperature (22.5$^{o}$C) was sufficient for the efficient binding of the antigens to the immobilized antibody layer. Extension of antigen binding time to 15 hours produced inconsequential differences in readings.

The binding efficiency of the quartz crystals after a storage period of 2 to 4 weeks at ambient room temperature (22.5$^{o}$C) fared better than the crystals that were refrigerated at 4$^{o}$C.

Results showed that 0.2M glycine hydrochloride is a poor reagent for the removal of the antigen layer on the quartz crystals for repeated assay use. The 16-mercaptohexadecanoic acid (MHDA) layer and adsorbed proteins on the quartz crystals can be removed by a mixture of sulphuric acid and hydrogen peroxide, known as a piranha process. This allows the crystals to be repeatedly recoated and reused.

Overall, this research provides new insights into the preparation process of the quartz crystals for the specific detection of *E. coli O157:H7*. Conclusive results have been obtained for several tested parameters and suggestions have been raised for further studies in the optimization of the QCM for the *E. coli O157:H7* detection process. With improved knowledge and recognition in the capability of the QCM as a biosensor, the QCM may soon be used in conjunction with conventional techniques for the rapid detection of *E. coli O157:H7*.

# - TABLE OF CONTENTS -

**Declaration** ..... **i**

**Acknowledgements** ..... **ii**

**Abbreviations** ..... **iv**

**Abstract** ..... **viii**

**Table of Contents** ..... **xi**

1. Introduction ..... 1

1.1 Aims of study ..... 2

1.2 Thesis structure ..... 5

2. Literature review ..... 6

2.1 Microbial Forensics ..... 6

2.2 Biosensors ..... 14

2.2.1 Biosensor industry ..... 14

2.2.2 Categorization of biosensors ..... 17

2.2.3 Quartz crystal microbalance (QCM) as a biosensor for microbial detection ..... 29

2.3 *Escherichia coli O157:H7* ..... 33

2.3.1 Pathogenesis ..... 33

2.3.2 Sources of infection ..... 39

2.3.3 Current detection and confirmation techniques ..... 43

3. Materials and methods ..... 47

3.1 *Escherichia coli O157:H7* ..... 47

3.1.1 Antigen purity and colony count ..... 47

3.1.2 Preparation of different antigen (*Escherichia coli O157:H7*) concentrations ..... 48

3.2 Polyclonal *Escherichia coli O157:H7* antibodies ..... 50

3.2.1 Preparation of different antibody concentrations ..... 50

3.2.2 Enzyme linked immunosorbent assay (ELISA) to ensure specificity of antibodies to attenuated antigens ..... 51

3.3 Quartz crystals ..... 54

3.3.1 Cleansing of quartz crystal surface ..... 54

3.3.2 Preparation of quartz crystal surface ..... 55

3.3.3 Coating of *Escherichia coli O157:H7* antibody layer ..... 57

3.3.4 Storage of antibody coated crystals ..... 58

3.3.5 Coating of antigen (*Escherichia coli O157:H7)* layer ..... 59

3.3.6 Removal of antigen (*Escherichia coli O157:H7)* layer ..... 60

3.3.7 Regeneration of quartz crystals ..... 60

4. Results ..... 62

4.1 *Escherichia coli O157:H7* ..... 62

4.1.1 Antigen purity ..... 62

4.1.2 Colony count ..... 63

4.2 *Escherichia coli O157:H7* antibodies ..... 64

4.2.1 Enzyme linked immunosorbent assay (ELISA) ..... 64

4.3 Quartz crystals ..... 68

4.3.1 Optimal antibody incubation duration ..... 70

4.3.2 Optimal antibody incubation concentration ..... 73

4.3.3 Optimal antigen incubation duration ..... 76

4.3.4 Optimal conditions for storage of antibody coated crystal ..... 79

4.3.5 Quartz crystal regeneration ..... 82

4.3.6 Quartz crystal reusability ..... 85

5. Discussion ..... 87

5.1 Optimal antibody incubation duration ..... 87

5.2 Optimal antibody incubation concentration ..... 89

5.3 Optimal antigen incubation duration ..... 91

5.4 Optimal conditions for storage of antibody coated crystal ..... 92

5.5 Quartz crystal regeneration ..... 94

5.6 Quartz crystal reusability ..... 95

6. Overall conclusion and future research ..... 96

7. References ..... 100

# 1. Introduction

## 1.1 Introduction

The recent surge in biologically-based threats has raised the need for improved methods in detection of microorganisms (Edelstein et al., 2000, Lei et al., 2005). This study is focused on the optimization of a device, the quartz crystal microbalance (QCM), for the detection of the bacterium, *Escherichia coli O157:H7*. Current techniques used in the detection of *E. coli O157:H7* in patients' stool samples and contaminated foods take at least two days. If the time required for testing can be reduced, lesser people will become infected via the consumption of these contaminated foods. Moreover, rapid detection of the bacteria will prove crucial in impeding the spread of an outbreak. Therefore, a biosensor, the QCM is suggested for use in the rapid detection of *E. coli O157:H7*.

Su and Li (2004) have successfully used the QCM for the detection of *E. coli O157:H7*. Using the QCM, they were able to detect *E. coli O157:H7* in the range of $10^3$ to $10^8$ $CFU.ml^{-1}$ within 30 to 50min (Su and Li, 2004). Although it had been shown that the accurate detection of *E. coli O157:H7* via the QCM was possible, there is a need to efficiently optimize the QCM procedures. This study aims to fill this gap in knowledge.

## 1.2 Aims of study

Several variables in the quartz crystal preparation process can be adjusted to produce optimal results with the minimal time and cost required. In this study, the following six aims will be examined.

*Aim 1: Optimal antibody incubation duration*

Time is required for the proper formation of an antibody layer on the crystal surface. Achieving a good antibody layer formation in the minimum time required will reduce the total preparation time needed.

*Aim 2: Optimal antibody incubation concentration*

Applying a high antibody concentration causes wastage of reagents and incurs a higher cost. Inversely, application of a low antibody concentration results in an incomplete antibody layer formation. The optimal antibody incubation concentration differs for each type of antibody due to antibody structural differences. The application of an optimal antibody incubation concentration allows the proper formation of the antibody layer with the minimum amount of antibodies required, making this procedure cost effective.

*Aim 3: Optimal antigen incubation duration*

Time is required for the binding of the antigen to the immobilized antibody layer. Knowing the duration required for the completion of the binding reaction reduces the overall preparation time needed.

*Aim 4: Optimal conditions for storage of coated crystal*

In this study, the effects of moisture and temperature on antibody pre-coated crystals will be carried out. If crystals pre-coated with antibodies can be stored for a period of time without any significant loss in antigen binding efficacy, crystals can then be prepared and stored beforehand until required. A supply of antibody pre-coated crystals readily available on demand will prove useful when time is of importance.

*Aim 5: Quartz crystal regeneration*

The removal of the antigen layer on the crystal, after the completion of an initial assay, allows the crystal to be reused for a second assay. This is provided that the antibody reactivity of the crystal is retained after the antigen removal process. Hence, the effect of glycine-hydrochloride, as a reagent for crystal regeneration, will be investigated in this study. The repetitive use of a single antibody coated crystal reduces the overall time and cost required per assay.

*Aim 6: Quartz crystal reusability*

If the chemical and protein layers formed on the quartz crystal can be completely removed without affecting the chrome/gold layer on the quartz crystal surface, the quartz crystal can be recoated and reused for more assays. This further reduces the overall cost per assay.

The optimization of the QCM procedures for *E. coli O157:H7* detection will improve the time and cost efficiency of the QCM as a biosensor for the specific detection of the bacterium.

In addition, research into the QCM as a device for detection of microorganisms paves the way for future forensic applications. The QCM can be potentially used in conjunction with conventional techniques in forensic analysis to rapidly determine the source in the event of a biological outbreak or threat. For example, the QCM has the potential to qualitatively and quantitatively detect microorganisms, be it *Shigella dyseneriae* in food contamination outbreaks or *Bacillus anthracis* in suspected biological threats. Moreover, the QCM is field deployable with the ability to perform real-time analysis; beneficial qualities when performing on-site forensic analysis. Its affordability, sensitivity and fast assay time add to the advantages of using the QCM in forensic work. With biological threats on the rise, biosensors similar to the QCM may prove a vital tool in the field of Microbial Forensics.

## 1.3 Thesis Structure

This thesis is divided into seven chapters. Chapter one consists of the introduction and aims of study. Chapter two reviews the relevant literature. Chapter three describes the materials and methods. Chapter four contains the results which are discussed in detail in chapter five. Chapter six concludes the study and includes suggestions for future research. Chapter seven lists the references used in this thesis.

# 2. Literature review

## 2.1 Microbial Forensics

History and group perpetrators of microbial agents

Microorganisms have long been used as biological weapons. The first recorded case of microbial attack was in 1346, during the siege of the city of Kaffa where the Tartar army catapulted plague-ridden corpses over the castle walls (Philips, 2005). The plague outbreak that ensued within the city ended in Kaffa's eventual downfall. However, it is important to note that the plague outbreak may not have been the result of the Tartar army's catapulting actions. This is because when an infected corpse cools down, the fleas which are the principle vectors of the plague bacteria, *Yersinia pestis*, leave the dead bodies to look for fresh blood (Mims, 2000). Unless the plague-infected dead bodies were relatively fresh, catapulting them over the city walls may not have caused the plague outbreak. A possible culprit for the infection within the city walls could have been rats, which are the preferred hosts for fleas. Rats carrying fleas infected with *Y. pestis* could have scurried into Kaffa and caused the outbreak (Mims, 2000). Nonetheless, *Y. pestis* played a crucial role in Tartar's victory.

Another historical example of biowarfare occurred during Pontiac's Rebellion of 1763-1764. Recovered documents revealed the intentions of the British army to use smallpox as a tactical weapon. Colonel Henry Bouquet and General Jeffrey Amherst apparently discussed their intention to inoculate blankets with the smallpox virus and send them to the warring Native American Indian tribes (Knollenberg, 1954). This was confirmed by a trader, William Trent, who recorded that gifts of two blankets and one handkerchief taken from a

smallpox hospital were given to two Delaware Indians that visited Fort Pitt (Knollenberg, 1954). There is no concrete evidence to prove that the British proceeded with their intentions to infect the American Indians. However soon after, a smallpox epidemic broke out amongst the tribes. The American Indians, who had limited to no immunity against smallpox, quickly succumbed to the infection. This outbreak had a severe impact on the ability of the American Indians to continue their fight against the British.

In World War II, biological weapons were developed by a Japanese army division, code named Unit 731 (Philips, 2005). Japanese occupied Manchuria was where experiments of viable biological warfare agents on human subjects were performed. The first successful use of microbes in warfare by Unit 731 was the contamination of the Soviet water supply with typhoid bacteria, *Salmonella typhi* (Phillips, 2005).

More recently in 1984, a sect known as the Rajneeshees, contaminated self-service salad restaurants in Oregon with *Salmonella typhimurium* (Carus, 1998). An estimated 751 people were infected in this attempt to sway the outcome of the elections being held at that time.

Aum Shinrikyo was a well-known sect who was responsible for the release of sarin gas into the Tokyo subway system in 1995. It was found that Aum Shinrikyo not only dabbled in chemical agents but also in biological agents. Reports have stated that the sect was actively experimenting with a range of biological agents including *Clostridium botulinum*, *Bacillus anthracis*, *Vibrio cholerae*, *Coxiella burnetii* and Ebola virus (Olsen, 1999). Information on groups like Aum Shinrikyo provides us with important insights, suggesting the possibility

of many unknown groups that have the financial capability and expertise to start a microbiological attack, be it to make a political statement or merely to inflict sheer terror.

Individual perpetrators of microbial agents

Attacks that involve microbiological agents have not been exclusively restricted to groups. The ability to grow relatively infectious agents is simple, to the point that individuals with personal intent have employed this mode of terrorism. In 1996, twelve laboratory staff in Dallas, Texas, fell ill after consuming pastries intentionally contaminated with *Shigella dysenteriae* type 2, obtained from the laboratory's stock culture. Eventually the perpetrator was found to be a disgruntled laboratory technician, Dianne Thompson (Kolavic et al., 1997).

In another case, a medical practitioner intentionally injected his former partner with Human Immunodeficiency Virus 1 (HIV-1) positive blood during an argument (Metzker et al., 2002). Phylogenetic analysis traced the virus back to a HIV-1 positive patient that was under his care. The doctor was charged with second degree murder.

Sources of microbial agents

To exacerbate the risk, it is not difficult to acquire microbial agents. There are four known ways of acquisition of these microbial agents: legitimate suppliers, natural source, theft or synthesis. The Rajneeshees owned a state certified laboratory which allowed them to purchase biological agents from unsuspecting legitimate suppliers (Carus, 1988). The medical practitioner obtained the HIV-1 positive blood from his patient, a natural source (Metzker et al., 2002). Dianne Thompson forged documents and stole the *Shigella dyseneriae* from her laboratory's stock culture (Budowle and Murch, 2005). Lastly, a

synthetic infectious poliovirus has been successfully created. Armed with just the genomic sequence of the poliovirus, scientists have managed to synthetically create the poliovirus from scratch (Cello et al., 2002). The publicly available database of the National Center for Biotechnology Information (NCBI) in the United States and other international genome facilities, store many genome sequences of various pathogens for research purposes. With the ease of public access to sequence information on a whole host of pathogenic microorganisms, synthetic biological weapons are now a possibility. One further potential route to obtain pathogens that are not readily available is probably from the black market.

Types of microbes that may be employed as biological agents

All pathogens in the past, present and even future can be used as biological weapons.

*Revival of past pathogens:*

The eradicated diseases caused by past viruses can make a comeback. The effect may be disastrous, as the present community, for the most part, will have no immunity against them. An example is smallpox, caused by a variola virus. Stringent vaccination resulted in the eradication of smallpox as a human disease by the 1970's. In 1972, the United States stopped smallpox vaccination and the World Health Organization (WHO) followed in 1979 (Michaeli, 2002). Current vaccination stocks available will be insufficient to cope with a smallpox epidemic (Michaeli, 2002). It is important to note that two officially recognized laboratories in the United States and Siberia still possess viral stocks (Mims, 2000). In addition, the complete sequence of the variola virus is readily available from the NCBI database. Consequently, the variola virus could potentially be synthesized.

*A plethora of present pathogens:*

There are a plethora of present-day virulent bacteria and viruses to choose from. Well-financed groups with the appropriate expertise can easily purchase and disperse pathogens. In the case involving the Rajneeshees, the dispersal of *Salmonella typhimurium* required no elaborate equipment. It was simply sprinkled on the salads. The Centers for Disease Control and Prevention has listed a number of existing pathogens which can be easily obtained to cause mass fatalities (CDC).

*The production of future pathogens:*

With molecular engineering, there is the potential for new pathogens to be manufactured in the laboratory using advanced cloning technologies. Creating new pathogens is unnecessary, as it is sufficient to mutate an existing strain to express virulent factors that will be harmful to humans. For example, the influenza virus naturally evolves by antigenic shifts and antigenic drifts (CDC, 2005a). People can be naturally infected with influenza more than once as the immunity obtained from prior infection may be inadequate in dealing with the newer strain. This is why flu vaccines are updated annually. Intentionally causing point mutations in certain loci of the viral genome to express different surface proteins could possibly create a new influenza viral strain.

Another possible area of molecular engineering involves the conversion of a virus or bacteria from a non-zoonotic to a zoonotic status. For example, researchers have evidence suggesting that the source of both the Ebola (Mims, 2000) and the Human Immunodeficiency Viruses (HIV) (Gao et al., 1999) can be traced back to monkeys. These natural examples highlight the possibility of using viruses which are harmless to monkeys but have crossed the species barrier, turning pathogenic in humans. The HIV and avian flu

pandemics are examples of natural variations that can serve as potential sources of new agents. The rapid rate at which these organisms mutate under natural selective pressure to develop more potent levels of virulence also highlights the ease by which new strains can be engineered.

The establishment of Microbial Forensics

However, amidst these occurrences of biologically-based crimes or "biocrimes" across the globe, the potential threat of biological weapons was largely ignored until 2001. The trigger was a series of anthrax scares in the United States between October and November 2001. A total of eleven confirmed cases of inhalation and eleven confirmed or suspected cases of cutaneous anthrax were reported (Sternbach, 2003); out of the 22 infected, 5 died. Soon after, the president of the United States, George Bush, signed a new bioterrorism bill amounting to US $4.3 billion. Subsequently, the Scientific Working Group on Microbial Genetics and Forensics (SWGMGF) was formed by the United States Federal Bureau of Investigation (FBI) in 2002. On June 20th 2003, SWGMGF established a set of quality assurance guidelines for laboratories performing microbial forensic work, recognizing that it was important to establish and implement guidelines for the appropriate handling of microbial evidence (SWGMGF, 2003). This would ensure the admissibility of the microbial evidence in court. Essential factors listed in the guidelines include security of evidence, chain of custody, detailed documentation of evidence, validation of procedures and laboratory audits (SWGMGF, 2003).

On June 27th 2006, the United States Department of Homeland Security (DHS) started the construction of the National Biodefense Analysis and Countermeasures Center (NBACC) comprising of key elements, the National Bioforensic Analysis Center (NBFAC) and the

Biological Threat Characterization Center (BTCC). NBFAC will, upon completion in 2008, be the lead federal facility in charge of facilitating the forensic analysis of evidence obtained from acts of biocrime or bioterrorism in the United States (US DHS, 2005). BTCC, on the other hand, will play a larger role in the research and understanding of current and future biological threats (US DHS, 2005). The BTCC will guide the development of countermeasure equipment including detectors and advances in medical technology such as new vaccines for the protection against biological attacks. Microbial Forensics has finally been recognized and established as a new field of forensics.

Challenges for Microbial Forensics

Microbial Forensics will face many challenges in the years ahead. Weaknesses will be gradually exposed and procedures and protocols improved upon before the discipline is well established. One area for thought is the source of pathogens. It may take time before symptoms of a biological attack appear. During this lapse or gestation period, the perpetrator has the opportunity to escape unscathed (Budowle and Murch, 2005).

The number of existing pathogens is also overwhelming. The Microbial Rosetta Stone Database has phylogenetically organized them into bacterial pathogens, eukaryotic pathogens, DNA viruses, single-stranded negative strand RNA viruses, single-stranded positive stand RNA viruses, retroid viruses and double-stranded RNA viruses (Ecker et al., 2005). The creation of the Microbial Rosetta Stone Database cannot replace the tools that NCBI provide, however it may prove to provide some assistance in the rapid determination of an undetermined biological agent.

Moreover, it is difficult to monitor the transportation of pathogens. With air travel on the rise, people within the window period of infection may unknowingly transport a pathogen from one country to another. It is also possible that bioterrorists carrying pathogens may go undetected by airport security. It is considerably easier to transport and deposit biological agents, compared to chemical agents or explosive devices.

Finally, an airborne pathogen that can be transmitted between humans may be disastrous. Pathogens that can be transmitted through the air will spread quickly if isolation measures are not adopted in time. Human to human transmission will provide a large number of hosts to house the replicating pathogens.

So, an important part in the strategy towards stopping or preventing widespread pathogenic infection will clearly involve improvements in detection methodology. Field-deployable biosensors with improved sensitivity, accuracy and speed will prove paramount in efforts to contain the spread of an outbreak.

## 2.2 Biosensors

### 2.2.1 Biosensor industry

A biosensor is a device for the detection and measurement of biological matter. The biosensor industry can be divided into four sectors: medical, environmental, food and military (Alocilja and Radke, 2003). The total biosensor industry is estimated to be worth US $10.8 billion by 2007 (Lin and Wang, 2005). Currently, the most profitable sector is the medical sector with its sale of blood glucose monitors. However in recent years, an increased emphasis has been placed on biosensors for evaluation of food safety and military use.

Biosensor developments for food safety

A study by Strategic Consulting showed that the United States food industry carried out approximately 144.3 million microbiological tests in 1999 (Alocilja and Radke, 2003). Numbers are expected to rise with increased regulatory attention in the United States. A testing program for *Escherichia coli O157:H7* was initiated in 1993 by the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) (Unnevehr et al., 2004). In 1996, food processing plants had to test for generic *Escherichia coli* as part of the Hazard Analysis Critical Control Point (HACCP) regulations and USDA FSIS started tests for *Salmonella* (Unnevehr et al., 2004). In 2003, *Listeria* testing was made compulsory for ready-to-eat meat and poultry products (Unnevehr et al., 2004). In addition, concerns of viral-infected poultry products were elevated when the avian influenza virus turned zoonotic in Asia and evidence of human-to-human transmission was observed. Human-to-

human transmissions occurred in Thailand in 2004 and Indonesia in 2006 (CDC, 2006a). In response, Georgia Tech Research Institute (GTRI) researchers developed a low-cost, rapid-screening optical waveguide biosensor with the potential to detect the H5N1 influenza virus (Sanders, 2005). The optical waveguide biosensor works on the principle that binding of the avian influenza antibody to the virus alters the speed of light through the waveguide as measured by an interferometer. This information is transmitted to a signal processing software which identifies and quantifies the antigen, in this case, the virus. The optical waveguide biosensor rivals virus isolation and polymerase chain reaction (PCR) technologies in sensitivity but is superior since it is faster, cheaper and comes in the form of a field-deployable direct immunological assay. The device was able to differentiate between H-antigen subtypes within a one hour test window and is estimated to cost US $1,000 excluding the chips and antibodies (Sanders, 2005). With a more rapid and affordable field detection device, the H5N1 influenza virus may be isolated faster in farms and the spread of infection kept in check. These various demands in food safety have propelled the need for newer, faster and more affordable means of food testing.

Biosensor developments for military use

With the introduction of Microbial Forensics and the increasing threat of microbes being used as biological weapons, biosensor development in this sector is expected to increase. The United States Naval Research Laboratory has designed a Bead Array Counter (BARC) based on giant magnetoresistance (GMR) effect, applicable in the detection of biological warfare agents (Edelstein et al., 2000). The phenomenon of GMR was first described in 1988 and it was found that when a magnetic field was applied to multilayers of ferromagnetic iron (Fe) and non-magnetic chromium (Cr), the resistance of the Fe/Cr superlattice drops significantly (Baibich et al., 1988). In the BARC prototype, an array of

GMR sensors was fabricated unto a chip. Thiolated deoxyribonucleic acid (DNA) probes for the specific biomolecule were patterned onto a gold layer on the chip directly above the GMR sensors. Biotin-labelled sample DNA strains were introduced into BARC, hybridizing with their complementary DNA probes. Streptavidin-labelled paramagnetic beads were introduced which would bind to the biotin-labelled DNA strains. Excess beads were removed by an in-built electromagnet. By passing a current through the GMR sensors and measuring the change in localized resistance, quantitative and qualitative analysis of the sample DNA strains could be determined (Edelstein et al., 2000).

Researchers of BARC suggest an adaptation of magnetoresistive memory (MRAM) for designing BARC chips with the potential to detect approximately 1,000 different analytes (Edelstein et al., 2000). The researchers were initially using DNA hybridization for the detection of the following biological warfare agents: *Bacillus anthracis*, *Yersinia pestis*, *Brucella sinus*, *Francisella tularenis*, *Vibrio cholerae*, *Clostridium botulinum*, *Campylobacter jejuni*, and Vaccinia virus (Edelstein et al., 2000). Proof-of-concept has been shown but much research is needed before BARC technology will be ready for use in field work. In addition to research undertaken in the naval laboratory, the construction of the Biological Threat Characterization Center (BTCC) is likely to fuel further research and developments on biosensors for military defense. Hence the biosensor industry in this sector is expected to thrive in the coming years.

### 2.2.2 Categorization of biosensors

There is a wide range of biosensors available to suit every purpose. Most types of biosensors are versatile and their uses overlap many fields. Biosensors can be subdivided into five categories based on the principle transducer, specifically: electrochemical, optical, piezoelectric, thermal and magnetic (see Figure 1).

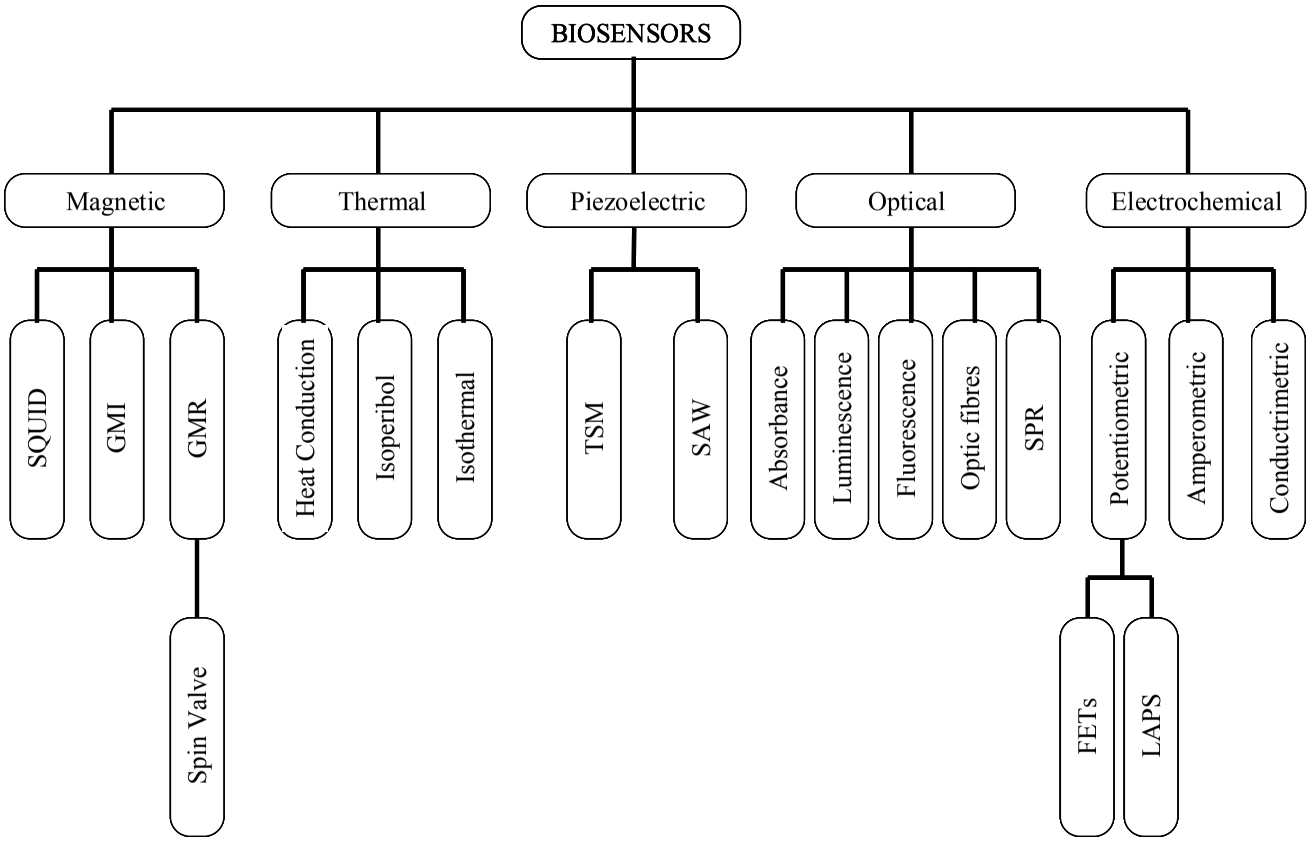


**Figure 1. Biosensors can be divided into five main categories which can be further subdivided**

Electrochemical biosensors

Electrochemical biosensors can be subdivided into potentiometric, amperometric and conductrimetric. Potentiometric biosensors measure the change in potential induced by the binding of free antigens or antibodies to its specific immobilized partner in the electrochemical cell at a constant current flow. Potentiometric biosensors can be further subdivided into two categories: Field-effect transistors (FETs) and light-addressable potentiometric sensors (LAPS).

*Field-effect transistors (FETs):*

There are several models of FETs, many using ISFETs (ion-selective field effect transistors) as its basic model. The concept of ISFETs was first introduced by Bergveld in the 1970s and its application has since taken off including the development of related devices ENFETs (EnzymeFETs), IMFETs (ImmunoFETs) and more (Bergveld, 2003). A potentiometric based immunosensor was developed for the detection of Hepatitis B surface antigen with a detection limit of 1.3ng.ml$^{-1}$ (Tang et al., 2004). A disposable potentiometric biosensor with a response time as low as 2 minutes was constructed for quality control in milk (Verma and Singh, 2003).

*Light-addressable potentiometric sensor (LAPS):*

LAPS is another FET, also known as a type of electrolyte insulator semiconductor. The chemical reaction produced by the binding of the reagent of interest results in a potential change in the electrolyte, creating a field effect across the insulator, registered via the flashing light emitting diodes, producing an alternating photocurrent. This recorded photocurrent can be back-calculated to determine the concentration of the reagent of interest that had reacted in the electrolyte solution. Calculations are carried out based on the

Nernst equation. LAPS in combination with an immunoligand assay had been used to detect *E. coli O157:H7* with a sensitivity of 7.1 x $10^2$ cells.ml$^{-1}$ of heat-killed or 2.5 x $10^4$ cells.ml$^{-1}$ of live *E. coli O157:H7* cells in under 45 minutes (Gehring et al., 1998). LAPS had also been used for the detection of generic *E. coli* in drinking water through the measurement of pH variations due to $NH_3$ production by a urease-*E. coli* antibody conjugate (Ercole et al., 2002).

*Amperometric:*

Amperometric biosensors work in opposite tangents to potentiometric biosensors. In amperometric devices, the potential is kept constant whilst the change in current is recorded as a result of redox reactions in the electrolyte. An amperometric immunosensor was modified for the detection of *Brucella melitensis*, an infectious disease that affects livestock and humans, with a detection limit of 3.5ng.ml$^{-1}$ (Li et al., 2002). Another amperometric biosensor was developed for the environmental detection of pesticides in lake water samples (Lei et al., 2005). Recently, the gastrointestinal bacterium, *Vibrio cholerae O1*, was detected using an amperometric immunosensor with a detection limit of $10^5$ cells.ml$^{-1}$ in 55 minutes (Rao et al., 2006).

*Conductrimetric/Impedance:*

The third type of electrochemical device, the conductrimetric or impedance biosensor involves the measurement of change in an electrical field. This includes the measurement of the capacitance between the interdigitated electrodes or in other cases, the electrode/solution interface during the binding reaction. An interdigitated array (IDA) in conjunction with an impedance immunosensor was used for *E. coli O157:H7* detection with a detection limit of $10^6$ CFU.ml$^{-1}$ (Yang et al., 2004). The amount of *E. coli O157:H7*

bonded onto the immobilized antibodies was indirectly measured as the amount of electron-transfer resistance encountered in the electrochemical reaction.

Optical biosensors

There are many variants of optical biosensors, from the basic to the complex, and they can be divided into several categories, which include: absorbance, luminescence, fluorescence, optic fibers, surface plasmon resonance (SPR) and more.

*Absorbance:*

Alkaline phosphatase conjugated *E. coli O157:H7* antibodies were used in a sandwich immunoassay for the detection of *E. coli O157:H7* through the hydrolysis of *p*-nitrophenyl phosphate to colourful *p*-nitrophenol (Liu and Li, 2002). The measurement of the absorbance of *p*-nitrophenol was an indirect measure of the amount of bonded *E. coli O157:H7*. The working range of this set-up was from $3.2 \times 10^2$ to $3.2 \times 10^4$ $CFU.ml^{-1}$ with a standard deviation of 2.5 to 9.9% for detection within two hours (Liu and Li, 2002).

*Luminescence:*

Engineered B cell lines which expressed a calcium sensitive bioluminescent protein in combination with antibodies specific to the antigen of interest known as cellular analysis and notification of antigen risks and yields (CANARY) had been developed and used for the detection of *Yersinia pestis*, orthopoxviruses, Venezuelan equine encephalitis virus , *E. coli O157:H7* and *B. anthracis* (Rider et al., 2003). The binding of the antigen to the antibody raises the amount of intracellular calcium, in turn resulting in the observed luminescence by the calcium sensitive protein.

*Fluorescence:*

Fluorescent semiconductor quantum dots, also known as nanocrystals are novel fluorescent labels made of cadmium selenide that have recently emerged into the biological market. Even though quantum dots are more expensive than regular fluorescent dyes, their advantages supersede their price. Biotin-labelled anti *Salmonella* antibodies were used in a sandwich immunoassay for the detection of *Salmonella typhimurium* in chicken carcass wash water using streptavidin coated quantum dots (Yang and Li, 2005). The measurement of the fluorescence of the quantum dots in solution due to the interaction between biotin and streptavidin indirectly measured the amount of *S. typhimurium* in the wash. The detection limit of this technique was 1 x $10^3$ CFU.ml$^{-1}$ (Yang and Li, 2005).

*Fiber-optic:*

Fiber-optic biosensors exploit the measurement of fluorescent light excited by an evanescent wave generated by a laser to quantitatively detect biomolecules immobilized on the fiber surface. An automated fiber-optic evanescent-wave biosensor, RAPTOR, was developed and used for the rapid detection of *S. typhimurium* in spent sprout irrigation water within 20 minutes (Kramer and Lim, 2004). Biotinylated *S. typhimurium* antibodies were immobilized on the surface of the optical fiber. When the spent irrigation water was added into RAPTOR, the *S. typhimurium* bacteria present in the irrigation water bonded onto the immobilized antibodies. Cyanine 5 (Cy5) labelled *S. typhimurium* was then added to complete the sandwich immunoassay. Cy5 was excited with a laser diode and the emitted fluorescence detected by the photodiode detector. The device was able to detect the bacterium in spent irrigation water, 3 to 5 days before the completion of the sprouting process, with a minimum detection limit of 5 x $10^5$ CFU.ml$^{-1}$ (Kramer and Lim, 2004).

*Surface plasmon resonance:*

The basic principle of all surface plasmon resonance (SPR) immunosensors is the measurement of the change in angle of reflected polarized light caused by the binding of molecules on the sensor surface which is proportional to the mass of the bound material of interest. BIAcore is one example of a well established commercialized SPR valued for its label-free, real time monitoring of biochemical reactions. A competitive immunoassay for the detection of Staphylococcal enterotoxin B (SEB) in fresh fluid milk using BIAcore had a detection range from 0.312 to 20 $ng.ml^{-1}$ with an analysis time of 25 minutes per sample (Medina, 2005). A different model of SPR, the SPR spectroscope was used to detect *Yersinia enterocolitica* with a detection range of $10^2$ to $10^7$ $CFU.ml^{-1}$. The Reichert SR 7000 SPR biosensor was used to detect *E. coli O157:H7* in a sandwich immunoassay with a detection limit of $10^3$ $CFU.ml^{-1}$ (Subramanian et al., 2006). A miniaturized version of BIAcore, Spreeta, has changed the face of SPR-based devices to a more affordable and portable form of immunosensor. Spreeta measures 2.2cm in height by 3.0cm in length and costs US $7,000 to US $10,000 compared to a few hundred thousand dollars for a BIAcore system. Spreeta had been used in the detection of human immunoglobulin G and 2,4-dinitrophenol (Suzuki et al., 2001).

Piezoelectric biosensors

Piezoelectricity was discovered by Pierre and Jacques Curie in 1880 (Curie and Curie, 1880). They found that upon applied mechanical stress, certain crystals generated an electrical voltage. This effect can be explained as when an acentric material is compressed, there is physical displacement of the atoms in the crystal lattice. The non-centrosymmetry of the lattice helps create a net dipole moment which results in a charge generation. Inversely, applying an electrical field across the crystal, rearranges the atoms in the lattice,

resulting in an altered structure. Examples of piezoelectric crystals include quartz ($SiO_2$), lithium tantalite ($LiTaO_3$), lithium niobate ($LiNbO_3$) and langasite (LGS). Pierre's student, Paul Langevin went on to develop piezoelectricity and piezoceramics. One of Langevin's notable developments was an underwater sonar for submarine detection during World War I. In 1918, using a transducer made from a mosaic of thin quartz crystals glued between two steel plates, echoes were received from a submarine as deep as 1500 meters (Graff, 1981).

In 1959, Sauerbrey proved that the change in resonant frequency was related to the change in mass deposited on the crystal (Sauerbrey, 1959). As described by the following formula:

$$\Delta F = -2.257 \times 10^6 F_1^2 \Delta M / A$$

$\Delta F$ is the change in frequency, $F_1$ is the resonant frequency of the crystal, $\Delta M$ is the change in mass on the crystal and A is the coated area of the crystal. The change in frequency of the crystal is inversely related to the change in mass deposited on the crystal. Therefore, frequency decreases with increasing mass deposited. The increased mass on the crystal places a restriction on the shear motion of the crystal, reducing its frequency. Sauerbrey's discovery was the first step towards the research of piezoelectric crystals for use as quantitative devices and consequently the development of the piezoelectric sensor. There are two main types of piezoelectric sensors categorized by the type of acoustic wave used, specifically: thickness shear mode (TSM) and surface acoustic wave (SAW).The different forms of acoustic waves are due to a combination of factors that include crystal symmetry, angle of cut and electrode configuration (Li, 2006).

*Thickness Shear Mode (TSM):*

The oldest and most common piezoelectric sensor is the quartz crystal microbalance (QCM). The QCM operates under the TSM and the piezoelectric crystal used is quartz. The QCM consists of a thin AT cut quartz crystal. AT is a term used to describe the angle of a plate cut from a crystal surface. In this case, a plate cut from a crystal of quartz such that the plate contains the X-axis and makes an angle of about 35 degrees with the Z-axis. The advantage of AT cut quartz crystal is that it gives negligible frequency drift at room temperature (Mecea et al., 1995). The quartz crystal is coated with metal electrodes usually gold, copper, nickel or platinum on both sides and inserted into a holder for easy manipulation. The holder connects the quartz crystal to an oscillator and a power supply. When an AC voltage is applied, the quartz crystal oscillates at its resonant frequency due to piezoelectric effect. The thickness of the AT-cut quartz crystal determines its resonant frequency and usually lies within the range of 5 to 10 MHz. Although quartz crystals with higher resonant frequencies can provide increased sensitivity, they are not usually used as the crystals become too thin and fragile to handle. The QCM had been used for the microbial detection of *Candida albicans* (Muramatsu et al., 1986).

*Surface acoustic wave (SAW):*

The SAW mode of propagation was discovered by Rayleigh in 1887 and has the highest sensitivity amongst acoustic sensors with an operational frequency up to 500MHz (Drafts, 2000). SAW biosensors use interdigital transducers (IDTs) to convert acoustic waves to electrical signals via the use of piezoelectric crystals. Unlike the QCM, where electrodes are coated on both sides of the crystal, metal IDTs are deposited on one side of the crystal for the SAW. Similarly, according to Sauerbrey, an increase in mass deposited on the IDTs obstructs a travelling SAW and is in turn recorded as a decrease in frequency. The use of

SAW as a biosensor is still in its infancy. A shear horizontal SAW (SH SAW) using langasite had been developed for the detection of *E. coli O157:H7* (Berkenpas et al., 2006). Recently in the medical field, a SAW device was investigated for use in early cancer detection (Wu, 2006).

Thermal biosensors

Thermal biosensors are based on the principle that during a biological reaction, heat is either absorbed or released. The total heat evolved during a catalytic reaction is proportional to the molar enthalpy and the number of moles of product molecules. Thermal biosensors can be categorized into three groups according to the mode of heat transfer between the reaction vessel and surroundings: heat conduction calorimetry, isoperibol calorimetry and isothermal calorimetry. In heat conduction calorimetry, the temperature change in the reaction vessel is recorded as a voltage difference between the reaction vessel and the surrounding isothermal heat sink via a thermoelectric transducer. The isoperibol calorimetry is the measurement of the temperature in the reacting vessel whilst keeping the temperature of the surroundings constant. In isothermal calorimetry, the temperature of the surroundings is heated or cooled to match that of the reaction vessel, minimizing any heat exchange between the two. Thermal biosensors was used for the monitoring of production of penicillin V in a fermentation pilot plant (Rank and Danielsson, 1992). Multiple analyte detection had been carried out using a flow injection thermal microbiosensor (Xie et al., 1995). A flow injection calorimeter biosensor was developed for the detection of an organophosphate pesticide, dichlorvos (Zheng et al., 2006).

Magnetic biosensors

Magnetic biosensors are the newcomers in the biosensor market. Since the introduction of magnetic beads by the company, Dynal known as Dynabeads, concentration and separation of reagents of interest have been carried out using immunomagnetic microbeads (Liu and Li, 2002 ;Ye et al., 2002; Yang and Li, 2005). Immunomagnetic beads have now been incorporated into magnetic biosensor research in combination with magnetic transducers. Using different magnetic properties, there are at present several magnetic biosensors under research.

One type of magnetic biosensor is the superconducting, quantum interference device (SQUID). SQUID uses the differential nature of the relaxation of the superparamagnetic nanoparticles after magnetization for detection purposes. Bonded nanoparticles undergo Néel relaxation, which is exponentially dependent on the particle volume. Free nanoparticles undergo Brownian rotation which is linearly dependent on the particle volume (Chemla et al., 2000). By appropriately selecting the size of the nanoparticles, the motion of the bonded and free nanoparticles can be manipulated. In SQUID, the free nanoparticles rotated for a few microseconds whilst the bonded nanoparticles, which were restricted in motion rotated for several seconds. This rotation of the bonded nanoparticles could be measured. Hence SQUID made use of the decaying magnetic field produced by the bonded superparamagnetic nanoparticles to determine the concentration of the bonded biomolecules of interest (Chemla et al., 2000). The detection limit was $5 \times 10^4$ magnetic particles (Chemla et al., 2000).

Magnetic biosensors are currently under development enabled by the giant magnetoimpedance (GMI) effect. GMI is the change in impedance of a conductive wire when a current is flowing through it in the presence of small magnetic fields. A study had

been carried out using CoFeSiB wire and its GMI effect for the detection of biomolecules (Chiriac et al., 2005). An immunoassay could be carried out on or near the CoFeSiB wire and detection of the target biomolecules through the use of biomolecules labelled with magnetic microparticles and the measurement of impedance via GMI effect.

As described earlier, another magnetic property known as the giant magnetoresistance (GMR) effect was used for the Naval Research Laboratory's development of Bead Array Counter (BARC) as a magnetic biosensor. GMR effect has also been used in the development of a magneto resistive spin valve sensor. The difference in a spin valve sensor is that one side of the magnetic layer is pinned down and the magnetic rotation of the unpinned layer rotates relative to the pinned layer under the influence of a weak magnetic field generating a significant change in the electrical resistance of the set-up due to GMR effect. Proof-of-concept using DNA hybridization and spin valve sensors for the genetic screening of cystic fibrosis has shown to be promising (Lagae et al., 2005). With further developments based on the concepts of GMR and GMI, the possibility of multi-analyte detection on a chip is slowly but surely becoming a reality.

## Conclusion

Biosensors in all five categories: electrochemical, optical, piezoelectric, thermal and magnetic have their individual advantages and disadvantages. With further research and development, these individual advantages can only increase. The prospects of biosensor research are exceptional as these biosensors can be adapted and applied across all fields. As work progresses, new categories of biosensors will emerge and the utilization of biosensors may become a staple in every trade.

### 2.2.3 Quartz crystal microbalance (QCM) as a biosensor for microbial detection

Applications of the quartz crystal microbalance

The quartz crystal microbalance (QCM) has been commercialized for several years with many applications in different fields. It was used in thin film deposition studies (Benes et al., 1989), in environmental detection of cyanide in industrial waste waters (Gomes et al., 1997), in biological cell-adhesion studies (Fredriksson et al., 1998), in chemistry for the quantification of potassium (Gomes et al., 2000), in electrochemical research (Eickes et al., 2000), in viral detection (Susmel et al., 2000), in plant research (Eun et al., 2002a), in industrial viscosity measurements (Gee et al., 2002), in drug delivery research (Byrne, 2003) and in bacterial detection (Su and Li., 2004).

Advantages of the Quartz Crystal Microbalance

There are several advantages of QCM technology. Firstly, the device is affordable with a basic set-up cost of approximately US $2,000. The quartz crystals are re-usable, lowering the cost of each sample test. Secondly, its sensitivity is within the nanogram (ng) scale which enables the detection of very fine mass changes. Thirdly, it is able to perform real-time analysis, an attractive quality for use in various applications. Fourthly, processes can be automated, reducing the amount of manpower required. Fifthly, it is portable and has the potential to be used in the field. Lastly, in comparison with other techniques, the QCM is comparable or much faster, with an assay time of approximately 30 minutes.

Types of probes used in the Quartz Crystal Microbalance for microbial detection

There are two tried and tested QCM probes for use in microbial detection. The immobilized probes on the QCM can be in the form of nucleic acids comprising of either single stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) complementary to the target DNA/RNA. The other alternative is immunological probes where antibodies specific to the target analyte are used. Figure 2 illustrates the two different probes used in QCM for microbial detection.

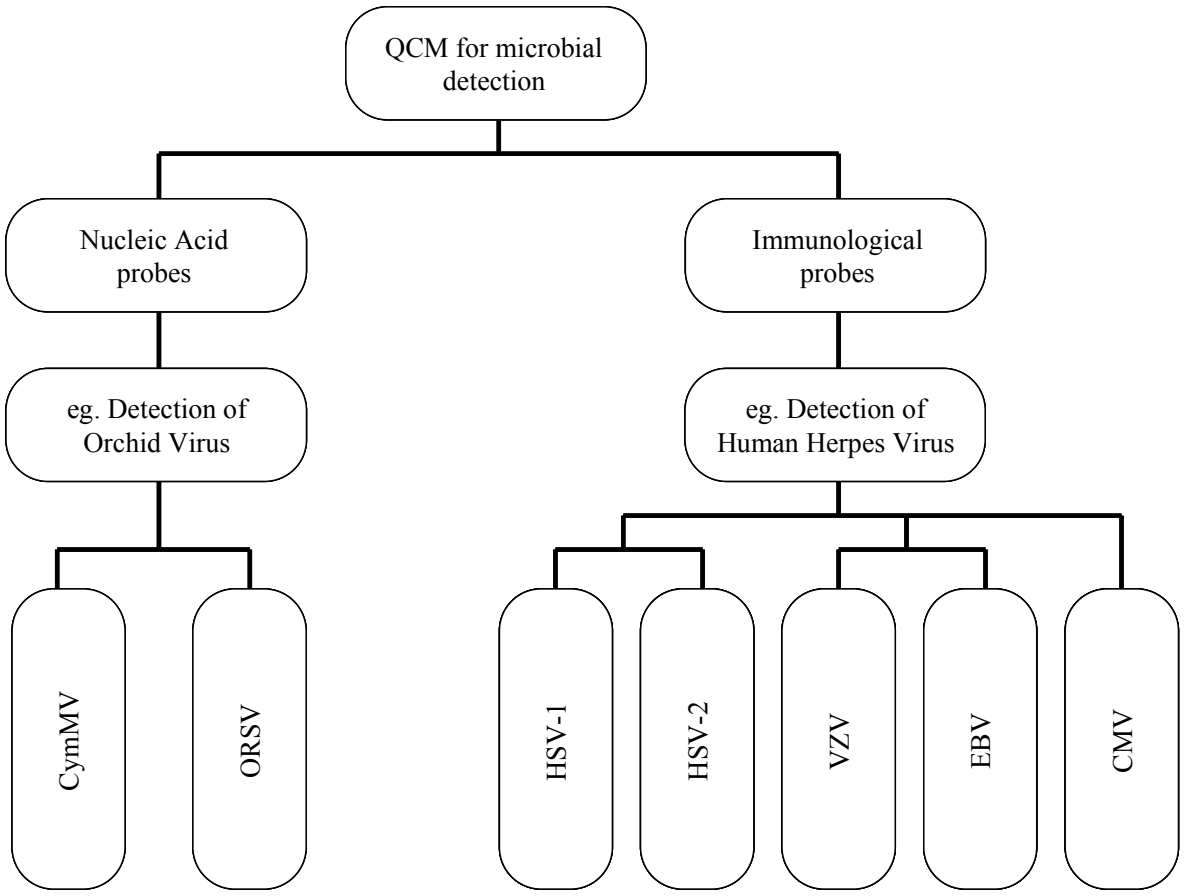


**Figure 2. Examples of nucleic acid or immunological probes in QCM for microbial detection**

Nucleic acid probes in the quartz crystal microbalance

A QCM-DNA based biosensor was used for the detection of two orchid viruses, *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) (Eun et al., 2002a). Two single stranded DNA probes complementary to CymMV and ORSV coat proteins were manufactured with a mercaptohexyl group attached to its 5'-phosphate terminal. The mercaptohexyl group aids in the attachment of the single stranded DNA probes to the gold surface of the quartz crystal. After cleansing the crystal, the single stranded DNA probes were immobilized onto the crystal surface. All unreacted aldehyde groups were blocked using 0.1M glycine. Excess DNA probes were rinsed off with buffer solution. Crude CymMV and ORSV infected sap samples were then introduced. A detection limit of 10ng CymMV or ORSV was obtained (Eun et al., 2002a).

Immunological probes in the Quartz Crystal Microbalance

A QCM-immunological based biosensor was used for the detection of five common types of human herpes viruses: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zooster virus (VZV), Epstein Barr virus (EBV) and cytomegalovirus (CMV) (König and Grätzel, 1994). In this study, Protein A was selected over (γ-aminopropyl)triethoxysilane (APTES) and Poly(ethylenimine) (PEI) as the immobilization aid of choice as it produced better results in terms of stability, sensitivity and reusability. Herpes virus specific antibodies were immobilized onto the Protein A-coated quartz crystal. The prepared QCM was then used to detect herpes virus in infected clinical samples. It was shown that the QCM was not only able to quantify the amount of virus present in the sample but was also able to distinguish between the five different types of structurally related herpes viruses (König and Grätzel, 1994).

Advantages of immunological probes

There are several advantages of immunological probes over nucleic acid probes. First of all, if specific antibodies are commercially available, the preparation of immunological probes is much simpler without the need for DNA/RNA sequencing or single stranded DNA/RNA probe construction. In addition, whole cell analyte detection can be carried out using immunological probes whereas cells need to be lyzed to release the nuclei acids in nucleic acid based probe detection. Overall, the simplification of the preparation processes for immunological probes provide a faster, cost-effective and simpler option for microbial detection.

## *2.3 Escherichia coli O157:H7*

### 2.3.1 Pathogenesis

*Escherichia coli O157:H7* is one of several hundred strains of gram-negative rod-shaped bacteria. The letter '*O*' represents the type of somatic antigens which are lipopolysaccharide (LPS) complexes that make up the cell wall of the bacterium. '*H*' represents the type of flagellin that makes up the flagella of the bacterium. This lettering and numbering system helps differentiate the hundreds of known strains of *E. coli* bacterium. The Centers for Disease Control and Prevention (CDC) in the United States plays a significant role in the combat against infectious diseases. In a 1999 study (published in 2005), the CDC estimated that 73,000 Americans were infected with *E. coli O157* annually. This resulted in a total loss of US $405 million each year, including US $370 million from premature deaths, US $30 million for medical expenses and US $5 million in lost productivity (Frenzen et al., 2005).

Mild symptoms of *E. coli O157:H7* infection

Upon infection with the bacterium, a 1 to 8 day incubation period was observed during which the bacterium proliferates internally. On average, it took three days before symptoms of abdominal cramps and bloody diarrhea were observed (Noris and Remuzzi, 2005). These symptoms are caused by the release of bacterial toxins into the small intestines and colon. Contrary to the norm, *E. coli O157:H7* was recovered from asymptomatic patients, as well as patients suffering from non-bloody diarrhea (Riley, 1987). The difference between *E. coli O157:H7* and other types of bacterial enterocolitis including *Shigellae, Salmonellae*

and *Campylobacter jejuni* is that *E. coli O157:H7* causes little or no fever. However, it should be noted that Riley (1987) cited a reference where it was observed that some elderly patients developed high fever in the later stages of the illness. With little or no treatment, mostly with intravenous or oral fluids and electrolyte replacement, patients recovered within 5 to 10 days.

Severe symptoms of *E. coli O157:H7* infection

The effects of *E. coli O157:H7* differ from person to person. People with a weakened immune system, the young and the elderly proved more susceptible to the toxins of the bacterium (Boyce et al., 1995). This suggests that individual immunity plays a crucial role in a person's yield to *E. coli O157:H7* toxins. Individuals with weaker immunity are more likely to progress from symptoms of bloody diarrhea to Hemolytic Uremic Syndrome (HUS). Approximately 5 to 10% of *E. coli O157:H7* infected patients progressed to HUS (Karmali, 1989). HUS has been recognized as the leading cause of kidney failure in children in the United Sates (Neill et al., 1987, Siegler et al., 1994), Canada (Karmali et al., 1985, Rowe et al., 1991) and Europe (Bitzan, 1993).

The virulence factors of *E. coli O157:H7*

HUS is caused by a group of virulence factors known as verotoxins which are produced by *E. coli O157:H7*. There are two main types of verotoxins, VT1 and VT2. VT1 is 50% homologous to VT2 but the similarity stops there. VT1 and VT2 adopt different strategies and cause different degrees of tissue damage. In a study of children infected with *E. coli O157:H7*, VT2 appeared to cause a higher degree of pathogenicity (Jenkins et al., 2003). Samples collected from children who had diarrhea alone or who were asymptomatic contained only VT1. Conversely, children suffering from HUS were VT2 carriers. Kidneys

from mice inoculated with strains containing VT2 showed damages in both epithelial and endothelial cells (Karpman et al., 1997). In addition, the study found that kidneys from mice pre-treated with anti- VT2 antibodies showed a lesser degree of histopathological change (Karpman et al., 1997). In a 2003 study using the baboon as a primate model, preliminary results indicated that VT2 strains are more likely to cause HUS than VT1 strains (Siegler, 2003). These studies collectively indicated that VT2 may play a more significant role in the progression of the *E. coli O157:H7* infection to full-blown HUS.

Charting the route of *E. coli O157:H7* infection

After ingestion, the bacterium will attempt to attach itself to the intestinal epithelial cells. It was shown that *E. coli O157:H7* belongs to a class of gastrointestinal pathogens that produce attaching and effacing lesions (Dytoc et al., 1993). Other factors, including the plasmid pO157 (Paton and Paton, 1998), the 94-kDa outer membrane protein (Sherman et al., 1991) and eaeA gene (Jerse et al., 1990) have been implicated in the attachment process of the bacterium to the epithelial cells. Once attached to intestinal epithelial cells, the bacterium releases toxins into the intestinal lumen and epithelium causing the painful abdominal cramps and watery diarrhea observed in patients (Paton and Paton, 1998). Within 1 to 2 days, this watery diarrhea progressed to bloody diarrhea (Riley, 1987). It was demonstrated *in-vitro* that human intestinal microvascular endothelial cells (HIMEC) are very sensitive to both VT1 and VT2 (Jacewicz et al., 1999). The exposed HIMEC showed considerable cytotoxicity when exposed to the toxins. This indicated that the probable cause of bloody diarrhea in HUS could be attributed to cell death and intestinal injury by the verotoxins. It is interesting to note that pretreatment of primary HIMEC with PDMP, an inhibitor of verotoxin receptor, globotriaosylceramide (Gb3), rendered the cells resistant to

VT1 (Jacewicz et al., 1999) even though to date, Gb3 had not yet been found in human enterocytes (Paton and Paton, 1998).

It is unclear how toxins cross the intestinal epithelial barrier into the circulation and travel to the target organs. The amount of circulating VT1 was below detection limit in blood plasma, with the highest concentration primarily at the kidneys (van Setten et al., 1996). Monocytes found at the site of damage have fueled the debate that monocytes might be the vector transporting the toxins from the intestine to the kidneys. Upon arrival at the target organs, the verotoxins cause coagulation of fibrin and platelets within the fine blood vessels of the kidney endothelium, forming clots. Clotting of these small vessels reduces the blood flow to the kidneys hampering the functional ability of the organ. Histopathology of the cortical tissue from a HUS patient showed severe damage to the tubular epithelial cells, cell nuclei fragmentation, presence of fibrin and enterocytes in the tubular luminae and obstruction of capillaries and blood vessels by thrombi formation (Karpman et al., 1998).

HUS is usually accompanied by a condition known as hemolytic anemia which is low red blood cell (RBC) count. This is because as the RBCs flow through these obstructed vessels, the RBCs are sheared and ruptured. This is associated with a reduction in the number of blood platelets (thrombocytopenia) in the blood stream. Most blood platelets in the blood stream have either been used up in the abnormal aggregation process or damaged travelling through the clots. Verotoxins have been shown to bind to blood platelets, presumably via glycosphingolipid receptors on the platelet cell membrane which could play a role in thrombocytopenia, platelet aggregation and microthrombus formation in HUS (Cooling et al., 1998). A low blood platelet count can result in further abnormal bleeding.

Molecular pathogenesis of HUS in the kidneys

The exact molecular basis of the bacterium that leads to the onset of HUS is still under research. However, because of intense *in-vitro* and *in-vivo* studies there has been an improved understanding of the sequence of events that leads to HUS. Studies have found that when activated by LPS, the availability of Gb3 receptors on monocytes for VT1 binding increases (van Setten et al., 1996). In addition, inflammatory mediators like TNF-α are produced and released by monocytes which increases the induction of Gb3 receptors on endothelial cells (van de Kar et al., 1992). This increase in the number of Gb3 receptors, in turn, caused a faster uptake of VT1 by the endothelial cells due to increased sensitivity of the endothelial cells to VT1. The verotoxins target certain areas of the body where there is abundant Gb3 receptors present on the cell surface membrane. In most HUS patients, endothelial damage is primarily in the cortical region of the kidney where a large number of Gb3 are present. Histopathological studies from three HUS patients revealed endothelial swelling and cell damage in the glomeruli and arterioles of the kidneys (Richardson et al., 1988).

In severe HUS patients, the endothelial damage might extend to the pancreas, adrenal gland, brain and heart (Hosler et al., 2003). The next step would be the attachment of the verotoxin B subunit to the galactose disaccharides of Gb3 receptors. The bonded verotoxins are internalized through retrograde transport through the Golgi complex (Noris and Remuzzi, 2005). SPR analysis showed that VT1 associated and disassociated more easily from Gb3 compared to VT2 (Nakajima et al., 2001). It was suggested that *in-vivo*, this stronger and longer period of VT2 association with Gb3 allowed sufficient time for the toxin to be internalized and thus was more potent than VT1 (Nakajima et al., 2001). In addition, lipopolysaccharides (LPS) in combination with VT1 increased the release of

cytokines, IL-1, IL-6 and TNF from glomerular epithelial cells (Hughes et al., 2001). These inflammatory cytokines aggravated the already injured kidney, exacerbating the degree of damage.

Recently the focus has shifted from the study of verotoxins on renal endothelial cell damage to the investigation of verotoxins on mesangial cells. Mesangial cells play a multifunctional role in the glomeruli of the kidneys. Mesangial cells are involved in structural support, regulation of blood flow, phagocytosis and may act as accessory cells in antigen presentation (Dark, 1997). The production of TNF-α by mesangial cells were believed to exacerbate the progress of HUS in the kidneys (Hruby et al., 1991). There was a debate that the inhibition of protein synthesis and mitogenesis, by the verotoxins, caused the death of the infected cells. However, van Setten et al (1997) has shown through the use of mesangial cells that both VT1 and VT2 inhibit protein synthesis but inhibition of mitogenesis is affected by the B-subunit of VT1 alone. Most importantly the experiments showed that the inhibited cells were still viable, this suggests that cell death induction might not be a by-product of protein synthesis suppression as many claim (van Setten et al., 1997). A recent study supported this finding and proposed that it was the Bcl-2-VT2 complex that induced cell death through caspase 3 activation (Suzuki et al., 2000). As research into HUS progresses and our understanding of this illness improves, it is likely that damage caused by the verotoxins can be alleviated and further deaths prevented.

### 2.3.2 Sources of infection

Consumption of contaminated food products

There are several known ways of transmission of *E. coli O157:H7*. The most common mode is through the consumption of contaminated food products. From 1982 to 2002, food accounted for 52% of 350 *E. coli O157* outbreaks and 61% of 8,598 *E. coli O157* outbreak-related cases (Rangel et al., 2005). In 2006, of 15 noted cases of *E. coli* outbreaks in the United States, 7 were food-related (Marler-Clark). Sources of *E. coli O157:H7* contamination include meat and milk products from the infected ruminants, vegetables fertilized with manure from infected animals and irrigation waters contaminated with faeces of infected ruminants (see Figure 3).

Contaminated products look, smell and taste the same as uncontaminated products, hence they tend to go undetected until an outbreak occurs. *E. coli O157:H7* has been reported in food products including apple cider (Besser et al., 1993), salami (CDC, 1995), unpasteurized juice (CDC, 1996b), alfafa sprouts (CDC, 1997), fresh cheese curds (CDC, 2000), ground beef (CDC, 2002) and spinach (CDC, 2006b). When infected products are not heated to a high enough temperature prior to consumption, the bacterium survives and is transmitted through ingestion.

The source of the most recent outbreak in the United States was from contaminated spinach. As of 1pm October 6, 2006, 199 persons from 26 states were infected with *E. coli O157:H7* from the consumption of contaminated spinach (CDC, 2006c). The United States Food and Drug Administration (FDA) advised cooking the spinach at $160^{o}$ F for 15 seconds to ensure

that the bacterium was killed (CDC, 2006c). In addition, the FDA emphasized the importance of practicing strict hygiene, as all other foods and contact surfaces that had come into contact with the infected raw product may be contaminated with the bacterium (CDC, 2006c).

Person to person transmission

The second most common route of infection is from person to person. When people who are symptomatic fail to practice proper hygiene, person-to-person transmission is common. The Minnesota Department of Health carries out regular surveillance for *E. coli O157:H7*. 29 cases of *E. coli O157:H7* infections in 9 child-care facilities were investigated and evidence of person-to-person transmission was uncovered in all 9 child-care facilities (Belongia et al., 1993). For the year 2006, there were two confirmed cases and one suspected case of *E. coli O157:H7* outbreak in daycare centers in the United States (Marler-Clark). The ease of transfer of *E. coli O157:H7* from person-to-person suggested that only a small amount of the bacterium was needed to cause an infection (Boyce et al., 1995). Data collected from the outbreaks that occurred indicated that the infectious dose might be as low as 10 organisms (Snyder, 1998).

Contact with infected animals

As discussed above, farm animals are a common source of infection because the primary reservoir for the growth of *E. coli O157:H7* is the intestines of ruminants (cows, sheep and goats). Animals infected with the bacterium exhibit no symptoms and hence are likely to go undetected. Another route of infection is via direct contact with the infected animals. Several outbreaks were reported in zoos and carnivals where visitors were allowed to pat the animals (CDC, 2005b). Visitors especially children upon playing with the animals, may

come into contact with the faeces of the infected animal. If hands are not washed after contact, transferal of the bacterium can occur from the infected animal to humans.

Swimming in infected waters

An unsuspecting route of infection is via swimming in contaminated and untreated water. A total of 12 cases, mostly children, were identified in Rockford, Illinois, USA during June/July of 2005 (CDC, 1996a). Interviews from parents in five cases indicated no common food source but they had all visited a lake swimming beach at an Illinois park in June. Stool samples from six persons who swam in the lake tested positive for Shiga toxins and pulsed field gel electrophoresis (PFGE) patterns were the same. After much investigation and analysis, it was suggested the likely route of infection was by taking the contaminated lake water into the mouth and swallowing of it (CDC, 1996a).

Suggested counter-measures

In the primary level of defense, detection of the bacterium in infected ruminants is important. Regular and randomized checks for the bacterium in the manure of ruminants help to circumvent the spread of the bacterium. Secondary measures may include food safety monitoring in key parts of food preparation processes. For example, regular checks for the presence of the bacterium in raw materials like fresh milk and ground meat before it is sold or processed. In addition, environmental monitoring must be carried out regularly in water bodies, like lakes and irrigation waters. These measures, when in place, allow detection of the bacterium before it reaches the population, minimizing *E. coli O157:H7* outbreaks. Hence, an affordable, easy-to-use, portable, sensitive assay for the rapid detection of the bacterium could prove fundamental in the prevention of further *E. coli*

*O157:H7* outbreaks. In addition, if an outbreak does occur, an assay with similar efficacy will prove critical in the containment of the spread of infection.

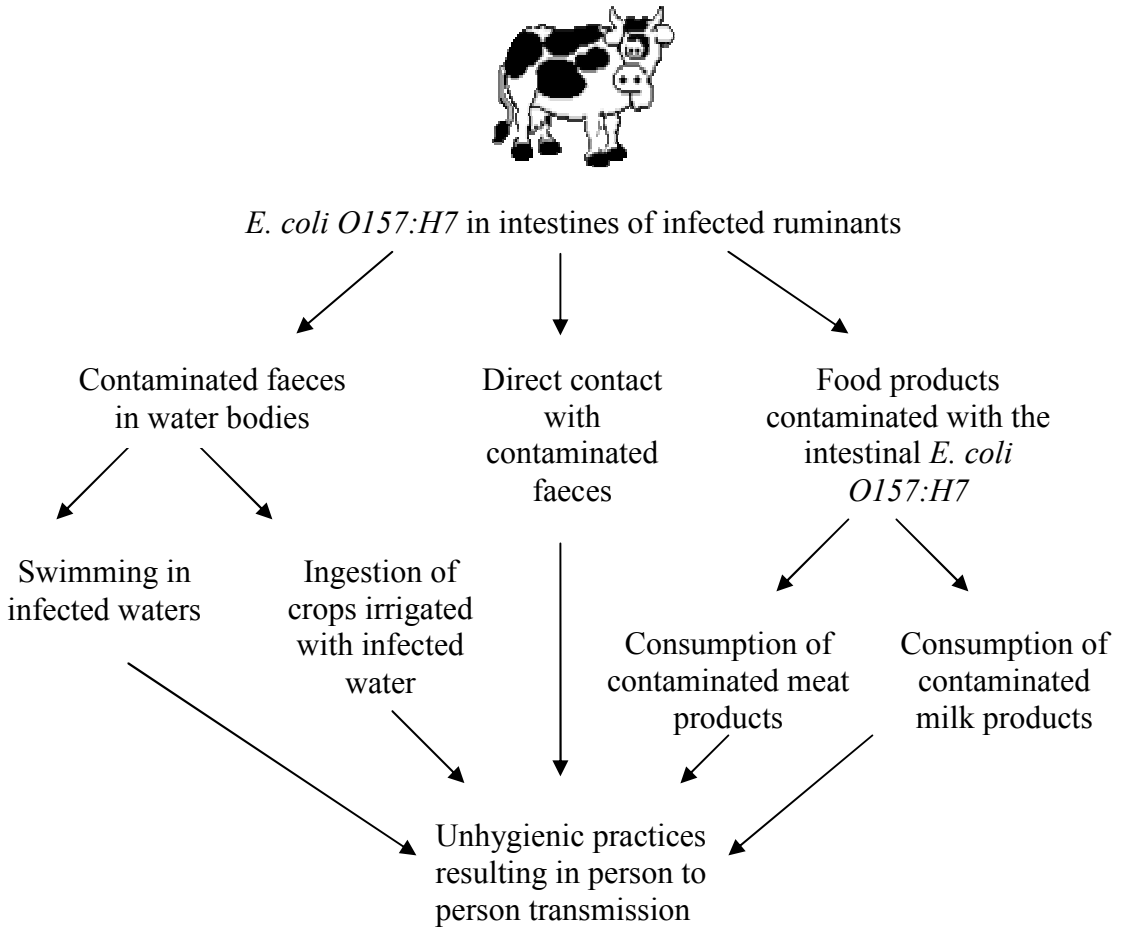


**Figure 3. Flowchart of *E. coli O157:H7* infection**

### 2.3.3 Current detection and confirmation techniques

The detection and confirmation of *Escherichia coli O157:H7* is a two-step process (see Figure 4):

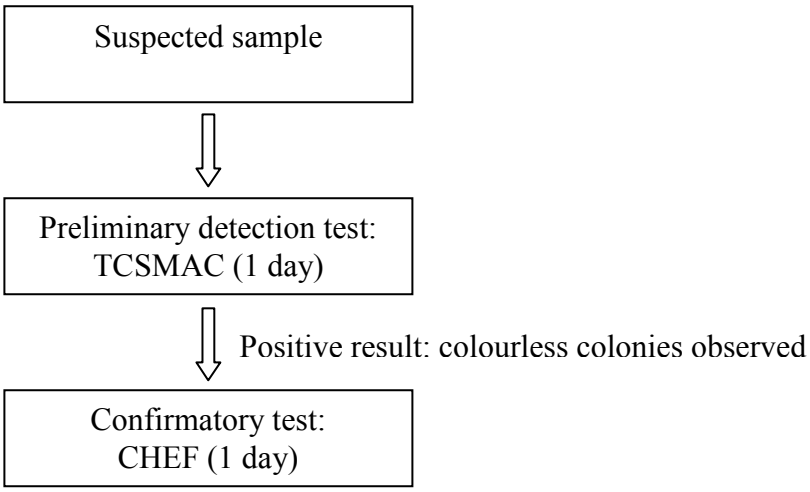


**Figure 4. Detection and confirmation of *E. coli* infection in samples require at least 48 hr testing time**

Preliminary detection of *E. coli O157:H7*

The United States Food and Drug Administration (US FDA) use an agar plating technique known as Tellurite-Cefixime Sorbitol-MacConkey agar (TCSMAC) for the detection of *E. coli O157:H7* in foods and in patients' stool samples (Feng and Weagant, 2002). The bacterium is first enriched for 6 hours at 37$^{o}$C in a modified soy broth supplemented with vancomycin, cefsulodin and cefixime, commonly referred to as the enterohemorrhagic *E. coli* Enrichment broth (EEC). The sample is streaked onto a TCSMAC agar and left to incubate at 37$^{o}$C for 18 to 24 hours. The supplements in both the enrichment broth and

SMAC effectively inhibit non-*O157 E. coli* and most sorbitol non-fermenting strains from proliferating. This reduces the number of sorbitol non-fermenting strains that needs to be tested. Most other strains of *E. coli* ferment the sorbitol in SMAC, acidifying the agar, in turn decreasing the pH of the medium. Conversely, *E. coli O157:H7* is unable to ferment sorbitol. Instead, it utilizes the peptones in the agar to produce alkali, increasing the pH of the medium. When used with a pH indicator, neutral red, *E. coli O157:H7* is observed as colourless colonies on TCSMAC after the incubation period. However, it is important to note that about 6% of isolates do not ferment sorbitol (Ewing, 1986). As such, a second confirmatory test is important to verify the presence of *E. coli O157:H7*. The good points of SMAC are that the test is simple and very affordable. However this test takes one day for results to be visualized and foods are generally consumed or sold by the time the testing regime is completed.

Confirmatory test for *E. coli O157:H7*

After a positive preliminary detection, a confirmatory test will be required to verify the identity of the bacterium. The most common confirmatory test used for the verification of positive isolates from TCSMAC is known as pulsed field gel electrophoresis (PFGE), an improved version of the conventional gel electrophoresis.

*Conventional gel electrophoresis:*

Conventional gel electrophoresis is the separation of DNA molecules according to its size through a gel matrix under a stationary electrical field. Conventional gel electrophoresis is used to separate deoxyribonucleic acid (DNA) molecules smaller than 20kbp (Birren and Lai, 1993). The gel matrix used in the electrophoresis process contains pores in which DNA molecules sieve through. Smaller sized DNA fragments will be able to move through these

pores at a faster rate than larger sized DNA fragments, producing size-based separation. Acrylamide gels are used for the separation of DNA molecules less than or around a few kilo base pairs (kbp) whilst agarose gels works best for DNA molecules that are 0.2kbp to 20kbp (Cantor et al., 1988). DNA molecules larger than 20kbp cannot be differentiated using conventional gel electrophoresis because DNA molecules above 20kbp migrate through the gel with the same mobility and merely appear as a single large concentrated band. Lowering the gel concentration or field strength are not solutions to the problem. There is a limit as to how dilute a gel can be. In addition, application of low field strength results in impractical running times.

*Pulsed field gel electrophoresis (PFGE):*

The first PFGE was built by Schwartz and Cantor in the 1980s (Birren and Lai, 1993). PFGE resolved the problem faced in conventional gel electrophoresis as it allows DNA molecules as large as 10Mbp (Cantor et al., 1988) to be differentiated with ease. The alternating electric fields applied at different angles in PFGE allow DNA molecules in the large concentrated band of conventional gel electrophoresis gels to separate from each other. The theory states that with each re-orientation of the electric field relative to the agarose gel, the smaller sized fragments within the large band will move in the new direction faster than the larger sized fragments within the large band, promoting separation. There have since been many versions of PFGE but all are based on the same principle of alternation of electric fields at different angles.

*A molecular subtyping network - PulseNet:*

PulseNet is a molecular subtyping network for foodbourne disease surveillance established by the Centers for Disease Control and Prevention (CDC) in 1996 (Swaminathan et. al.,

2001). The CDC standardized the protocols for PFGE to a one day PulseNet procedure in selected state public health laboratories, increasing comparability of PFGE results amongst these laboratories. The contour-clamped homogeneous electric field (CHEF) gel electrophoresis is the form of PFGE that is used at CDC for the separation of bacterial DNA fragments (NCID, 2006). Presently, PFGE patterns for *E. coli O157:H7* are stored on the PulseNet server, providing epidemiological and clinical information for individual bacterial isolates (Swaminathan et al., 2001). The advantage of PFGE is its reproducibility. The disadvantages are that PFGE is an expensive, labour intensive and time consuming method. It takes a minimum of one day for results to be obtained. In addition, CDC raised further limitations they had observed of PFGE in terms of pattern analysis, optimization of DNA separation and possible misrepresentation of bands in the gel matrix (NCID, 2006). Taking into account these limitations, an alternative technique should be considered for use as a confirmatory test for *E. coli O157:H7* verification.

# 3. Materials and methods

## 3.1 *Escherichia coli O157:H7*

### 3.1.1 Antigen purity and colony count

Non-toxigenic *Escherichia coli O157:H7* (NCTC12900) was purchased from Oxoid (Hampshire, UK) in the form of culti-loops. Using a pair of sterilized scissors, one loop shaft of *E. coli O157:H7* was cut from the handle into a vial containing 10ml of triptocase soy broth (Oxoid, Hampshire, UK). A volume of 1ml was drawn from the mixture and inoculated on blood agar (Sigma-Aldrich, St. Louis, USA) and incubated at 37$^{o}$C in an inverted position for at least 24hr. This was to ensure that the bacterium purchased was a pure culture. The remainder of the mixture in the vial was placed on a shaker at 37$^{o}$C for at least 24hr to allow bacterial growth. Serial dilutions of the broth culture were carried out as outlined in Table 1. Each vial was vortexed before 1ml of culture was added to a 9ml vial of saline that was labelled $10^{-1}$. The $10^{-1}$ vial was vortexed before 1ml of contents was added to another 9ml vial of saline that was labelled $10^{-2}$. Dilutions were repeated until a concentration of $10^{-8}$ was obtained.

**Table 1. Serial dilutions of *E. coli O157:H7* culture**

| Labelled Vial | Contents |
|---|---|
| **TSB** | 1 cultiloop in 10ml triptocase soy broth |
| **$10^{-1}$** | 1ml of TSB in 9ml saline |
| **$10^{-2}$** | 1ml of $10^{-1}$ in 9ml saline |
| **$10^{-3}$** | 1ml of $10^{-2}$ in 9ml saline |
| **$10^{-4}$** | 1ml of $10^{-3}$ in 9ml saline |
| **$10^{-5}$** | 1ml of $10^{-4}$ in 9ml saline |
| **$10^{-6}$** | 1ml of $10^{-5}$ in 9ml saline |
| **$10^{-7}$** | 1ml of $10^{-6}$ in 9ml saline |
| **$10^{-8}$** | 1ml of $10^{-7}$ in 9ml saline |

Solutions of 100µl in the $10^{-4}$ to $10^{-8}$ dilution range was spread onto individual nutrient agar plates (Oxoid, Hampshire, UK) and incubated at 37$^{o}$C in an inverted position for at least 24hr. All remaining solutions were placed into a cold room at 4$^{o}$C to cease the continued growth of the bacterium. After incubation, colony counts were carried out on the agar plates.

### 3.1.2 Preparation of different antigen (*Escherichia coli O157:H7*) concentrations

Antigen concentrations of 1 x $10^{8}$ and 1 x $10^{7}$ CFU.ml$^{-1}$ were prepared from heat-killed stock antigen solution (1 x $10^{9}$ CFU.ml$^{-1}$ *E. coli O157:H7*) using phosphate buffered saline (0.01M PBS consists of 0.138M NaCl and 0.0027M KCl, pH 7.4) as a dilution medium. *E. coli O157:H7* solutions were vortexed each time before pipetting to ensure adequate mixing. Table 2 summarizes the dilution steps.

**Table 2. Preparation of antigen solutions**

| **Concentration of attenuated *E. coli O157:H7* (CFU.ml$^{-1}$)** | **Contents** |
|---|---|
| **$10^8$** | 7ml of stock solution in 63ml PBS |
| **$10^7$** | 10ml of $10^8$ in 90ml PBS |

All antigen solutions were stored at 4$^{o}$C until further use. Prior to use, antigen solutions were well mixed on a vortex.

## 3.2 Polyclonal *Escherichia coli O157:H7* antibodies

### 3.2.1 Preparation of different antibody concentrations

Polyclonal antibodies (70-XG13) specific for *E. coli O157:H7* of concentration 5.4mg.ml$^{-1}$ was purchased from Fitzgerald (MA, USA). When required, small amounts of *E. coli O157:H7* antibodies of different concentrations were prepared in autoclaved microfuge tubes using PBS as a dilution medium (see Table 3). Preparations of the required antibody concentrations were repeated as required.

**Table 3. Preparation of *E. coli O157:H7* antibody solutions**

| **Concentration of *E. coli O157:H7* antibodies (mg.ml$^{-1}$)** | **Contents** |
|---|---|
| **0.05** | 428µl PBS + 4µl stock antibody |
| **0.1** | 212µl PBS + 4µl stock antibody |
| **0.2** | 104µl PBS + 4µl stock antibody |
| **0.3** | 102µl PBS + 6µl stock antibody |
| **0.4** | 100µl PBS + 8µl stock antibody |
| **0.5** | 98µl PBS + 10µl stock antibody |

.

Prior to use, antibody solutions of concentrations 0.05mg.ml$^{-1}$ to 0.5mg.ml$^{-1}$ were stored at 4$^{o}$C. Antibody stock solution (5.4mg.ml$^{-1}$) was stored in the freezer at -20$^{o}$C until further use.

### 3.2.2 Enzyme linked immunosorbent assay (ELISA) to ensure specificity of antibodies to attenuated antigens

An enzyme linked immunosorbent assay (ELISA) was performed to test the affinity of the purchased goat *E. coli O157:H7* antibodies (Fitzgerald, MA, USA) to the attenuated *E. coli O157:H7* antigens (Oxoid, Hampshire, UK). Antigen dilutions of 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600 were prepared from attenuated $10^8$ CFU.ml$^{-1}$ *E. coli O157:H7* using coating buffer (3.78g $NaCO_3$ and 3.35g Na $(CO_3)_2$ made up to 250ml with MilliQ water, pH 9.6) as a diluent. Respective antigen concentrations of 100µl were pipetted into the wells of a microtiter plate as detailed in Table 4. Wells A7 to H7 were incubated with 100µl of coating buffer. The microtiter plate was placed in the fridge at 4$^o$C and incubated overnight. After the incubation period, the wells of the microtiter plate was rinsed three times with phosphate buffered saline (PBS) and 0.5% Tween. The plate was then incubated with 0.5% bovine serum albumin (BSA) and placed at 37$^o$C for 90min. Afterward, the wells on the plate were rinsed three times with PBS and 0.5% Tween.

Antibody dilutions of 1:1000, 1:2000, 1:3000, 1:4000 were prepared from 5.4mg.ml$^{-1}$ goat *E. coli O157:H7* antibody solution in wash buffer (PBS + 0.5% Tween) solution. Respective antibody dilutions of 100µl were added to the well as indicated in Table 4. Wells B5, B6, D6, F5, F6, H5 and H6 were without antibody and incubated with 100µl of wash buffer (PBS + 0.5% Tween) solution. The plate was incubated at 37$^o$C for 90min and then rinsed three times with PBS and 0.5% Tween.

Donkey anti-goat alkaline phosphatase conjagate (Promega, Madison, USA) of 100µl at concentration of 1:2500 was pipetted into each well. The plate was incubated for another 90min at 37$^{o}$C. The plate was rinsed three times with PBS and 0.5% Tween after incubation. *P*-nitrophenyl phosphate (PNPP) (Sigma-Aldrich, St. Louis, USA) of concentration 250µg.ml$^{-1}$ was diluted to a concentration of 1:100 with a commercial diethanolamine stock buffer (Sigma-Aldrich, St. Louis, USA). A volume of 100µl of diluted PNPP was added to each well. PNPP in combination with alkaline phosphatase undergo hydrolysis reactions to form a yellow water-soluble product with absorbance at a wavelength of 405nm. Absorbance was recorded using an ELISA plate reader (Biorad, CA, USA) after 10min and 20min of reaction time. The intensity of the yellow colour observed in each well would be proportional to the amount of antigen-antibody reaction that had taken place. A volume of 50µl of 3.75M NaOH was added to each well after 20min to cease further reaction.

**Table 4. Microtitre plate set-up for ELISA**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Antibody Concentration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | Negative Control | - | - | - | - | - | 1:1000 |
| **B** | 1:50 | 1:100 | 1:200 | 1:400 | Antibody excluded | Antibody excluded | Negative Control | - | - | - | - | - | 1:1000 |
| **C** | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | Negative Control | - | - | - | - | - | 1:2000 |
| **D** | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | Antibody excluded | Negative Control | - | - | - | - | - | 1:2000 |
| **E** | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | Negative Control | - | - | - | - | - | 1:3000 |
| **F** | 1:50 | 1:100 | 1:200 | 1:400 | Antibody excluded | Antibody excluded | Negative Control | - | - | - | - | - | 1:3000 |
| **G** | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | Negative Control | - | - | - | - | - | 1:4000 |
| **H** | 1:50 | 1:100 | 1:200 | 1:400 | Antibody excluded | Antibody excluded | Negative Control | - | - | - | - | - | 1:4000 |

Antigen concentrations
(1:50, 1:100, 1:200, 1:400, 1:800, 1:1600)

Wells were unused.

## 3.3 Quartz crystals

### 3.3.1 Cleansing of quartz crystal surface

Methods were adapted from Su and Li (2004). Quartz crystals were immersed in 1M NaOH for 20min, rinsed thoroughly with MilliQ water and air-dried. Crystals were then immersed in 1M HCl for 3min, rinsed thoroughly with MilliQ water and air-dried. Finally, crystals were subjected to a piranha process where both sides of the crystal were coated with a mixture of sulphuric acid ($H_2SO_4$) and hydrogen peroxide (30% $H_2O_2$) at a ratio of 3:1 (150µl $H_2SO_4$ and 50µl 30%$H_2O_2$) for 1min. Sulphuric acid was added directly to the crystal, followed by hydrogen peroxide. After incubation with the piranha mixture for 1min, the crystal was rinsed thoroughly with MilliQ water and air-dried. This cleansing process was performed to remove contaminants from the surface. In addition, the oxidizing piranha solution produced a hydrophilic surface which assisted in the formation of the subsequent 16-mercaptohexadecanoic acid (MHDA) monolayer. Frequency reading of the cleansed crystal was recorded using the quartz crystal microbalance, QCM200 (Stanford Research Systems, CA, USA). Each crystal was placed into the crystal holder, which was connected to the crystal oscillator. Frequency recordings were measured by the digital controller using SRS QCM200 LabVIEW Data Acquisition Software Version 1.00 provided by Stanford Research Systems. The frequency of the crystal was recorded as the first stable plateau maintained for at least 5min.

### 3.3.2 Preparation of quartz crystal surface

Methods were adapted from Su and Li (2004). A solution of 10mM 16-mercaptohexadecanoic acid (MHDA) was prepared in ethanol. Crystal was placed onto a holder and immersed in a beaker containing 10mM MHDA for 24hr at ambient room temperature (22.5$^{o}$C). The beaker was sealed with parafilm to prevent evaporation of solution. 16-mercaptohexadecanoic acid forms a monolayer on the gold surface of the crystal as illustrated in Figure 5. The sulphide groups of the MHDA have an affinity to gold and will be adsorbed onto the surface forming Au-SH bonds. This affinity is strong enough to remove any contaminants not completely washed off in the cleansing process. The MHDA takes at least 24hr to stabilize and form a compact and even monolayer. After 24hr, the crystal was rinsed thoroughly with consecutive washes of 100% ethanol and MilliQ water. The crystal was air-dried at ambient room temperature (22.5$^{o}$C) and placed onto the QCM holder for a frequency reading. The first stable frequency plateau maintained for at least 5min was recorded as the resonant frequency of the MHDA-coated quartz crystal.

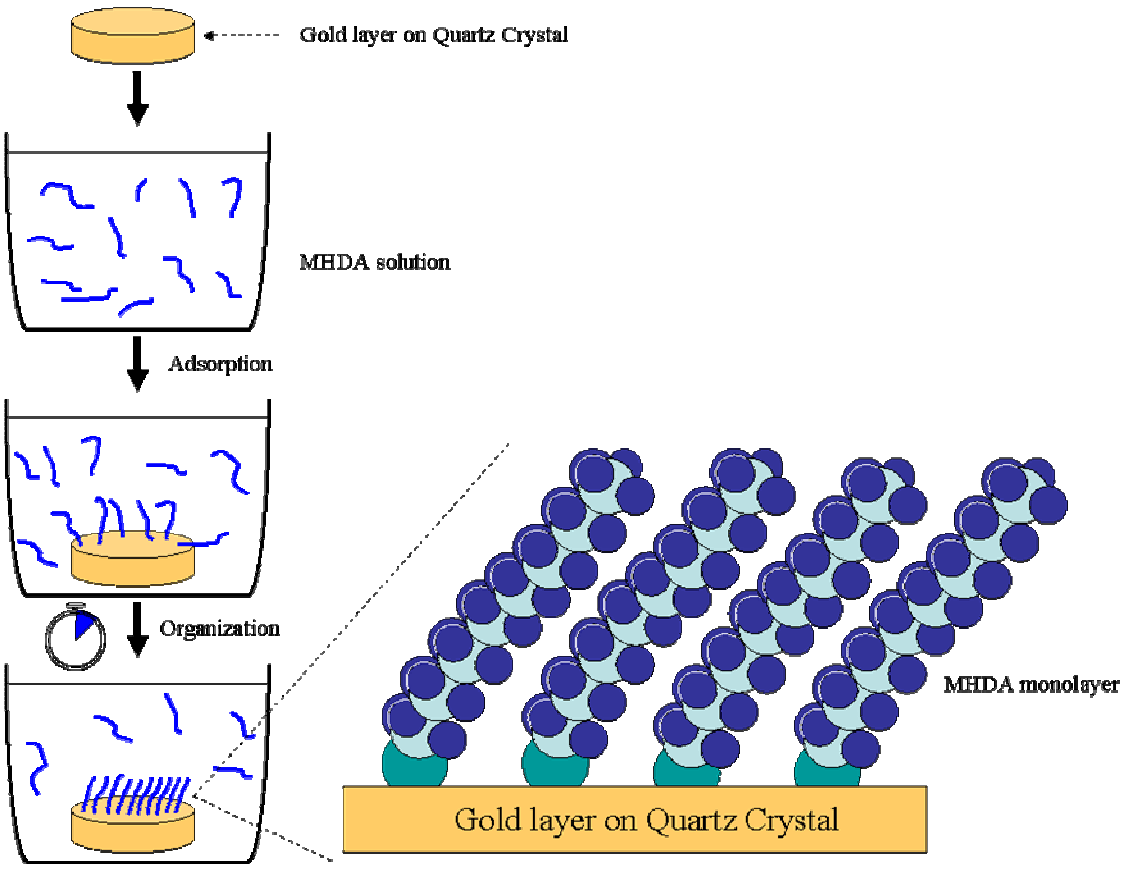


**Figure 5. A monolayer of MHDA is laid on the gold coating of the quartz crystal. The crystal is immersed in a solution of MHDA. MHDA has an affinity for the gold layer. Over time, a monolayer matrix of MHDA is laid on the surface of the crystal (adapted from www.ifm.lie.se/applphys/ftir/sams.html).**

75mM N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide (EDC) and 15mM N-Hydroxy-succinimide (NHS) solutions were prepared in PBS. The MHDA monolayer was activated with a 1:1 ratio of EDC and NHS (100µl 75mM EDC and 100µl 15mM NHS) and left to incubate at ambient room temperature (22.5$^{o}$C) for 1hr. The terminal carboxylic group of the MHDA monolayer was converted by EDC and NHS to form a NHS ester. The terminal NHS ester aids in the formation of the antibody layer. After activation, crystals were rinsed

thoroughly with PBS followed by MilliQ water and air-dried at ambient room temperature (22.5$^{o}$C). Using the QCM, frequency readings after EDC-NHS were recorded as the first stable frequency plateau maintained for at least 5min.

### 3.3.3 Coating of *Escherichia coli O157:H7* antibody layer

Methods were adapted from Su and Li (2004). 90µl of PBS and 10µl of antibody solution was added to both sides of the crystal in a petri dish. The gold surface of the crystal was completely covered with the mixture. The crystal was then refrigerated at 4$^{o}$C and left to incubate for the specified amount of time (1hr, 7hr or 15hr). After incubation, the crystal was rinsed thoroughly with PBS, followed by MilliQ water and air-dried. Frequency of the antibody coated crystal was recorded using the QCM. The frequency of the crystal was recorded as the first stable plateau maintained for at least 5min. Overall process of crystal preparation is illustrated in Figure 6.

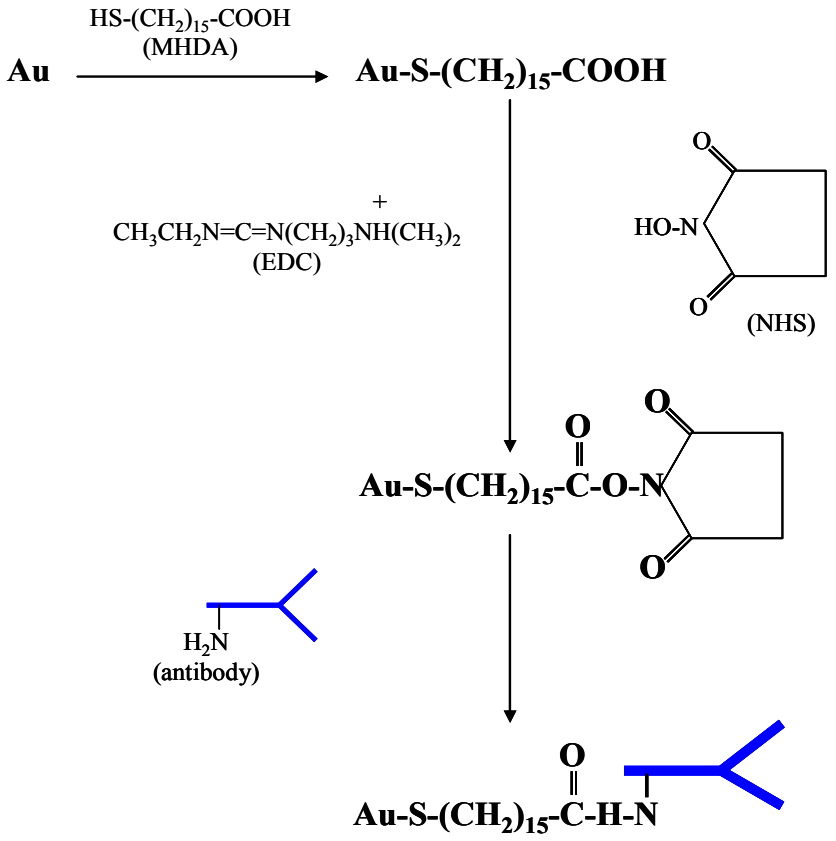


**Figure 6. Process of crystal preparation for the detection of *Escherichia coli O157:H7* using the quartz crystal microbalance adapted from Su and Li (2004)**

### 3.3.4 Storage of antibody coated crystals

Methods were adapted from Eun et al (2002b). After recording the frequency of the antibody layered crystal, 200µl of 1% bovine serum albumin in PBS (1% BSA-PBS) was applied to both sides of the crystal and left to incubate for at least 1hr. BSA blocks the remaining terminal NHS-esters that had not reacted with the antibodies, preventing non-specific antigen binding. After incubation, the crystal was rinsed thoroughly with consecutive rinses of PBS and MilliQ water. The crystal was then air dried at ambient room

temperature (22.5$^{o}$C). Frequency measurement of the dried crystal was taken by placing the crystal onto the holder and recorded as the first stable frequency plateau maintained for at least 5min.

Three methods of storage of BSA blocked, antibody coated crystals were carried out as outlined in Table 5. Crystals were either stored at ambient room temperature (22.5$^{o}$C) or refrigerated at 4$^{o}$C. Crystals stored dried were placed in a dessicator covered with a base layer of $SiO_2$ and air in the dessicator was removed using a vacuum pump. Vacuum was re-applied every two days. Crystals stored undried were left uncovered on a stand in a fumehood.

**Table 5: Method of storage of antibody coated crystals**

| Method | Temperature | Condition |
|---|---|---|
| **RT, undried** | 22.5$^{o}$C | Stored uncovered in a fumehood. |
| **RT, dried** | 22.5$^{o}$C | Stored in a vacuum dessicator. |
| **4$^{o}$C, dried** | 4$^{o}$C | Stored in a vacuum dessicator. |

### 3.3.5 Coating of antigen (*Escherichia coli O157:H7)* layer

Methods were adapted from Su and Li (2004). Each MHDA coated crystal was immersed in 3ml of antigen ($10^7$ CFU.ml$^{-1}$ *E. coli O157:H7*) and left to incubate at ambient room

temperature (22.5$^{o}$C) for the specified amount of time (1hr or 15hr). After incubation, crystal was rinsed thoroughly with consecutive washes of PBS and MilliQ water and left to air dry at ambient room temperature (22.5$^{o}$C). Each dried crystal was placed onto the QCM holder and the frequency reading was taken. The frequency of the crystal was recorded as the first stable plateau maintained for at least 5min.

### 3.3.6 Removal of antigen (*Escherichia coli O157:H7)* layer

Methods were adapted from Su and Li (2004). After the completion of each assay, the quartz crystal was immersed in 0.2M Gly-HCl for 5min. Immersion in 0.2M Gly-HCl would break the bonds between the antibodies and antigens, releasing the antigens into the solution. Thereafter, crystal was rinsed consecutively with PBS and MilliQ water and left to air dry at ambient room temperature (22.5$^{o}$C) before it was placed onto the QCM holder for a frequency reading. The frequency of the crystal was recorded as the first stable plateau maintained for at least 5min. The crystal was then ready to be reused for another antigen binding assay. Each antibody coated crystal was used for a maximum of two times.

### 3.3.7 Regeneration of quartz crystals

Methods were adapted from Wu et al (2003). Used quartz crystals were washed with piranha solution (150µl $H_2SO_4$ and 50µl 30% $H_2O_2$) three times for 3min to completely remove all traces of the MHDA monolayer and proteins bonded onto the crystal surfaces. After each set of piranha cleansing, each crystal was rinsed thoroughly with MilliQ water

and air dried at ambient room temperature (22.5$^{o}$C). Upon completion of the treatment process, the resonance frequency of the crystals was found to revert back to the average resonance of 5MHz, ready to be reused for a new assay.

# 4. Results

## 4.1 *Escherichia coli O157:H7*

*Escherichia coli O157:H7* was purchased from Oxoid (Hampshire, UK) in the form of culti-loops. The purchased *Escherichia coli O157:H7* was plated on blood agar to ensure that the bacteria were of a pure culture. To determine the bacteria concentration in each loop, serial dilutions of the dissolved bacteria loop were plated and colony counts carried out. (See Table 6 of section 4.1.2)

### 4.1.1 Antigen purity

The plated stock bacteria solution on the blood agar showed a single bacterium growth. This confirmed that the culti-loops purchased were pure and free from contaminating microbes.

### 4.1.2 Colony count

**Table 6. Colony counts of serially diluted *Escherichia coli O157:H7* culture**

| Labelled nutrient agar plate | Colony counts |
|---|---|
| **$10^{-4}$** | Confluent |
| **$10^{-5}$** | Confluent |
| **$10^{-6}$** | 235 |
| **$10^{-7}$** | 30 |
| **$10^{-8}$** | 3 |

Confluent growth was observed on plates $10^{-4}$ and $10^{-5}$ and there were too few colonies on plate $10^{-8}$ to be used in calculations (see Table 6). Therefore, only colony counts from nutrient agar plates labelled $10^{-6}$ and $10^{-7}$ were used for calculation of concentration in $CFU.ml^{-1}$ of stock culture labelled TSB:

- Using results from $10^{-6}$ nutrient agar plate:

  $CFU.ml^{-1}$ of stock culture = $235 \times 10 \times 10^{6} = 2.35 \times 10^{9}$ $CFU.ml^{-1}$

- Using results from $10^{-7}$ nutrient agar plate:

  $CFU.ml^{-1}$ of stock culture = $30 \times 10 \times 10^{7} = 3.00 \times 10^{9}$ $CFU.ml^{-1}$

- Average $CFU.ml^{-1}$ of stock culture = $(2.35 + 3.00)/2 \times 10^{9}$ $CFU.ml^{-1}$

  $= 2.675 \times 10^{9}$ $CFU.ml^{-1}$

  $= 3 \times 10^{9}$ $CFU.ml^{-1}$ (to nearest whole number)

The concentration of stock *E. coli O157:H7* culture was recorded as $3 \times 10^{9}$ $CFU.ml^{-1}$.

## 4.2 *Escherichia coli O157:H7* antibodies

The viable *E. coli O157:H7* strains (antigen) purchased were inactivated by heat (120$^{o}$C) prior to use in further experiments. The ability of the goat polyclonal antibodies specific for *E. coli O157:H7* purchased from Fitzgerald (MA, USA) to bind to the antigen strains were tested using an enzyme linked immunosorbent assay (ELISA). The ELISA test would ensure that any negative binding results obtained were not due in any part to the affinity of the antibodies for the attenuated antigens.

### 4.2.1 Enzyme linked immunosorbent assay (ELISA)

An ELISA test was carried out using the *Escherichia coli O157:H7* specific antibodies and attenuated *E. coli O157:H7* antigens. Reactions were stopped after 20min and absorbance recorded in Table 7. Values of varying antigen concentration (1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600) and varying antibody concentrations (1:1000, 1:2000 and 1:3000) were plotted against absorbance (Figure 7). Where multiple absorbance readings were taken, the average value was plotted.

Keeping the antigen concentration constant (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600), the absorbance decreased with decreasing antibody concentration (1:1000, 1:2000, 1:3000). When the antibody concentration was kept constant (1:1000, 1:2000, 1:3000), the

absorbance decreased with decreasing antigen concentration (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600).

It is good practice to not use the peripheral wells of the ELISA plate due to possible edge effect. However, for the purpose of this experiment, edge effect is of minimal consequence. Hence, from the results, it can be concluded that the purchased goat *E. coli O157:H7* antibodies had a good affinity to the attenuated *E. coli O157:H7* antigens. This showed that although the *E. coli O157:H7* antigens were attenuated, their binding affinity to the respective *E. coli O157:H7* antibody had not been compromised.

**Table 7. Absorbance readings taken after 20min of reaction showing the affinity of the commercial antibody preparation for the attenuated *E. coli O157:H7***

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Antibody Concentration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 1.070 | 0.887 | 0.806 | 0.729 | 0.478 | 0.361 | 0.109 | - | - | - | - | - | 1:1000 |
| **B** | 1.025 | 0.762 | 0.630 | 0.531 | 0.116 | 0.111 | 0.110 | - | - | - | - | - | 1:1000 |
| **C** | 0.961 | 0.628 | 0.557 | 0.477 | 0.350 | 0.255 | 0.108 | - | - | - | - | - | 1:2000 |
| **D** | 0.963 | 0.671 | 0.536 | 0.394 | 0.251 | 0.109 | 0.109 | - | - | - | - | - | 1:2000 |
| **E** | 0.790 | 0.539 | 0.447 | 0.340 | 0.268 | 0.211 | 0.107 | - | - | - | - | - | 1:3000 |
| **F** | 0.781 | 0.538 | 0.420 | 0.379 | 0.109 | 0.110 | 0.106 | - | - | - | - | - | 1:3000 |
| **G** | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **H** | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Antigen Concentration** | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | - | - | - | - | - | - | - |

* All highlighted values were negative controls (i.e. no antigen was added into the respective wells).

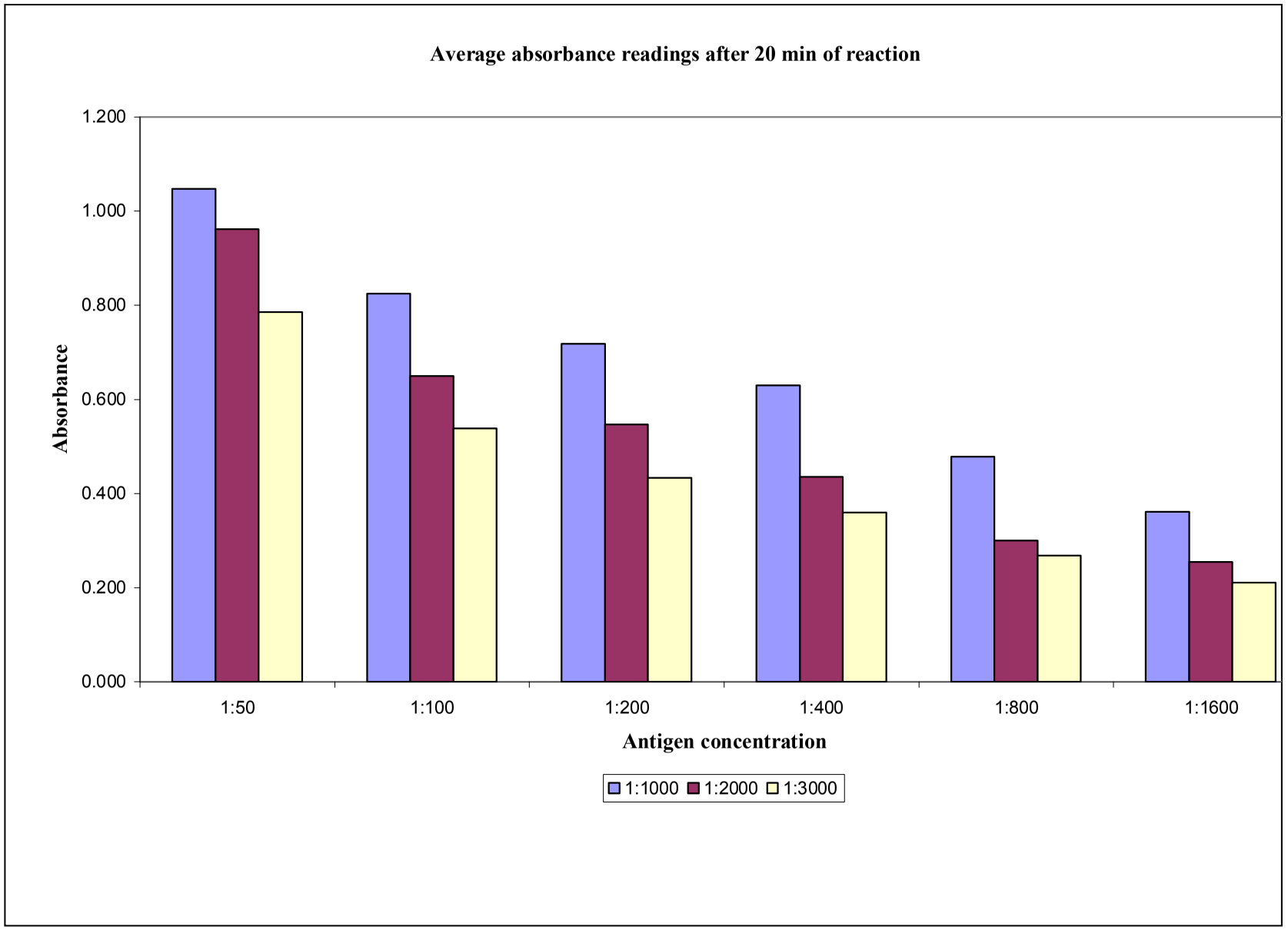


**Figure 7. Varying antigen concentrations (1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600) and varying antibody concentrations (1:1000, 1:2000 and 1:3000) plotted against absorbance after 20min of reaction**

## 4.3 Quartz crystals

The following factors were studied using quartz crystals purchased from Stanford Research Systems (CA, USA):

(1) Optimal antibody incubation duration

(2) Optimal antibody incubation concentration

Determining the optimal antibody incubation duration provides an estimate of the time required for completion of antibody binding to the 16-mercaptohexadecanoic acid (MHDA) monolayer surface. This will be useful in cases where time available is a constraint. Data relating to the optimal antibody incubation concentration would provide an estimate of the minimal antibody concentration needed to fully saturate the crystal surface, determining the minimal cost required to produce an optimum antibody coating.

(3) Optimal antigen incubation duration

Establishing the optimal antigen incubation duration would provide the minimal time required for the completion of the antibody-antigen binding reaction. The optimal condition would allow the determination of the minimum time required for the binding reaction without time being a limiting factor in the binding process.

(4) Optimal coated crystal storage conditions

Three different conditions for storage of antibody coated crystals for a period of 2 to 4 weeks were investigated. If antibody coated crystals can be stored for a period of time without a significant loss in antigen binding efficacy, antibody coated crystals can then be prepared beforehand and stored until required. This allows pre-coated crystals to be readily available on demand, a useful means when time is of constrain.

(5) Quartz crystal regeneration

After an initial assay, if the antigen layer can be removed efficiently without a significant loss in antibody reactivity, the crystal can then be reused for a second assay. This will allow the repetitive use of a single antibody-coated crystal, reducing time and cost per assay reaction.

(6) Quartz crystal reusability

Ten chrome/gold plated quartz crystals costs US $295. If the MHDA layer and proteins can be completely stripped without affecting the chrome/gold layer, the quartz crystal can be recoated and reused. The reusability of the quartz crystal will further reduce the unit cost of each assay.

### 4.3.1 Optimal antibody incubation duration

Quartz crystals were incubated with 0.1mg.ml$^{-1}$antibody concentration for 1hr, 7hr and 15hr. This procedure was repeated using 0.5mg.ml$^{-1}$ antibody concentration. For this particular section, only one round of rinsing was carried out with phosphate buffered saline (PBS) and MilliQ water after the specified period of antibody incubation. Results were plotted time (hr) against frequency (Hz). Refer to Figure 8.

A time dependent binding of the antibody to the 16-mercaptohexadecanoic acid (MHDA) layer was observed for crystals incubated with an antibody concentration of 0.1mg.ml$^{-1}$. The frequency drop for the first 7hr was an average of 150Hz.hr$^{-1}$. The average frequency drop between 7hr to 15hr was an average of 41Hz.hr$^{-1}$. This showed that the binding of antibodies in the initial hours of incubation was rapid, followed by a declining rate of antibody binding. The same pattern of antibody binding to the MHDA layer was observed for crystals incubated with 0.5mg.ml$^{-1}$. The frequency drop for the first 7hr was an average of 172Hz.hr$^{-1}$ and the frequency drop between 7hr to 15hr was 30Hz.hr$^{-1}$. Rate of antibody binding in the first few hours was rapid followed by a decreasing rate of antibody binding.

Comparing the graphs of 0.1mg.ml$^{-1}$ and 0.5mg.ml$^{-1}$, the frequency decrease of 0.5mg.ml$^{-1}$ throughout the 15hr incubation period was more rapid than that of 0.1mg.ml$^{-1}$. This showed that for the first 15hr of incubation, the rate of antibody binding to the MHDA layer was faster for crystals incubated with the higher antibody concentration of 0.5mg.ml$^{-1}$ than crystals incubated with the lower antibody concentration of 0.1mg.ml$^{-1}$.

At 7hr, the difference between the recorded 0.1mg.ml$^{-1}$ and 0.5mg.ml$^{-1}$ values was 153Hz and at 15hr was 61Hz. This showed that by increasing the antibody incubation duration, the difference in the amount of bonded antibody using the two different antibody incubation concentrations (0.1mg.ml$^{-1}$ and 0.5mg.ml$^{-1}$) was decreasing and converging to a common value, which is the point of crystal surface saturation.

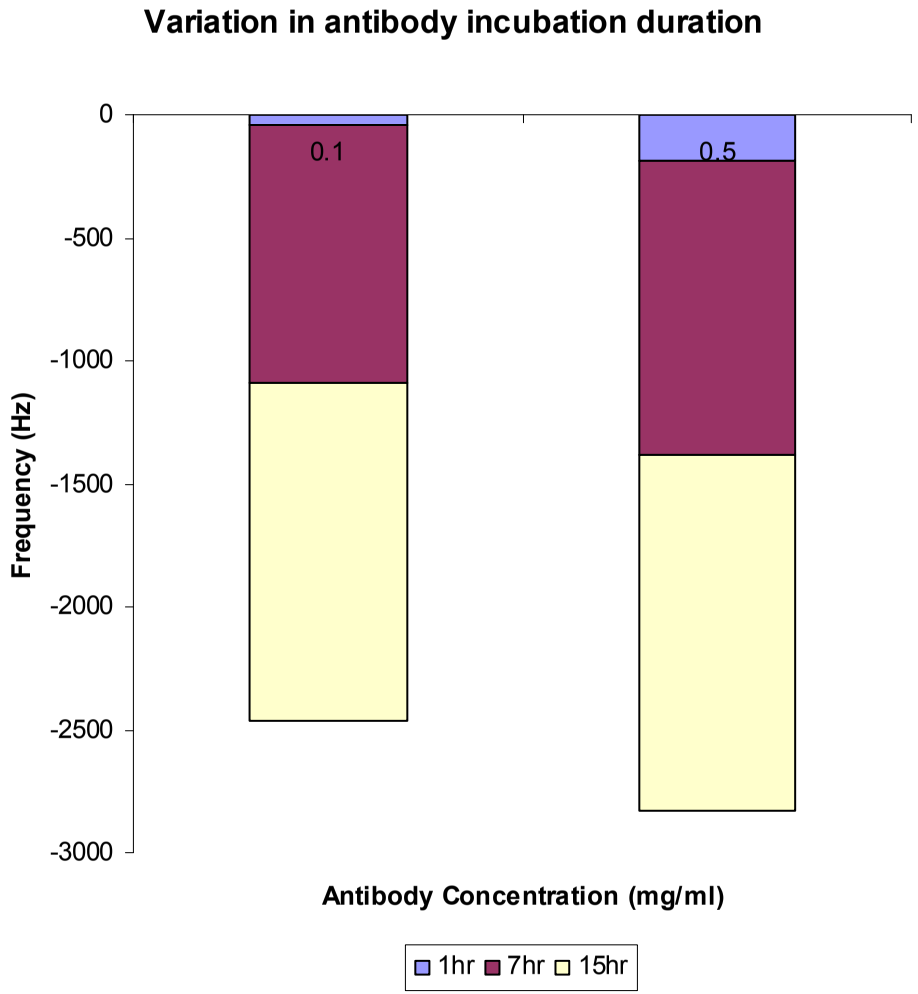


**Figure 8. Differential antibody incubation duration (1hr, 7hr or 15hr) for crystals coated with antibody concentration of either 0.1mg.ml$^{-1}$ or 0.5mg.ml$^{-1}$. (n=1)**

### 4.3.2 Optimal antibody incubation concentration

Crystals were coated with antibody solutions of varying concentrations (0.05mg.ml$^{-1}$, 0.1mg.ml$^{-1}$, 0.2mg.ml$^{-1}$, 0.3mg.ml$^{-1}$, 0.4mg.ml$^{-1}$, 0.5 mg.ml$^{-1}$) and incubated for 15hr, allowing binding of the antibodies to the 16-mercaptohexacedanoic acid (MHDA) layer. Frequency readings after incubation were recorded in Table 8 and the average frequency change plotted in Figure 9.

Crystals incubated with antibody concentration 0.2mg.ml$^{-1}$ registered the largest mean frequency drop. However it cannot be concluded that the optimum antibody concentration for incubation of crystals is 0.2mg.ml$^{-1}$ because the standard deviation for 0.2mg.ml$^{-1}$ is too large (see Figure 9).

Although the standard deviation for 0.05mg.ml$^{-1}$ was relatively small, the mean frequency drop for antibody concentrations 0.1mg.ml$^{-1}$, 0.2mg.ml$^{-1}$, 0.3mg.ml$^{-1}$ and 0.5mg.ml$^{-1}$ exceeded that of 0.05mg.ml$^{-1}$. This indicates that 0.05mg.ml$^{-1}$ is not the optimum antibody incubation concentration.

**Table 8. Frequency readings of crystals coated with varying antibody concentrations ($0.05mg.ml^{-1}$, $0.1mg.ml^{-1}$, $0.2mg.ml^{-1}$, $0.3mg.ml^{-1}$, $0.4mg.ml^{-1}$, $0.5mg.ml^{-1}$) in a 15hr incubation period**

| Reading | Antibody Concentration ($mg.ml^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| **1** | -66 | -3 | -142 | -103 | 4 | -200 |
| **2** | -86 | -90 | -157 | -136 | 6 | -84 |
| **3** | -21 | -85 | -82 | -37 | -83 | -124 |
| **4** | -73 | -7 | -46 | -78 | -51 | -88 |
| **5** | 3 | -91 | -69 | -95 | -46 | -7 |
| **6** | | -100 | 9 | -78 | -1 | -12 |
| **7** | | -25 | -186 | 13 | -94 | -5 |
| **8** | | -185 | | | -54 | -11 |
| **9** | | 3 | | | | |
| **10** | | -21 | | | | |
| **11** | | 19 | | | | |
| **12** | | -46 | | | | |
| **13** | | -58 | | | | |
| **14** | | -51 | | | | |
| **15** | | -49 | | | | |
| **16** | | 24 | | | | |
| **17** | | -165 | | | | |
| **Mean frequency (Hz)** | -48.6 | -54.7 | -96.1 | -73.4 | -39.9 | -66.4 |
| **Standard deviation** | 33.8 | 57.8 | 63.7 | 44.9 | 36.6 | 66.4 |

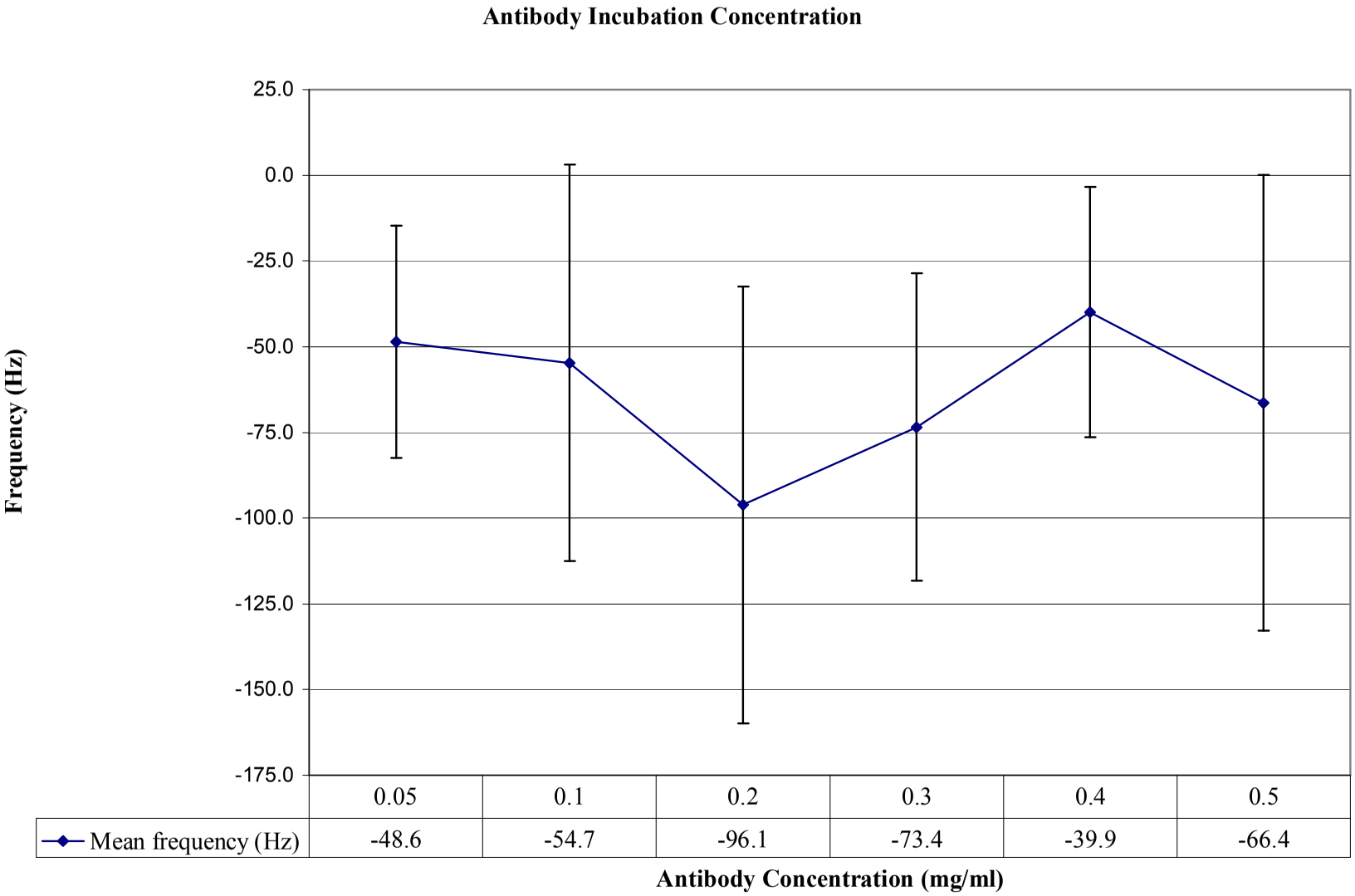


**Figure 9. Graph of varying antibody concentration (0.05mg.ml$^{-1}$ to 0.5mg.ml$^{-1}$) plotted against average crystal frequency (Hz) after 15hr of incubation at 4$^{o}$C**

### 4.3.3 Optimal antigen incubation duration

Crystals coated with antibody concentration of 0.1mg.ml$^{-1}$ were incubated with $10^7$ CFU.ml$^{-1}$ antigen concentration at ambient room temperature (22.5$^{o}$C) for either 1hr or 15hr. Results were tabulated in Table 9 and mean frequency values were plotted in Figure 10.

The mean frequency decrease when crystals were incubated for 1hr was comparatively close to the mean frequency decrease when crystals were incubated for 15hr, with a difference of only 5.2Hz. It can be concluded that a 1hr antigen incubation time at ambient room temperature (22.5$^{o}$C) is sufficient for efficient binding of the antigens to the immobilized antibody layer.

**Table 9. Antibody concentration, antibody incubation duration and antigen concentration were kept constant whilst antigen incubation time was varied (1hr or 15hr)**

| Reading | Time (hr) | |
|---|---|---|
| | 1 | 15 |
| **1** | -126 | 11 |
| **2** | -14 | 14 |
| **3** | 7 | -85 |
| **4** | -22 | -221 |
| **5** | -37 | 63 |
| **Mean Frequency (Hz)** | -38.4 | -43.6 |
| **Standard deviation** | 46.0 | 100.8 |

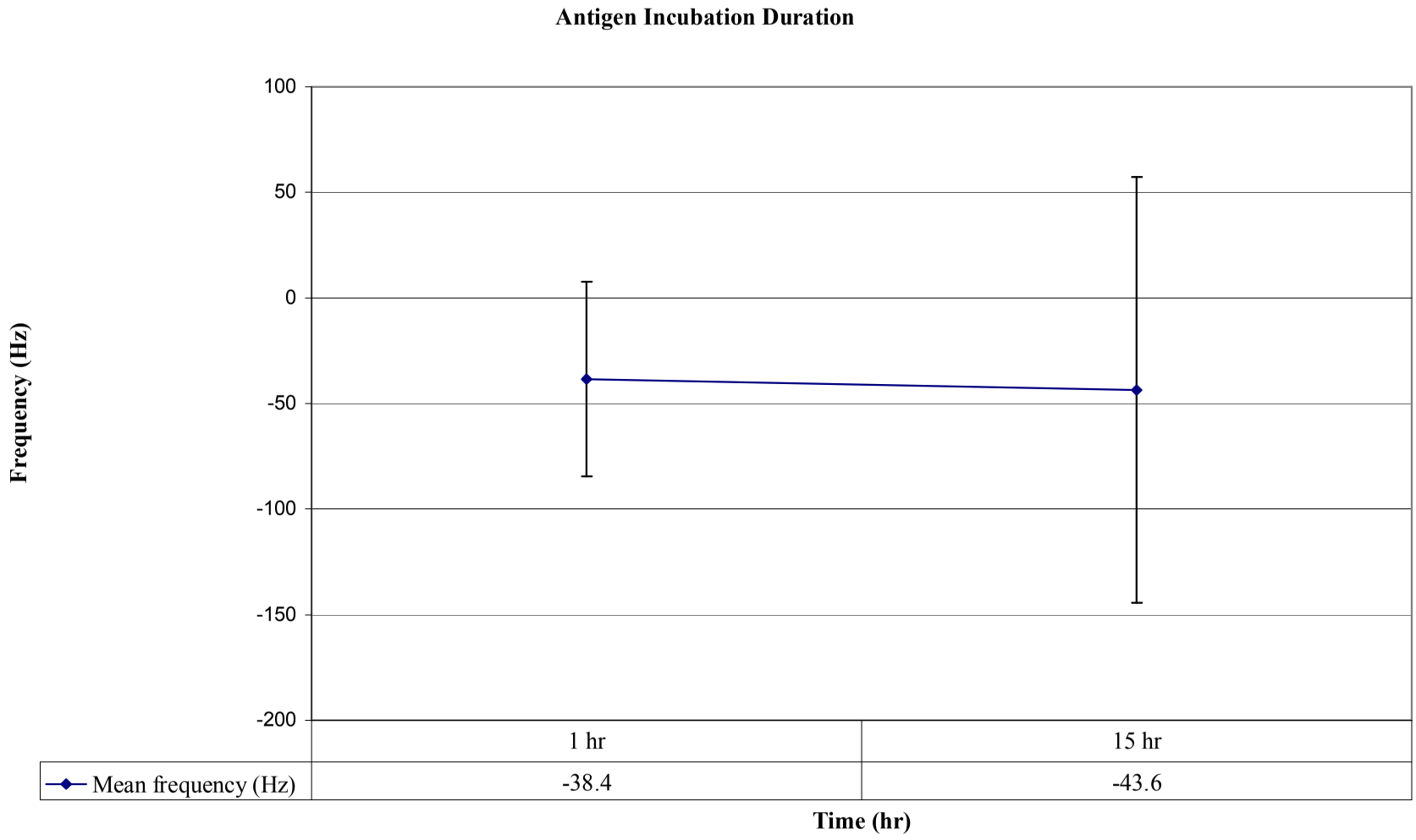


**Figure 10. Graph of varying antigen incubation time (1hr or 15hr) plotted against average crystal frequency (Hz)**

### 4.3.4 Optimal conditions for storage of antibody coated crystal

Crystals were stored for 2 to 4 weeks in three different conditions:

(1) Dried and stored at ambient room temperature (22.5$^{o}$C)

(2) Undried and stored at ambient room temperature (22.5$^{o}$C)

(3) Dried and stored in the refrigerator at 4$^{o}$C

The viability of stored crystals was compared against the viability of freshly prepared crystals. After the specified storage period, crystals were incubated with antigen and frequency readings recorded in Table 10.

Immediate use of freshly coated crystals produced good results across the different antibody concentrations (0.1mg.ml$^{-1}$ to 0.5mg.ml$^{-1}$) tested.

Overall, the immunochemical efficiency of the crystals after storage for 2 to 4 weeks was reduced regardless of the nature of the storage conditions used. Dry storage of crystals at 4$^{o}$C fared worst with only 1 out of 5 crystals registering a frequency drop (see Table 10). This showed poor viability of the pre-coated antibody crystal stored dry at 4$^{o}$C for a storage period of 2 to 4 weeks. Three of the five crystals that were stored at 22.5$^{o}$C (dried and undried) registered a frequency drop, reflecting a significant reduction in the immunochemical efficiency of the stored crystals.

Comparing the three different storage conditions, crystals stored at an ambient room temperature of 22.5$^{o}$C fared better than crystals stored at 4$^{o}$C. In addition, crystals stored

dried at 22.5$^{o}$C and crystals stored undried at 22.5$^{o}$C both had 3 out of 5 crystals registering a frequency drop after 2 to 4 weeks storage time. Hence, there was no relative difference in the viability of crystals kept at a constant 22.5$^{o}$C when either stored dry in a vacuum (dried) or left uncovered in room conditions (undried). This set of results suggests that the best and most convenient method amongst the three storage conditions tested is undried at the ambient room temperature of 22.5$^{o}$C.

**Table 10. A comparison of the viability of crystals stored under varying conditions after a storage period of 2 to 4 weeks**

| Antibody Concentration ($mg.ml^{-1}$) | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
|---|---|---|---|---|---|
| **Immediate use (0 week storage)** | -221 | -138 | -107 | -80 | -118 |
| **Dried at ambient room temperature of 22.5°C (2 to 4 weeks storage)** | 12 | -167 | -8 | -162 | 59 |
| **Undried at ambient room temperature of 22.5°C (2 to 4 weeks storage)** | -8 | -67 | 43 | 72 | -16 |
| **Dried at 4°C (2 to 4 weeks storage)** | 151 | 150 | 28 | 45 | -49 |

Frequency change (Hz) under different storage conditions

### 4.3.5 Quartz crystal regeneration

After an initial assay, each coated crystal was immersed in 0.2M Gly-HCl for 5min to remove the antigen layer without affecting the immobilized antibody layer. The frequency reading taken after the Gly-HCl wash was compared against the previous frequency recording of the antibody coated crystal. Results were tabulated in Table 11 and illustrated in Figure 11.

Three of the 16 crystals had negative frequency differences which indicated an under-removal of proteins from the crystal surface. Thirteen of the 16 crystals registered positive frequency readings which indicated an over-removal of proteins from the crystal surface. Overall, the results showed that the removal of the antigen layer by immersion in 0.2M Gly-HCl for 5min resulted in an over-removal of proteins from the crystal surface. The average frequency difference was in excess of +132 Hz (see Table 11).

**Table 11. Calculation of the effectiveness of 0.2M Gly-HCl as a reagent for antigen layer removal**

| | Frequency of crystal coated with antibody + BSA (Hz) | Frequency of crystal after 5min Gly-HCl (Hz) | Frequency Difference (Hz) |
|---|---|---|---|
| **1** | 5,005,835 | 5,005,867 | +32 |
| **2** | 5,005,673 | 5,005,539 | -134 |
| **3** | 5,004,947 | 5,005,667 | +720 |
| **4** | 5,000,849 | 5,001,671 | +822 |
| **5** | 5,005,439 | 5,005,648 | +209 |
| **6** | 5,005,648 | 5,005,903 | +255 |
| **7** | 5,001,183 | 5,001,231 | +48 |
| **8** | 5,010,144 | 5,010,196 | +52 |
| **9** | 5,010,196 | 5,010,034 | -162 |
| **10** | 5,005,785 | 5,005,716 | -69 |
| **11** | 5,004,252 | 5,004,294 | +42 |
| **12** | 5,010,113 | 5,010,164 | +51 |
| **13** | 4,997,268 | 4,997,371 | +103 |
| **14** | 5,006,298 | 5,006,334 | +36 |
| **15** | 5,006,334 | 5,006,364 | +30 |
| **16** | 4,995,530 | 4,995,609 | +79 |
| | | Mean frequency difference | +132.1 |

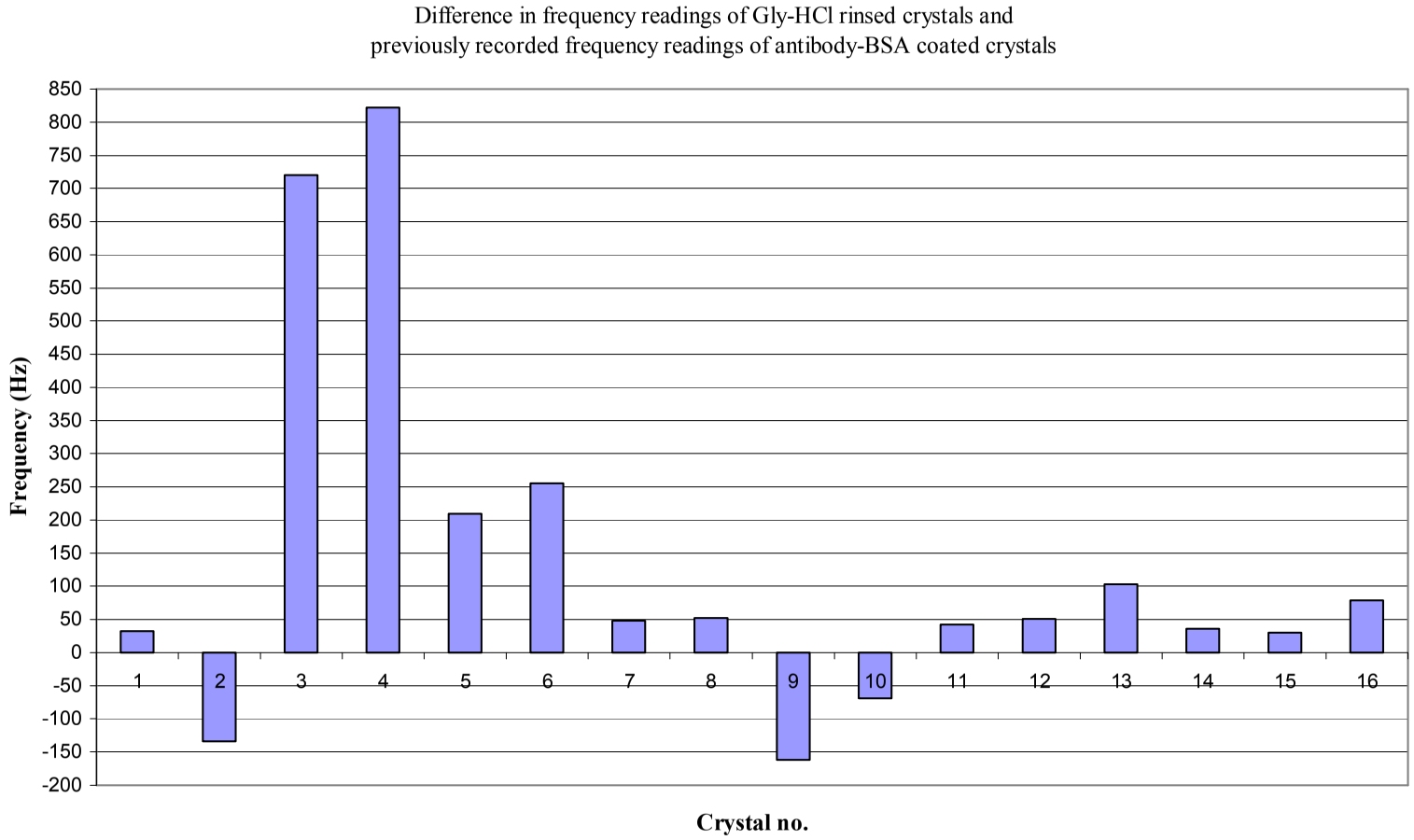


**Figure 11. Effectiveness of 0.2M Gly-HCl as a reagent for antigen layer removal for crystal regeneration**

### 4.3.6 Quartz crystal reusability

The 16-mercaptohexadecanoic acid layer, bonded antibodies, bovine serum albumin and antigens immobilized on the crystal were removed by a piranha process where crystals were immersed in a mixture of sulphuric acid ($H_2SO_4$) and hydrogen peroxide (30% $H_2O_2$) at a ratio of 3:1 ($H_2SO_4$ and 30% $H_2O_2$) for 1min three times. Frequency of the cleansed crystal reverted back to the resonant frequency of approximately 5MHz and was recoated and reused. Referring to Table 12, crystals used in this study had been reused up to 9 times without any reduction in binding efficacy.

**Table 12. The number of repeated uses per crystal**

| Crystal | Number of times reused |
|---|---|
| **1** | 8 |
| **2** | 9 |
| **4** | 9 |
| **5** | 9 |
| **6** | 8 |
| **7** | 8 |
| **8** | 6 |
| **9** | 7 |
| **10** | 1 |
| **11** | 1 |
| **12** | 3 |
| **13** | 1 |
| **14** | 2 |
| **15** | 7 |
| **16** | 3 |
| **17** | 7 |
| **18** | 3 |
| **19** | 4 |
| **20** | 3 |
| **21** | 4 |

# 5. Discussion

## 5.1 Optimal antibody incubation duration

There is a specific pattern of antibody binding to the 16-mercaptohexadecanoic acid (MHDA) layer on the quartz crystal regardless of the antibody concentration used. There was an initial rapid binding of antibody to the MHDA layer (see Figure 8 of section 4.3.1). The rate of binding was gradually reduced with time after 7 hours of incubation. To better characterize the binding pattern, incubation times can be spaced out at hourly intervals. This will provide a more detailed binding pattern and the exact point of change between rapid and reduced rate of binding can be determined.

Results show that antibody incubation duration of the crystals may become a limiting factor should insufficient time be allowed for the binding process to occur. Time is required for the optimal binding of antibodies to the MHDA layer. However, results did show that incubation with a higher antibody concentration bring about a greater amount of antibody uptake. The frequency drop at every incubation time interval was greater for crystals incubated with $0.5mg.ml^{-1}$ than $0.1mg.ml^{-1}$ (see Figure 8 of section 4.3.1). This indicates that saturation of crystal surfaces with antibodies can be accomplished faster with a higher antibody concentration. Therefore, should time be a constraint, incubating the crystal with a higher antibody concentration may prove more efficient.

Results showed that with increasing incubation time, the total amount of antibodies bonded on the crystal surface using $0.1mg.ml^{-1}$ was gradually equalizing the total amount of antibodies bonded on the crystal surface using $0.5mg.ml^{-1}$ (see Figure 8 of section 4.3.1). Therefore should time not be a constraint, a lower antibody concentration with a prolonged incubation time will be sufficient to construct a complete antibody layer.

Further tests can be carried out through microscopy to view the differences (if any) of an antibody layer constructed rapidly with a higher antibody concentration and an antibody layer constructed gradually with a lower antibody concentration.

## 5.2 Optimal antibody incubation concentration

Results tabulated in Figure 9 of section 4.3.2 showed large standard deviations and hence no conclusive trend for antibody incubation duration could be made. This variation in data is most likely attributed to the inconsistency derived from the repeated manual rinsing of the crystals. Crystals that were only rinsed once after antibody application gave consistent results as shown in Figure 8 of section 4.3.1. However it is important to note that repeated rinsing is required for the efficient removal of excess antibodies as this would affect the frequency readings recorded for the subsequent steps. Hence an alternative method for the gentle yet efficient rinsing of the crystals after each incubation step has to be adopted. The attachment of a flow cell to the crystal holder is suggested for use in further experiments. The flow cell will provide well-defined flow conditions whereby rinsing processes of all the crystals can be kept at a consistent level. In addition, by using a flow cell, flow-induced shear forces that can be induced via the rinsing processes can be kept to a minimum. Consequently, the introduction of a flow cell in tandem with the quartz crystal microbalance could give a more reproducible set of results.

With the higher level of sensitivity using a flow cell, the antibody solution can be further titrated to cover a broader spectrum of antibody concentrations. It is suggested that further experiments be carried out using the following range of antibody concentrations $0.05mg.ml^{-1}$, $0.10mg.ml^{-1}$, $0.15mg.ml^{-1}$, $0.20mg.ml^{-1}$, $0.25mg.ml^{-1}$, $0.30mg.ml^{-1}$, $0.35mg.ml^{-1}$, $0.40mg.ml^{-1}$, $0.45mg.ml^{-1}$ and $0.50mg.ml^{-1}$. Results obtained from a broader

range of antibody concentrations will provide a more precise indication of the optimal antibody incubation concentration when incubation time is kept constant at 15 hours.

Optimal antibody concentration is linked with optimal antibody incubation duration and hence further studies will have to be conducted to expound the relationship between the two inter-twined factors, presenting the most efficient conditions for the construction of the antibody layer.

## 5.3 Optimal antigen incubation duration

The one hour incubation time required for the efficient binding of the antigen to the antibody layer is a promising result. When compared to the antigen-antibody binding steps carried out in section 3.2.2 for the enzyme linked immunosorbent assay (ELISA), the antigen-antibody binding process via the quartz crystal microbalance proved much simpler and faster. Efficient antigen-antibody binding in ELISA required 90min of incubation at 37$^{o}$C whilst via the QCM process, it took 1hr of incubation at ambient room temperature (22.5$^{o}$C). ELISA required further labelling steps for the visualization of the antigen-antibody binding reaction which required more reagents and another 90min of reaction time. On the other hand, no additional labelling was required for the QCM and results could be taken directly after completion of the antigen-antibody binding process, saving both time and money.

## 5.4 Optimal conditions for storage of antibody coated crystal

Immediate use of antibody coated, bovine serum albumin (BSA) blocked crystals had a 100% antigen binding efficiency as shown in Table 10 of section 4.3.4. Regardless of storage conditions, crystals that were stored for a period of 2 to 4 weeks showed a reduction in antigen binding efficiency. The loss of immunochemical activity with time was suggested by Suleiman and Guilbault (1994) as the gradual detachment of immobilized antibodies from the quartz crystal surface.

Eun et al (2002b) managed to successfully store antibody precoated, BSA-blocked crystals by keeping the crystals at 4$^{o}$C dried in dessicators for an approximate period of 6 months without any significant loss in immunochemical activity. Yet in this study, under the same conditions, crystals coated with *Escherichia coli O157:H7 antibodies*, BSA-blocked, stored at 4$^{o}$C and kept dried in a dessicator produced the largest loss in immunochemical activity amongst the three storage conditions tested. König and Grätzel (1993) showed that storage of coated crystals at room temperature and not at 4$^{o}$C gave the best results. Results obtained in this study were consistent with that of König and Grätzel (1993) with crystals stored at ambient room temperature (22.5$^{o}$C) faring better than crystals stored at 4$^{o}$C. König and Grätzel (1993) suggest that this surprising result may be due to the destabilization of the antibody-antigen interaction due to the subjection of the antibody coated crystals to repeated cooling and warming. Similarly, in this experiment, the coating and binding reactions are conducted at the ambient temperature of 22.5$^{o}$C but storage of crystals is at 4$^{o}$C. Hence, it is agreed on a partial note that the

cause of crystal storage at ambient room temperature faring better than crystal storage at 4$^{o}$C may be due to the cooling and warming process. However, essentially the affected mechanism may be the structure of the antibody itself rather than the antibody-antigen interaction as suggested. Thus by subjecting the antibody coated crystal to a warming and cooling process, the antibody structure may have incurred damage, resulting in an overall reduction in antibody activity.

Studies from both Eun et al (2002b) and König and Grätzel (1993) showed that keeping the antibody coated crystals dry produced better storage results. Data obtained in this study were inconclusive, further experiments will have to be carried out on the effect of moisture in the storage of antibody coated crystals.

## 5.5 Quartz crystal regeneration

There are three common reagents employed in the regeneration of the quartz crystal for repeated assay use, namely: 0.2M glycine hydrochloride (Gly-HCl), 0.2M ethanolamine or 8M urea. In this study, regeneration of the crystal was carried out using 0.2M Gly-HCl. Results showed that Gly-HCl tend to over-strip the proteins on the crystal surface, removing not only the antigen layer but also part of the antibody layer. Generally, studies employing these three regeneration reagents produced poor results.

A different method for the regeneration of the quartz crystal in two previous studies proved promising (König and Grätzel, 1993; König and Grätzel 1994). Short antigen specific synthetic peptides were constructed for the regeneration of the crystal surface via competitive binding. Short peptides have a low binding constant making it easy to remove the synthetic peptide from the antibody by a simple rinsing process. The disadvantage was that a high synthetic peptide concentration was required to overcome the high binding constant of the bound antigen. However the cost savings on the reduction in antibody usage far outweighs that of the increase in usage of competitive synthetic peptides. Using this method, König and Grätzel (1994) managed to use a single antibody coated crystal for 18 times without any significant loss in antibody activity. This method of regeneration can possibly reduce the average cost of each assay, making the quartz crystal microbalance an even more cost-effective biosensor. Competitive synthetic peptide elution should be considered in further studies for the regeneration of the quartz crystals used in *Escherichia coli O157:H7* detection.

## 5.6 Quartz crystal reusability

The 16-mercaptohexadecanoic acid and adsorbed proteins on the crystal are easily removed using piranha solution. Crystals undergo the piranha process three times before they are recoated and reused for further experiments. Wu et al (2003) repeatedly cleansed and reused a crystal for 50 cycles without any significant loss in crystal efficacy. In this study, crystals had been cleansed and recoated up to 9 cycles without any reduction in crystal efficacy (see Table 12 of section 4.3.6). The ability to clean and reuse the crystals reduces the cost per assay test, making the quartz crystal microbalance biosensor a cost-effective option.

# 6. Overall conclusion and future research

The following 6 quartz crystal microbalance (QCM) procedures for use in the detection of *Escherichia coli O157:H7* were tested.

(1) Optimal antibody incubation duration

(2) Optimal antibody incubation concentration

Antibody incubation duration is interdependent on antibody incubation concentration. Tests have concluded that incubation with a higher antibody concentration results in a faster rate of antibody uptake. Should time be a constraint, incubation with a higher antibody concentration will prove to be an efficient option. Conversely, incubation with a lower concentration of antibody for a longer period of time would prove a cost-effective option. Tests have also shown that binding of antibodies to the 16-mercaptohexadecanoic acid (MHDA) layer follows a distinct pattern. There is a point in incubation time at which the rapid rate of antibody uptake decreases. Knowing the exact point at which the rate of antibody uptake decreases will help in the further optimization of the procedure for antibody layer formation. Further studies can include testing the structural differences in the antibody monolayer formation between the rapidly formed antibody layer using a high antibody concentration and the gradually formed antibody layer using a low antibody concentration. In addition, further tests will have to be carried out to better characterize the pattern of antibody binding. Results from 1 hour antibody incubation intervals will reveal a more distinct antibody binding pattern. This would assist in

optimizing the antibody incubation duration for a specific antibody incubation concentration in future work.

(3) Optimal antigen incubation duration

This study has shown that antigen incubation for one hour is sufficient for the efficient binding of the antigens to the antibody layer. Extension of antigen incubation duration produced insignificant results. This is a promising outcome because a short antigen incubation duration accelerates the overall time required for the completion of the microbial detection process.

(4) Optimal conditions for storage of coated crystal

The viability of the antibody coated, BSA blocked crystals after 2 to 4 weeks of storage was markedly reduced. Crystals stored at ambient room temperature (22.5$^{o}$C) fared better than crystals stored at 4$^{o}$C. In this study, the effect of moisture on the storage of antibody pre-coated crystals was inconclusive. A vacuum was reapplied to the dessicator every two days. In addition, a layer of $SiO_2$ crystals was placed at the base of the dessicator as an indicator for moisture entry into the dessicator. These steps ensured that the crystals were stored dry. However, results showed no differences between crystals stored dried or undried. It may be that the storage time of 2 to 4 weeks was too short to show the effect of moisture on the storage viability of antibody pre-coated crystals. Further research will

have to be carried out for longer storage durations to study the effect of moisture on the viability of antibody pre-coated crystals.

Also, additional ELISA tests can be conducted on the effect on antibody activity caused by storage under the different conditions. This will enable us to know if the reduction in activity is due to the loss of bound antibody or the inactivation of antibody activity.

(5) Quartz crystal regeneration

Quartz crystals regenerated by 0.2M Gly-HCl proved inefficient. This finding supported other studies that had used 0.2M Gly-HCl as a regeneration agent where Gly-HCl had not only stripped off the antigen layer but also part of the antibody layer. Future research using an alternative method of crystal regeneration is suggested – competitive synthetic peptide elution. A high concentration of short *E. coli O157:H7* specific synthetic peptides will be used to compete against bonded *E. coli O157:H7*, eluting the bonded *E. coli O157:H7* in the process. This alternative method for crystal regeneration may prove better than the use of harsh chemicals that damage the antibody layer.

(6) Quartz crystal reusability

It is concluded that the MHDA layer and adsorbed proteins can be removed by a piranha process. This gives each crystal the potential to be repeatedly recoated and reused. The ability to recycle each crystal reduces the cost incurred per assay.

Additional suggestion

It is suggested that for future work, a flow cell be included in the QCM set-up. The attachment of a flow cell to the QCM will prove invaluable for the efficient rinsing of the crystals improving result reproducibility. A flow cell can provide consistent flow conditions throughout all experiments, reducing flow-induced shear forces that can be caused by manual rinsing of the crystals.

Finally, the utilization of the quartz crystal microbalance (QCM) for *Escherichia coli O157:H7* detection has proved to be an efficient, rapid and cost-effective technique. In critical situations where speed of detection is a necessity, QCM trumps the conventional techniques of Tellurite-Cefixime Sorbitol-MacConkey agar (TCSMAC) and pulsed field gel electrophoresis (PFGE). More studies on the use of the QCM for microbial detection will be required. With the improved knowledge and recognition in the capability of the QCM as a biosensor, the QCM may soon be used in conjunction with conventional techniques for the detection of *E. coli O157:H7*. In the immediate future, biosensors may even replace conventional techniques as the forefront in microbial detection.

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

**Marie Curie**
*French (Polish-born) chemist & physicist (1867 - 1934)*