The development of a Real Time Polymerase Chain Reaction using TaqMan probes to determine the burden of multi drug resistant tuberculosis (MDR-TB) in Kwa-Zulu Natal

By

Anura Ganas

202500272

Submitted in partial fulfillment of the requirements for the degree of Master of Medical Science

In the Department of Medical Microbiology

Nelson R Mandela School of Medicine

Faculty of Health Sciences

University of Kwa-Zulu Natal

Durban

2008

Declaration

This study represents the original work by the author and has not been submitted in any form to another University.

Where use was made of work of others it has been duly acknowledged in the text.

The research described in the study was carried out in the Department of Medical Microbiology, under the supervision of Professor Alexander Pym.

Anura Ganas

Acknowledgements

Every step that you take in your life becomes easier with the presence of angels who guide and encourage you along the way.

To the following celestial beings without whom the completion of my MSc would not have been possible, my heartfelt appreciation goes out to you:

- Professor A.S.Pym for his advice, encouragement,expertise and editing of the drafts of this thesis
- Professor A.W.Sturm and Professor P.Moodley for their assistance, encouragement and support
- Dr.M.Pillay for providing isolates, assistance, support and encouragement
- The staff of the TB lab for their assistance
- Jenny Allen for her assistance, advice and encouragement
- Loshnee Ganas for assistance with the location of stored isolates
- Kasavan , Prathna , Mona , Sheryll and Yoshan for making the tough times bearable
- Sheryll for her amazing photographic skills
- The staff of the Pfizer Molecular Biology Department for their assistance with the NanoDrop ND 1000
- Bongiwe for providing sequencing results, support, advice and encouragement
- Zama and Mbali from the MRC for their help with orders and queries
- Gerald for assistance and advice
- Athish and Ushira for who have 'strengthened me with their prayers, blessed me with their love and encouraged me with their hope.'
- My mum and dad, my pillars of strength for inspiring me to do my best and showing me that the sun never stops shining, even on the darkest day.

- Sanjay for being there in the way that only you could be
- The numerous individuals who made dark days seem bright by just providing smiles and words of encouragement
- And last, but not least to God, for giving me the strength to successfully complete my MSc

Declaration of Ethics

This study has obtained approval from the University of Kwa-Zulu Natal Biomedical Research Ethics committee.

Abstract

The aim of this study was to develop a technique for the surveillance of Multi Drug Resistance tuberculosis (TB) in Kwa Zulu Natal. Conventional drug resistance surveys are often limited by laboratory capacity to culture *Mycobacterium tuberculosis*, so the development of a high through-put molecular technique for use on clinical samples could expand the reach of drug resistance surveillance. Initially allele-specific PCR using either linear or hairpin primers was evaluated in a SYBR green based T_m shift assay targeting two drug resistance conferring mutations in *rpoB* and *katG*. The reproducibility of this assay was poor so a real time PCR assay using TaqMan probes was then developed and evaluated. This method showed reliable and reproducible results on culture isolates but was found not to be sensitive when used on clinical isolates. Further optimization of the method of DNA extraction from sputum is required, but the assay has potential to become a rapid surveillance technique for both MDR- and XDR-TB. A commercially available Genotype ® MTBDR*plus* assay was also evaluated for the surveillance of MDR-TB but was also found to have low sensitivity when used on smear negative sputum samples.

THE DEVELOPMENT OF A REAL-TIME POLYMERASE CHAIN REACTION USING TAQMAN PROBES TO DETERMINE THE BURDEN OF MULTI DRUG RESISTANT TUBERCULOSIS (MDR-TB) IN KWA-ZULU NATAL

List of Abbreviations

Δ Rn – normalized reporter signal

ABI Prism 7000 SDS – ABI Prism 7000 Sequence Detection System

AFB – Acid Fast Bacteria

AIDS – Acquired Immunodeficiency Syndrome

AC – Amplification control zone

AR – Auramine-Rhodamine staining

ARMS – Amplification Refractory Mutation

ASP – allele-specific PCR

BAC – Bacterial Artificial Chromosome

bp base pair

CC – conjugate control zone

CFA – Freund's complete adjuvant

Ct – threshold cycle

CTAB – N, N, N-trimethyl ammonium bromide

DC – Dendritic cells

DC SIGN – DC specific intercellular adhesion molecule-3 grabbing nonintegrin

DDGE – Denaturing Gradient Gel Electrophoresis

DNA – Deoxyribonucleic Acid

dNTP – deoxyribonucleotide triphosphates

DOH – Department of Health

DOTS – Directly Observed Therapy, short course

DRS – Drug Resistance Surveillance

dsDNA – double-stranded DNA

DST - Drug Susceptibility Testing

EDTA – Ethylene Diamine Trichloro Acetic Acid

FAM – 6-carboxy-fluoroscein

FAST-Rif – fluorometric assay for susceptibility testing of rifampicin

Fc – complement receptors

FP – forward primer

FRET – fluorescent resonance energy transfer

GC - Guainidine-cytosine content

GTC – Guanidine Thiocyanate

HCl – hydrochloric acid

HIV – Human Immunodeficiency Virus

HPI – hydroperoxidase I

HYB – Hybridization buffer

IDI - Infectio Diagnostics, Inc

INH isoniazid

IRS – Inhibitor Removal Solution

KCl – potassium chloride

LAM - mycobacteria-specific lipoglycan lipoarabinomannan

MGIT – Mycobacterial growth Indicator Tube

LJ – Lowenstein Jensen media

MADGE microtiter array diagonal gel electrophoresis

MDR – multi drug resistance

MDR-TB – multi-drug resistance tuberculosis

MGIT – Mycobacterial growth Indicator Tube

$MgCl_2$ – Magnesium chloride

MGB – Minor Groove Binding probes

MICs – Minimum Inhibitory Concentrations

MRC – Medical Research Council

MTBDR*plus* – *Mycobacterium tuberculosis* Drug Resistant plus

Mut - mutant

NALC N Acetyl L Cysteine

NaCl – Sodium Chloride

NaOH - Sodium hydroxide

NFQ – non-fluorescent quencher

NTC – non template control

PCR – Polymerase Chain Reaction

PNM – primer nucleotide mix

RFLP Restriction Fragment Length Polymorphism

RIF - rifampicin

RIN – rinse solution

RNA ribonucleic acid

RP – reverse primer

RRDR – Rifampicin Resistance Determining Region

RT PCR – real time PCR

SCC – Short Course Chemotherapy

ssDNA – single stranded DNA

SDS – Sodium Dodecyl Sulphate

Ser - Seronine

SNP – Single Nucleotide Polymorphism

SOP – Standard Operating Procedure

SSCP Single-Strand Conformational Polymorphism Analysis

STR – stringent wash buffer

TAMRA – 6-carboxy-tetramethyl-rhodamine

TB – Tuberculosis

TBE - Tris-Borate-EDTA Buffer

TE - 10 mM Tris-HCl [ph 8.0], 1 mM EDTA

Thr - Threonine

T_m – melting temperature

tRNA – transcription RNA

WHO – World Health Organization

WT – wild type

XDR TB – extensively drug resistant tuberculosis

ZN- Ziehl Neelsen

Lists of Tables

List of Figures

Chapter One

1. Introduction

Tuberculosis (TB) is a major health problem in South Africa with annually over 250,000 new cases being reported. The estimated annual incidence rate of TB is amongst the highest in the world, in excess of 500/100 000 population and in some areas the rate exceeds 1,000/100,000 (1%) [1-4]. TB is currently one of the leading causes of death in adults and children in South Africa. It is predicted that there will be 3.5 million new cases of TB over the next decade in SA, and at least 90,000 deaths. A major reason for the escalation of the TB epidemic is the evolution of the Human Immunodeficiency Virus (HIV) epidemic [5]. It is estimated that approximately 60% of adult TB cases aged 15-49 years are HIV co-infected [6]. The HIV epidemic has more than tripled the annual number of new TB cases in South Africa since 1990 and is continuing to rise. The World Health Organization (WHO) Regional Committee for Africa has declared TB to be an emergency in the African region, at its 55th session in Maputo, Mozambique, in August 2005.

Under most circumstances, treatment for TB using the DOTS (directly observed therapy, short course) strategy in which a rifampin based four drug regimen is given under direct observation leads to high rates of cure, shortening of the period of infectiousness, and when broadly applied, can reduce the incidence of the disease. The effectiveness of the DOTS strategy is, however, threatened in areas in which there is a high prevalence of resistance to the first-line anti-TB drugs, particularly isoniazid (INH) and rifampicin (RIF), so-called multiple drug resistance (MDR) [7]. The development of resistance to these drugs reduces the efficacy of standard anti-TB treatment to 77% [8]. This results in lower cure rates leading to persistent infectiousness and, thus, greater potential for transmission. The resulting spread of Multi-Drug Resistant Tuberculosis (MDR-TB) has potentially devastating consequences. Even in a clinical situation where drug susceptibility testing, secondary-line drugs and good

adherence are present, the disease carries a significant mortality, which can be over 50% in HIV co-infected individuals [6].

During 1994–2002, the WHO Global Project on Anti TB Drug Resistance Surveillance coordinated data collection on more than 250 000 patients' from 109 countries. From the data collected, WHO estimated the annual burden of MDR-TB to be approximately 300 000–600 000 cases and the prevalence of MDR TB to be threefold higher than the annual incidence, mainly in low and middle-income countries [9] . However these figures are based on surveys conducted in a limited number of countries. Recently the emergence of XDR-TB or extensively drug resistant TB (defined as a MDR strain that also is resistant to any fluoroquinolone and at least one of the three following injectable drugs: capreomycin, kanamycin, and amikacin) [10, 11] suggests that the spread of drug resistant TB can be rapid. Population based surveillance for drug resistance is therefore urgently required to describe the magnitude and trends of MDR- and XDR-TB worldwide in order for appropriate action to be taken [10, 12].

A series of surveys conducted by WHO and the International Union against Tuberculosis and Lung Disease has shown that MDR strains of *Mycobacterium tuberculosis* are widespread [13]. However the global distribution of MDR strains is not uniform, and only in certain countries has MDR rates reached levels above 5% in previously untreated patients. A major limitation of these surveys has been the selectivity of the sampling and Africa in particular has not been extensively studied. Only five countries from the African continent were included in the last WHO Global Project on Anti TB Drug Resistance Surveillance carried out between 1999 and 2002 [2].

The relative lack of surveillance data is due to a number of factors, not least the logistics of processing large numbers of samples for drug susceptibility testing (DST). *Mycobacterium tuberculosis* is a slow growing organism with a generation time of over 12 hours, and even in the faster broth based culture systems at least a week is required to isolate the organism from clinical specimens, before sub culturing in the presence of antibiotic to determine specific drug susceptibilities. The cost and laboratory infrastructure requirements mean DST is not routinely performed in resource-poor countries and few possess national drug resistance surveillance (DRS) systems. These limitations also impede research efforts to determine the prevalence of drug resistance in areas where TB is endemic because it is usually only feasible to test a small fraction of TB cases. Focal epidemics of drug resistance can therefore be easily missed if cluster sampling is adopted.

Surveillance techniques based on the genetic determination of resistance present an attractive alternative to the traditional culture based phenotypic methodology, but have not been formally developed for this purpose. In South Africa, in particular, a commercially available molecular line-probe assay for rapid detection of rifampicin and isoniazid resistance was only recently assessed to determine its performance and feasibility in a high burden setting [14]. The genetic basis of resistance to isoniazid and rifampicin, was first described over a decade ago, and has been extensively studied in different populations [15, 16]. Various techniques have already been proposed for detecting drug resistance mutations. These include denaturing gel electrophoresis (DGGE) [17], multiplex allele-specific polymerase chain reaction (PCR) (MAS-PCR) [18, 19], fluorometric assay for susceptibility testing of rifampicin (FAST-rif) [20], restriction fragment length polymorphism PCR (RFLP-PCR) [21], PCR and deoxyribose nucleic acid (DNA) sequencing [22], , and single strand conformation polymorphism analysis

(SSCP PCR) [23]. Many of these techniques require initial isolation of *Mycobacterium tuberculosis* and formal DNA preparations so cannot be used directly on clinical samples. Others require cumbersome detection techniques not amenable to high throughput analysis or expensive solid phase detection systems. However homogenous or "closed-tube" techniques that do not require further processing after addition of the clinical sample are available for detecting single nucleotide polymorphisms (SNPs). Furthermore, real-time PCR shows sufficient specificity and sensitivity to detect drug-resistant *Mycobacterium tuberculosis* even with sputum samples from TB patients without culture. The aim of this study was to develop such a molecular test for population based surveillance.

Chapter Two

2. Literature Review

2.1 Future of MDR-TB

Multi-Drug Resistant TB represents a very important threat to TB control. As with all TB, 99% of MDR-TB occurs in high-burden resource-poor countries. However, factors such as a continuing rise in the size of the world population and population mobility will mean an increase in MDR-TB in developed countries as well. It is therefore quite clear that without both political backing and sufficient financing, the number of MDR-TB cases in all countries, whether already developed or still developing countries, will continue to rise [24].

Another very important threat discovered through molecular epidemiology in the past decade is that some *Mycobacterium tuberculosis* strains are spreading more quickly than others and therefore create problems irrespective of how effective the TB control program is [25].

In theory, a solution to this problem seems simple enough. By curing new TB cases (the majority of which are susceptible strains), with short course chemotherapy (SCC), through the implementation of the DOTS strategy will prevent such cases from becoming resistant and multi-drug resistant. Also, since MDR TB cases respond poorly to SCC, a careful introduction and close monitoring of second-line drugs to treat these cases will prevent the further transmission of these strains [26].

Although MDR-TB requires prolonged treatment with more costly and less effective agents, some have suggested that in the long run, MDR strains may not be able to replace drug-susceptible strains unless a major resistant 'super-bug' arises globally [26, 27] [9]. Others have contested that MDR-TB strains are less "fit" that drug susceptible strains [23, 24].

However, MDR-TB still remains a serious threat to TB control programs. The elevated levels of MDR-TB in certain settings and the high estimates of MDR-TB in some of the TB high-burden countries should not and cannot be ignored. Some form of action needs to be taken by the international community to prevent a global MDR TB problem. These include: 1) improvement of surveillance to gather data that will give a better representation of the magnitude of the problem and predict how MDR-TB strains may spread in the future; 2) follow-up and better understanding through further studies of the fitness of resistant strains and 3) expansion/strengthening of TB control efforts [26]. So, although genotypic testing cannot totally replace phenotypic methods for the detection of antibiotic resistance, a molecular screen capable of use on primary clinical samples to detect most mutations associated with resistance could be invaluable [28]. Thus, there is a need to develop a surveillance technique that is not time consuming, cheap and applicable in low-technology environments [29].

2.2 Drug Resistance Surveillance in KwaZulu-Natal

Renewed surveillance of multi-drug resistance is urgently required in KwaZulu-Natal. South Africa was included in the last WHO survey of multi-drug resistance, as a result of a nationwide project carried out by the South African Medical Research Council. This was a population-based, cross sectional study conducted according to WHO protocols. In KwaZulu Natal province 595 drug susceptibility tests were available from patients with no history of prior treatment, recruited in 9 different centers. Of these, only 1.7% was found to be MDR and low rates were also found in the other South African provinces. However in the last 3 years since the study was carried out there is mounting evidence to suggest there has been a significant deterioration of the situation. The number of MDR cases treated at the regional referral centre in Durban (King George V Hospital) has increased from 202 in the year 2000 to over 3000 in the year 2007, and is continuing to rise.

More recent data have revealed shocking facts with regard to both MDR-TB and XDR-TB. A study was carried out to assess the burden of XDR-TB and MDR-TB in a rural area of KwaZulu Natal [11]. The study was carried out from January 2005 - March 2006 and sputum samples from 1539 patients were analyzed. MDR-TB was detected in 221 patients. Of these patients, 53 had XDR-TB. All 44 patients with XDR-TB that had been tested for HIV were found to be HIV positive. There have been many hypotheses with regard to the origin of the spread of XDR-TB. One of them was thought to be as a result of the default of treatment of MDR-TB. However, this study showed that 55% of these patients had never been previously treated for TB. Another interesting factor to note was that these 53 patients had no known contact with each other and did not live in close proximity of each other. The only common factor was that 67% of these patients had received treatment from the same hospital. This study thus revealed that the prevalence of both MDR-TB and XDR-TB was significantly

higher that previously thought [12]. Furthermore, the similarity amongst the strains as shown by genotyping data shows that these strains are being very easily transmitted from one individual to the next. It is thus very likely that the transmission of XDR-TB occurred nosocomially. This study was therefore found to be very useful in enforcing the seriousness of the problem of the drug resistant forms of TB in a resource limited area that also has a high prevalence of HIV [11]. However, there is still a need to assess the distribution of these forms of TB within the province.

It is critical to determine whether multi-drug resistance is generalized throughout Kwa Zulu Natal, or restricted to certain facilities as well as to determine the risk factors for transmission of MDR TB [30] so that the appropriate interventions can be more efficiently targeted. There are now over 90,000 reported cases of TB in the province making a conventional DST difficult to conduct. The development of a novel technique to rapidly estimate levels of multi-drug resistance in large populations would therefore be of great benefit to health care in KwaZulu Natal Province as well as South Africa in general as early detection of drug resistance in TB allows starting of an appropriate treatment, which has an impact in the better control of the disease [31].

2.3 Biology of *Mycobacterium tuberculosis*

2.3a General Characteristics

Mycobacterium tuberculosis is the etiologic agent of TB in humans [32]. It was first isolated in 1882 by Robert Koch [33]. *Mycobacterium tuberculosis* is a fairly large non-motile rod-shaped bacterium. The bacterium is a facultative intracellular pathogen, usually of macrophages, and has a slow generation time of 15-20 hours, a physiological characteristic that may contribute to its virulence [34].

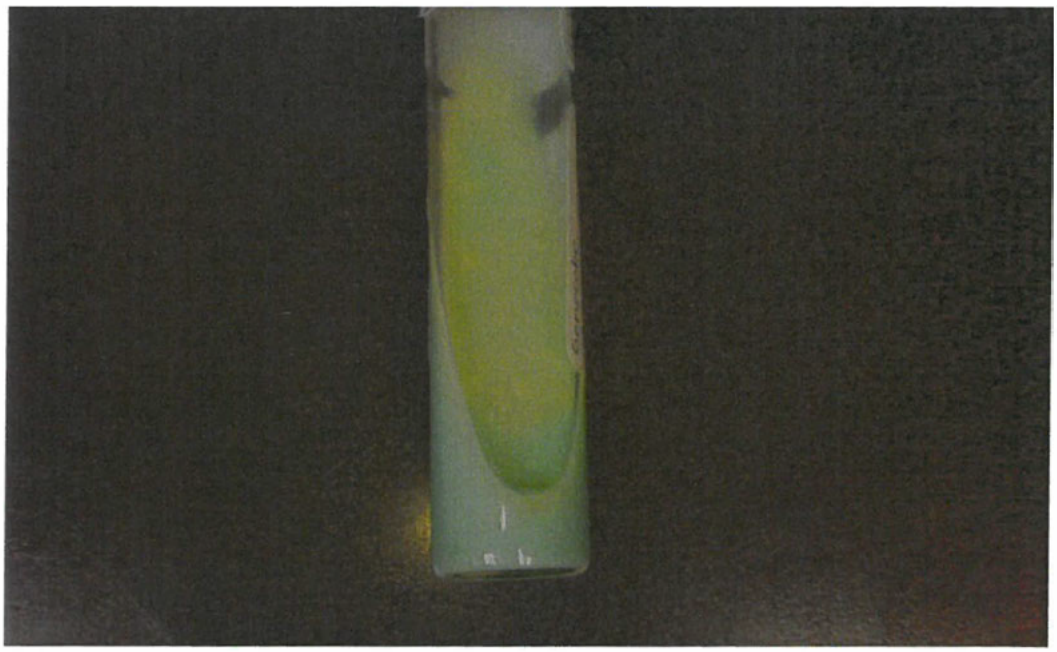


Figure 2.1: Colonies of *Mycobacterium tuberculosis* on Lowenstein-Jensen medium

Mycobacterium species are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds, and this forms the most commonly used acid-fast staining method for *Mycobacterium tuberculosis*, the Ziehl-Neelson (ZN) stain.

2.3b Cell Wall Structure

Although it has been difficult to correctly describe the exact constituents of the cell envelope of mycobacteria due to difficulties in conventional electron microscopy [35], it is known that the cell wall structure of the *Mycobacterium tuberculosis* is unique among prokaryotes and it is a major determinant for virulence for the bacterium [34]. The study by Zuber B. *et al.* (2008) has been a breakthrough since the group has been able to show the different layers of the mycobacterial cell envelope including for the first time, an outer membrane, similar to that found in gram negative bacteria. It has been found that the cell envelopes of mycobacteria are composed of a plasma membrane, an inner wall zone, a medial wall zone and an outer membrane. The importance of mycolic acids have once again been re-emphasized for the survival of the mycobacterial species.

Mycolic acids are unique alpha-branched lipids found in the cell wall of mycobacteria. They are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. These acids may also be a significant determinant of virulence in *Mycobacterium tuberculosis.* They also protect extracellular bacteria from complement deposition in serum and are also thought to protect the mycobacteria from cationic proteins, lysosyme and oxygen radicals in the phagocytic granules [34].

There is also a high concentration of lipids in the cell wall of *Mycobacterium tuberculosis* that has been associated with the following properties of the bacterium: impermeability of stains and dyes, resistance to many antibiotics, resistance to killing by acidic and alkaline compounds, resistance to osmotic lysis via complement deposition and resistance to lethal oxidations and survival of macrophages [34].

2.3c Virulence Mechanisms and Virulence Factors

A number of structural and physiological properties of the bacterium contribute to bacterial virulence and the pathology of TB. These include:

a) Special mechanisms for cell entry. The tubercle bacillus can bind directly to mannose receptors on macrophages via the cell wall-associated mannosylated glcolipid, mycobacteria specific lipoglycan lipoarabinomannan (LAM) or indirectly via certain complement receptors or Fc receptors. The bacteria is also capable of entering dendritic cells (DC) after binding with the lectin DC specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) [36]

b) *Mycobacterium tuberculosis* can grow intracellularly, which is an effective means of evading the immune system. Once *Mycobacterium tuberculosis* is phagocytosed, it can inhibit phagosome-lysosome fusion. The exact mechanism used by *Mycobacterium tuberculosis* to accomplish this is not known but it is thought to be the result of a complex interaction between bacterial proteins and the phagosome. The bacterium may then remain in or escape from the phagosome.

c) *Mycobacterium tuberculosis* possesses specific mechanisms to protect against the toxic effects of reactive oxygen and nitrogen intermediates, such as *katG*.

2.3d The Genome of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis contains a single chromosome. The genome is very much like other eubacterial species in terms of its structure and organization. *Mycobacterium tuberculosis* has a sequence consisting of 4, 411, 529 base pairs (bp) and a GC content of 65.6%. It has also been found that that the GC content was relatively constant throughout the genome [34, 37].

2.4 Multi drug resistance of *Mycobacterium tuberculosis*

In 1943, Selman Waksman and his colleagues discovered streptomycin. This discovery was important since it provided a chemotherapeutical approach to TB treatment. However, soon after the initiation of this chemotherapy era, it was found that not all cases of TB could be treated with streptomycin due to the emergence of resistant strains of TB [34]. The potential impact of drug resistance in the treatment of TB thus became apparent [38]. In the following years, other drugs were discovered including isoniazid, pyrazinamide, ethambutol and rifampicin. Although it was shown that administering these drugs simultaneously could prevent the emergence of resistant mutants as the rate of spontaneous mutations against two or more drugs is very low [34, 38], inadequate treatment and other factors can lead to the emergence of resistance to all of these drugs.

Multi-drug resistant TB is the result of the stepwise accumulation of chromosomal mutations and is not due to a single event such as the acquisition of a plasmid or transposon [39]. Possible reasons for the acquisition of mutations include inefficient prescription and follow-up of chemotherapy by the clinician, poor compliance, or the administration of an in inappropriate treatment regimen [5, 40, 41]. The use of anti-TB drugs of inferior quality is another confounder, as is the sale of these medications over the counter and on the black market [13].

Rifampicin resistance conferring mutations occur spontaneously at an estimated rate of 1 in 10^8 bacilli, while isoniazid resistance arises in approximately 1 in 10^6 bacilli [38, 42]. Due to selective pressure, the susceptible bacteria are slowly eliminated while the resistant bacteria, that have an obvious advantage, begin to dominate the population giving rise to "acquired

resistance" [43]. Transmission of these drug-resistant *Mycobacterium tuberculosis* strains leads to new TB cases or 'primary resistance' (defined as the development of active TB after infection with resistant strains) which eventually led to the emergence of multi drug resistance [38].

Thus, as postulated many years ago, it appears as though random mutations conferring resistance occur naturally during microbial replication and are then selected by incorrect drug use [34, 39].

Although, it is reassuring that multidrug resistance does not occur through a new resistance mechanism, it is imperative that suitable measures are taken to effectively contain the spread of MDR-TB. To do this, research needs to be carried out on a continual basis, especially in two important areas. Firstly, molecular tests that allows for the early detection of resistance, thereby allowing more efficient patient treatment and management, needs to be improved and evaluated. Secondly, new, improved drugs that are more efficacious and has fewer side effects than those currently available must be identified. It is also extremely important to discover new compounds that would be active on dormant *Mycobacterium tuberculosis.* The availability of such a compound or compounds would prevent relapse while also playing a vital role in eliminating *Mycobacterium tuberculosis* from the huge latently infected population in the world. This would, effectively reduce the "size of the potential reservoir" [39].

In the 1980's, a presumptive diagnosis of TB was usually made on the basis of patient history, clinical and radiological findings and on the presence of acid fast bacilli in smears [44]. Although, the tuberculin skin test was also available, isolation of the bacterium was required

to confirm this diagnosis. Routine culture was found to be the most reliable method. This method is, however, highly technical and costly both in terms of time and reagents [44]. Thus, in resource poor countries especially, cultures are not readily available [45]. A slightly faster method was the examination of direct smears for acid fast bacteria. Another method used was direct microscopy but this requires a skilled technician for interpreting the smears and the presence of a large number of acid-fast bacteria. The introduction of the BACTEC system followed which greatly reduced the time for detection, final identification and susceptibility testing for *Mycobacterium tuberculosis.* However, the drawback of this system is that it depends on growth of the bacteria [44]. Non cultural methods for the diagnosis of TB soon followed. These include the direct detection of specific nucleotide sequences in clinical material by using nucleic acid probes [45]. These nucleic acid amplification based genotypic assays are extremely sensitive and rapid for the detection of drug resistance and include DNA micro arrays and Strand displacement amplification. The advantage that these systems offer is that they greatly shorten the time taken from diagnosis to the delivery of effective chemotherapy [46, 47].

2.5 Mechanisms of Drug Resistance in *Mycobacterium tuberculosis*

2.5a Overview of resistance mechanisms

Naturally occurring plasmids are not found in clinical isolates of *Mycobacterium tuberculosis.* So although plasmids are known to be the carriers of antibiotic resistance genes in other bacteria, they are not thought to play a role in this phenomenon in *Mycobacterium tuberculosis* [34].

Although insertion sequences are found in the *Mycobacterium tuberculosis* genome, there is no evidence that is suggestive of their involvement in the transfer of DNA among strains. Furthermore, since the bacteria infect and live within the macrophage and do not have a free-living stage, this minimizes the opportunity for the bacteria to exchange DNA [48].

However, mycobacteria display low-levels of resistance to most common antibiotics and chemotherapeutic agents which is probably due to the hydrophobic cell envelope, as described earlier. *Mycobacterium tuberculosis* also has other mechanisms that make them naturally resistant to drugs such as drug-efflux systems. Therefore, the drugs that can act against these bacteria are quite limited [37]. Despite this fact, effective chemotherapy for TB was derived in the 1960s.

Mycobacterium tuberculosis displays three major mechanisms of resistance to antimicrobial agents. These are: altered cell wall permeability or drug efflux, drug titration due to target overproduction and alteration of the target by mutation. The fourth mechanism of resistance, inactivation of the drug, is not a method that is currently thought to occur in *Mycobacterium tuberculosis* as no enzymes that are capable of doing this has been found in this pathogen [39] Resistance can also occur as a result of loss of activation of pro drugs such as isoniazid. The

most common resistance mechanism is alteration of the target. This causes a decrease in drug binding and occurs as a result of mutations in chromosomal genes. This technique is employed by these bacteria both in the case of anti-TB drugs isoniazid, and ethionamide as well as in resistance to the broad spectrum antibiotics rifampicin, streptomycin and fluoroquinolones. Overproduction of the drug target also appears to lead to resistance to isoniazid and ethionamide whereas changes in permeability or the activation of antibiotic-efflux systems, may contribute to the low-level resistance of the tubercle bacillus to streptomycin and fluoroquinolones [39].

The acquisition of resistance to a specific drug is of no benefit to the bacterium unless it is exposed to that drug [16]. It is only in the presence of that drug, that the drug susceptible bacteria are killed while the mutants that are resistant to the drug survive. If a patient undergoes a second course of chemotherapy with another drug and the same scenario occurs, it eventually happens that you have bacilli that are resistant to more than one drug. This is the predominant mechanism for the development of multi drug resistance, where there is an accumulation of chromosomal mutations in individual target genes [16]. Multi-drug resistant TB is defined as a strain of TB that is resistant to at least isoniazid and rifampicin, the backbone of the standard regimen for treating TB [49]. Thus only these two drugs and their targets will be discussed in more detail.

2.5b Resistance to Isoniazid

Isonicotinic acid hydrazide, 4- pyridinecarboxylic acid hydrazide [16] or isoniazid was synthesized for the first time in 1912 by two Czech biochemists [34]. However, it was only in 1951 that its anti-TB activity became known. Since isoniazid was further proved to be a powerful anti-TB agent with relative efficacy and low toxicity, its discovery was a huge advancement in the chemotherapy of TB [39, 50]. In terms of its structure, isoniazid has a very simple chemical structure consisting of a hydrazide group attached to a pyridine ring [51].

Isoniazid enters the tubercle bacillus by passive diffusion and has been found to be a very effective bactericidal agent since it inhibits fatty acid metabolism as well as nucleic acid and protein synthesis [39, 51, 52]. However, it is a prodrug that needs to be converted to an active form before exerting a toxic effect on the bacillus [53]. The enzyme responsible for this activation is *katG*, a 2223-bp gene encoding an 80-kDa hemoprotein of 744 amino acids that has a catalase-peroxidase (a heme-containing enzyme) [53, 54]. *katG* oxidizes isoniazid which transforms the drug into a nucleophilic radical. This radical then reacts with the co-factor NAD^+ thereby yielding a potent inhibitor of the enoyl-ACP reductase InhA. The inhibition of InhA affects the synthesis of mycolic acid, an important component of the bacterial cell wall [55]. This results in the loss of acid fastness in the tubercle bacillus [34].

The genetic basis of resistance to isoniazid is complex and involves multiple loci. The principal mechanism of high level resistance is due to mutations in *katG* [54]. Although *katG* insertions and frameshifts do occur and induce complete loss of the functional gene product, most mutations identified in clinical isolates are single-point mutations [53, 56]. In some isoniazid resistant strains that had completely lost their catalase-peroxidase activity, the *katG*

gene had been deleted from the chromosome. However, this phenomenon is quite rare (~5%). Most isoniazid resistant strains have missense mutations in the *katG* gene that affect their catalase-peroxidase activity and result in variable levels of resistance [34].

These mutations generally affect the catalytic domain (which contains the active site) of catalase peroxidase and in some cases change the structure of the active site. These substitutions have a negative effect on the activity of the enzyme. Other substitutions that affect a glycine rich loop that is close to the substrate diffusion channel of catalase peroxidase also leads to a loss of enzyme activity [39].

As mentioned previously, mutations in codon 315 of the *katG* gene are considered to be the most prevalent mutations encoding higher levels of resistance to isoniazid [57]. Since this mutation is associated with a low fitness cost, easier transmission of such a strain allows evolution of this strain to an MDR strain as a result of its maintained fitness. It is interesting to note that the distribution of isoniazid resistance-conferring mutations has been reported as different in isoniazid-monoresistant isolates from that in multidrug resistant isolates. Mutations in *katG*315 were significantly more common in the multidrug-resistant isolates [58]. This evidence thus supports the notion that *katG315* mutations have been found to be a marker for MDR [57, 59].

Although numerous isoniazid resistance conferring mutations in *katG* have been described [15, 54], it has been found that the Ser315Thr mutation is the most prevalent mutation encoding higher levels of resistance to isoniazid (30% to 90% of resistant strains), depending on geographical areas [41, 46, 57, 60-62]. A population based survey has also demonstrated that amongst *katG* mutations only this particular mutation is effectively transmitted [7], which is compatible with it being the most frequently encountered isoniazid resistance-conferring

mutation (60% to 100% of surveys) [53, 63]. The Ser315 residue that acts as a heme ligand, when replaced by Thr affects both heme-binding and catalysis thus causing severely impaired enzyme activity [39]. In a study carried out in the Middle East, 60% of mutations occurring at codon 315 of the *katG* gene were found to be Ser315Thr mutations [64]. A similar result was also found in a study carried out in South Africa by Haas W.H. *et al.* (1997) [60]. An even higher percentage of this particular mutation was found in a study carried out in Germany which investigated resistance mutations of MDR strains (84.5% of MDR strains) [65]. Biochemical evidence obtained from studies involving purified wild type and mutant proteins suggest that this mutation results in a competent catalase peroxidase that has a reduction of about 50% in its ability to metabolize isoniazid [66, 67]. This mutation also causes a significant decrease in the INH mediated inhibition of the activity of the NADH dependent enoyl [acylcarrier protein] reductase, InhA, compared to wild type *katG* in an *in vitro* assay [66]. The high frequency with which variants with this mutation are recovered from patients all over the world suggest that *Mycobacterium tuberculosis* cells achieve a good balance between the need to maintain catalase peroxidase activity and the need to reduce the conversion to the prodrug isoniazid to its activated form without a significant loss of bacterial fitness [68, 69]. There is also laboratory and clinical evidence suggesting that the Ser315Thr substitution is a low cost mutation that does not reduce the virulence or transmissibility of the bacterial strain [50, 69, 70]. Furthermore, a study carried out by Martilla *et al.* (1998) [71], in the St. Petersburg area of Russia found that the Ser315Thr substitutions in the *katG* gene were predominant (92%) among 27 multidrug resistant *Mycobacterium tuberculosis* isolates. Another very interesting point to note is that this mutation was also found to be the most prevalent mutation (77 out of 79 isoniazid resistant isolates) in a study carried out in Kwa-Zulu Natal by Kiepiela P. *et al* (2000) [61] thus making it a very important mutation to target.

Other isoniazid resistance conferring mutations also occur. After *katG* mutations, mutations in the promoter region of *inhA* are the most common. These result in upregulation of *inhA*, a target for activated isoniazid but only confer low level resistance. Other genetic loci implicated in isoniazid resistance include *kasA*, *ahpC* and *ndh* [39]. It is conceivable that different mutations lead to differences in the degree of resistance and to differences in the ability to generate next generation cases [57].

2.5c Resistance to Rifampicin

Rifampicin (a lipophilic ansamycin) was first isolated from *Streptomyces mediterranei* in 1963 and is a broad-spectrum antibiotic [39, 72]. It was introduced as an anti-TB agent in 1972 and since then it has been a crucial drug in short course multi-drug anti-TB therapy [73]. It is highly active against mycobacteria since it diffuses rapidly across the hydrophobic cell envelope [34]. Rifampicin is a bactericidal agent and is a potent inhibitor of bacterial DNA dependant RNA polymerase. It works by binding to the β subunit of the polymerase encoded by the *rpoB* gene, which is involved in chain initiation and elongation. This inhibits the initiation of transcription [39, 73].

Rifampicin resistance is particularly amenable to detection by rapid genotypic assays because, as 95% to 98% of all rifampicin-resistant strains contain mutations localized in an 81-bp core region of the bacterial RNA polymerase gene, *rpoB* (between bases 1276 and 1356 or codons 507-533) [15, 19, 74, 75]. Mutations that occur in this region confer resistance to rifampicin and rifampicin susceptible *Mycobacterium tuberculosis* isolates have the same wild-type nucleotide sequence in this region which is highly conserved. Thus, the detection of a mutation in the *rpoB* core region is usually sufficient to state that the bacilli are rifampicin resistant [76]. It was observed that resistance to rifampicin follows a "single-step, high level" resistance pattern in which mutations occur spontaneously at a rate one mutation per 10^{-7} to 10^{-9} organisms [77, 78]. Studies carried out on the cost of rifampicin-resistance conferring mutations to mycobacteria have shown that there are certain mutations such as the Ser531Trp that confer a high level of resistance but are not commonly isolated from patients since they confer a high fitness cost to the bacteria thereby preventing these strains from being easily transmitted [79, 80].

Most of the mutations in the *rpoB* gene are missense mutations but insertions and small deletions also occur. The result of these genetic adaptations is an enzyme that is still functional but now represents an altered target for rifampicin. The more common mutations, His526Tyr and Ser531Leu, represent more than 70% of the mutations found in clinical isolates [39]. Since there is a lot of information available on mutations responsible for rifampicin resistance, the development of molecular tests for the rapid detection of rifampicin resistance has been made a lot easier [39].

Studies carried out have shown that mutations in codon 531 of the *rpoB* gene are one of the most frequently encountered mutations [81]. The detection of specific *rpoB* alleles in this codon can predict high-level resistance and simultaneously provide indirect information about susceptibility to other rifamycins [82]. The Ser531Leu mutation has also been found to account for 54% of rifampin-resistant isolates. The high frequency of this mutation is probably due to the low fitness cost associated with this mutation [27].

Rifampicin resistance is an excellent marker for MDR-TB because all MDR strains are resistant to rifampicin and rifampicin monoresistance is uncommon [19, 83, 84]. In most populations 90% of all strains of *Mycobacterium tuberculosis* that are resistant to rifampicin are also resistant to isoniazid [85]. Therefore, it is not necessary for a screening assay to test susceptibility to all anti-TB drugs.

2.6 Cost of Antibiotic Resistance on the Fitness of Bacteria

Studies using model systems have shown that, although there is a range, most resistance conferring mutations convey some cost to bacteria in terms of their fitness (a composite measure of an infectious organism's ability to survive, reproduce, and be transmitted) [56, 79, 86]. The extent of this fitness 'cost' and resultant effect on bacterial virulence [87] is however dependent on the specific drug resistance-conferring mutation and the genetic background of the strain [7, 26]. In some cases, these costs can also be partially compensated for by secondary or compensatory mutations that restore levels of fitness towards those of sensitive strains [27, 88-90] and this appears to be the case for *Mycobacterium tuberculosis.*

Antibiotic resistance is commonly associated with a reduced competitive ability against antibiotic-sensitive strains in the absence of the relevant antibiotic [27]. It is thus thought that a reduction in the use of antibiotics will lead to a reduction in the frequency of resistant bacteria in the population. The rationale for this idea is that since resistance is associated with a reduced bacterial fitness and that in the absence of the drug, susceptible strains will thrive and out compete resistant bacteria and as a result the frequency of resistance will decline [79].

2.7 Methods for detecting Single Nucleotide Polymorphisms

Variation at the genomic level consists mostly of sequence differences in single nucleotide positions. These differences are commonly referred to as SNPs [91]. Single nucleotide polymorphism analysis has been found to be very informative with regard to the study of drug resistance, evolution and molecular epidemiology not only in *Mycobacterium tuberculosis*, but in other organisms as well [67, 76, 87, 92]. This type of analysis can also play a crucial role in confirming associations between specific SNPs and a phenotype of interest, such as drug resistance [92]. Another interesting property of SNPs is that they are stably inherited thus making them excellent targets for the detection of a phenotype of interest [91].

Thus far, various methods have been described to detect SNPs. These methods involve target sequence amplification followed by the detection of variations in the DNA sequences by short hybridization probes or by restriction enzymes; discrimination of mismatched DNA substrates by polymerases or ligases; or by observing the template dependant choice of nucleotide incorporated by a polymerase. These molecular strategies include the following:

2.7a Assay Hybridization: These are assays which rely on the differences in hybridization stability of short oligonucleotides to target sequence variants (either mismatched or perfectly matched sequences). The problems with this assay includes the limitations of multiplex PCR and oligonucleotide hybridization to complex DNA samples as well as lack of flexibility caused by hardwiring of markers on a chip that is not easy to redesign [91].

2.7b Restriction Digestion: If a SNP alters the recognition sequence for a restriction enzyme, the products produced after a restriction enzyme cleavage will differ in size to those obtained when using a native DNA template. When these products are electrophoresed, their migration patterns will differ. This is the principle used in the microtiter array diagonal gel

electrophoresis technique (MADGE). The problem with this process is that an estimated 50% of all SNPs do not alter any restriction enzyme recognition sequence [91].

2.7c Mismatch Distinction by Polymerases and Ligases: The polymerization reactions in PCR require correct base pairing on the 3' end of the hybridizing primers. This is the principle used as the basis on the design of Allele-Specific PCR (ASP). But in this method, a DNA ligase is used to join two oligonucleotides that are hybridized to a target DNA sequence. Mismatches at or near the 3' end of the ligation site can then be detected by either the oligonucleotides ligation assay or the ligation chain reaction. The disadvantage of these assays is that a lot of optimization is required [91].

2.7d Minisequencing: In this method, DNA polymerase is used to add a specific nucleotide to a single primer. The nucleotide added is target dependant. This method allows a more accurate distinction between variable nucleotides located immediately downstream of the primer [91].

2.7e Pyrosequencing: This method involves the amplification of the gene of interest, followed by subjecting the PCR products to Pyrosequencing analysis using sequencing primers in overlapping regions. This allows for the detection of mutations in the gene of interest through the detection of pyrophosphate release upon incorporation of a nucleotide. This technique differs from standard sequencing, in that no fluorochromes or radioactivity is used and that no post-reaction step is required. Other advantages include the fact that shorter DNA sequences are generated. The technique is also easy to automate and has been determined to be accurate in the determination of specific mutations. Since the reaction can be carried out in a 96 well format, it allows fast prediction of mutations and can be used for the simultaneous screening of a large number of samples. However a potential problem of this technique is that looping and self-priming do commonly occur [93].

2.7f Homogenous Hybridisation: Two assays have been developed that allow hybridization based allele-discrimination during PCR. These are the TaqMan assay and the Molecular Beacon assay.

The most important point to take into consideration is that the ideal SNP detection method, as stated by Hazbón M.H and Alland D. (2004) [92] should be "simple to design, easy to perform under uniform assay conditions, easy to automate and inexpensive". Of the methods mentioned above, only the PCRs that can be used for the detection of SNPs will be discussed in more detail.

2.7g Types of PCR that can also be used for this purpose

2.7g (i) Allele-Specific PCR

This method is very similar to real-time PCR (RT-PCR) that relies on fluorescent oligonucleotides probes [46, 47, 59, 76, 81, 85]. However the modified oligonucleotides used in RT- PCR can be quite expensive. Allele-Specific PCR on the other hand, is an uncomplicated and inexpensive method of SNP genotyping that does not rely on these probes [94].

Allele-specific PCR is dependant on the non detected allele having a mismatch at or near the 3' nucleotide of the primer which, as can be expected, considerably reduces the efficiency of the amplification process [94]. This homogenous melting temperature or T_m-shift genotyping method uses two allele-specific primers each of which contains a 3' terminal base that corresponds to either the wild-type nucleotide or the expected mutated nucleotide, a reverse primer that amplifies both allelic variants and a fluorescent dye that can detect a double stranded DNA (dsDNA) product, such as SYBR green [94, 95]. Therefore whether just one of the primers or both of them amplify the sample is dependent on the sample genotype [95]. Usually amplification is more efficient and occurs more quickly with the perfectly complementary primer than that which contains the mismatched primer [92]. Since the temperature at which primer template duplexes dissociate, depends largely on product length and GC content, the PCR product will have a distinct T_m, depending on which one of the two primers is responsible for the amplification [96]. By adding GC-rich tails of differing lengths to each primer, the difference of the T_m of each PCR product can be altered to be even more

distinct. The genotypes can then be determined by the generation of a melting curve on a real-time PCR instrument [95].

Optimization of this assay can be done by altering the lengths of the GC tails added to the primers or by altering the concentration of primers added to the PCR [95]. However, this may be a time consuming exercise. Although it is possible for a primer to amplify template DNA despite a 3' terminal mismatch, the amplification of the non-matching template is usually delayed. This can be seen through a comparison of threshold cycle (Ct) values [94]. The Ct value is the cycle at which there is a significant increase in fluorescence, and this value is associated with exponential growth of the PCR product during the log-linear phase [97].

The advantages of using this assay are that, as mentioned previously, it is a simple and inexpensive method and does not rely on modified oligonucleotides probes. In terms of material requirements, this system requires just a few materials in addition to what is required for standard PCR amplification. Since it is also a high throughput method, a large number of samples can be processed in a very short period of time. The assay is also very flexible. It can be performed in a single closed tube and requires no post-PCR processing, thereby drastically reducing the risk of contamination. Since both alleles are detected in a single reaction, the T_m-shift assay allows an internal amplification control thereby eliminating the risk of false-negative results. The T_m shift genotyping method is also a very accurate genotyping method [95].

Allele-specific PCR has been used to distinguish between the wild type and mutated alleles of the *katG*315 of *Mycobacterium tuberculosis*. It was found that this multiplex allele-specific (MAS-PCR) assay could be performed on both purified DNA preparations as well as DNA

samples from crude cell lysates and sputum slide preparations. However, there was a slight difference in the method used in this study as restriction enzymes were used to digest the amplified *katG*315 fragment. If a mismatch occurred at this position, an additional restriction cleavage site was created. The resultant bands produced by restriction enzyme cleavage would then differ from the bands produced by restriction cleavage of a non-mutated allele. These products were then viewed on an agarose gel to determine the presence or absence of a mutation. This is in contrast to the method of detection of mutations by generating and analyzing melting profiles of the ASP described before [18]. A similar study was then carried out to detect mutations in codons 516, 526 and 531 of the *rpoB* gene of *Mycobacterium tuberculosis.* The study proved highly useful in detecting rifampicin resistant and hence MDR-TB in regions with high burdens of MDR TB [19].

The concept of MAS PCR was then taken a step further by developing a MAS-PCR that targeted codon 315 of the *katG* gene, codons 516, 526 and 531 of the *rpoB* gene, the *mabA-inhA* promoter region and codon 306 of the *embB* gene. This method as compared to culture based phenotypic drug susceptibility testing showed a sensitivity of 77.3% and a specificity of 99%. This assay was an improvement to the MAS PCRs described previously as it combines three tests in a single assay, thereby increasing the efficiency and decreasing the detection cost. This method therefore provides a rapid screening tool for a majority of resistant *Mycobacterium tuberculosis* isolates [98]. However, the one disadvantage of this method is that since SYBR green (a non-specific dye) is used for the detection of PCR products, a lot of optimization is required before the method can be considered to be reliable.

2.7g (ii) Detection of SNPs using Hairpin primers

This is an alternative ASP-PCR method that uses hairpin primers. Hairpin primers (so-called due to their shape) are modified primers that are used to detect SNPs through the addition of a 5' tail complementary to the 3' end of the linear primer [92]. Hairpin primers are very similar to molecular beacons. The loop sequence is complementary to the target sequence while the stem is formed by the annealing of the complementary arm sequences of between 5 – 7 nucleotides long on the ends of the oligonucleotide. Due to the fluorescent label and the quencher chromophore being in such close proximity to each other, little or no fluorescence occurs. During hybridization, the stem-loop structure undergoes a conformational change that allows it to adopt a linear format, thus causing a separation of the fluor and quencher from each other and the emission of maximum fluorescence. The use of hairpin primers is thought to increase the specificity of PCR assays by reducing primer dimer formation as well as mispriming. [99].

A study was carried out by Hazbon M.H and Alland D. (2004) [92] to determine the efficiency of these primers. This study was based on a similar principle to the Amplification Refractory Mutation (ARMS) method which distinguishes between the wild type and mutated nucleotide by making use of the relative inability of *Taq* polymerase to extend primers that are mismatched at their 3' ends. Primers are thus designed to be identical except for the 3' end nucleotide. The study compared hairpin primers with linear primers, and it was found that the assay which used hairpin primers was more sensitive for SNPs as the Ct values were greater.

2.7h Real-Time PCR

2.7h (i) Goal of RT PCR

Real-Time PCR has many applications in research since it combines the sensitivity of conventional PCR to the simultaneous detection of the PCR products while the amplification reaction is running [81]. This is achieved by incorporating a fluorescent DNA binding agent or probe into the PCR reaction, and the quantity of DNA can be assessed as the reaction proceeds by quantifying the amount of fluorescence.

The speed at which the fluorescent signal reaches a threshold level depends on the amount of original target sequence, thus allowing quantification. If required, the PCR product or amplicon can then be further analyzed by determining its melting temperature through the generation of a melting curve [100].

2.7h (ii) Advantages and Limitations of RT-PCR

One of the important advantages of this method is its ability to quantify nucleic acids over a wide range. The method has also been found to be extremely sensitive, allowing the analysis of small quantities of DNA such as in clinical biopsies. This method is also very quick due to reduced cycle times and earlier product detection and also allows a high-throughput format. Since the PCR is performed in a 'closed' system that does not require any post-amplification manipulations, there is a great reduction in the possibility of the occurrence of any cross-contamination [101].

As with any molecular technique, RT-PCR does have its limitations. One of these is the presence of PCR inhibitors in clinical samples that may not allow amplification to take place.

However, this problem may be overcome by choosing a DNA extraction technique, from the sample of interest, which allows for any possible PCR inhibitor to be removed. Another limitation is that only certain fluorescent chemistries can be used, depending on the machine used. This would be problematic in certain multiplex reactions. The biggest limitation is however, the cost involved in the design and manufacture of oligonucleotide probes and the price of the real-time instrument.

2.7h (iii) Amplicon Detection and Acquisition of Fluorescence

It is the detection of PCR products that distinguishes real-time PCR from conventional PCR. Real time chemistries consist of different specific and non-specific fluorescence reagents that bind to DNA, allowing monitoring of the PCR through the measurement of the fluorescent signal [81].

Non specific fluorescence reagents include the dyes ethidium bromide or SYBR green which bind to any dsDNA. However if specific fluorescence reagents such as labeled probes are used, the PCR signal corresponds specifically to the amplification of the target.

2.7h (iv) Types of probes that can be used

Different kinds of probes can be used. These include TaqMan or 5' nuclease probes, Molecular Beacons, hybridization probes and Scorpions which allow multiple DNA species to be measured in the same sample (multiplex PCR). Amplification products can be identified either by their different fluorescence spectra or by their melting characteristics or a combination of both [102]. Probes can also be designed to discriminate between wild type and mutant sequences within the same DNA template [81]. Thus, although each probe has its own

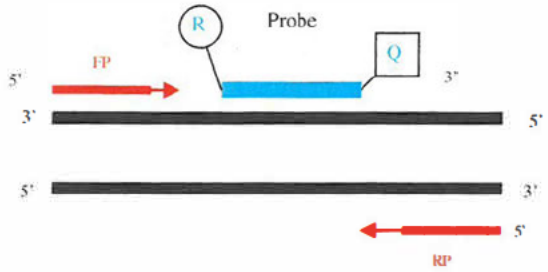
unique characteristics, each works on the same basic concept [100]. The data generated by these probes are usually collected from PCR cycles that occur during the exponential phase of the amplification reaction. During this phase, conditions in all aspects are optimal for the assay [101].

1. Taqman probes

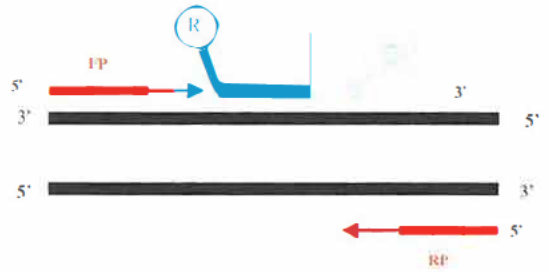
Conventionally, TaqMan probes used to be labeled with a fluorophore such as 6-carboxy-fluoroscein (FAM) on the 5' end and a quencher fluorophore such as 6 carboxy tetramethyl rhodamine (TAMRA) on the 3' end. However, an advancement of this technology includes the replacement of the TAMRA molecule with a minor groove binder (MGB), non-fluorescent quencher (NFQ) on the 3' end. The advantage that this replacement offers is threefold. Firstly, this results in the incorporation of a molecule that stabilizes the probe-target duplex [101, 103]. Secondly, due to the configuration of the probes, they are efficiently quenched. Thirdly, as a result of the interaction of the MGB with the DNA helix, short probes can be designed (thereby increasing the specificity of binding) due to them having a higher T_m. These probes are thus the probes of choice for detecting SNPs since they are easily destabilized by nucleotide substitutions within the target site [101].

This donor acceptor dye pair of a TaqMan probe interacts via fluorescence resonance energy transfer (FRET). The probe anneals to the amplified target in each of the annealing steps of the PCR. While bound to its homologous sequence, the proximity of the fluor and quench molecules causes the quencher to absorb the signal from the reporter thereby preventing the detection of the fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' nuclease activity of the polymerase cleaves the probe, causing a separation of the quencher from the dye. This allows the reporter's energy and fluorescent signal to be liberated. This fluorescence is measured by

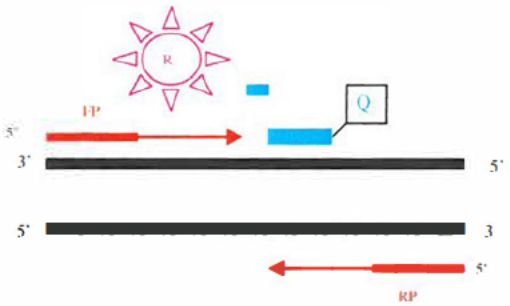
the RT PCR instrument. However, fluorescence is only emitted if the probe is correctly anchored to its homologous site to allow the *Taq* polymerase to release the dye. This is the basis for the detection of mutations using TaqMan probes, if a mismatch occurs, this would prevent binding of the probe, resulting in no fluorescence being emitted. So depending on whether the probes are designed to hybridize to a wild type or mutant sequence, fluorescence may or may not occur, indicating the presence or absence of a sequence [100, 104].



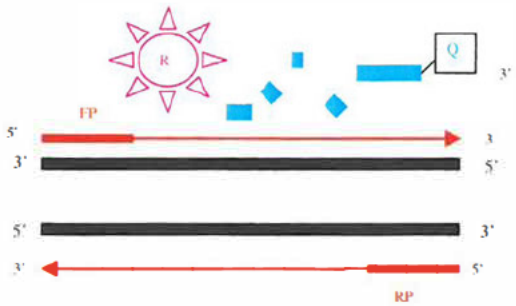
If the target of interest is present, the probe specifically anneals between the forward (FP) and reverse primer (RP) sites. In the unhybridised state, the proximity of the fluor (R) and the quench (Q) molecule prevents the detection of the fluorescent signal from the probe.



During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'-nuclease activity of the polymerase cleaves the probe.



This decouples the fluorescent and quenching dyes and FRET no longer occurs. This allows for the emission of fluorescence.



The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3′ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product.

Figure 2.2: A schematic representation of how TaqMan probes work (Adapted from TaqMan® One-Step RT-PCR Master Mix Reagents Kit, Protocol {Applied Biosystems}).

2. Molecular Beacons

Molecular Beacons form a stem loop structure when free in solution, with the loop consisting of the sequence which is homologous with the target DNA. In this configuration, the close proximity of the fluor and quench molecules prevents the probe from fluorescing. The stem-loop structure is disrupted by the binding of the beacon to its homologous sequence in the target DNA thus separating the quencher from the dye which enhances the fluorescence. The principle is thus, in essence, very similar to the TaqMan probes. If a mutation occurs in the region covered by the probe, its binding to the target is impaired and the beacon remains in its stem loop conformation. Thus no fluorescence is emitted [104]. Molecular Beacons are able to discriminate between DNA sequences that differ from one another by as little as a single nucleotide substitution [46].

3. FRET Probes

Fluorescence resonance energy transfer probes are paired probes which are designed to anneal to the DNA template in a head-to-tail orientation with each probe close to the next. One probe referred to as the anchor is fluorescence labeled at its 3'end and the adjacent probe or sensor probe is labeled at the 5'end with a dye which can be excited by the fluorescence of the anchor. The sensor probe is designed to overlap with the mutation which is to be searched for. When the FRET probes are correctly bound to their homologous target, by a process of energy transfer, excitation of the anchor dye stimulates the sensor dye, which in turn emits fluorescence. Mutation analysis, using this system, is performed in a melting step at the end of the PCR. The melting of the probe is represented as a melting peak with a melting temperature value. This T_m is constant for a pair of probes when bound to the wild type sequence. If a mutation occurs in the region covered by the probe, the mismatch between template and

probe, leads to a decrease in the T_m of the probes. The presence of a mutation is thus detected by a deviation in the melting temperature of the probe [104].

2.7h (v) Quantitation of Results

Normalization

The Passive Reference Dye is a dye that does not participate in the 5' nuclease assay. It provides an internal reference to which the reporter dye signal can be normalized during data analysis. For the ABI system, the dye used is a ROX dye which is a component of the Universal Master Mix used in the system. This process of normalization is required to correct for fluorescent fluctuations due to changes in DNA concentration or volume. Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the Passive Reference to obtain a ratio defined as the normalized reporter (Rn) for a given tube. Rn+ is the Rn value of a reaction containing all components including the template. Rn is the Rn value of an unreacted sample. ΔRn is the difference between the Rn+ and the Rn- value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions. The ΔRn is plotted against cycle numbers to produce amplification curves and give the Ct value.

2.7h (vi) Applications of RT-PCR

A rapid closed tube method was developed to detect common *Mycobacterium tuberculosis* mutations associated with resistance to isoniazid and rifampicin. The fluourogenic reporter molecules used in the study were molecular beacons. This method was found to be highly reliable as the overall sensitivity and specificity of the assay for isoniazid resistance was 85% and 100% respectively and for rifampicin resistance was 98% and 100% respectively [46].

With regard to the *rpoB* gene, a single-tube PCR assay was developed to detect all mutations that occur in the *Mycobacterium tuberculosis rpoB* core region. Five different molecular beacons were used to assess resistance mutations that occurred at any point in the core region. The assay was found to be particularly useful as it was used on DNA extracted from sputum samples. The method also proved to be sensitive and rapid. It was also found to be very specific and easy to perform. Furthermore, from the results obtained, it was also possible to determine if a patient was infected with rifampicin-resistant TB and the concentration of bacilli present in the sample. All these findings pointed to a huge advancement in the treatment of TB, through an improvement of diagnostic and screening procedures for not only TB but MDR-TB as well [76].

A study was also carried out by de Viedma *et al* (2002) to develop a real time based PCR assay that would be able to detect rifampicin and isoniazid resistant bacteria in a single reaction tube. The method was advantageous in that it doesn't only search for the most prevalent resistance-conferring mutations in the *rpoB* core region but for most other resistance-conferring mutations as well. The assay also allows for the detection for the most prevalent resistnce conferring-mutations in the *katG* gene. However, the limitations of this study are firstly, that not all possible mutations that encode resistance for rifampicin are detected by the assay and secondly, some of the mutations detected may not be related to resistance, These limitations prompted the investigators of this study to encourage more work to be done on improving this method. Further improvements have been achieved using probes capable of detecting eleven different mutations that confer resistance to both rifampicin and isoniazid within a period of just 2 hours. Furthermore, the T_ms of each of the probes varied to such an extent that no overlap was found between susceptible and resistant strains [47].

Prior to 2004, most of the RT-PCR assays were designed to detect mutations in DNA from cultured isolates [76, 104]. A study was therefore carried out to determine whether rifampicin and isoniazid resistance could be detected in DNA extracted from positive Auramine Rhodamine staining (AR) sputum samples. The results of this study showed that in comparison to other molecular methods also used for this purpose, a lack of sensitivity was not observed. However, the one problem that was evident from this method is that for isoniazid resistance, the sensitivity of this method is only 53.8% [105]. A similiar study was then carried out by Marin M. *et al* (2004). This study was, however, a little more ambitious as it aimed at detecting multiple resistance mutations directly from clinical samples. In order to do this, two major changes were implemented in the research design. The first change required a change in the design of the RT-PCR assay so that the DNA strands complementary to the probes were preferentially amplified. The second change was that the three PCRs that amplified the 5' end of the *rpoB* gene, the 3' end of the *rpoB* gene and the *katG* gene were performed in three separate reaction tubes. This study was different from the study by Ruiz *et al* in two respects. Firstly, a wider variety of resistance-conferring mutations were assessed and secondly, sequence data was available, thereby allowing a comparison of RT PCR data with the 'gold standard' method of mutation detection. This method showed a 100% correlation with DNA sequencing and was also able to detect a much wider variety of mutations, including those that were not so common. [59].

Mixed populations of *Mycobacterium tuberculosis* occur frequently. This concept of a combination of both susceptible and resistant bacteria may therefore give a false perception of the phenotype of a particular culture [16]. Real-time PCR was therefore used to evaluate whether or not resistance-conferring SNPs could be identified in the presence of sensitive or

wild type alleles. The three different types of probes mentioned above were evaluated in the study. All probes were designed to target the wild-type or mutant allele at codon 531 of the *rpoB* gene or the wild-type or mutant allele at codon 315 of the *katG* gene. From the results obtained, it was found that molecular beacons and TaqMan probes can specifically detect individual alleles in a mixture but this depended on the ratio of the alleles and on the absolute quantity of each allele (1:4.8 for low concentrations of allele and 1:48 for high concentrations of allele). The detection levels reached by FRET probes could only compare to those reached by molecular beacons and TaqMan probes, after target enrichment by primary PCR thus making this technique a little less useful [43].

Another study was conducted by Hillemann D. *et al* (2006) [106] to determine the sensitivity and specificity of RT PCR to detect Beijing and non-Beijing strains in a collection of MDR and fully susceptible isolates. Strains were analyzed using RT PCR, spoligotyping and the 'gold standard' of TB IS*6110* fingerprinting. In this study, 103 MDR isolates were analyzed and the real-time assay correctly identified 62 isolates as Beijing genotypes and 41 isolates as non-Beijing genotypes. These results were based on the fact that Beijing genotype strains have a different distribution of resistance mutations as compared to non Beijing strains. There was also a 100% concordance between RT PCR and the results obtained using the other two methods.

In another study carried out by Sajduda A. *et al* (2004) [107] where clinical specimens were analyzed both by sequence analysis and RT-PCR technology, it was shown that the results found using both methods were comparable. Thus this group also reached the conclusion that RT-PCR is a fast and reliable method for the detection of isoniazid and rifampicin resistance-associated mutations in clinical isolates of *Mycobacterium tuberculosis*.

Table 2.1: Showing the frequency of mutations in the *rpoB* and *katG* genes, in various parts of the world, as determined by RT-PCR

Mutation	Group								
	Telenti A. et al (1993) Spain	Torres M.J. *et al* (2000) Spain	Edwards K.J. *et al* (2001) [108] (United Kingdom	De Viedma D.G. *et al* (2002) (Spain)	Varma-Basil M. *et al* (2004) India and Mexico	Wada T. *et al* (2004) Japan	*Marin M. *et al* (2004) Spain	Kocagoz T. *et al* (2005) Turkey	Espasa M. *et al* (2005) Spain
rpoB 533	ctg → ccg (1.6 %)				ctg → ccg (2.8 %) ctg → gcg (1.4 %)				
rpoB 531	tcg → ttg (48.4 %) tcg → cag (1.6 %) tcg → tgg (1.6 %)	tcg → ttg (50 %)	tcg → ttg (48.9 %) tcg → tgg (8.5 %)	tcg → ttg (27.8 %)	tcg → ttg (42.3 %) tcg → tgg (12.7 %) tcg → ttc (2.8 %)	tcg → ttg (53.3 %) tcg → tgg (8.8 %)	tcg → ttg (45.5 %)	tcg →ttg (60.4 %)	tcg →ttg (10.9 %)
rpoB 530					ctg → gct (1.4 %)				

* Denotes results from clinical respiratory specimens

Mutation	Group								
	Telenti A. et al (1993) Spain	Torres M.J. *et al* (2000) Spain	Edwards K.J. *et al* (2001) [108] (United Kingdom	De Viedma D.G. *et al* (2002) (Spain)	Varma-Basil M. *et al* (2004) India and Mexico	Wada T. *et al* (2004) Japan	*Marin M. *et al* (2004) Spain	Kocagoz T. *et al* (2005) Turkey	Espasa M. *et al* (2005) Spain
rpoB 526	cac → tac (12.5 %) cac → gac (7.8 %) cac → cgc (3.1 %) cac → acc (1.6 %) cac → ccc (1.6 %) cac → cag (1.6 %)	cac → gac (5.5 %)	cac → tac (12.8 %) cac → gac (6.4 %) cac → ctc (2.1 %) cac → tgc (2.1 %)	cac → tac (5.6 %) cac → gac (11.1 %) cac → ctc (5.6 %)		cac → tac (2.2 %) cac → gac (2.2 %) cac → cgc (4.4 %) cac → agc (2.2 %)	cac → gac (18.2 %)	cac →caa (20.8 %)	cac → gac (3.6 %)
rpoB 522	Deletion (1.6 %)					tcg → ttg (8.8 %)			
rpoB 520						ccg → tcg (4.4 %)			

Mutation	Group								
	Telenti A. et al (1993) Spain	Torres M.J. *et al* (2000) Spain	Edwards K.J. *et al* (2001) [108](United Kingdom	De Viedma D.G. *et al* (2002) (Spain)	Varma-Basil M. *et al* (2004) India and Mexico	Wada T. *et al* (2004) Japan	*Marin M. *et al* (2004) Spain	Kocagoz T. *et al* (2005) Turkey	Espasa M. *et al* (2005) Spain
rpoB 518	Deletion (1.6%)								
rpoB 516	gac → gtc (9.4 %)		gac → gtc (12.8 %) gac → ttc (2.1 %)	gac → gtc (5.6 %) Deletion tg – g (5.6 %)		gac → gtc (20 %)		gac → gtc (5.2 %) gac → tac (4.2 %)	gac → gtc (3.6 %) gac → ttc (1.8 %)
rpoB 515				Deletion tg – g (5.6 %)			Deletion tgg (9.1 %)		
rpoB 514							Deletion tca → tgg (9.1 %)		
rpoB 513								caa → cta (2.1 %)	
rpoB 511			ctg → ccg (2.1 %)		ctg → ccg (4.2 %)				
katG 315		agc → acc (50 %) agc → aac (5.5 %)		agc → acc (44.4 %) agc → aca (11.1 %)		agc → acc (48.9 %)	agc → acc (45.5 %)		agc → acc (32.7 %)

The alleles detected in the above listed studies have also been reported from South Africa [109-111].

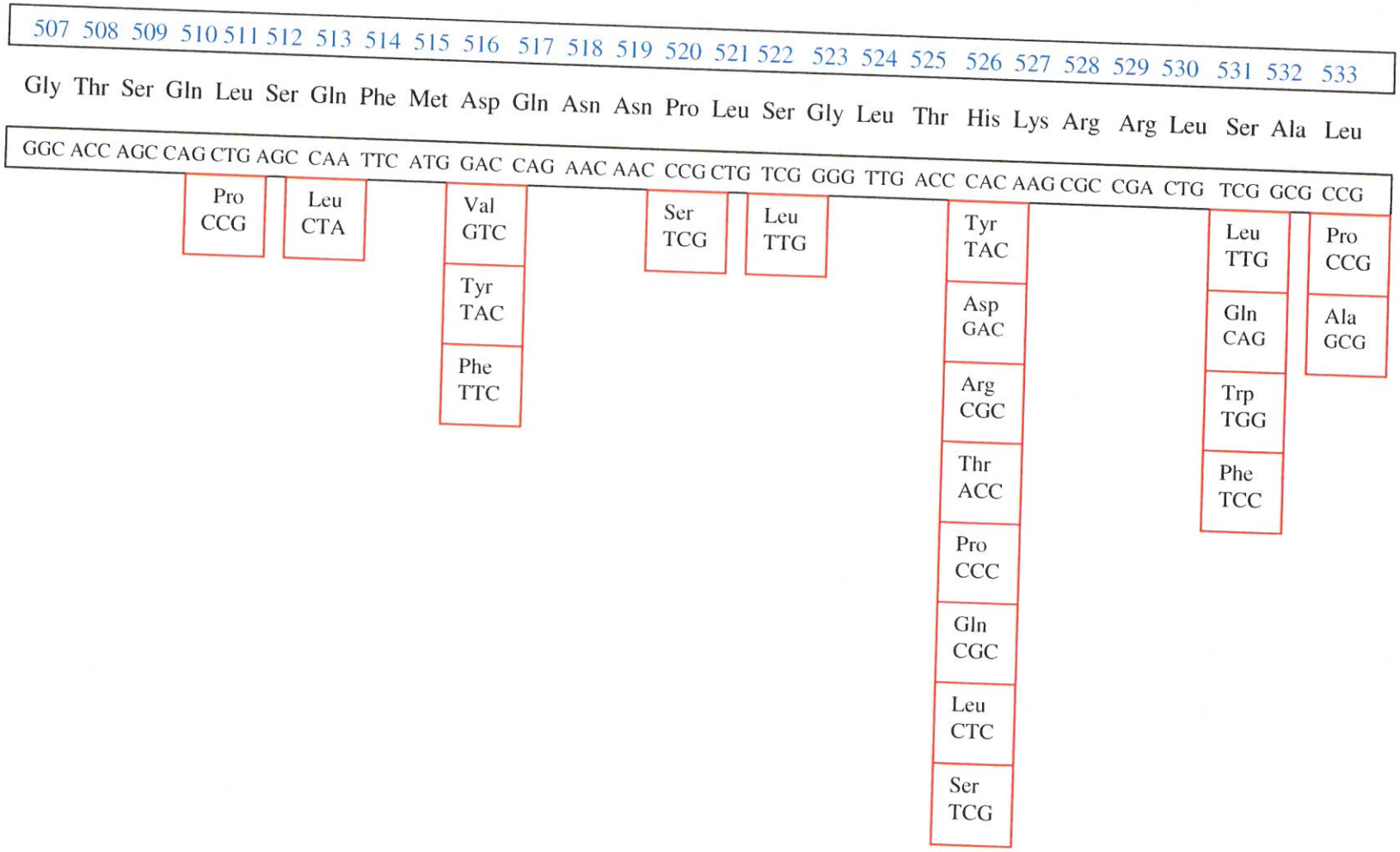


Figure 2.3: Mutations occurring in the Rifampicin Resistance Determining Region (RRDR) (codons 507 through 533) (shown in blue) of the *M.tuberculosis rpoB* gene as described in the table above. The data are a compilation of the single nucleotide substitutions as determined by the studies, summarized in table 2.1. The missense mutations are shown below the wild-type sequence. (Adapted from Musser J.M. 1995. Antimicrobial Agent Resistance in Mycobacterial Molecular Genetic Insights. Clin. Microbiol, Reviews. 8: 496-514)

Chapter Three

3. Materials and Methods

3.1. *Mycobacterium tuberculosis* strains

The assay was initially optimized using 15 *Mycobacterium tuberculosis* isolates obtained from Dr M Pillay and Mrs K.Ganas of the Department of Medical Microbiology at the University of Kwa Zulu Natal. These isolates were originally isolated as part of the Rapid Detection study and from the OFLOTUB Phase III clinical trial and were collected from Stanger, Umlazi, King George V hospital and from King Edward VIII hospital. All isolates had previously been tested for resistance to rifampicin and isoniazid and their susceptibility profiles for these two drugs are shown in table 4.1. In addition H37Rv (reference) was used as a reference strain. Spoligotypes and IS*6110* patterns were available for 7 of these isolates and they represented 3 genotypes including 2 KZN strains.

3.2 Culturing of *Mycobacterium tuberculosis*

Isolates which had been preserved in media containing Tryptic Soya Broth and glycerol at - 80^{0}C, were thawed out and cultured in 5 ml of liquid 7H9 broth at 37^{0}C until visible growth occurred. Isolates were also subcultured onto Lowenstein-Jensen (LJ) media at 37^{0}C from existing cultures for a period of between 6-8 weeks.

3.3 DNA Extraction

3.3a DNA extraction from Lowenstein-Jensen media

A loopfull of cells were transferred from the Lowenstein-Jensen (LJ) media into a microcentrifuge tube containing 500 µl 1 X TE (10 mM Tris-HCl [ph 8.0], 1 mM EDTA) buffer. The cultures were then heat-killed by incubation at 80^{0}C for 30 minutes. 50 µl of

10mg/mL lysozyme (Appendix 1) was then added to these heat killed bacteria. The mixture was then left to incubate for 1 hour at 37^0C. 75 µl of a mix containing proteinase K (10mg/mL) and 10% sodium dodecyl sulphate (SDS) (Appendix 1) was then added to the lysosyme treated samples. After vortexing and 20 minute incubation at 65^0C, 100 µl of 5M sodium chloride (NaCl) was added, followed by 100 µl of a mix of N, N, N trimethyl ammonium bromide (CTAB) and NaCl (Appendix 1). The mixture was then vortexed until it became 'milky' and then incubated for 20 minutes at 65^0C. 750 µl of chloroform isoamyl alcohol was then added followed by vortexing and centrifugation at 12 000 *g* for 15 minutes. The aqueous supernatant was then transferred into fresh microcentrifuge tubes. 500 µl isopropanol was then used to precipitate the nucleic acids. These tubes were then left at -20^0C for 30 minutes followed by a 30 minute centrifugation step. 1 mL of cold 70% ethanol was then used to wash the pellet followed by a 5 minute centrifugation. The supernatant was then discarded and the pellet left to dry at room temperature. The pellet was then dissolved in 20 µl of 1 X TE buffer [112, 113].

3.3b DNA extraction from 7H9 Broth

Cultures were first transferred to 50 mL sterilin tubes and centrifuged at 12 000 *g* for 25 minutes. The supernatant was discarded and the pellet re dissolved in 500 µl 1 X TE buffer. The cells were then heat killed by boiling at 95^0C for 20 minutes. A DNA extraction was carried out using a similar method to the one described for the cultures from the LJ media, as described above, with the following exceptions:

1. 70 µl instead of 50 µl of lysozyme was added to each sample.
2. 100 µl instead of 75 µl of a Proteinase K amd 10% SDS mix was added to each sample.
3. 25 µl RNase was then added following the addition of the mix described above.
4. 200 µl of a mix of CTAB and NaCl was added instead of 100 µl.

DNA precipitation was then carried out exactly as described above.

3.3c DNA extraction from Ziehl Neelsen stained microscopic preparations

The mineral oil was first removed from the slide with the addition of approximately 2 mL of xylene. Ziehl Neelsen-stained material was scraped off from the microscopic slides (using a clean, sterile blade for each slide) following the addition of 25 µl of triple distilled sterile water. The scraped material was then added to 25 µl of sterile distilled water in a microcentrifuge tube. 75 µl of Chelex suspension [114] was added. After a thorough vortexing of the samples, the samples were incubated at 97^0C for 30 minutes. The samples were then centrifuged at 13 000 *g* for 10 minutes. The supernatant was then transferred to a 1.5 mL microcentrifuge tube and used directly for PCR [30].

3.3d DNA extraction from sputum samples

Three methods of extracting DNA from sputum samples were evaluated.

i) DNA Extraction using Chelex

Sputum samples were first decontaminated and liquefied using the NALC/NAOH method (Appendix 2). The samples were then heat-killed for 15 minutes at 100^0C. An equal volume of Inhibitor Removal Solution (IRS) {Appendix 4} was then added and the samples were incubated at 37^0C for 10 minutes. Samples were then centrifuged at 12 000 *g* for 10 minutes and the supernatant discarded. The resulting pellet was washed with 1 mL sterile triple distilled water and centrifuged at 12 000 *g* for 10 minutes. The supernatant was then discarded. 75 µl of DNA Extraction solution (Appendix 4) was then added. The samples were

then incubated for 30 minutes at 95^{0}C followed by a centrifugation step at 12 000 *g* for 10 minutes. The supernatant was then used as a template in RT-PCR (modified from [115]).

ii) DNA Extraction using the Silica Method

Samples processed by the NALC/NAOH method were first heat-killed by incubation for 15 minutes at 100^{0}C. 1 mL of lysis buffer (Appendix 5) was then added followed by the addition of 40 µl of acid washed silica (Appendix 5). The samples were then incubated for 15 minutes at room temperature with constant vortexing. This was followed by a centrifugation step at 12 000 *g* for 30 seconds. The supernatant was then discarded. The resulting pellet was the washed twice with 1 mL wash buffer, vortexed and centrifuged for 30 seconds at 12 000 *g*. The supernatant was then discarded. The pellet was then washed twice, first with 1 mL and then with 500 µl of 70% ethanol and then centrifuged for 30 seconds at 12 000 *g*. Following the removal of the supernatant, the resulting pellet was washed once with 1 mL acetone and centrifuged for 30 seconds at 12 000*g*. The pellet was then air dried and 95 µl of 1 X TE buffer was added. The samples were then vortexed briefly followed by an incubation step at 55^{0}C for ~10 minutes. The samples were then vortexed and centrifuged for 2 minutes at 12 000 *g*. The supernatant was then transferred to a clean microcentrifuge tube and used as a template in RT-PCR (adapted from [116]).

iii) Genotype® MTBDR DNA Extraction

Sputum samples were first decontaminated and liquefied using the NALC/NAOH method. 500 µl of pretreated sputum samples were first centrifuged at 10 000 *g* for 15 minutes. The supernatant was then discarded and the pellet re-suspended in 100 µl sterile triple distilled

water. The samples were then heat inactivated at 100^0C for 20 minutes. DNA was then extracted by ultrasonication using a Transsonic Digital S ultrasonic waterbath (60^0C for 15 minutes). Samples were then centrifuged at 10 000 *g* for 5 minutes. The supernatant was then used as a template for PCR [117].

3.4 Polymerase chain reaction amplification of drug resistance loci

For the 15 isolates that was to be used in the development and the optimization of the RT-PCR, amplification of both the *katG* and *rpoB* genes was carried out using oligonucleotides primers that have been previously designed for the amplification and sequencing of the codon 315 region of the *katG* locus and the 'rifampicin resistance determining region' or RRDR region of the *rpoB* gene [87, 93] (table 3.1). Each reaction tube contained in a total volume of 25 µl, 1 X PCR buffer (Lucigen), 1.5 mM Magnesium chloride ($MgCl_2$), 100 µM of each deoxyribonucleotide triphosphates (dNTP) [Fermentas], 1.25 U of EconoTaq DNA polymerase (Lucigen), 0.2 µM of each primer (Inqaba) and 2.5 µl of Template DNA. Amplification was performed in the GeneAmp PCR System 9700 machine. Conditions for cycling were 94^0C for 2 minutes, followed by 35 cycles of 94^0C for 1 minute, 55^0C for 1 minute and 72^0C for 2 minutes, followed by an additional step at 72^0C for 5 minutes.

Table 3.1: Primers used to amplify *rpoB* and *katG* genes for sequencing

Target	Oligonucleotides	Product size
katG	*katG*315.F: CCGCAGCGAGAGGTCAGTGG *katG*315.R: TCGGCGATGAGCGTTACAGC	542 bp
rpoB	*rpoB*.F: TCGGCGAGCTGATCCAAAACCA *rpoB*.R: ACGTCCATGTAGTCCACCTCAGA	601 bp

3.5 DNA Sequencing

The PCR products (*katG* gene or *rpoB* gene) were evaluated using agarose gel electrophoresis (Appendix 6). The amplified fragments (5 µl) were electrophoresed on a 2% agarose gel for 1.5 hours at 100 V. Ethidium bromide was used to stain the gel. The PCR products were then viewed under ultra violet (UV) light on the Vacutec Bio-Imaging system. 12.5 ul of successful PCR reactions were sent to Inqaba biotech in Pretoria for sequencing (www.inqababiotec.co.za). Analysis of these gene sequences was then carried out by comparing them to the H37Rv genome (available at www.genolist.pasteur.fr/Tuberculist). It was then determined whether the relevant mutation was present or absent in each gene.

3.6 Allele-Specific PCR

3.6a) Primer Design

Allele-Specific Primers were designed with the 3' end matching either the wild-type or mutant allele of the *rpoB* or *katG* gene and a reverse common primer. Primers were blasted against the H37Rv (reference) genome to ensure that the target sequences were in the correct region (www.genolist.pasteur.fr/Tuberculist) Primers were also analyzed to check for hairpins, homo and heterodimers on www.idtdna.com. Primers were designed to have a T_m between 58.3^0C – 63.5^0C and a length ranging from 14-27 base pairs. Common primers were designed with a T_m from 62^0C – 75^0C with a length ranging from 22-30 base pairs. In the case of the *rpoB* gene primers, a AG tail comprising a 12 bp nucleotide sequence was added to the wild type allele specific primer to favor allele discrimination.

Table 3.2: Primers used in allele-specific PCR

	Name of primer	**Sequence**	
rpoB gene	*rpoB*.531.ttg.rev	CGGGCCCCAGCGCCA	All primers designed in this study
	rpoB.531.wt.tail.rev	GGAGGAGGAGGACGGGCCCCAGCGCCG	
	rpoB.CP	GGTGGTCGCCGCGATCAAGGAG	
katG gene	*katG*.315.acc	CGGTAAGGACGCGATCACCAC	
	katG.315.wt	CGGTAAGGACGCGATCACCAG	
	katG.CP.rev	ACTCGTAGCCGTACAGGATC	

3.6b) PCR conditions for allele specific PCR

Initially each reaction tube contained in a total volume of 25 µl, nuclease free water, 1 X PCR buffer, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 1.25 U of EconoTaq DNA polymerase, 0.2 µM of each primer and 2.5 µl of Template DNA. Amplification was performed in the GeneAmp PCR System 9700 machine. Conditions for cycling were 95^0C for 12 minutes, followed by 40 cycles of 95^0C for 20 sec, 58^0C for 1 minute and 72^0C for 30 sec, followed by an additional step at 72^0C for 5 minutes [95]. Experiments were subsequently carried out to optimize the annealing temperature, dNTPs concentration, $MgCl_2$ concentration, primer concentration and template concentration. These were as follows.

i) Annealing Temperature - In order to determine the optimal annealing temperature, the initial annealing temperature used was estimated by calculating the T_m of the allele-specific primer. As a general rule, an annealing temperature 5^0C lower than the T_m is often satisfactory. However, the annealing temperature was varied whilst other conditions were kept constant in order to determine the optimal annealing temperature.

ii) dNTPs and $MgCl_2$ - Magnesium concentration is a crucial factor that can affect the success of amplification. In order for DNA polymerase to be active, adequate magnesium is required to form complexes with dNTPs, a substrate that the polymerase recognizes. The optimal $MgCl_2$ concentration varies between 0.5 mM and 5 mM and the final dNTP concentration should be between 50 µM and 500 µM. To determine the optimal $MgCl_2$ and dNTP concentration, experiments were carried out using varying concentrations of each component as shown in Table 3.3 while keeping all other reagent concentrations, in the PCR, constant. Thus each tube had a different concentration of dNTPs and $MgCl_2$ while the concentrations of all other PCR reagents were kept constant.

iii) Primers - Optimally 20 pmol of primers in a 50 µl reaction mix is the amount used. A primer concentration higher than optimal may lead to mis-priming and accumulation of non-

specific product while a concentration lower than optimal may lead to exhaustion of the primer before the reaction is completed. For optimization purposes, the PCR was carried out using the following concentrations of primers: 2 μM, 1 μM, 0.5 μM, 0.25 μM. The concentration of $MgCl_2$ and dNTPs that produced optimal results was used with the above primer concentrations. The concentration producing the best result was chosen.

iv) Template DNA - The amount of template required for successful amplification depends on the complexity of the DNA sample. The DNA concentration was therefore varied to determine the optimal DNA concentration. The following concentrations of DNA were evaluated: 195.64 ng/μl, 14.64 ng/μl, 2.48 ng/μl, 0.54 ng/μl and 0.20 ng/μl.

v) Cycling conditions - After the above optimization experiments were carried out further modifications to the conditions for cycling were made and the following conditions were used: 95^0C for 12 minutes, followed by 35 cycles of 95^0C for 20 sec, 55^0C for 1 minute and 72^0C for 30 sec, followed by an additional step at 72^0C for 5 minutes.

Table 3.3: Optimization of $MgCl_2$ and dNTPs

50 μM dNTPs with:	100 μM dNTPs with:	150 μM dNTPs with:	200 μM dNTPs with:
1.5 mM $MgCl_2$	1.5 mM $MgCl_2$	1.5 mM $MgCl_2$	1.5 mM $MgCl_2$
2.0 mM $MgCl_2$	2.0 mM $MgCl_2$	2.0 mM $MgCl_2$	2.0 mM $MgCl_2$
2.5 mM $MgCl_2$	2.5 mM $MgCl_2$	2.5 mM $MgCl_2$	2.5 mM $MgCl_2$
3.0 mM $MgCl_2$	3.0 mM $MgCl_2$	3.0 mM $MgCl_2$	3.0 mM $MgCl_2$
3.5 mM $MgCl_2$	3.5 mM $MgCl_2$	3.5 mM $MgCl_2$	3.5 mM $MgCl_2$

3.7 Real-Time PCR using allele-specific primers

Reactions were performed in 96-well microtiter plates (MicroAmp 96-well optical reaction plate, Applied Biosystems). Each well contained a total volume of 25 µl of 1 X PCR buffer (Lucigen), 0.2 µM of each primer, 100 µM of each dNTP, 1 X SYBR green (Cambrex), 0.25 µM ROX dye and 2.5 µl of template DNA. Amplification was performed in the ABI Prism 7000 Sequence Detection System (ABI Prism 7000 SDS). Intitially the following conditions for cycling were used: 94^0C for 3 minutes, 70^0C for 30 sec, 72^0C for 30 sec, 94^0C for 45 sec, 68^0C for 30 sec, followed by 35 cycles of 72^0C for 30 sec, 94^0C for 45 sec and 64^0C for 30 sec, followed by an additional step at 72^0C for 2 minutes.

Cycling parameters were altered in subsequent experiments. There were 7 stages involved: Stage 1: 94^0C for 3 minutes, 70^0C for 30 sec, 72^0C for 30 sec; Stage 2: 94^0C for 45 sec , 69^0C for 30 sec, 72^0C for 30 sec; Stage 3: 94^0C for 45 sec, 68^0C for 30 sec, 72^0C for 30 sec; Stage 4: 94^0C for 45 sec, 67^0C for 30 sec, 72^0C for 30 sec; Stage 5: 94^0C for 45 sec, 66^0C for 30 sec, 72^0C for 30 sec; Stage 6: 94^0C for 45 sec, 65^0C for 30 sec, 72^0C for 30 sec; Stage 7: 94^0C for 45 sec, 64^0C for 30 sec, 72^0C for 30 sec (repeated for 35 cycles), followed by an additional 72^0C for 2 minutes.

3.8 Real-time PCR using hairpin primers

A different set of conditions were used to test a set of hairpin and linear primers. The primers tested for the Ser315Thr mutation are listed in table 3.4. All reactions were carried out in an ABI Prism 7000 SDS (Applied Biosystems) in a 96-well optical reaction plate. Reactions were carried out in 25 µl volumes containing nuclease free water, 1 X polymerase buffer, 1.25 U of Taq polymerase, 0.2 µM of each primer, 100 µM of each dNTP and 1 X SYBR green (Cambrex). 2.5 µl of DNA was added to each tube to give a final reaction volume of 25 µl.

Two master mixes for each assay (hairpin and linear primer) were used, one that contained the mutant primer and the common reverse primer and another that contained the wild-type primer and the common reverse primer. The primers used in these experiments were designed using the Primer Express Software version 2 (Applied Biosystems). Primers were blasted against the H37Rv (reference) genome to ensure that the target sequences were in the correct region (www.genolist.pasteur.fr/Tuberculist). Thermal conditions were as follows for hairpin primers and linear primers: Stage 1: 94^0C for 3 min, 70^0C for 30 sec, 72^0C for 30 sec, Stage 2-6, only the annealing temperature was changed by lowering by one degree for every subsequent cycle, Stage 7, 94^0C for 45 sec, 64^0C for 30 sec and 72^0C for 30 sec (repeated for 35 cycles) and Stage 8: 72^0C for 2 min.

Table 3.4: Primers used in the Hairpin Assay

	Name of Primer	Sequence	
Hairpin primers	HP_KatGS315T_C	AAACTGTTGTCCCATTTCGTCGG	Primers were designed in this study
	HP_KatGS315T_WT	CTGGTGATCGGTAAGGACGAGATCACCAG	
	HP_KatGS315T_MUT	GTGGTGATCGGTAAGGACGAGATCACCAC	
Linear primers	KatGS315T_WT	GGTAAGGACGAGATCACCAG	
	KatGS315T_Mut	GGTAAGGACGAGATCACCAC	

3.9 Real Time PCR using TaqMan probes

3.9a) Design of TaqMan MGB probes and primers

Two TaqMan MGB probes were designed for each gene to hybridize with either wild type DNA or mutant DNA. Primers for the *rpoB* gene were designed using the Primer Express Program, version 2 (Applied Biosystems). Primers for the *katG* gene were taken from published literature [118]. The MGB probes [118] were synthesized by Applied Biosystems while the primers were synthesized by Inqaba Biotech. A list of primers and probes are shown in table 3.5. Probes that hybridized with wild-type DNA were labeled with 6-carboxyfluoroscein (FAM) (emission wavelength, 518 nm) at the 5' end and both with a MGB, NFQ at the 3' end. Probes that hybridized with the mutant sequence were labeled with VIC (emission wavelength, 552 nm) at the 5' end and with a MGB, NFQ at the 3' end. The advantage of using two dyes that emit luminescence of different wavelengths is that they can be individually distinguished in one tube thus allowing multiplexing by color to be carried out.

3.9b) Amplification-hybridization assay

Real-time PCR was performed in the ABI 7000 Sequence Detection System (Applied Biosystems) in a 96-well optical reaction plate format (Applied Biosystems). For a uniplex reaction, the PCR mixture was prepared in a final volume of 25 µl with 12.25 µl of Universal Master Mix (Applied Biosystems), 0.5 µM of each primer, 0.1 µM of the necessary probe and 10 µl of purified DNA or extracted DNA. The initial conditions of PCR amplification were 50^0C for 2 minutes, 95^0C for 10 minutes and then 40 cycles of 95^0C for 15 s and 60^0C for 1 minute. To increase discrimination between the two probes annealing temperature from 60^0C to 65^0C was changed for the first set of *katG* probes that were tested The PCR for the second

set of *katG* probes tested used the following conditions: 50^0C for 2 minutes, 95^0C for 10 minutes and then 40 cycles of 95^0C for 15 s and 60^0C for 1 minute.

For the multiplex reaction, the pair of probes, wild-type and mutant, designed for each resistance codon were mixed in the same amplification reaction. The PCR mixture was prepared in a final volume of 25 µl with 12 µl of Universal Master Mix (Applied Biosystems), 0.5 µM of each primer, 0.1 µM of each probe and 10 µl of purified DNA or extracted DNA. The conditions of PCR amplification were 50^0C for 2 minutes, 95^0C for 10 minutes and then 40 cycles of 95^0C for 15 s and 65^0C for 1 minute.

Fluorescence of hybridized probes was expressed as ΔRn (normalized reporter signal). The number of amplification signals required for emission of a certain luminescence intensity by each probe (ΔRn = 0.2) reflected the amount of DNA in the sample. The cycle number is called the threshold cycle. Therefore the presence of a mutation would result in an increase in the Ct value.

Table 3.5: Primers and Probes used in RT- PCR

Target	Oligonucleotide primers	Oligonucleotide probes
rpoB gene	Forward primer: AGCCAGCTGAGCAATTCAT Reverse primer: TCGATCGGGCACATCCGG	*Wild-type probe: 5' – FAM AGCGCCGACAGTCGGCG- MGB-NFQ-3' *Mutant probe: 5' – FAM-CAGCGCCAACAGTCGGCG MGB-NFQ-3'
katG gene	*Forward primer: GGAAACTGTTGTCCCATTTCG *Reverse primer: GGGCTGGAAG AGCTCGTATG	*Wild-type probe: 5' FAM-CGACCTCGATG CCGCTGGTGAT MGB-NFQ 3' *Mutant probe: 5'-VIC- CGACCTCGATGCCGGTGGTGAT- MGB-NFQ-3'
		#Wild-type probe: 5'–FAM-TCACCAGCGGCATC MGB- NFQ-3' Mutant probe: 5' VIC TCACCACCGGCATC-MGB NFQ-3'

#Taken from Wada T. *et al.*, 2004

*Taken from Espasa M.A. *et al.*, 2005

3.10 Genotype® MTBDR*plus*

Genotype® MTBDR*plus* is a commercially available multiplex PCR DNA strip assay (Hain Lifescience GmbH, Nehren, Germany) that is capable of simultaneously detecting mutations in the *rpoB* gene, the *katG* gene and in the regulatory region of the *inhA* gene. It thus allows for a rapid detection of not only the *Mycobacterium tuberculosis* complex but also its resistance to rifampicin and isoniazid in a single procedure.

The principle of the test is based on the combination of a multiplex PCR and a reverse hybridization between the *katG* and *rpoB* biotin-labelled amplicons to membrane bound probes [117]. The DNA strip covers eight *rpoB* WT probes, four *rpoB* mutant probes, one *katG* WT probes, two *katG* mutant probes, two *inhA* WT probes and four *inhA* mutant probes. The strip is designed so that the most significant mutations in the *rpoB* gene (between codons 507 533) are detected, for the testing of high-level isoniazid resistance, codon 315 of the *katG* is examined and for low level isoniazid resistance, the promoter region of the *inhA* gene is examined.

The assay was carried out according to the manufacturer's instructions. In brief after DNA extraction (as outlined under the sputum DNA extraction methods above) amplification was carried out in a 50 µl reaction volume consisting of 35 µl primer-nucleotide mix (PNM) (provided with the kit), 1 X PCR reaction buffer (Roche Applied Science) containing 1.5 mM $MgCl_2$, 2 U of Taq polymerase (Roche Applied Science), 5 µl nuclease free water and 5 µl of extracted DNA using a GeneAmp PCR 9700 system (Applied Biosystems). The amplification profile consisted of a denaturation step (95^0C for 5 min), 10 cycles of 95^0C for 30 sec and 58^0C for 2 min, followed by 40 cycles of 95^0C for 25 sec, 53^0C for 40 sec and 70^0C for 40 sec followed by a final extension step of 70^0C for 8 min. This was followed by reverse

hybridization. 20 µl of denaturation solution (provided with the kit) was added each of the wells followed by 20 µl of PCR product. After incubate for 5 min at room temperature, 1 mL of hybridization (HYB) buffer (pre-warmed to 45^0C) was then added to each well, followed by a gentle shaking. A DNA strip was then added to each well and left to incubate in a shaking water bath/TwinCubator® for 30 min at 45^0C. After complete aspiration of the HYB buffer, 1 mL of stringent wash solution (STR) (pre-warmed to 45^0C) was then added to each well and left to incubate for 15 min at 45^0C in the shaking water bath. The STR was then poured out and 1 mL of rinse solution (RIN) was added and left to incubate for 1 min at room temperature in the shaking water bath. Following removal of the RIN, 1 mL of diluted conjugate was added to each well and left to incubate for 30 min at room temperature in the shaking water. The solution was then removed and each strip was then washed twice for 1 min with 1 mL RIN and once with 1 mL of distilled water on the shaking platform. 1 mL of diluted substrate (provided with the kit) was added to each strip and incubated at room temperature for 5 min without shaking. The reaction was stopped by briefly rinsing twice with distilled water. The strips were then removed from the tray using tweezers, dried on absorbent paper and pasted onto an evaluation sheet. Interpretation of the DNA strip was then carried out using the provided template according to manufacturer's instructions. All solutions were supplied by the manufacturer.

Chapter Four

4. Results

4.1 Identification of *Mycobacterium. tuberculosis* isolates with specific *katG* and *rpoB* mutations

A 601 bp fragment spanning the *rpoB* rifampicin resistance conferring region and a 542 bp fragment spanning the codon 315 region of *katG* were amplified from 15 clinical isolates (Figure 4.1). The *katG* gene from 12 isolates and the *rpoB* gene from 10 isolates were sequenced. The results of the sequence analysis are shown in Table 4.1. According to sequencing results, for the *katG* gene, 10 isolates (83%) had the Ser315Thr mutation and for the *rpoB* gene, 6 isolates (60%) had the Ser531Leu mutation.

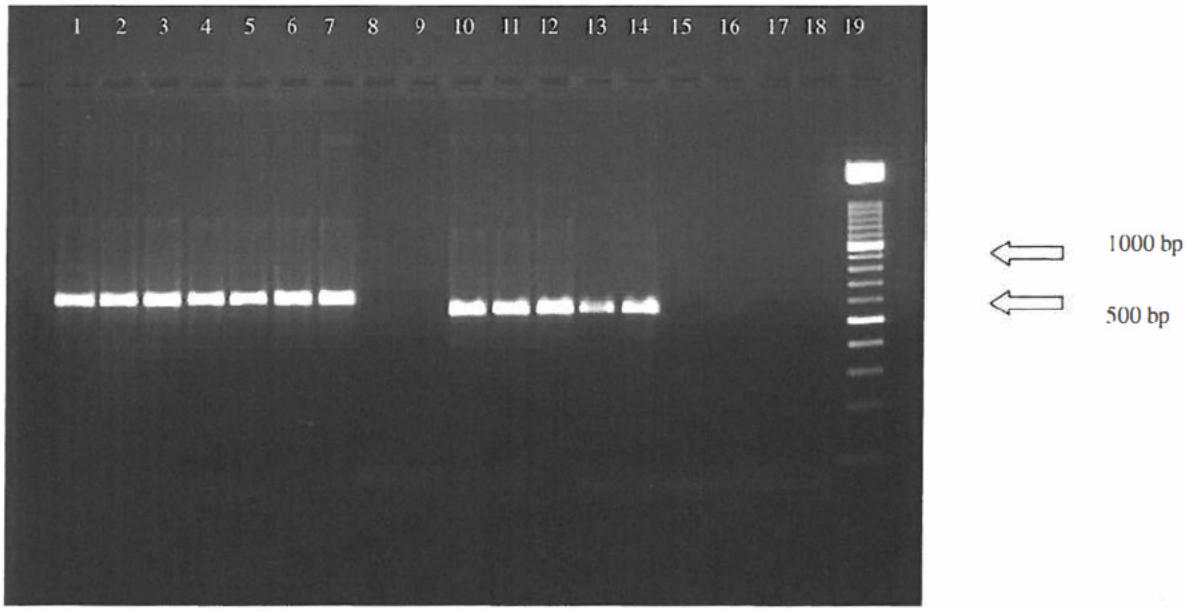


Figure 4.1: Gel picture showing the amplified *rpoB* gene (lanes 1-7) using primers *rpoB*.F and *rpoB*.R and the amplified *katG* gene (lanes 10-14) using primers *katG*315.F and *katG*315.R. PCR product sizes for the *rpoB* gene are 601 bp and for the *katG* gene are 542 bp. Lanes 8

and 15 show negative controls. Lane 19 shows the molecular weight marker (Marker XIV (100 bp ladder), Roche Diagnostics).

Table 4.1 A comparison between susceptibility profiles and sequencing results for samples used in the optimization of the assay

Isolate No	Susceptiblilty profile		Sequencing Results	
	Isoniazid	Rifampicin	*katG* (Isoniazid)	*rpoB* (Rifampicin)
R376	R	R	Ser315Thr	-
R377	R	R	no mutation	Leu533Leu
R380	R	R	Ser315Thr	Ile572Phe
R383	S	S	-	-
R387	R	R	Ser315Thr	Ser531Leu
R389	R	R	-	Asp516Gly Leu533Pro
KEH 65688	R	S	Ser315Thr	-
KEH 75311	R	S	Ser315Thr	-
052450 (KLM)	R	R	Ser315Thr	Ser531Leu
053338 (MTN)	R	R	Ser315Thr	Leu533Pro
052951(NOMI)	R	R	Ser315Thr	Ser531Leu
052872(RIM)	R	R	Ser315Thr	Ser531Leu
053051 (SHN)	S	R	-	Ser531Leu
051298 (TBD)	R	R	Ser315Thr	Ser531Leu
WP 68137	R	R	Ser315Thr	-

denotes that strain was not sequenced

As can be seen from the table, the sequencing results for samples that were sequenced correlated with the results as determined by susceptibility testing for all except one sample.

4.2 Allele-Specific PCR

4.2.1 Conventional PCR

As an initial step to developing the ASP assay, primer pairs for the *katG* gene were tested under the same conditions as those outlined in the study carried out by Wang *et al* (2005) with the exception of the initial annealing temperature being at 60^0C, due to a slightly higher T_m of the primers. The primer pairs used were katG.315.acc and katG.CP.rev for detection of the Ser315Thr mutation and katG.315.wt with katG.CP.rev for detecting the wild type sequence. PCR reactions were set up using genomic DNA from strains with or without the Ser315Thr mutation. No amplification was seen at this temperature so further experiments were carried out at annealing temperatures of 58^0C and 55^0C. Amplification was detected at the lower annealing temperature, but was inefficient as only faint bands were detected with gel electrophoresis.

In order to improve the efficacy of the PCR, optimization of the dNTP and magnesium concentrations was carried out as explained in the methods section. Briefly, in each set of experiments, the dNTP concentrations and $MgCl_2$ concentration were altered and the efficacy of the PCR was determined using agarose gel electrophoresis. According to these experiments the optimal concentration of dNTPs was found to be 100μM and the concentration of $MgCl_2$, 1.5 mM.

Although improved amplification occurred using the altered parameters as described above, the PCR was not specific as both the WT as well as the mutant primers were amplifying all samples, irrespective of whether the samples had mutations or not. In order to increase the specificity of the primers the cycling parameters used were altered, and it was found that the mutant primer worked as it amplified only the samples with the Ser315Thr mutations.

However the wild-type primer still amplified with genomic DNA from H37Rv as well as DNA harboring the Ser315Thr mutation.

A similar set of experiments was carried out using the *rpoB* primers that were designed as described in the material and methods section. In these experiments primer rpoB.531.ttg.rev was used in conjunction with primer rpoB.CP for the Ser531Leu and primer rpoB.531.wt.tail.rev with rpoB.CP for the wild type. Various annealing temperatures were tested between 65^0C and 55^0C, and the lower temperature of 55^0C was found to be the optimum (Figure 4.2) and gave adequate amplification when combined with the magnesium and dNTPs concentrations utilized for the *katG* gene. However the specificity of the *rpoB* primers for the mutation was excellent but the WT primers still amplified the samples with the mutation.

This lack of specificity of both wild type primer sets could have been due to a number of factors. Contamination of the PCR reactions could be ruled out because negative controls consistently showed no amplification. An alternative explanation is that patients with drug resistant TB have been shown to harbor mixed populations of strains [119] which would result in templates for both primer sets coexisting in a single sample. To overcome this problem it was therefore decided to adopt a quantitative PCR methodology.

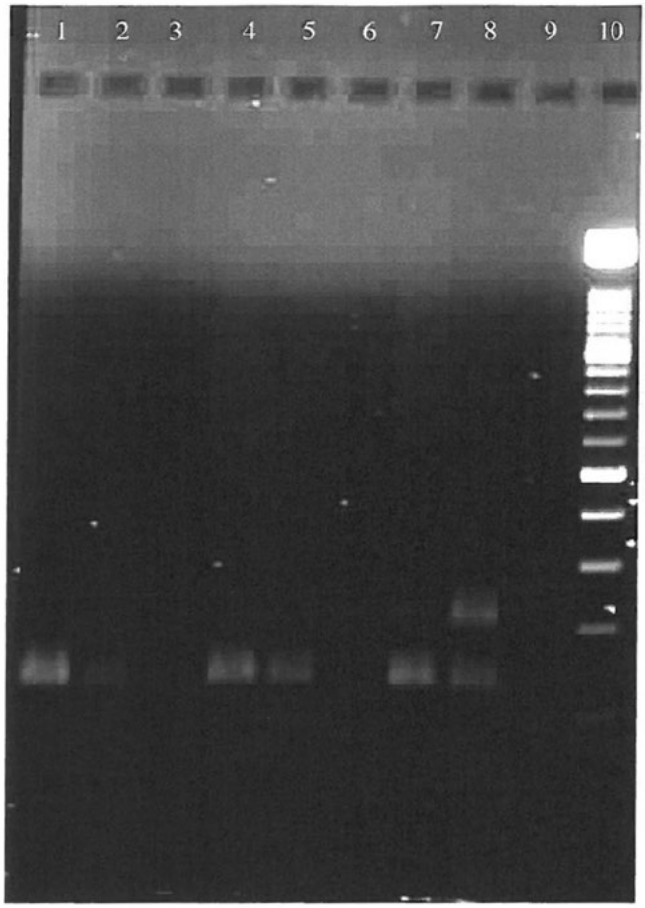


Figure 4.2: Optimization of the DNA concentration and annealing temperatures of the *rpoB* gene ASP assay using primers *rpoB*.CP and *rpoB*.531.wt.tail.rev.

Lanes 1, 4 & 7 show the products obtained using a 1:10 dilution of H37Rv DNA while lanes 2, 5 & 8 show the products obtained using a 1:100 dilution of H37Rv DNA. Lanes 1 & 2 show PCR products obtained at an annealing temperature of 58^0C, lanes 4 & 5 show PCR products obtained at an annealing temperature of 55^0C and lanes 7 & 8 show PCR products obtained at an annealing temperature of 65^0C. Lanes 3, 6 & 9 show the negative controls. Lane 10 shows the marker (Marker XIV (100 bp ladder, Roche Diagnostics).

4.2.2 Quantitative PCR using SYBR Green

The primer pairs' *katG*.315.acc and *katG*.CP.rev and *katG*.315.wt and *katG*.CP.rev were trialed in a quantitative PCR system using the ABI 7000 Sequence Detection System (Applied Biosystems) in conjunction with SYBR green. Conditions and reagent concentrations used were the same as those used in the conventional PCRs with the exception of the addition of a ROX reference dye and SYBR green. Although SYBR green is the most economical choice for RT-PCR product detection, it lacks specificity as its fluorescence is based on non-specific binding to any dsDNA

However, the results from these experiments were similar to those with the conventional PCR. The WT primers showed amplification for both the wild type sample as well as the mutant sample while the mutant primers amplified only samples that contained the mutation These results were evident both from the amplification plot produced by the ABI 7000 SDS as well as when these products were run on a 1.5% agarose gel (Figure 4.3 and Figure 4.4).

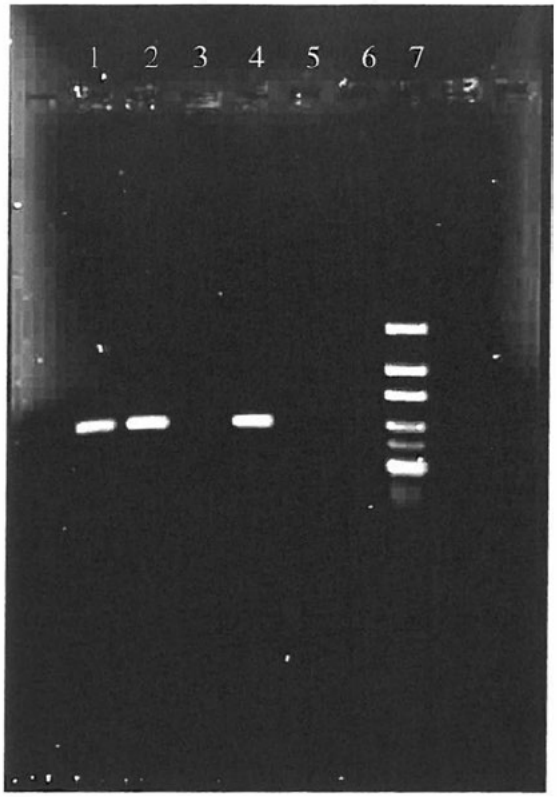
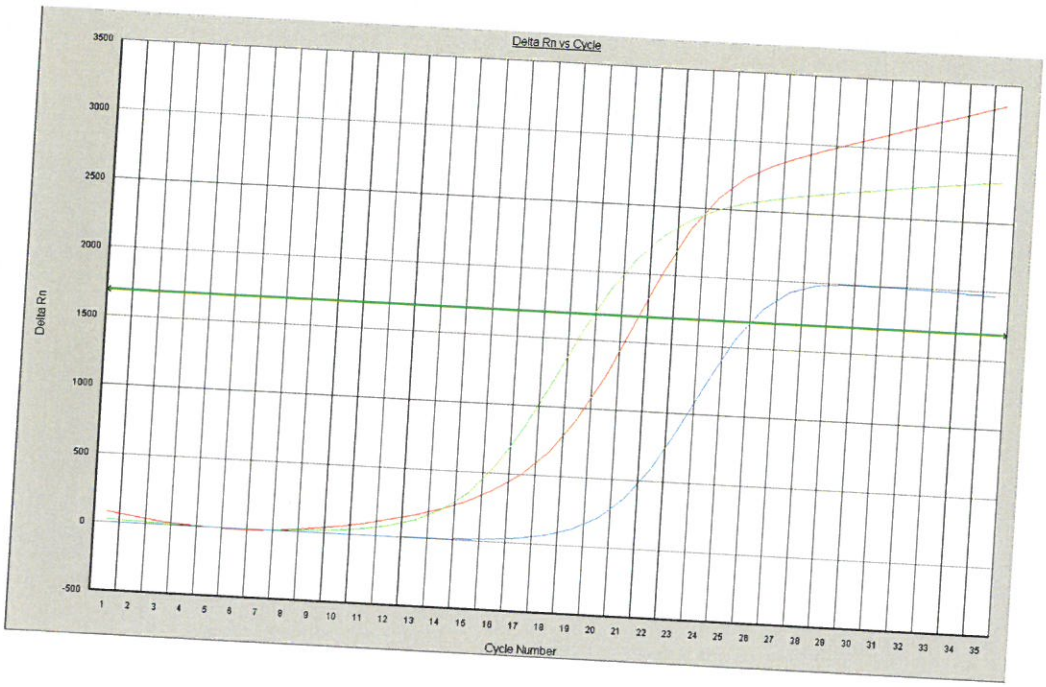


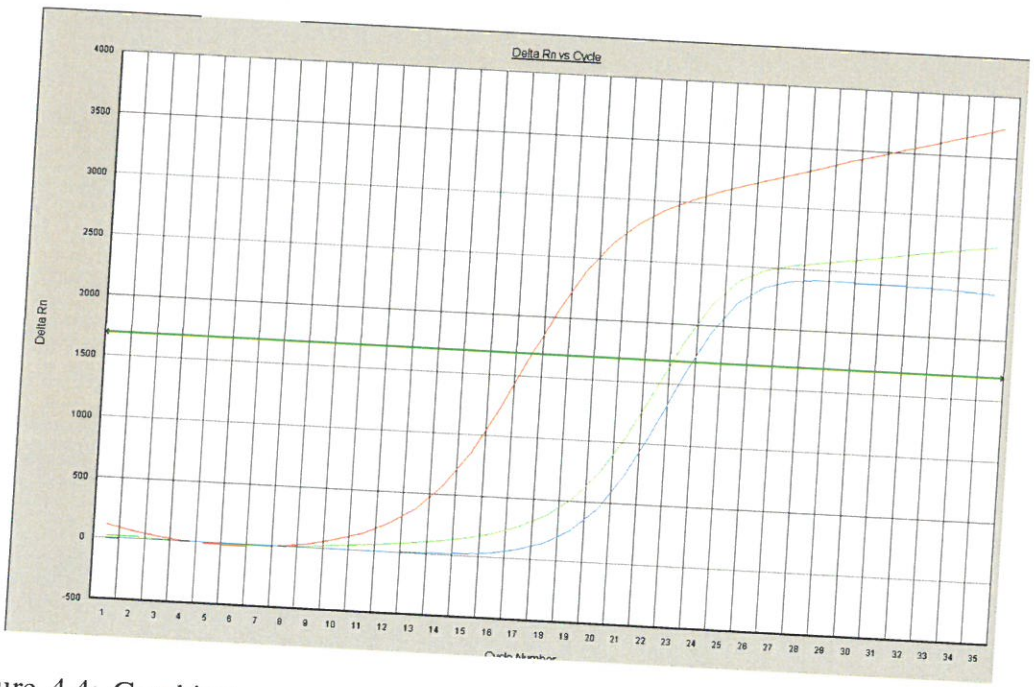
Figure 4.3: Results of an allele specific RT-PCR using SYBR green and primers for the *katG* gene, codon 315. Lanes 1-2 shows products obtained using primers *katG*.315.wt & *katG*.CP.rev. Lanes 4-5 shows products obtained using primers *katG*.315.ACC & *katG*.CP.rev. Lanes 3 & 6 shows the negative controls. Lane 7 shows the molecular weight marker (GeneRuler DNA Ladder, low range). Lanes 1 & 4 show products obtained using a template harboring the Ser315Thr mutation (R376). Lanes 2 & 5 show products obtained using a wild type template (R377).

A)



Sample no	Ct Value
R376 (mut)	21.16 (red graph)
R377 (WT)	19.44 (green graph)
NTC	25.56 (blue graph)

B)



Sample no	Ct Value
R376	17.11 (red graph)
R377	22.60 (green graph)
NTC	23.38 (blue graph)

Figure 4.4: Graphical representation of changes in fluorescent signal with increasing cycle number using allele-specific PCR targeting the Ser315Thr mutation.

A) Amplification plot obtained using primers *katG*.315.wt and *katG*.CP.rev targeting the wild type sequence of *katG* codon 315. The red graph was obtained using a template harboring wild type sequence and green graph obtained with a Ser315Thr template. The blue line

corresponds to the negative control. B) Amplification plot obtained using primers *katG.315*.acc and *katG*.CP.rev targeting the Ser315Thr mutation in the *katG* codon. The red graph was obtained using a template harboring the mutant sequence and green graph obtained with a wild type template. The x axis indicates the number of cycles of the PCR and the y axis is the Delta Rn.

A comparison of figure 4.3 and figure 4.4 shows that for the same experiment, the negative control does not show any contamination when run on the gel but amplification is seen on the Delta Rn vs cycle curve from the ABI Prism 7000 SDS (blue curve). A possible explanation for this is that due to the lack of specificity of SYBR green, DNA other than the PCR product of interest was being detected at low levels by the ABI Prism 7000 SDS. However for the primers detecting the mutant sequence there was no difference between the negative control and amplification plot for the wild type template, but the plot for the mutant template was well separated with a threshold value 6 cycles earlier. In contrast the plots for the wild type primers indicated that there was no difference in amplification found with the wild type and mutant templates, again indicating poor specificity of this primer pair.

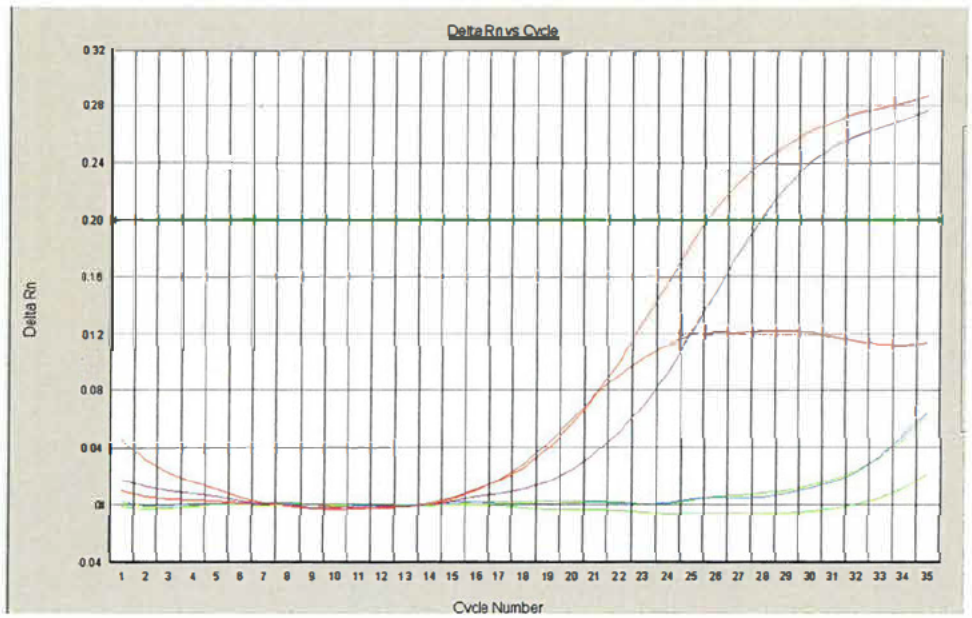
Further optimization was therefore carried out, first by completely changing the cycling parameters. Further changes were made to the annealing conditions (for stages 2-6 the annealing temperature was dropped by one degree at each stage beginning at a temperature of 69^0C and ending at a temperature of 65^0C), which still resulted in good specificity for the mutant probes which only amplified from the Ser315Thr samples but the WT primers amplified the DNA irrespective of whether the mutation was present or not.

Since there was no improvement in the specificity of the PCR, it was therefore decided to increase specificity by using modified primers called hairpin primers for the detection of SNPs.

4.3 Hairpin Primers

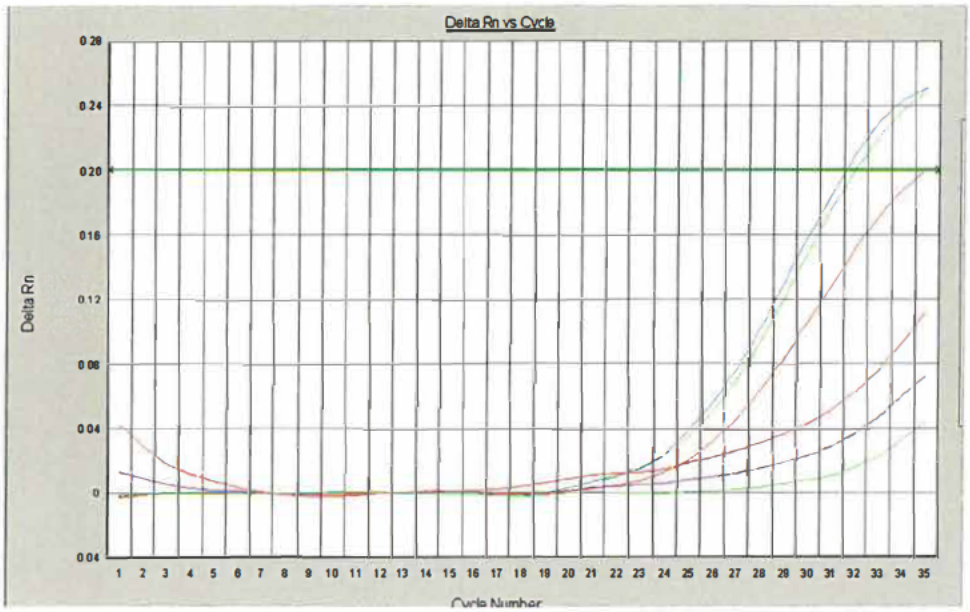
A RT-PCR assay was attempted using hairpin primers to identify the AGC→ACC mutation in codon 315 of the *katG* gene. Hairpin primers have the addition of a tail to the 5'-end of the primer, such that a stem-loop structure is produced (see methods section), and this can improve the SNP detecting ability of the SNP-detecting primer [92]. Samples, whose sequencing results were known, were used and the reactions carried out in the ABI Prism 7000 SDS using standard conditions. Figure 4.5 shows the result of an experiment using the hairpin primers for wild type and Ser315Thr in separate simplex reactions. This experiment worked well and easily identified samples that had the relevant mutation or the wild type sequence.

A)



Sample no	Ct value
KLM (mut)	-
NOMI (mut)	-
RIM (mut)	-
R383 (WT)	27.89 (purple graph)
TF 2065 (WT)	25.62 (red graph)
NTC	-

B)



Sample no	Ct value
KLM (mut)	34.92 (red graph)
NOMI (mut)	31.96 (green graph)
RIM (mut)	31.58 (blue graph)
R383 (WT)	-
TF 2065 (WT)	-
NTC	-

Figure 4.5: Amplification profiles on the ABI Prism 7000 SDS, for five samples using hairpin primers. A) Results using primers HP KatGS315T WT and HP_KatGS315T C targeting the wild type sequence. B) Results using primer HP KatGS315T MUT and HP KatGS315T C targeting the Ser315Thr mutation

Primers whose sequences were exactly the same as that of the hairpin primers, with the exception of the addition of a 5' tail complementary to the 3' end were also designed, resulting in the production of a linear primer. Experiments using these linear primers were then run in parallel with those using the hairpin primers, in order to determine whether the addition of the "tail" was critical for the functioning of the primers. These experiments (data not shown) found the hairpin primers to be more specific as they accurately identified whether a sample had a mutation or not in more of the samples than the linear primers did. The hairpin primers also in general showed lower Ct values than the linear primers.

Further optimization was attempted by changing the concentration of the primers in the assay. All reagent concentrations remained the same, only the primer concentrations were altered over a range of 2μM to 0.25μM. The optimal primer concentration was found to be 0.5μM.

However despite these promising results when the assay was tried on a larger number of samples the results were inconsistent and not reproducible. One possibility for this assay not working was the fact that the fluorogenic dye used, was SYBR green. Due to its lack of specificity, it binds to any dsDNA including primer dimers and other non-specific reaction products, thus any dsDNA present in the tube would have been detected. If specificity was difficult to achieve on genomic DNA extracted from culture it was unlikely that the assay would work directly on sputum. This prompted the change in methodology from ASP-PCR to a RT-PCR using TaqMan probes, which are innately more specific because fluorescence is contingent on the specific binding of a probe.

4.4 TaqMan Assay

4.4.1 Development of Assay

Initial development of the assay was using TaqMan probes and primers from published literature [118]. The assay was first attempted using H37Rv DNA. The assay was first run separately, using two master mixes, one for the WT probe and one for the mutant probe.

An experiment was carried out to determine the optimal DNA concentration for this assay. H37Rv was used exactly as it was extracted, then a 1:10 dilution was used and finally a 1:100 dilution was used. The 'neat' DNA showed no amplification. A possible reason for this is that the DNA contained inhibitors. The ten fold dilution made no significant impact on the results of the RT-PCR assay. It was therefore decided to use a 1:100 dilution for the assay.

4.4.2 Optimization of TaqMan assay for Ser315Thr and Ser531Leu mutations

These initial experiments used the same conditions as outlined in the study by Espasa *et al* (2005) [118]. However when the assay was carried out on genomic DNA from 15 isolates that were used in the development phase of the assay, a Ct value difference of just 1 was observed. To increase the discriminatory power of the probes, the annealing temperature was increased to 65^0C since the probes were longer (22 bp) than the maximum length recommended by Applied Biosystems (20 bp). The concentration of the probe was also reduced from 0.2 μM to 0.1 μM. Results, using these conditions were shown to be a lot more favorable when compared to results produced by sequencing (Table 4.2). For the *katG* gene probes, all but 1 sample showed a correlation between the TaqMan results and sequencing results. One possible cause for this discrepancy between these two results is that of a mixed infection, in

which the sample contains two strains with different susceptibilities or one strain with both resistant and susceptible genotypes.

Table 4.2: A comparison of the results of DNA sequencing with a TaqMan assay using *rpoB* probes for the Ser531Leu mutation and wild type *rpoB* sequence, and *katG* probes for Ser315Thr mutation and wild type *katG* sequence.

Sample no	***rpoB* probe Ct value**		***rpoB* TaqMan Assay Result**	***rpoB* gene mutation (sequencing)**	***katG* probe Ct value**		***katG* TaqMan Assay Result**	***katG* gene mutation (sequencing)**
	Wt	**Mut**			**Wt**	**Mut**		
R376	21.53	-	Wt	ND	23.76	19.35	Mut	Ser315Thr
R377	23.51	-	Wt	Leu533Leu	23.25	34.25	Wt	no mutation
R380	22.15	-	Wt	Ile572Phe	23.77	19.17	Mut	Ser315Thr
R383	22.43	-	Wt	^{S}ND	22.37	30.67	Wt	^{S}ND
R387	-	15.44	Mut	Ser531Leu	24.34	20.29	Mut	Ser315Thr
R389	22.12	-	Wt	Asp516Gly Leu533Pro	24.41	22.48	Mut	^{R}ND
KEH 65688	21.86	32.79	Wt	^{S}ND	28.85	23.54	Mut	Ser315Thr
KEH 75311	21.66	27.80	Wt	^{S}ND	24.35	21.11	Mut	Ser315Thr
KLM 052450	-	19.47	Mut	Ser531Leu	25.27	22.87	Mut	Ser315Thr
MTN 053338	26.44	-	Wt	Leu533Pro	29.20	-	Wt	no mutation
NOMI 052951	20.83	-	Wt	Ser531Leu	24.96	22.52	Mut	Ser315Thr
RIM 052872	-	18.18	Mut	Ser531Leu	24.96	22.52	Mut	Ser315Thr
SHN 053051	-	17.17	Mut	Ser531Leu	22.80	32.62	Wt	^{S}ND
TBD 05128	-	18.39	Mut	Ser531Leu	27.47	24.95	Mut	Ser315Thr
WP 68137	-	21.65	Mut	Ser531Leu	27.32	32.72	Wt	Ser315Thr

^{R}ND corresponds to samples not sent for sequencing that were resistant on DST and ^{S}ND to samples not sent for sequencing where DST found them to be susceptible. Mut corresponds to mutation detected by TaqMan assay and WT to wild-type detected by the assay. Samples whose TaqMan assay results did not correlate with sequencing results are shown in red.

Although the probes used for the *rpoB* assay were slightly shorter than the probes used in the *katG* assay, the GC content of the probes were quite similar. Therefore an annealing temperature of 65^0C was also used in this assay. All other reagent concentrations and cycling conditions were also the same as those used in the *katG* assay

For the 15 isolates mentioned previously, all except for 1 isolate showed a correlation between the TaqMan assay and sequencing results. The sample was shown to be susceptible by the TaqMan assay but resistant by sequencing. The same result was seen when the assay was repeated.

At this point a multiplex PCR (using both probes in one master mix) was also carried out. Experiments were run in parallel using the same samples in a uniplex reaction and a multiplex reaction. Although the multiplex reactions worked, results from these experiments showed that the Ct values obtained in the multiplex reaction were a lot higher than those obtained in uniplex reactions and the difference in Ct values were a lot smaller too. It was therefore decided to run uniplex reactions instead of multiplex reactions.

4.4.3 Evaluation of TaqMan Assay on genomic DNA

Genomic DNA was obtained from 24 more isolates whose susceptibility profiles were available. Of these samples, 10 were susceptible samples. For the *katG* gene, the remaining 14 samples were sent to Inqaba biotech for sequencing. These samples were then used to further assess the TaqMan assay (Table 4.3). Once these samples were run on the TaqMan assay, all samples from which a result could be obtained correlated with sequencing results. 4 samples showed inconclusive results as no amplification occurred using either probe. A repeat of these samples showed the same results. A possible explanation for these results could be that there were PCR inhibitors present in the samples.

For the same 24 isolates, for the *rpoB* gene, sequencing results for 21 isolates were available from a previous study. The remaining 3 isolates could not be sequenced as no DNA was available. Susceptibility results were however, available for all 24 isolates. According to the TaqMan assay 2 samples did not give expected results, and further 3 samples gave indeterminate results.

Table 4.3: Results from culture samples using the *rpoB* and *katG* TaqMan assay and sequencing results

Sample no	*rpoB* probe Ct values		*rpoB* TaqMan assay Result	*rpoB* gene results (sequencing)	*katG* probe Ct values		*katG* TaqMan assay Result	*katG* gene results (sequencing)
	Wt	Mut			Wt	Mut		
R01	35.67	26.06	Mut	Ser531Leu	-	34.57	Mut	Ser315Thr
R02	44.20	16.01	Mut	Ser531Leu	-	20.72	Mut	Ser315Thr
R18	34.80	41.50	Wt	^{R}ND	-	38.19	Mut	Ser315Thr
R23	-	21.18	Mut	no mutation	-	31.69	Mut	Ser315Thr
R24	35.08	-	Wt	^{R}ND	44.43	-	Wt	no mutation
R26	22.87	-	Wt	no mutation	-	21.23	Mut	Ser315Thr
R32	-	21.93	Mut	Ser531Leu	-	27.98	Mut	Ser315Thr
R43	30.84	-	Wt	no mutation	-	31.99	Mut	Ser315Thr
R47	28.40	49.55	Wt	no mutation	-	28.87	Mut	Ser315Thr
R54	-	-		no mutation	*	*		no mutation
R62	-	-		^{R}ND	*	*		no mutation
R79	24.01	24.48	Wt	no mutation	31.36	33.23	Wt	no mutation
R103	30.48	33.61	Wt	no mutation	46.12	46.12	Wt	no mutation
R104	39.79	39.85	Wt	no mutation	*	*		no mutation
R125	37.32	46.46	Wt	no mutation	58.17	-	Wt	no mutation
R126	22.03	29.73	Wt	no mutation	29.38	34.23	Wt	no mutation
R151	23.56	37.74	Wt	no mutation	31.00	35.44	Wt	no mutation
R152	23.17	32.99	Wt	no mutation	28.97	34.99	Wt	no mutation
R226	-	31.51	Mut	Ser531Leu	-	46.00	Mut	Ser315Thr
R300	29.53	18.26	Mut	Ser531Leu	-	25.61	Mut	Ser315Thr
R375	-	-		^{S}ND	*	*		Ser315Thr
R443	-	15.85	Mut	S531L Ser531Leu	-	22.05	Mut	Ser315Thr
R503	26.38	31.96	Wt	^{S}ND	-	26.86	Mut	Ser315Thr
R513	44.28	28.32	Mut	^{S}ND	53.89	56.58	Wt	no mutation

* denotes samples who did not amplify despite 3 attempts with each sample.

^{S}ND and ^{R}ND denote samples which were not sent for sequencing or were no more DNA was available. ^{S}ND corresponds to susceptible by DST and ^{R}ND corresponds to resistant. Wt denotes that no mutation was detected by the assay; Mut denotes that the Ser315Thr mutation was detected and no mutation denotes that sequencing results showed no mutation present in

codon concerned (531 or 315). Samples whose TaqMan assay results did not correlate with sequencing results are shown in red.

These promising results encouraged the attempt of a multiplex TaqMan assay. However, this assay did not produce satisfactory results as the Ct values obtained with both the WT and mutant probes were very similar. It was therefore decided to do each assay (one with the wild-type probe and one with the mutant probe) separately.

4.4.4 TaqMan assay using DNA extracted from slides

The ability to detect resistance mutations in material extracted from a slide prepared for routine AFB microscopy would be an extremely useful surveillance tool. To determine if the TaqMan assay could be employed in this way, DNA was extracted from 20 slides by the method described by van der Zanden A.G.M. *et al* (2003) [30] and used as a template in the TaqMan assay. All the slides had been found to be smear positive. However, no amplification occurred in any of the samples. It was initially thought that there was not enough of DNA present in the sample, so a spiking experiment (with 14.64 ng/µl H37Rv DNA) was carried out on some of the DNA samples. Amplification did occur in two of the four samples when these spiked DNA samples were used as a DNA template in the TaqMan assay. This spiking experiment suggested that the failure of amplification was not only due to the presence of PCR inhibitors in the DNA and that the concentration of DNA extracted, was not high enough to be amplified by the ABI Prism 7000 SDS.

4.4.5 TaqMan assay on DNA from Sputum samples using different extraction methods

The assay was then attempted using DNA from sputum samples. Eight decontaminated sputum samples were subjected to DNA extraction using both the silica method and the Chelex method. The samples were split into two 500 µl aliquots and the respective extraction methods performed.

Prior to conducting a RT-PCR on these samples, their concentrations were first obtained using the Nandrop ND 1000 (table 4.4)

The *rpoB* gene of all these samples were then amplified using conventional PCR and the products run on a gel. No products were detected from the samples extracted using the silica method, but four out of eight, although of differing band intensity, were detected from the samples extracted using the Chelex method. The DNA extracted using the Chelex method was then amplified by RT-PCR using the TaqMan assay and amplification did occur as a fluorescent signal was seen. Reagent and cycling conditions were the same as those used in the assay on culture isolates.

Table 4.4: Nanodrop results for DNA extracted from sputum samples using the Silica and Chelex methods.

Isolate no	Chelex	Silica
	Concentration (ng/µl)	Concentration (ng/µl)
5052	10.55	8.31
5053	12.65	21.50
5072	23.87	40.46
5073	36.87	32.71
5115	16.65	58.16
5116	37.85	14.69
5132	37.76	11.17
5133	18.03	7.66

The third method that was evaluated, the Genotype® MTBDR*plus* DNA Extraction also did not show any amplification with the TaqMan assay when tried on 6 different sputum samples This is a very crude DNA extraction method, which has been optimized specifically for the Genotype® MTBDR*plus* assay where no inhibitor removal was required. It was therefore decided to use the Chelex method to extract DNA from all clinical isolates used in the study.

4.4.6 Evaluation of TaqMan assay on DNA extracted from sputum samples using the Chelex DNA extraction method

A collection of 140 sputum samples (which had already been decontaminated using the NALC/NAOH method) were obtained from Mrs Jenny Allen from the Department of Microbiology at the Nelson R Mandela School of Medicine. These samples were collected from TB suspects in the Department of Health (DOH) Drug resistance surveillance study carried out by the Medical Research Council (MRC). Susceptibility results and smear results for these samples were thus readily available. DNA was then extracted from these samples using the Chelex method. The DNA was then stored at -20^0C until needed.

Of the 140 samples, 120 of were then run using the optimized TaqMan assay for both the Ser315Thr mutation and the Ser531Leu mutation. 15 of these samples were AFB smear positive by auramine staining, and 32 of these samples were culture positive and DST results revealed 17 of the 32 were MDR and 2 were INH monoresistant. For each gene, 2 assays were run, one containing the WT probe and the other containing the mutant probe. The threshold value for these samples was set at 0.2 (Appendix 8)

For the assays run on 87 samples that were culture negative 0/87 (0%), 1/87 (1.15%), and 3/87 (3.44%) and 1/87 (1.15%) of the *rpoB* WT, *rpoB* Mut, *katG* WT and *katG* Mut and gave positive results respectively. These may correspond to false positive results although it is possible that they could represent true TB infection that failed to grow in culture.

Amongst the 33 culture positive samples there were 12 samples that were smear positive, 18 smear negative and three with unknown result because of the culture being contaminated. 9 of these gave a result with the *rpoB* probes of which 8 were smear positive and one of unknown

smear status. Of the 9 samples, 8 were MDR and 1 was isoniazid monoresistant. Results of the *rpoB* assay on the INH monoresistant strain were correct in that it was negative. Amongst the 8 MDR strains five were positive for the Ser531Leu mutation and three were wild-type, which gives an allele frequency of 62.5% amongst the MDR, similar to the results with the sequencing.

For the *katG* assay, 9 out of the 33 culture positive samples gave results. Six samples of these 9 were smear positive and 1 sample was smear negative. Furthermore, 2 samples gave results for both probes and both these samples were also smear positive. There were 5 MDR samples, 1 isoniazid monoresistant and 1 susceptible sample amongst the culture positive samples which gave results. The isoniazid monoresistant sample was also correctly identified by the assay. Amongst the 5 MDR samples, 2 or 40% had the Ser315Thr mutation which is slightly lower than the allele frequency as determined by sequencing.

Table 4.5: Results of the RT-PCR assay and DST.

	DST Results					
		RIF resistant	RIF sensitive	INH resistant	INH sensitive	Culture Negative
TaqMan assay Results	*rpoB* Ser531Leu	6/19 (31.58%)	0/22			1/99 (1.01%)
	rpoB WT	3/19 (15.79%)	5/22 (22.73%)			0/99 (0%)
	katG Ser315Thr			3/21 (14.29%)	0/20 (0%)	1/99 (1.01%)
	katG WT			5/21 (23.81%)	7/20 (35.0%)	4/99 (4.04%)

For each of the probes the number of positive tests over the total number of tests done is shown for the samples with the different susceptibility profiles. Percentages are the percent of postive results for each category of drug susceptibility.

Table 4.6: Results of the RT PCR assay and DST (smear positive samples)

	DST Results					
		RIF resistant	RIF sensitive	INH resistant	INH sensitive	Culture Negative
TaqMan assay Results	*rpoB* Ser531Leu	5/9 (55.56%)	0/6			0/3 (0%)
	rpoB WT	3/9 (33.33%)	3/6 (50.0%)			0/3 (0%)
	katG Ser315Thr			3/11 (27.27%)	0/4 (0%)	0/3 (0%)
	katG WT			6/11 (54.54%)	1/4 (25%)	0/3 (0%)

For each of the probes the number of positive tests over the total number of tests done is shown for the samples with the different susceptibility profiles. Percentages are the percent of postive results for each category of drug susceptibility.

An additional 20 samples were also tested using a multiplex assay for evaluation. Of these, 12 were culture negative, and 8 were culture positive but no amplification was seen with any of the samples (Appendix 8)

The results produced showed that the *rpoB* Ser531Leu assay was specific, with only 1 of 99 culture negative samples testing positive. However only 31.58% of rifampicin resistant samples were accurately detected, indicating low sensitivity. This sensitivy did improve to 55.56% on smear positive samples, an alleleic frequency compatible with the sequencing results in this study as well as previous studies. The *rpoB* WT probe faired less well as 15.79% of drug resistant samples tested gave a positive result. Interestingly in the case of the *katG* probes the resistant probe also outperformed the sensitive probe. The probe to detect the Ser315Thr mutation was highly specific, but the wild type probe still gave positive results in 35% of INH susceptible samples. It is unlikely that simple contamination could account for these discrepancies because there were very few positives results in the culture negative category, so it is more likely due to either mixed infection or binding of the probe to both resistant and susceptible sequences. In an attempt to improve the performance of the *katG* probe it was decided to redesign the probe as its length was 22 nucleotides which is 2 longer than the maximum recommended by Applied Biosystems.

These probes were taken from a study by Wada T. *et al* (2004) and were shorter than the probes used previously in the study. The assay required no optimization as it was run under the same conditions as those outlined by the investigators of that study.

The probes were first tested on 6 genomic DNA samples whose sequencing results were available. The probes identified 3 samples as being wild type and 3 samples as having the Ser315Thr mutation which corresponded with sequencing results. Further work will be required to determine if this probe could improve on the performance of the assay.

4.5 Genotype® MTBDR*plus* assay

The aims of this thesis were to develop a high throughput tool for drug resistance surveillance. During the period of the optimization of the assay a commercially available Genotype® MTBDR*plus* assay became available for testing. This has been previously been shown to work well as a diagnostic test for MDR-TB [14], but has yet to be evaluated for use in surveillance. The results of this assay are interpreted by examining the banding patterns that form on each strip. These banding patterns are as a result of the hybridization of the single-stranded, biotin-labelled amplicons, following a denaturation step, to membrane bound probes. Either the omission of a wild type band or the appearance of bands of DNA signals representing specific mutations indicates the existence of a resistant strain [65].

To evaluate the Genotype® MTBDR*plus* assay as a tool for surveillance 459 samples that were collected as part of a surveillance study for MDR-TB in KwaZulu-Natal were subjected to the Genotype® MTBDR*plus* assay. The results of these are presented below in table 4.7.

Table 4.7: Results for the Genotype® MTBDR*plus* assay

Genotype® MTBDR*plus* result		**Number**
Rifampicin Result	**Isoniazid Result**	
Resistant	Resistant	18
Resistant	Susceptible	6
Susceptible	Susceptible	71
Susceptible	Resistant	5
Indeterminate	Susceptible	4
Resistant	Indeterminate	1
Indeterminate	Indeterminate	354
Total		459

88 of the 459 samples were found to be positive by auramine staining. Of these 88 smear positive samples, 23 gave an indeterminate result for both rifampicin and isoniazid resistance. Among the samples with a valid Genotype® MTBDR*plus* result 9 were found to be MDR, 48 susceptible to both rifampicin and isoniazid, 4 RIF-monoresistant and 4 INH monoresistant. From the 459 samples *Mycobacterium tuberculosis* was isolated in 96 cases. The results of the Genotype® MTBDR*plus* assay by culture and smear results are displayed in table below.

Table 4.8: Results of the Genotype® MTBDR*plus* assay by culture and smear results

Genotype® MTBDR*plus* result		**Culture Positive**	**Smear Positive**	**Culture Negative**
Rifampicin Result	**Isoniazid Result**			
Resistant	Resistant	10	9	4
Resistant	Susceptible	4	4	0
Susceptible	Susceptible	49	48	10
Susceptible	Resistant	3	4	0
Indeterminate	Susceptible	1	0	0
Resistant	Indeterminate	0	0	0
Indeterminate	Indeterminate	29	23	122
Total		96	88	136

Full DST results were available for 36 samples all of which were found to be fully susceptible to both rifampicin and isoniazid. The Genotype® MTBDR*plus* assay results for these 36 susceptible isolates was susceptible in 24 cases, indeterminate in 8 cases, MDR in 1 case and RIF-monoresistant in 3 cases.

These results differ from previous published studies in that the indeterminate rate was high for both culture and smear positive cases, where the suspicion of TB is high. One possible explanation for this was the use of Roche Taq Polymerase, rather than the Qiagen Taq polymerase. Alternatively sample transit time from distant clinics might have led to DNA degradation.

The results of the Genotype® MTBDR*plus* assay (table 4.9) also confirm that the Ser315Thr mutation could be used for surveillance purposes since all isoniazid resistant isolates had this mutation. In the case of rifampicin resistance the Ser531Leu mutation was only found in 38.89% of the MDR strains which is less than in most previous studies and suggests other mutations may need to be targeted for surveillance purposes.

Table 4.9: Results for samples found to be positive for TB by the Genotype® MTBDR*plus* assay

	Mutation (as determined by Genotype® MTBDR*plus* assay)	
	Ser531Leu	**Ser315Thr**
MDR-TB	7/18 (38.89%)	18/18 (100%)
INH monoresistant		5/5(100%)
RIF monoresistant	1/6 (16.67%)	
Total	8/24 (33.33%)	23/23(100%)

Chapter Five

5. Discussion

5.1 Allele Specific PCR

Allele specific PCR has been demonstrated to be a technique capable of detecting point mutations in *Mycobacterium tuberculosis* when used on DNA extracted from cultured bacilli [71] [18, 19]. It is also a low cost method. However for the experiments run for the purposes of this study, it has also been shown that considerable optimization is required to ensure that the assay works in the particular setting that one is working in. Assays run using the same reagent concentrations and cycling parameters as those outlined in these studies did not produce optimal results as expected. Furthermore all problems that were encountered occurred in assays that were run on genomic DNA only. It is important to note that more challenges will inevitably arise before the assay can be successfully run on crude DNA samples extracted from sputum. Thus, although the assay showed promise, the extensive optimization required for it to be applicable, suggested this type of assay might not be robust enough for use on clinical specimens.

5.2 Hairpin Assay

Hairpin assays have also been proven to be a successful way of improving the specificity of allele specific PCR [92]. Due to their unusual shape and the addition of a 'tail' to the 3' end of the primer, the primers need to be very carefully designed. Results presented in this study confirmed that these primers can be used successfully in allele-specific PCR and could be incorporated into an allele detection assay using differences in T_m. However given the difficulties in optimization that were encountered using this assay with SYBR green it was

thought that it was not appropriate for the detection of mutations in sputum samples. One possibility would be to use a different fluorescent DNA binding reagent as has been successfully used by another group [120] but it remains to be seen if this type of mutation detection system can be successfully employed on sputum.

5.3 TaqMan Assay

As has been shown in previous studies both the *rpoB* and *katG* probes worked well on genomic DNA. The two mutations targeted in this study are very common in South Africa. In Kwa Zulu Natal, 97.5% of resistance-conferring mutations in the *katG* gene are Ser315Thr mutations [121], while for the *rpoB* gene, in a study carried out in Cape Town, the Ser531Leu mutation accounts for 76% of these mutations [14]. These mutations are therefore ideal for surveillance purposes. Ideally, a surveillance method should be high throughput method that can work on sputum specimens.

5.3.1 DNA Extraction on Sputum samples

The DNA extraction should be easy to perform, should not be too time consuming so as to allow a large number of samples to be completed in a day and deliver clean DNA on which a PCR can be performed. It should also be able to extract sufficient template from smear negative patients. For the purposes of carrying out both a conventional and RT PCR assay, many methods of DNA extraction for *Mycobacterium tuberculosis* have thus far been carried out. These include a TE boil extraction, an extraction using SDS and 10% Triton X, PrepMan extraction, Infection Diagnostics, Inc. (IDI) lysis extraction, Silica method and the Qiagen QIAmp DNA mini kit [122]. Problems encountered with these methods include the cost

effectiveness of the methods, the labor intensity as well as the time taken and the presence of inhibitory factors that may have been extracted along with the DNA. With regard to PCR inhibitors, a solution to this problem may be the use of magnetic particle-based technologies. Studies done thus far [123, 124]}[125] have indicated that magnetic bead DNA extraction is a reliable, simple, sensitive and safe method that removes any PCR amplification/enzyme inhibitors that might create problems for the following detection.

For the purposes of this assay, as mentioned previously, three methods of DNA extraction were carried out. Of these, the Chelex method did give the best results but the method is still labor intensive and do not produce a very high yield of DNA. Work therefore needs to be done in a possible optimization of the Chelex method so as to reduce the time involved. Ways also needs to be found to improve the yield of DNA obtained. In this way, it is possible for the Chelex method to become a high throughput method. Another factor that could possibly be further looked into is the further optimization of the PCR. A more sensitive PCR would require a smaller volume of DNA. In this way it would possible to start off with a smaller volume of sputum deposit at the beginning of the extraction procedure. This would allow the extraction of DNA in a 96-well format using a multi-pipette. This would reduce the time, labor and reagents involved by processing a large number of samples at once.

The Genotype® MTBDR*plus* assay was also found to be a very simple extraction procedure. However, it is a crude extraction method and was not found to work with the PCRs used in this study. It may be possible to co optimize the extraction and the TaqMan assay in such a way that both techniques can be used together and on a smaller volume of sputum. It should be noted that the Genotype® MTBDR*plus* assay does not yet perform well on smear negative samples.

5.3.2 *katG* PCR Optimization

The sensitivity for any type of PCR is important. For the real-time assay, the probes initially used were from published literature yet still not produce satisfactory results. It is thus important to pay careful attention to the guidelines for probe and primer design as set out by the real-time machine that one is using. If design specifications such as melting temperatures, the GC content of the probe and the length of the probe are not strictly adhered to, then problems will arise.

The other factor that may also be considered is that it may be necessary to work out the exact constituents of the master mix supplied by Applied Biosystems. This may enable one to design primers that are more sensitive, depending on the components of the master mix. This would allow a more sensitive PCR that uses a smaller volume of DNA and would also reduce the cost of the assay

5.3.3 Sensitivity and Specificity

Ideally a test should have a high sensitivity and specificity. However for a surveillance tool the sensitivity and specificity do not necessarily need to be as exacting as for a diagnostic tool. The aim is to be able to monitor drug resistance levels over time and across regions. If the assay performs consistently then valid trends and comparisons can be made. For further assay developed, a few areas need to be worked on or improved on, in order to increase the sensitivity and specificity of the assay. These include: 1) Improving the DNA extraction method used, as mentioned previously, and searching for other DNA extraction methods that will improve the yield of DNA obtained; 2) The possibility of using a nested PCR in order to increase the target for the probe; 3) Further optimization of cycling conditions or a deviation

from default conditions; 4) The use of a custom made master mix using modified primers. This has been clearly done with success with the GenoType ® MTBDR*plus* assay since although a crude DNA extraction method is employed, a successful PCR reaction can still be carried out; 5) For the samples used in this study, half of the sputum sample was cultured and the other half was used for the assay. Once the assay is proven to be working properly, and culture is not required for surveillance purposes, more sputum will be available for the assay. This will significantly improve the yield of DNA obtained and 6) it should be taken into consideration that smear positive samples gave better results than smear negative samples but the results were not high enough for the assay to be used for diagnostic purposes. It is not yet clear if MDR-TB is associated with smear status, but it may be possible to design a surveillance strategy based only on smear positive samples.

Another factor that should be taken into consideration, which has been shown in the assays run, is that a sample can show 'negative results' in spite of the sample actually being positive. Of the 140 samples run using the TaqMan assay using both *katG* probe set 1 and the *rpoB* probe set, 28 samples although shown to be positive according to culture and susceptibility testing, did not give a result using any probe used in the assay. For samples that did not produce any results, it is possible that there were low quantities of DNA present initially so that undetectable levels of DNA remained. Another possible explanation for this is DNA degradation. If the samples are not stored at the correct temperature or left out at room temperature for too long periods of time such as in transit from the clinics then DNA degradation can occur prior to sample processing.

This assay focused on mutations only in codon 315 of the *katG* gene and codon 531 of the *rpoB* gene. Besides being the most frequently encountered mutations, for the *katG* gene: 1)

mutations in this codon were clearly associated with high isoniazid minimum inhibitory concentrations (MICs) [57, 71] and 2) the mutations in this codon were found to be markers of MDR-TB and strains with these mutations are easily transmitted [57]. The results of the Genotype® MTBDR*plus* assay in this study also confirm that the Ser315Thr mutation is a good marker for surveillance in KwaZulu-Natal. For the *rpoB* gene, mutations in both codon 531 and 526 are associated with high level resistance (MIC > 64 μg/mL) [126, 127] [83] [42] [85]. However the Genotype® MTBDR*plus* assay suggested that the Ser531Thr mutation was less common than in previous studies and surveillance would therefore need to target additional mutations in KwaZulu Natal. Another important factor to note is that this mutation is not only associated with resistance to rifampicin but all other rifamycins [85]. The above aspects were considered in the design of this assay in order to minimize the need to search for not-so relevant mutations but still search for the most relevant ones.

Finally although the Genotype® MTBDR*plus* assay performed better that the RT PCR assay described in this thesis, there were still significant discrepancies when compared to DST results as well as indeterminate results in samples. In addition the need for a hybridization step and visualization of banding patterns may be a limitation to making the test genuinely high throughput. So although the RT PCR cannot yet replace the culture based phenotypic susceptibility test, it still has shown potential to become a rapid surveillance tool for MDR and potentially XDR-TB.

References

1. Corbett, E., et al., *Tuberculosis in sub-Saharan Africa: opportunities, challenges and change in the era of antiretroviral treatment.* Lancet, 2006. **367**: p. 926-937.

2. *World Health Organisation:Anti-tuberculosis drug resistance in the world: Report Number 3. The WHO/IUALTD global project on anti-tuberculosis drug resistance surveillance,.* WHO/CDS/TB/2004343, 2004. **Geneva**.

3. *World Health Organization: Anti-tuberculosis drug resistance in the world. Report no 2: Prevalance and trends.* Geneva, 2000.

4. Zager, E. and R. McNerney, *Multidrug-resistant tuberculosis.* BMC Infectious Diseases, 2008. **8:10**.

5. Heym, B., et al., *Implications of multi-drug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study.* Lancet, 1994. **344**: p. 293-298.

6. *World Health Organisation: Global tuberculosis control: Surveillance, planning, financing.* WHO/HTM/TB/2004.331. 2004. **Geneva**.

7. Gagneux, S., et al., *Impact of Bacterial Genetics on the Transmission of Isonoazid-Resistant Mycobacterium tuberculosis.* PLoS Pathogens, 2006. **2**: p.????

8. Herrera, L., et al., *Molecular Analysis of RIF-resistant Mycobacterium tuberculosis isolated in Spain (1996-2001). Description of new mutations in the rpoB gene and review of the literature.* International Journal of Antimicrobial Agents, 2003. **21**: p. 403-408.

9. Dye, C. and M.A. Espinal, *Will TB become resistant to all antibiotics?* Proc.R.Soc.Lond.B, 2001. **268**: p. 45-52.

10. Raviglione, M.C. and I.M. Smith, *XDR TB - Implications for Global Public Health.* New England Journal of Medicine, 2007. **356**: p. 656-659.

11. Gandhi, N.R., et al., *Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa.* Lancet, 2006. **368**: p. 1575-1580.

12. CDC, *Emergence of Mycobacterium with Extensive Resistance to Second-line drugs Worldwide, 2000-2004.* Morbidity and Mortality Weekly Report, 2006. **55**: p. 301-305.

13. Aziz, M. and A. Wright, *The WHO/IUATB and Lung Disease Global Project on Surveillance for Anti-TB Drug Resistance: A Model for other Infectious Diseases.* Clinical Infectious Diseases, 2005. **41**: p. 5258-5262.

14. Barnard, M., et al., *Rapid Molecular Screening for Multidrug-Resistant Tuberculosis in a High-Volume Public Health Laboratory in South Africa.* American Journal of Respiratory and Critical Care Medicine, 2008. **177**: p. 787-792.

15. Musser, J.M., *Antimicrobial agent resistance in mycobacteria: molecular genetic insights.* Clinical Microbiology Reviews, 1995. **8**: p. 496-514.

16. Rattan, A., A. Kalia, and N. Ahmad, *MDR-TB: Molecular Perspectives.* Emerging Infectious Diseases, 1998. **4**: p. 195-209.

17. McCammon, M., et al., *Detection of rpoB mutations associated with rifampicin resistance in Mycobacterium tuberculosis using Denaturing Gradient Gel Electrophoresis.* Antimicrobial Agents and Chemotherapy, 2005. **49**: p. 2200-2209.

18. Mokrousov, I., et al., *Detection of Isoniazid-Resistant Mycobacterium tuberculosis Strains by a Multiplex Allele Specific PCR Assay Targeting katG codon 315 Variation.* Journal of Clinical Microbiology, 2002. **40**: p. 2509-2512.

19. Mokrousov, I., et al., *Allele-specific rpoB PCR Assays for Detection of Rifampin-Resistant Mycobacterium tuberculosis in Sputum Smears.* Antimicrobial Agents and Chemotherapy, 2003. **47**: p. 2231-2235.

20. Hoek, K.G.P., et al., *Fluorometric Assay for Testing Rifampin Susceptibility of Mycobacterium complex.* Journal of Clinical Microbiology, 2008. **46**: p. 1369-1373.

21. Ahmad, S., E. Mokaddas, and A. Jaber, *Rapid detection of Etambutol-resistant Mycobacterium tuberculosis strains by PCR-RFLP targetting embB codons 306 and 497 and iniA codon 501 mutations.* Molecular and Cellular probes, 2004. **18**: p. 299-306.

22. Yam, W., et al., *Direct detection of Rifampicin-resistant Mycobacterium tuberculosis in respiratory specimens by PCR-DNA sequencing.* Journal of Clinical Microbiology, 2004. **42**: p. 4438-4443.

23. Cheng, X., et al., *A new multi-PCR-SSCP assay for simultaneous detection of isoniazid and rifampicin resistance in Mycobacterium tuberculosis.* Journal of Microbiological Methods, 2007. **70**: p. 301-305.

24. Ormerod, L.P., *Multidrug-resistant TB (MDR-TB): epidemiology, prevention and treatment.* British Medical Bulletin, 2005. **73&74**: p. 17-24.

25. van Soolingen, D. and *Molecular epidemiology of TB and other mycobacterial infections: main methodologies and achievements.* Journal of Internal Medicine, 2001. **249**: p. 1-26.

26. Espinal, M.A., *The global Situation of MDR-TB.* Tuberculosis, 2003. **83**: p. 44-51.

27. Gagneux, S., et al., *The Ecology of Isoniazid-Resistant Mycobacterium tuberculosis in a Human Population: Impact of Drug Resistance Mutations and Strain.* submitted, 2006.

28. Arnold, C., et al., *Single Nucleotide-based differenitiation and drug resistance detection in Mycobacterium tuberculosis from isolates or directly from sputum.* Clinical Microbial Infection, 2004. **11**: p. 122-130.

29. Caws, M., et al., *Mutations prevalent among Rifampin and Isoniazid-Resistant Mycobacterium tuberculosis Isolates from a hospital in Vietnam.* Journal of Clinical Microbiology, 2006. **44**: p. 2333-2337

30. van Der Zanden, A.G.M., et al., *Use of DNA extracts from Ziel-Neelsen-stained slides for molecular Detection of Rifampin resistance and Spoligotyping of Mycobacterium tuberculosis.* Journal of Clinical Microbiology, 2003. **41**: p. 1101-1108.

31. Palomino, J.C., *Newer diagnostics for Tuberculosis and Multidrug-resistant TB.* Current Opinion in Pulmonary Medicine, 2006. **12**: p. 172-178.

32. Grassi, M., et al., *An improved real-time PCR assay for the detection of GC-rich and low abundance templates of Mycobacterium tuberculosis.* Journal of Microbiological Methods, 2006. **64**: p. 406 410.

33. Whitney, J.B. and M.A. Wainberg, *Isoniazid, the Frontline of Resistance in Mycobacterium tuberculosis.* McGill Journal of Medicine, 2002. **6**: p. 114 123.

34. Shinnick, T.M., in *Tuberculosis.* 1996, Springer Publication: Atlanta, USA. p. 1-49.

35. Zuber, B., et al., *Direct visualization of the outer membrane of native mycobacteria and corynebacteria.* Journal of Bacteriology, 2008. **190**: p. 5672 5680.

36. Tailleux, L., et al., *DC-SIGN is the major mycobacterium tuberculosis receptor on human dendritic cells.* Journal of Experimental Medicine, 2003. **197**: p. 121 127.

37. Cole , S.T., et al., *Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence.* Nature, 1998. **393**: p. 537-544.

38. Loddenkemper, R., D. Sagebiel, and A. Brendel, *Strategies against MDR-TB.* European Respiratory Journal, 2002. **Suppl 36**: p. 66s 77s.

39. Heym, B. and S.T. Cole, *Multidrug resistance in Mycobacterium tuberculosis* International Journal of Antimicrobial Agents, 1997. **8**: p. 61-70.

40. Pablos Mendez, A., et al., *Global Surveillance for Anti-Tuberculosis-drug resistance 1994-1997.* The New England Journal of Medicine, 1998. **338**: p. 1641-1649.

41. Wada, T., et al., *Dual-Probe Assay for rapid detection of Drug-Resistant Mycobacterium tuberculosis by Real-Time PCR.* Journal of Clinical Microbiology, 2004. **42**: p. 5277 5285.

42. Riska, P.F., W.R. Jacobs, and D. Alland, *Molecular determinants of drug resistance in tuberculosis.* International Journal of Tuberculosis, 2000. **4**: p. S4-S10.

43. Yesilkaya, H., et al., *Evaluation of Molecular Beacons, TaqMan and Flourescence Resonance Energy transfer probes for detection of Antbiotic Resistance-Conferring Single Nucleotide Polymorphisms in mixed Mycobacterium tuberculosis DNA Extracts.* Journal of Clinical Microbiology, 2006. **44**: p. 3826 3829.

44. Eisenach, K.D., J.Y. Crawford, and J.H. Bates, *Repetitive DNA Sequences as Probes for Mycobacterium tuberculosis* Journal of Clinical Microbiology, 1988. **26**: p. 2240-2245.

45. Schluger, N.W., *Changing Approaches to the Diagnosis of Tuberculosis.* American Journal of Respiratory and Critical Care Medicine, 2001. **164**: p. 2020 2024.

46. Piatek, A.S., et al., *Genotypic Analysis of Mycobacterium tuberculosis in Two Distinct Populations Using Molecular Beacons: Implications for Rapid Susceptibility Testing.* Antimicrobial Agents and Chemotherapy, 2000. **44**: p. 103-110.

47. Torres, M.J., et al., *Improved real-time PCR for rapid detection of rifampin and isoniazid resistance in Mycobacterium tuberculosis clinical isolates.* Diagnostic Microbiology and Infect Disease, 2003. **45**: p. 209 212.

48. Billington, O.J., T.D. McHugh, and S.H. Gillespie, *Physiological Cost of Rifampin Resistance Induced in Vitro in Mycobacterium tuberculosis.* Antimicrobial Agents and Chemotherapy, 1999. **43**: p. 1866-1869.

49. Grace-Lin, S.Y., et al., *Rapid Detection of Isoniazid and Rifampin Resistance Mutations in Mycobacterium tuberculosis Complex from Cultures or Smear-Positive Sputa by Use of Molecular Beacons.* Journal of Clinical Microbiology, 2004. **42**: p. 4204-4208.

50. Cohen , T., M.C. Becerra, and M.B. Murray, *Isoniazid resistance and the future of Drug-Resistant Tunerculosis. Microbial Drug Resistance.* 2004. **10**: p. 280-285.

51. Cardoso, R.F., et al., *Screening and Characterisation of mutations in Isoniazid-resistant Mycobacterium tuberculosis isolates obtained in Brazil.* Antimicrobial Agents and Chemotherapy, 2004. **48.**: p. 3373 3381.

52. Bardou, F., et al., *Mechanism of INH uptake in Mycobacterium tuberculosis.* Microbiology, 1998. **144**: p. 2539-2544.

53. Slayden, R.A. and C.E. Barry, *The Genetics and Biochemistry of Isoniazid Resistance in Mycobacterium tuberculosis.* Microbes and Infection, 2000. **2**: p. 659 669.

54. Zhang, Y., et al., *The catalase peroxidase gene and Isoniazid resistance of Mycobacterium tuberculosis.* Nature, 1992. **358**: p. 591-593.

55. Wiliming, M. and K. Johnson, *Inter and Intramolecular domain interactions of the catalase-peroxidase KatG gene from Mycobacterium tuberculosis.* Federation of European Biochemical Societies, 2001. **509**: p. 272-276.

56. Cohen, T., B. Sommers, and M. Murray, *The effect of drug resistance on the fitness of Mycobacterium tuberculosis.* 2003. **3**: p. 13-21.

57. van Soolingen, D., et al., *Mutations at Amino Acid Position 315 of the katG gene are Associated with High-Level Resistamce to Isoniazid, Other Drug Resistance and Successful Transmission of Mycobacterium tuberculosis in the Netherlands.* The Journal of Infectious Diseases, 2000. **182**: p. 1788-1790.

58. Hazbón, M.H., et al., *Population genetics study of Isoniazid Resistance Mutations and Evolution of Multi-drug Resistant Mycobacterium tuberculosis.* Antimicrobial Agents and Chemotherapy, 2006. **50**: p. 2640-2649.

59. Marin, M., et al., *Rapid Detection of Multiple Rifampin and Isoniazid Mutations in Mycobacterium tuberculosis in Respiratory samples by Real-Time PCR.* Antimicrobial Agents and Chemotherapy, 2004. **48**: p. 4293-4300.

60. Haas, W.H., et al., *Molecular Analysis of katG gene Mutations in Strains of Mycobacterium tuberculosis Complex from Africa.* Antimicrobial Agents and Chemotherapy, 1997. **41**: p. 1601-1603.

61. Kiepiela, P., et al., *Genomic Mutations in the katG, inhA and aphC genes are useful for the prediction of Isoniazid resistance in Mycobacterium tuberculosis isolates from Kwazulu Natal, South Africa.* Tubercle and Lung Disease, 2000. **80**: p. 47-56.

62. Rindi, L., et al., *A real-time PCR assay for detection of Isoniazid resistance in Mycobacterium tuberculosis clinical isolates.* Journal of Microbiological Methods, 2003. **55**: p. 797-800.

63. Pym, A.S. and S. Cole, *Drug resistance and tuberculosis chemotherapy – from concept to genomics.*, in *Bacterial resistance to antimicrobials: mechanisms, genetics, medical practice, and public health*, S.A. Lewis K., Taber H. and Wax R, Editor. 2000, Marcel Dekker: New York, in press.

64. Ahmad, S. and E. Mokaddas, *Contribution of AGC to ACC and other mutations at codon 315 of the katG gene in INH-resistant Mycobacterium tuberculosis isolates from the Middle East.* International Journal of Antimicrobial Agents, 2004. **23**: p. 473-479.

65. Hillemann, D., et al., *Disequilibrium in Distribution of Resistance Mutations among Mycobacterium tuberculosis Beijing and Non-Beijing Strains isolated from patients in Germany.* Antimicrobial Agents and Chemotherapy, 2005. **49**: p. 1229-1231.

66. Saint-Joanis, B., et al., *Use of site-directed mutagenesis to probe the structure, function and isoniazid activation of the catalase-peroxidase, katG, from Mycobacterium tuberculosis.* Biochem J, 1999. **Pt 3**: p. 753-760.

67. Ramaswamy, S.V., et al., *Single nucleotide Polymorphisms in Genes Associated with Isoniazid Resistance in Mycobacterium tuberculosis.* Antimicrobial Agents and Chemotherapy, 2003. **47**: p. 1241-1250.

68. Pym, A.S., et al., *Regulation of catalase-peroxidase (katG) expression, Isoniazid sensitivity and virulence by furA of Mycobacterium tuberculosis.* Molecular Microbiology, 2001. **40**: p. 879-889.

69. Pym, A.S., B. Saint-Joanis, and S.T. Cole, *Effect of katG mutations on the virulence of Mycobacterium tuberculosis and the implication for transmission in humans.* Infection and Immunity, 2002. **70**: p. 4955-4960.

70. Heym, B., B. Saint Joanis, and S.T. Cole, *The Molecular Basis of Isoniazid Resistance in Mycobacterium tuberculosis.* Tubercle and Lung Disease, 1999. **79**: p. 267-271.

71. Marttila, H.J., et al., *A Ser315Thr Substitution in katG is predominant in Genetically Heterogonous Multidrug-Resistant Mycobacterium tuberculosis Isolates Originating from the St. Petersburg Area in Russia.* Antimicrobial Agents and Chemotherapy, 1998. **42**: p. 2443-2445.

72. Zhang, Y., D. Young, and . *Molecular Genetics of drug resistance in Mycobacterium tuberculosis.* Journal of Antimicrobial Chemotherapy, 1994. **34**: p. 313 319.

73. Kocagoz, T., Z. Saribas, and A. Alp, *Rapid determination of Rifampin Resistance in Clinical Isolates of Mycobacterium tuberculosis by Real-Time PCR.* Journal of Clinical Microbiology, 2005. **43**: p. 6015-6019.

74. Miller, L.P., J.T. Crawford, and T.M. Shinnick, *The rpoB gene of Mycobacterium tuberculosis.* Antimicrobial Agents and Chemotherapy, 1994. **38**: p. 805-811.

75. Jenkins, C., et al., *Rifampin Resistance in Tuberculosis Outbreak, London, England.* Emerging Infectious Diseases, 2005. **11**: p. 931 934.

76. El Hajj, H.H., et al., *Detection of Rifampin Resistance in Mycobacterium tuberculosis in a Single Tube with Molecular Beacons.* Journal of Clinical Microbiology, 2001. **39**: p. 4131 4137.

77. Mani, C., et al., *Mutations in the rpoB gene of MDR Mycobacterium tuberculosis Clinical Isolates from India.* Journal of Clinical Microbiology, 2001. **39**: p. 2987-2990.

78. Telenti, A., et al., *Genotypic Assessment of Isoniazid and Rifampin resistance: a blind study at reference level.* Journal of Clinical Microbiology, 1997. **35**: p. 719-723.

79. Mariam, D.H., et al., *Effect of rpoB mutations conferring rifampin resistance on fitness of Mycobacterium tuberculosis.* Antimicrobial Agents and Chemotherapy, 2004. **48**: p. 1289-1294.

80. O'neil, A.J., et al., *Molecular genetic and structural modeling studies of Staphylococcus aureus RNA polymerase and the fitness of rifampin resistance genotypes in relation to clinical prevalence.* Antimicrobial Agents and Chemotherapy, 2006. **50**: p. 298-309.

81. de Viedma, D.G., et al., *New Real-Time PCR able to detect in a single tube multiple rifampin resistance mutations and High-level Isoniazid resistance mutations in Mycobacterium tuberculosis.* Journal of Clinical Microbiology, 2002. **40**: p. 988-995.

82. Chaves, F., et al., *rpoB mutations as an epidemiologic marker in Rifampin-resistant Mycobacterium tuberculosis.* International Journal of Tuberculosis and Lung Disease, 2000. **4**: p. 765 770.

83. Telenti, A., et al., *Detection of rifampin resistance mutations in Mycobacterium tuberculosis.* Lancet, 1993. **341**: p. 647 650.

84. Varma Basil, M., et al., *Rapid Detection of Rifampin Resistance in Mycobacterium tuberculosis Isolates from India and Mexico by a Molecular Beacon Assay.* Journal of Clinical Microbiology, 2004. **42**: p. 5512 5516.

85. Williams, D.L., et al., *Contribution of rpoB mutations to Development of Rifamycin Cross-Resistance in Mycobacterium tuberculosis.* Antimicrobial Agents and Chemotherapy, 1998. **42**: p. 1853-1857.

86. Andersson, D.I. and B.R. Levin, *The biological cost of antibiotic resistance.* Current Opinion in Microbiology, 1999. **2**: p. 489-493.

87. Ramaswamy, S., J.M. Musser, and *Molecular Genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 Update.* Tubercle and Lung Disease, 1998. **79**: p. 3 29.

88. Schrag, S.J. and V. Perrot, *Reducing antibiotic resistance.* Nature, 1996. **381**: p. 120-121.

89. Bjorkman, J., J.D. Hughes, and D.I. Andersson, *Virulence of antibiotic-resistant Salmonella typhimurium.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**: p. 3949-3953.

90. Maisnier-Patin, S., et al., *Compensatory adaptation to the deleterious effect of antibiotic resistance in Salmonella typhimurium.* Molecular Microbiology, 2002. **46**: p. 355 366.

91. Landegren, U., M. Nilsson, and K. Pui-Yan, *Reading Bits of Genetic Information: Methods for Single Nucleotide Polymorphism Analysis.* Genome Research, 1998. **8**: p. 769-776.

92. Hazbón, M.H. and D. Alland, *Hairpin primers for Simplified Single Nucleotide Polymorphism Analysis of Mycobacterium tuberculosis and Other Organisms.* Journal of Clinical Microbiology, 2004. **42**: p. 1236 1242.

93. Jureen, P., et al., *Rapid detection of rifampin resistance in Mycobacterium tuberculosis by Pyrosequencing Technology.* Journal of Clinical Microbiology, 2006. **44**: p. 1925-1929.

94. Germer, S. and R. Higuchi, *Single-tube genotyping without oligonucleotide probes.* Genome Research, 1999. **9**: p. 72 78.

95. Wang, J., et al., *High-throughput SNP genotyping by single-tube PCR with Tm-shift primers.* Biotechniques, 2005. **39**: p. 885 893.

96. Sheffield, V.C., et al., *Attachment of a 40-base pair G+C-rich sequence (GC-Clamp) to genomic DNA fragments by the PCR results in Improved Detection of Single-base changes.* Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**: p. 232-236.

97. Cleary, T.J., et al., *Rapid and Specific Detection of Mycobacterium tuberculosis by using the Smart Cycler Instrument and a specific Fluorogenic Probe.* Journal of Clinical Microbiology, 2003. **41**: p. 4783 4786.

98. Yang, Z., et al., *Simultaneous Detection of Isoniazid, Rifampicin and Ethambutol Resistance of Mycobacterium tuberculosis by a single multiplex allele-specific polymerase chain reaction (PCR).* Diagnostic Microbiology and Infectious Diseases, 2005. **53**: p. 201-208.

99. Kaboev, O.K., et al., *PCR hot start using primers with the structure of Molecular Beacons (hairpin-like structure).* Nucleic Acids Research, 2000. **28**(e93 e94).

100. Valasek, M.A., J.J. Repa, and *The power of real-time PCR.* Advances in Physiology Education, 2005. **29**: p. 151-159.

101. Mackay, I.M., *Real-Time PCR in the microbiology Laboratory.* Clinical Microbial Infection, 2004. **10**: p. 190 212.

102. Wittwer, C.T., et al., *Real-Time PCR Multiplex Assays.* Methods, 2001. **25**: p. 430 442.

103. Kutyavin, I.V., et al., *Minor groove binding-DNA probes increase sequence specificity at PCR extension temperatures.* Nucleic Acids Research, 2000. **28**: p. 655 661.

104. de Viedma, D.G., *Detection of resistance in Mycobacterium tuberculosis: A Review discussing molecular approaches.* Clinical Microbiology and Infectious Diseases, 2003. **9**: p. 349 359.

105. Ruiz, M., et al., *Direct Detection of Rifampin- and Isoniazid-Resistant Mycobacterium tuberculosis in Auramine-Rhodamine-Positive Sputum Specimens by Real-Time PCR.* Journal of Clinical Microbiology, 2004. **42**: p. 1585 1589.

106. Hillemann, D., et al., *Rapid detection of Mycobacterium tuberculosis Beijing Genotype Strains by Real-Time PCR.* Journal of Clinical Microbiology, 2006. **44**: p. 302 306.

107. Sajduda, A., et al., *Molecular Characterization of Rifampin and Isoniazid-Resistant Mycobacterium tuberculosis Strains isolated in Poland.* Journal of Clinical Microbiology, 2004. **42**: p. 2425 2431.

108. Edwards, K.J., et al., *Detection of rpoB mutations in Mycobacterium tuberculosis by Biprobe Analysis.* Journal of Clinical Microbiology, 2001. **39**: p. 3350-3352.

109. Post, F.A., et al., *Genetic polymorphism in Mycobacterium tuberculosis from patients with chronic multidrug-resistant tuberculosis.* Journal of Infectious Disease, 2004. **190**: p. 99-106.

110. Kiepiela, P., et al., *Comparison of PCR-heteroduplex characterization by automated DNA sequencing and line probe assay for the detection of rifampicin resistance in Mycobacterium tuberculosis isolates fro Kwa-Zulu Natal, South Africa.* Microbial Drug resistance, 1998. **4**: p. 263-269.

111. Schilke, K., et al., *Universal pattern of rpoB mutations among multi-drug resitant isolates of Mycobacterium tuerculosis complex from Africa.* International Journal of Tuberculosis Lung Disease, 1999. **3**: p. 620-626.

112. Somerville, W., et al., *Extraction of Mycobacterium tuberculosis DNA: a Question of Containment.* Journal of Clinical Microbiology, 2005. **43**: p. 2996-2997.

113. Van Soolingen, D., et al., *DNA Fingerprinting of Mycobacterium tuberculosis.* Methods in Enzymology, 1994. **235**: p. 196-205.

114. van Der Zanden, A.G.M., et al., *Simultaneous detection and strain differentiation of Mycobacterium tuberculosis complex in paraffin wax embedded tissues and in stained microscopic preparations.* Journal of Clinical Pathology, 1998. **51**: p. 209-214.

115. Chakravorty, S. and J. Tyagi, *Novel use of Guanidium Isothiocyanate in the isolation of Mycobacterium DNA from clinical material.* Federation of European Microbiological Societies: Microbiology Letters, 2001. **205**: p. 113-117.

116. Boom, R., et al., *Rapid and Simple method for the purification of Nucleic Acids.* Journal of Clinical Microbiology, 1990. **28**: p. 495-203.

117. Bang, D., D. Andersen, and V. Thomsen, *Rapid Genotypic detection of rfiampicin and isoniazid-resistant Mycobacterium tuberculosis directly in clinical specimens.* Journal of Clinical Microbiology, 2006. **44**: p. 2605 2608.

118. Espasa, M., et al., *Direct detection in clinical samples of multiple gene mutations causing resistance of Mycobacterium tuberculosis to isoniazid and rifampicin using fluorogenic probes.* Journal of Antimicrobial Chemotherapy, 2005. **55**: p. 860-865.

119. Post, F., et al., *Genetic polymorphisms in Mycobacterium tuberculosis isolates from patients with chronic multi-drug resistant tuberculosis.* Journal of Infectious Diseases, 2004. **190**: p. 99-106.

120. Warren, R.M., et al., *Patients with active tuberculosis often have different strains in the same sputum specimen.* American Journal of Respiratory and Critical Care Medicine, 2004. **169**: p. 610-614.

121. Kiepiela, P., et al., *Genomic mutations in the katG, inhA and aphC genes are useful for the prdiction of isoniazid resistance in Mycobacterium tuberculosis from Kwazulu Natal, South Africa.* Tubercle and Lung Disease, 2000. **80**: p. 47-56.

122. Aldous, W.k., et al., *Comparison of six methods for extracting Mycobacterium tuberculosis DNA from processed sputum for testing by Quantitative real-time PCR.* Journal of Clinical Microbiology, 2005. **43**: p. 2471-2473.

123. Chui, L.W., et al., *Evaluation of four DNA extraction methods for the detection of Mycobacterium avium subsp. paratuberculosis by PCR.* Diagnostic Microbiology and Infectious Disease, 2004. **48**: p. 39-45.

124. Caldarell-Stefano, R., et al., *Use of magnetic beads for tissue DNA extraction and IS6110 Mycobacterium tuberculosis PCR.* Journal of Clinical Pathology: Molecular Pathology, 1999. **52**: p. 180-192.

125. Barken, K.M., T. Tolker-Nielsen, and J.A.J. Haagensen, *Advances in nucleic acid-based diagnostics of bacterial infections.* Clinica Chimica Acta, 2007. **384**: p. 1-11.

126. Qian, L., et al., *rpoB genotypes of Mycobacterium tuberculosis Beijing Family Isolates from East Asian Countries.* Journal of Clinical Microbiology, 2002. **40**: p. 1091 1094.

127. Shin, S., et al., *rpoB gene mutation in Clinical Isolates of MDR-TB in Northern Lima, Peru.* Microbial Drug Resistance, 2005. **11**: p. 26-30.

Appendices

Appendix 1: DNA Extraction from cultures (Somerville W *et al*; Van Soolingen D *et al*)

10 X TE buffer (10 mM Tris-HCl [ph 8.0], 1 mM EDTA)

- Dissolve 1.211 g Tris in 80 mL of triple distilled water.
- pH to 8.0 with concentrated HCl (hydrochloric acid).
- Add 0.3722 g EDTA (1M). Mix until it dissolves. Check final pH (must be 8.0).
- Make up to 100 mL in measuring cylinder. Autoclave at 121^0C for 15 minutes.

Lysozyme (10 mg/mL)

Dissolve 10 mg (0.01 g) in 1 mL triple distilled water.

Proteinase K (10 mg/mL)

Dissolve 10 mg (0.01 g) in 1 mL triple distilled water. Store at -20^0C.

10% SDS (Sodium Dodecyl Sulphate)

- Dissolve SDS in ~ 9 mL of triple distilled water.
- Heat at 65^0C for ~ 10 minutes with gentle swirling. Do not shake.
- Bring up to 10 mL in measuring cylinder. Filter sterilize before use.

SDS/Proteinase K Mix (per sample)

5 µl Proteinase K (10mg/mL)

70 µl 10% SDS

Prepare just before use.

5M NaCl

- Dissolve 14.6 g of NaCl in ~ 45 mL of triple distilled water with heating.
- Bring up to 50 mL. Autoclave solution at 121^{0}C for 15 minutes.

CTAB/NaCl

- Dissolve 4.1 g of NaCl in 80 mL of triple distilled water.
- Add 10 g of CTAB (N, N, N-trimethyl ammonium bromide).
- Heat solution at 65^{0}C until dissolved. Make up to 100 mL with triple distilled water. Do not sterilize.

Chloroform Isoamyl Alcohol

Chloroform: Isoamyl Alcohol

24: 1

Add 2 mL of isoamyl alcohol to 48 mL of chloroform and mix thoroughly.

70% Ethanol

Add 70 mL of absolute ethanol to 30 mL of triple distilled water and mix thoroughly.

Appendix 2: NALC/NaOH Sodium Citrate decontamination (TB lab SOP)

Principle of Method

N Acetyl-L-Cysteine (NALC) is a mucolytic agent that dissolves mucous material and cellular material. This allows for the release of any mycobacteria present in the sputum sample. Sodium hydroxide (NaOH) decontaminates the sample while sodium citrate is added to stabilise the NALC by chelating any heavy metal ions that may be present in the specimen.

Method

- First wipe the outside of the sputum jar with disinfectant.
- Pour the specimen into a 50 mL centrifuge tube.
- Wipe the lip of the container.
- Add an equal volume of NALC working solution.
- Vortex the mixture for 15 minutes, until liquefied.
- If required, add extra NALC solution.
- Leave the mixture to stand for 15 minutes with regular vortexing, followed by the addition of buffer to the 35 mL mark on the tube. (Buffer must be added to solution within 20 minutes as mycobacteria are killed off if exposed to NaOH for too long a period).
- Process negative and positive controls as samples.
- Centrifuge at 3000 *g* for 20 minutes.
- Decant the supernatant into concentrated phenol disinfectant.
- Resuspend the sediment by vortexing and inoculate 500 µl into a MGIT tube.
- Add 1 2 mL of 7H11 buffer to the remaining deposit and vortex.
- 500 µl of this decontaminated sputum sample was then used for the DNA extraction as described below.

Appendix 3: Susceptibility testing of *Mycobacterium tuberculosis*: Modified proportion method (TB lab SOP)

Principle of Method

In this method, dilutions of inoculums are seeded onto both a control or drug free and a drug containing media so that countable colony forming units (CFU) of 50 100 are obtained on the control media. The numbers of CFU that grow on the drug containing medium is then compared on the control media. From this the population of bacilli resistant to a given drug can be calculated and expressed as a percentage of the total population tested. For the purposes of this study, only two drugs were looked at, isoniazid and rifampicin with drug susceptibilities of 1.0 and 1.0 respectively.

Method

Only positive cultures from 7H11 agar plates and positive (mycobacterial growth indicator tube) MGIT tube (uncontaminated) are used in DST.

3A) Setting up of susceptibilities from a No 1 MacFarland

- 7H10 plates need to be brought to room temperature.
- Harvest several colonies with a sterile applicator stick.
- Inoculate into 4 mL tween buffer with beads and vortex for 1 2 minutes.
- Allow heavy particles of organisms to settle (15-20 minutes).
- Adjust supernatant to No 1 MacFarland Standard using sterile distilled water and vortex to homogenize the solution.
- Make 4 10 fold serial dilutions ($10^{-1} - 10^{-4}$).

- Use Pasteur pipettes to make the dilutions by adding 0.5 mL of the 10^{-1} dilution tube to the next tube to make a 10^{-2} dilution. Continue in this fashion until a 10-^{4}dilution is reached.
- Inoculate the 1% control quadrant with the 10^{-4} dilution with 0.1 mL (3 drops with Pateur pipette).
- Inoculate one plain plate (extra drug-free quadrant) with 10^{-3} dilution for extra growth should it be required for repeating the susceptibility.
- Inoculate all drug quadrants with 10^{-2} dilution tube by adding 0.1 ml to each drug quadrant (3 drops).
- Tilt plates gently to spread the inoculums.
- Allow to air dry in the biosafety cabinet for ~2 hours or until dry.
- Pack into CO_2 permeable plastic packets and seal.
- Incubate in the 37^0C CO_2 incubator for at least one week but not longer than two weeks.
- Read susceptibilities after 21 days of inoculation.

3B) Setting up of susceptibilities if turbidity is less than a No 1 MacFarland

- 7H10 plates need to be brought to room temperature.
- Only positive cultures from 7H11 agar plates and positive (mycobacterial growth indicator tube) MGIT tube (uncontaminated) are used in DST.
- Harvest several colonies with a sterile applicator stick.
- Inoculate into 4 mL tween buffer with beads and vortex for 1-2 minutes.
- Allow heavy particles of organisms to settle (15-20 minutes).

If, after pipetting the supernatant, the turbidity is less than a No 1 MacFarland, omit the 4th dilution. The rest of the procedure is exactly as described above.

3C) Setting up of susceptibilities from a positive MGIT culture

- If susceptibilities are to be done from positive MGIT cultures, the MGIT tubes must have been inoculated for 1-2 days after becoming instrument positive.
- The MGIT tube acts as the 10^{-1} dilution tube.
- Make 2 10-fold serial dilutions (10^{-2} – 10^{-3}).
- Use Pasteur pipettes to make the dilutions by adding 0.5 mL of the 10^{-1} dilution tube to the next tube to make a 10^{-2} dilution. Continue in this fashion until a 10-^{4}dilution is reached.
- Inoculate the 1% control quadrant with the 10^{-3} dilution with 0.1 mL (3 drops with Pasteur pipette).
- Inoculate one plain plate (extra drug free quadrant) with 10^{-2} dilution for extra growth should it be required for repeating the susceptibility.
- Inoculate all drug quadrants with 10^{-2} dilution tube by adding 0.1 ml to each drug quadrant (3 drops).
- Tilt plates gently to spread the inoculums.
- Allow to air dry in the biosafety cabinet for ~2 hours or until dry.
- Pack into CO_2 permeable plastic packets and seal.
- Incubate in the 37^{0}C CO_2 incubator for at least one week but not longer than two weeks.
- Read susceptibilities after 21 days of inoculation.

Interpretation of results

- Appropriate dilutions of inoculums are seeded onto both control and rug containing media so that countable 50 100 CFU are obtained on at least one of the control media.
- The numbers of CFU that grow on the drug containing medium is then compared wit the number on the control media.
- A resistant strain will have growth on the drug containing media equal to or more than 10% of colonies compared with the control media.
- A susceptible strain will have no growth on the drug containing media or less than 10% of colonies compared with the control media.
- An isoniazid intermediate strain will have no growth on the drug containing media (isoniazid 1.0µl/mL) but will have growth on the isoniazid 0.2µl/mL drug containing media when compared with the control media.
- Cultures of TB are usually obviously susceptible or obviously resistant and only rarely are the results ambiguous.

Appendix 4: DNA Extraction Using IRS and Chelex

The Inhibitor Removal Solution (IRS)

5M Guanidine Thiocyanate (GTC): 30 g

50nM Tris HCl (pH 7.5): 5 mL of 500 nM Tris HCl

25nM EDTA: 4.47 g

0.5% Sarcosyl (N Laurylsarcosine): 0.25 g

0.2M L-Mercaptoethanol: 0.78 mL

Add GTC, EDTA, sarcosyl and mercaptoethanol to ~ 45 mL triple distilled water. Add 5 mL of 500 mM Tris-HCl, pH 7.5 to a final volume of 50 mL.

NB: 1) IRS is stable for 2 months at room temperature if stored in an airtight container.

2) IRS is prepared in a fume hood.

3) GTC containing waste is disposed of in 10M NaOH.

Extraction Solution

Chelex: 1.2 g

Tween 20: 36 µl

Triton X 100: 3.6 µl

Mix the Chelex, Tween and Triton X 100 in triple distilled water to a final volume of 10 mL.

500 mM Tris-HCl

Dissolve 3.028 g Trizma base in 30 mL triple distilled water and then adjust to a pH of 7.5 and a final volume of 50 mL.

0.2M EDTA (Ethylene Diamine Trichloro Acetic Acid)

Dissolve 37.22 g of EDTA in 500 mL and adjust pH to 7.5 using NaOH.

Appendix 5: DNA Extraction using the Silica Method

Preparation of Acid Washed Silica: 2 day procedure (stable for 6 months at room temperature)

- Add 6 g of SiO_2 in 50 mL of triple distilled water, vortex thoroughly and allow to settle at room temperature for 24 hours.
- Remove 43 mL of liquid, fill up to 50 mL, allow to settle for 5 hours at room temperature.
- Remove 44 mL of fluid.
- Add 60 µl of 10M HCl to adjust the suspension to to pH 2.0.
- Store at room temperature in the dark.

Lysis Buffer

- Dissolve 120 g of GTC in 100 mL of 0.1M Tris HCl, pH 6.4.
- Heat to 60^0C to dissolve.
- Add 4.4 mL of 0.2M EDTA (pH 8.0).
- Add 500 µl TritonX 100.

Mix the solution by inverting.

Wash Buffer

- Dissolve 24 g GTC in 20 mL of 0.1M Tris-HCl, pH 6.4.
- Dissolve at 60^0C.

Washing Ethanol

Add 36.84 mL absolute ethanol to 0.125 mL 4M NaCl and fill up to 50 mL with triple distilled water.

Appendix 6: Gel Electrophoresis

6A. 10X TBE (Tris-Borate-EDTA) Buffer

- Measure out 108g Tris, 55g Boric Acid and 9.3g EDTA into a flask.
- Add 700 mL distilled water and dissolve at low heat.
- pH to pH 7.0 using HCl or NaOH as required
- Adjust volume to 1L with distilled water.
- Filter and store at room temperature.

Sample Loading Buffer

- Measure out 5 ml Tris-HCl, 1 ml EDTA (500 mM), 0.05g Bromophenol Blue and 300µl RNAse (10 mg/mL).
- Add distilled water to a final volume of 100 mL
- Boil for 15 min at 100°C and cool overnight.

6B. Procedure to make Agarose Gel

For 2 % Gel (20 wells)

- Measure out 1.4 g of agarose into a flask, add 70 ml 0.5XTBE (Electrophoresis buffer) and swirl.
- Place into a microwave oven, swirling occasionally until the agarose has dissolved.
- Cool to 40-50 °C and add 70 µl Ethidium Bromide

(Gloves must be worn when handling Ethidium bromide as it is mutagenic).

- Set the gel casting tray and comb as required.
- Pour the warm agarose carefully ensuring that no bubbles form and allow approximately 30 min to set at room temperature.

Procedure to run the Gel

- Remove combs from the gel
- Immerse gel into the buffer-filled (0.5X TBE buffer) tank.
- Add more 0.5X TBE buffer into the gel tank to ensure that the gel is completely covered.
- Pipette 3 µl of gel loading buffer per sample onto a piece pf parafilm.
- Add 5 µl of sample to the loading gel, mix well and add into the gel wells.
- Place the lid of the gel tank into position and ensure the electrodes are correctly connected, switch on the power supply.
- Since DNA is negatively charged, the gel runs towards the positive electrode.
- Leave the gel to run at 100 Volts for approximately 60 to 90 min.
- Remove gel from tank.
- View gel under the Vacutec Bio-Imaging system.

Appendix 7: Gel pictures showing products from RT-PCR using TaqMan probes for the *katG* gene and *rpoB* gene

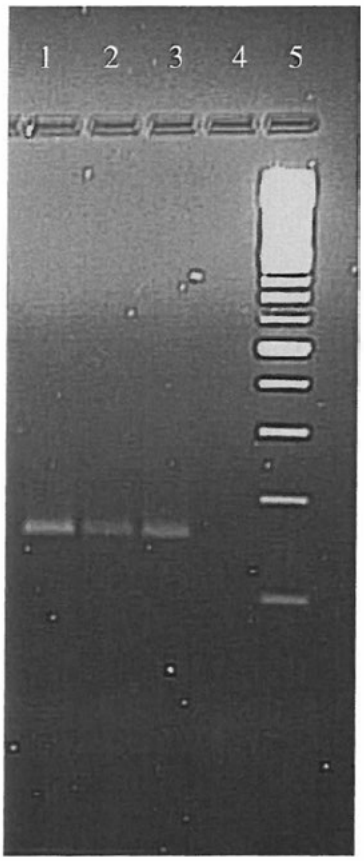


Figure 7A: Gel picture showing RT-PCR products on a gel using primers rpoB.SD.F and rpoB.SD.R which produce a product that is 160 bp long, and probes Mut_531_probe (Wada et al) and WT 531 probe. Lane 1 shows the PCR product of a sample that has the wild-type *rpoB* gene and lane 2 shows the PCR product of a sample that has the C-T mutation in codon 531 of the *rpoB* gene. Lane 3 shows the positive control (H37Rv) and lane 4 shows the negative control. Lane 5 shows the molecular weight marker (Marker XIV).

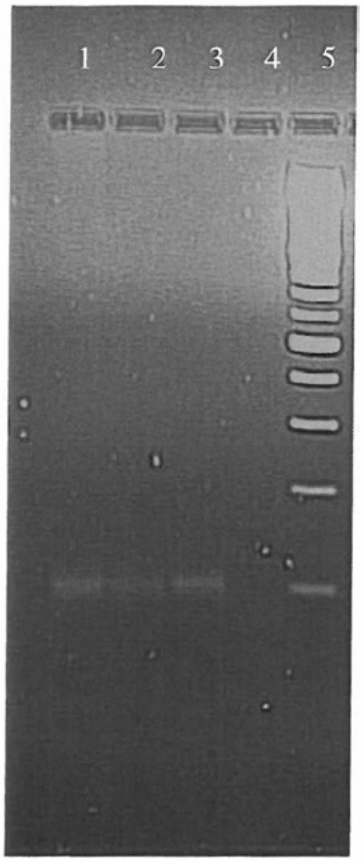


Figure 7B: Gel picture showing RT-PCR products on a gel using primers katG.SD.F and katG.SD.R which produce a product that is 103 bp long, and probes WT_315 probe (Espasa et al) and Mut 315 probe. Lane 1 shows the PCR product of a sample that has the wild-type *katG* gene and lane 2 shows the PCR product of a sample that has the G-C mutation in codon 315 of the *katG* gene. Lane 3 shows the positive control (H37Rv) and lane 4 shows the negative control. Lane 5 shows the molecular weight marker (Marker XIV).

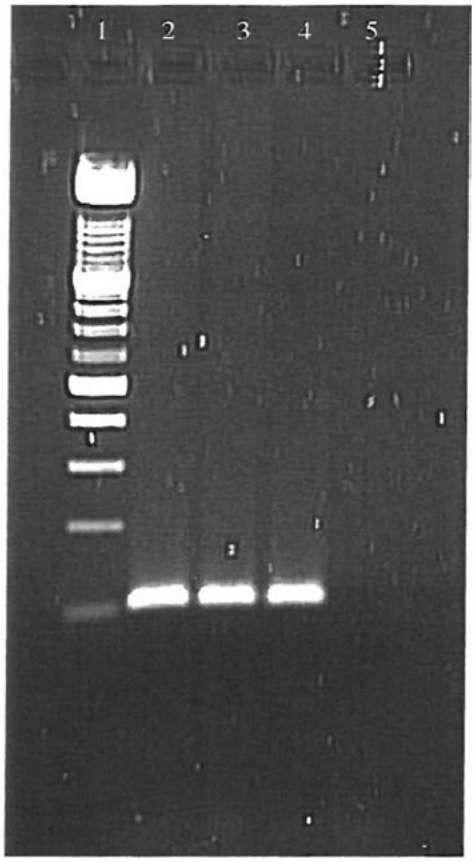


Figure 7C: Gel picture showing RT-PCR products on a gel using primers katG.SD.F and katG.SD.R which produce a product that is 103 bp long, and probes katG.315.WT (Wada et al) and katG.315.Mut. Lane 1 shows the molecular weight marker (Marker XIV). Lane 2 shows the PCR product of a sample that has the wild-type *katG* gene. Lane 3 shows the PCR product of a sample that has the G-C mutation in codon 315 of the *katG* gene. Lane 4 shows the negative control. Lane 5 shows the positive control (H37Rv).

Appendix 8: Table showing results of sputum samples analyzed using TaqMan assay

Table 8A : Table showing results of sputum samples analyzed using TaqMan assay: *rpoB* and first set of *katG* probes, smear results and susceptibility results

Laboratory No	Patient Initial	MOS No	Smear result	Susceptibilty result		*rpoB*		*katG*	
				RIF	INH	Wt	Mut	Wt	Mut
5245	SBM	MOS 1009	3+	R	R	-	38.30	37.93	-
5238	BLG	MOS 1014	NEG	R	R	-	-	-	-
5451	NL	MOS 1029	3+	R	R	-	-	-	-
5277	NN	MOS 1035	NEG	-	-	-	-	-	-
5265	HG	MOS 1036	NEG	-	-	-	-	-	-
5267	PN	MOS 1037	NEG	S	S	-	-	-	-
5259	MN	MOS 1038	-	-	-	-	-	-	-
5262	MP	MOS 1039	NEG	-	-	-	-	-	-
5260	MF	MOS 1040	NEG	-	-	-	-	-	-
5261	BM	MOS 1041	NEG	-	-	-	-	-	-
5269	MS	MOS 1043	NEG	-	-	-	-	-	-
5273	TN	MOS 1046	2+	-	-	-	-	-	-
5258	SG	MOS 1047	1+	-	-	-	-	-	-
5266	IM	MOS 1049	NEG	-	-	-	-	-	-
5257	AM	MOS 1050	NEG	-	-	-	-	-	-
5274	PM	MOS 1051	2+	-	-	-	-	-	-
5264	HM	MOS 1053	NEG	S	S	-	-	-	-
5272	DN	MOS 1054	2+	S	S	-	-	-	-
5291	MG	MOS 1055	NEG	-	-	-	-	-	-
5287	NM	MOS 1056	NEG	-	-	-	-	-	-

Laboratory No	Patient Intitials	MOS No	Smear results	Susceptibility results		*rpoB*		*katG*	
				RIF	INH	Wt	Mut	Wt	Mut
5284	TM	MOS 1057	NEG	-	-	-	-	-	-
5290	BM	MOS 1058	NEG	-	-	-	-	-	-
5282	MM	MOS 1059	NEG	-	-	-	-	-	-
5289	FG	MOS 1060	NEG	-	-	-	-	-	-
5280	LM	MOS 1061	NEG	-	-	-	-	-	-
5281	NM	MOS 1062	NEG	S	S	-	-	-	-
5288	BM	MOS 1064	NEG	-	-	-	-	-	-
5285	NM	MOS 1065	NEG	-	-	-	-	-	-
5283	MJ	MOS 1066	NEG	S	S	-	-	-	-
5342	MM	MOS 1067	NEG	-	-	-	-	-	-
5335	TN	MOS 1068	NEG	-	-	-	-	-	-
5337	DG	MOS 1069	NEG	-	-	-	-	-	-
5345	MT	MOS 1070	NEG	-	-	-	-	-	-
5339	JM	MOS 1071	NEG	-	-	-	-	-	-
5338	ME	MOS 1072	NEG	-	-	-	-	-	-
5346	SM	MOS 1073	NEG	-	-	-	-	-	-
5333	SS	MOS 1074	NEG	-	-	-	-	-	-
5341	MT	MOS 1075	NEG	-	-	-	-	-	-
5340	MN	MOS 1076	NEG	-	-	-	-	-	-
5336	MM	MOS 1077	NEG	-	-	-	-	-	-
5334	GP	MOS 1079	NEG	-	-	-	-	-	-
5344	TZ	MOS 1080	1+	S	S	-	-	-	-

Laboratory No	Patient Initials	MOS No	Smear results	Susceptibility results		*rpoB*		*katG*	
				RIF	INH	Wt	Mut	Wt	Mut
5343	MJ	MOS 1081	NEG	S	S	-	-	-	-
5371	MB	MOS 1082	NEG	-	-	-	-	-	-
5380	MN	MOS 1083	NEG	-	-	-	-	-	-
5375	MT	MOS 1084	NEG	-	-	-	-	-	-
5365	LM	MOS 1085	NEG	-	-	-	-	-	-
5373	SN	MOS 1086	NEG	-	-	-	-	-	-
5370	SS	MOS 1087	NEG	-	-	-	-	-	-
5367	NM	MOS 1088	NEG	-	-	-	-	-	-
5364	BN	MOS 1089	NEG	-	-	-	-	-	-
5374	SM	MOS 1090	NEG	-	-	-	-	-	-
5368	SN	MOS 1091	NEG	-	-	-	-	-	-
5379	LN	MOS 1092	NEG	-	-	-	-	-	-
5381	MM	MOS 1093	NEG	-	-	-	-	-	-
5361	VD	MOS 1094	NEG	-	-	-	-	-	-
5372	MT	MOS 1095	NEG	-	-	-	-	-	-
5378	MM	MOS 1096	NEG	-	-	-	-	-	-
5362	ST	MOS 1097	NEG	-	-	-	-	-	-
5366	NZ	MOS 1098	NEG	-	-	-	-	-	-
5369	VS	MOS 1099	NEG	S	S	-	-	38.15	-
5377	MT	MOS 1100	NEG	-	-	-	-	-	-
5363	NL	MOS 1101	NEG	-	-	-	-	-	-
5459	BG	MOS 1104	NEG	-	-	-	-	-	-

Laboratory No	Patient Initials	MOS No	Smear results	Susceptibility results		*rpoB*		*katG*	
				RIF	INH	Wt	Mut	Wt	Mut
5466	TN	MOS 1105	NEG	-	-	-	-	-	-
5474	SN	MOS 1106	NEG	-	-	-	-	-	-
5473	MN	MOS 1107	NEG	-	-	-	-	-	-
5462	KM	MOS 1108	NEG	-	-	-	-	-	-
5461	MR	MOS 1109	NEG	-	-	-	-	-	-
5470	MS	MOS 1111	NEG	-	-	-	-	-	-
5475	KM	MOS 1112	NEG	-	-	-	-	-	-
5472	SM	MOS 1114	NEG	-	-	-	-	-	-
5460	MT	MOS 1116	NEG	-	-	-	-	-	-
5465	NM	MOS 1117	SC	S	S	-	-	-	-
5469	KB	MOS 1119	SC	-	-	-	-	-	-
5468	AH	MOS 1120	NEG	-	-	-	-	-	-
5467	TZ	MOS 1121	NEG	-	-	-	-	-	-
5484	MS	MOS 1122	NEG	-	-	-	-	-	-
5483	MJ	MOS 1123	NEG	-	-	-	-	-	-
5488	MM	MOS 1124	NEG	S	S	-	-	-	-
5489	DD	MOS 1129	NEG	S	S	-	-	-	-
5527	NB	MOS 1130	NEG	-	-	-	-	41.35	-
5518	NI	MOS 1134	NEG	-	-	-	-	40.48	-
5587	ZD	MOS 1151	NEG	R	R	-	-	-	-
5622	RM	MOS 1161	NEG	-	-	-	-	-	-

Laboratory No	Patient Initials	MOS No	Smear results	Susceptibility results		*rpoB*		*katG*	
				RIF	INH	Wt	Mut	Wt	Mut
5623	LT	MOS 1162	NEG	S	S	-	-	-	-
5616	TM	MOS 1163	NEG	-	-	-	-	-	44.09
5620	GM	MOS 1164	NEG	S	S	-	-	-	-
5619	SN	MOS 1165	NEG	-	-	-	-	-	-
5617	MM	MOS 1166	NEG	-	-	-	-	-	-
5618	SM	MOS 1167	NEG	-	-	-	-	-	-
5704	LN	MOS 1168	NEG	-		-	-	42.95	-
5701	BM	MOS 1169	NEG	-	-	-	-	-	-
5699	PM	MOS 1170	NEG	-	-	-	-	-	-
5703	MO	MOS 1171	NEG	-	-	-	-	-	-
5705	BG	MOS 1173	NEG	-	-	-	-	-	-
5700	SN	MOS 1174	NEG	-	-	-	-	-	-
5707	ZN	MOS 1175	NEG	-	-	-	-	-	-
5698	SM	MOS 1176	NEG	-	-	-	-	-	-
5699	TS	MOS 1177	NEG	-	-	-	-	-	-
5706	ZN	MOS 1179	NEG	-	-	-	-	-	-
5702	TT	MOS 1180	2+	S	R	41.23	-	39.82	-
5702	TT	MOS 1180	2+	S	R	-	-	-	-
5719	ML	MOS 1181	NEG	-	-	-	-	-	-
5718	MS	MOS 1182	NEG	-	-	-	42.99	-	-

Laboratory No	Patient Initials	MOS No	Smear results	Susceptibility results		*rpoB*		*katG*	
				RIF	INH	Wt	Mut	Wt	Mut
5717	BP	MOS 1183	NEG	-	-	-	-	-	-
5797	NB	MOS 1196	NEG	R	R	-	-	-	-
5857	PM	MOS 1229	2+	R	R	-	33.39	40.07	-
5849	SN	MOS 1232	NEG	R	R	-	-	-	-
5850	MN	MOS 1233	3+	R	R	-	34.82	44.18	-
5879	DN	MOS 1242	NEG	R	R	-	-	-	-
5876	BN	MOS 1246	3+	R	R	29.51	-	-	-
5883	SD	MOS 1248	NEG	R	R	-	-	-	-
6010	SM	MOS 1281	?	R	R	-	-	-	-
6015	TF	MOS 1283	NEG	R	R	-	-	-	-
6076	TD	MOS 1301	2+	R	R	38.24	-	41.60	43.79
6093	KN	MOS 1307	?	R	R	-	33.52	-	-
0289	MS	MOS 1354	?	S	S	-	-	-	-
0860	NM	MOS 1376	1+	R	R	43.64	-	42.56	42.73
1999	MN	MOS 1470	1+	R	R	-	35.84	-	-

Table 8B: Table showing results of sputum samples analyzed using TaqMan assay: *rpoB* and first set of *katG* probes (multiplex PCR), smear results and susceptibility results

Laboratory No	Patient Initials	MOS No	Smear results	Susceptibility results		*rpoB*		*katG*	
				RIF	INH	Wt	**Mut**	**Wt**	**Mut**
5241	MM	MOS 1001	3+	S	S	33.16	-	-	-
5244	PG	MOS 1002	NEG	S	S	-	-	-	-
5243	JB	MOS 1003	NEG	S	S	-	-	-	-
5236	GM	MOS 1004	3+	S	S	36.50	-	38.62	-
5237	CD	MOS 1005	NEG	-	-	-	-	-	-
5240	MS	MOS 1007	NEG	S	S	-	-	-	-
5235	DS	MOS 1008	NEG	-	-	-	-	-	-
5245	SBM	MOS 1009	3+	R	R	-	30.38	-	33.08
5246	DM	MOS 1010	NEG	-	-	-	-	-	-
5247	SM	MOS 1011	NEG	-	-	-	-	-	-
5248	EM	MOS 1012	NEG	-	-	-	-	-	-
5239	MZ	MOS 1013	NEG	S	S	-	-	-	-
5238	BLG	MOS 1014	NEG	R	R	-	-	-	-
5242	ZT	MOS 1015	NEG	-	-	-	-	-	-
5234	TG	MOS 1016	NEG	-	-	-	-	-	-
5252	SM	MOS 1031	NEG	-	-	-	-	-	-
5253	PN		NEG	-	-	-	-	-	-
5256	MB	MOS 1044	NEG	-	-	-	-	-	-
5254	SN	MOS 1048	NEG	-	-	-	-	-	-
5255	AP	MOS 1052	NEG	-	-	-	-	-	-

Appendix 9: Figure showing *rpoB* gene, with position of relevant primers and probes

M. tuberculosis H37Rv|Rv0667|rpoB: 3519 bp - DNA-DIRECTED RNA POLYMERASE (BETA CHAIN) RPOB (TRANSCRIPTASE BETA CHAIN) (RNA POLYMERASE BETA SUBUNIT)

```
    1 - ttg gca gat tcc cgc cag agc aaa aca gcc

   31 - gct agt cct agt ccg agt cgc ccg caa agt

   61 - tcc tcg aat aac tcc gta ccc gga gcg cca

   91 - aac cgg gtc tcc ttc gct aag ctg cgc gaa

  121 - cca ctt gag gtt ccg gga ctc ctt gac gtc

  151 - cag acc gat tcg ttc gag tgg ctg atc ggt

  181 - tcg ccg cgc tgg cgc gaa tcc gcc gcc gag

  211 - cgg ggt gat gtc aac cca gtg ggt ggc ctg

  241 - gaa gag gtg ctc tac gag ctg tct ccg atc

  271 - gag gac ttc tcc ggg tcg atg tcg ttg tcg

  301 - ttc tct gac cct cgt ttc gac gat gtc aag

  331 - gca ccc gtc gac gag tgc aaa gac aag gac

  361 - atg acg tac gcg gct cca ctg ttc gtc acc

  391 - gcc gag ttc atc aac aac aac acc ggt gag

  421 - atc aag agt cag acg gtg ttc atg ggt gac

  451 - ttc ccg atg atg acc gag aag ggc acg ttc

  481 - atc atc aac ggg acc gag cgt gtg gtg gtc

  511 - agc cag ctg gtg cgg tcg ccc ggg gtg tac

  541 - ttc gac gag acc att gac aag tcc acc gac

  571 - aag acg ctg cac agc gtc aag gtg atc ccg

  601 - agc cgc ggc gcg tgg ctc gag ttt gac gtc

  631 - gac aag cgc gac acc gtc ggc gtg cgc atc

  661   gac cgc aaa cgc cgg caa ccg gtc acc gtg

  691 - ctg ctc aag gcg ctg ggc tgg acc agc gag

  721 - cag att gtc gag cgg ttc ggg ttc tcc gag

  751 - atc atg cga tcg acg ctg gag aag gac aac

  781 - acc gtc ggc acc gac gag gcg ctg ttg gac
```

811 - atc tac cgc aag ctg cgt ccg ggc gag ccc

841 - ccg acc aaa gag tca gcg cag acg ctg ttg

871 - gaa aac ttg ttc ttc aag gag aag cgc tac

901 - gac ctg gcc cgc gtc ggt cgc tat aag gtc

931 - aac aag aag ctc ggg ctg cat gtc ggc gag

961 - ccc atc acg tcg tcg acg ctg acc gaa gaa

991 - gac gtc gtg gcc acc atc gaa tat ctg gtc

1021 - cgc ttg cac gag ggt cag acc acg atg acc

1051 - gtt ccg ggc ggc gtc gag gtg ccg gtg gaa

1081 - acc gac gac atc gac cac ttc ggc aac cgc

1111 - cgc ctg cgt acg gtc ggc gag ctg atc caa

1141 - aac cag atc cgg gtc ggc atg tcg cgg atg

1171 - gag cgg gtg gtc cgg gag cgg atg acc acc

1201 - cag gac gtg gag gcg atc aca ccg cag acg

1231 - ttg atc aac atc cgg ccg gtg gtc gcc gcg

1261 - atc aag gag ttc ttc **ggc acc agc cag ctg**

1291 - **agc caa ttc atg gac cag aac aac ccg ctg**

1321 - **tcg ggg ttg acc cac aag cgc cga ctg tcg**

1351 - **ctg** ggg ccc ggc ggt ctg tca cgt gag

1381 - cgt gcc ggg ctg gag gtc cgc gac gtg cac

1411 - ccg tcg cac tac ggc cgg atg tgc ccg atc

1441 - gaa acc cct gag ggg ccc aac atc ggt ctg

1471 - atc ggc tcg ctg tcg gtg tac gcg cgg gtc

1501 - aac ccg ttc ggg ttc atc gaa acg ccg tac

1531 - cgc aag gtg gtc gac ggc gtg gtt agc gac

1561 - gag atc gtg tac ctg acc gcc gac gag gag

1591 - gac cgc cac gtg gtg gca cag gcc aat tcg

1621 - ccg atc gat gcg gac ggt cgc ttc gtc gag

1651 - ccg cgc gtg ctg gtc cgc cgc aag gcg ggc

1681 - gag gtg gag tac gtg ccc tcg tct gag gtg

1711 - gac tac atg gac gtc tcg ccc cgc cag atg

Targeted sequence where mutations are found from codon 507 533

→ and ← indicate position of primers used for sequencing of gene.

→ and ← indicate position of primers used in the RT PCR.

The highlighted portion shows the position of the probes used to detect the WT codon and the Ser531Leu mutation.

1741 - gtg tcg gtg gcc acc gcg atg att ccc ttc

1771 - ctg gag cac gac gac gcc aac cgt gcc ctc

1801 - atg ggg gca aac atg cag cgc cag gcg gtg

1831 - ccg ctg gtc cgt agc gag gcc ccg ctg gtg

1861 - ggc acc ggg atg gag ctg cgc gcg gcg atc

1891 - gac gcc ggc gac gtc gtc gtc gcc gaa gaa

1921 - agc ggc gtc atc gag gag gtg tcg gcc gac

1951 - tac atc act gtg atg cac gac aac ggc acc

1981 - cgg cgt acc tac cgg atg cgc aag ttt gcc

2011 - cgg tcc aac cac ggc act tgc gcc aac cag

2041 - tgc ccc atc gtg gac gcg ggc gac cga gtc

2071 - gag gcc ggt cag gtg atc gcc gac ggt ccc

2101 - tgt act gac gac ggc gag atg gcg ctg ggc

2131 - aag aac ctg ctg gtg gcc atc atg ccg tgg

2161 - gag ggc cac aac tac gag gac gcg atc atc

2191 - ctg tcc aac cgc ctg gtc gaa gag gac gtg

2221 - ctc acc tcg atc cac atc gag gag cat gag

2251 - atc gat gct cgc gac acc aag ctg ggt gcg

2281 - gag gag atc acc cgc gac atc ccg aac atc

2311 - tcc gac gag gtg ctc gcc gac ctg gat gag

2341 - cgg ggc atc gtg cgc atc ggt gcc gag gtt

2371 - cgc gac ggg gac atc ctg gtc ggc aag gtc

2401 - acc ccg aag ggt gag acc gag ctg acg ccg

2431 - gag gag cgg ctg ctg cgt gcc atc ttc ggt

2461 - gag aag gcc cgc gag gtg cgc gac act tcg

2491 - ctg aag gtg ccg cac ggc gaa tcc ggc aag

2521 - gtg atc ggc att cgg gtg ttt tcc cgc gag

2551 - gac gag gac gag ttg ccg gcc ggt gtc aac

2581 - gag ctg gtg cgt gtg tat gtg gct cag aaa

2611 - cgc aag atc tcc gac ggt gac aag ctg gcc

2641 - ggc cgg cac ggc aac aag ggc gtg atc ggc

Appendix 10: Figure of the *katG* gene, with position of relevant primers and probes

M. tuberculosis H37Rv|Rv1908c|katG: 2223 bp - CATALASE-PEROXIDASE-PEROXYNITRITASE T KATG

```
   1 - gtg ccc gag caa cac cca ccc att aca gaa
  31 - acc acc acc gga gcc gct agc aac ggc tgt
  61 - ccc gtc gtg ggt cat atg aaa tac ccc gtc
  91   gag ggc ggc gga aac cag gac tgg tgg ccc
 121 - aac cgg ctc aat ctg aag gta ctg cac caa
 151 - aac ccg gcc gtc gct gac ccg atg ggt gcg
 181 - gcg ttc gac tat gcc gcg gag gtc gcg acc
 211 - atc gac gtt gac gcc ctg acg cgg gac atc
 241 - gag gaa gtg atg acc acc tcg cag ccg tgg
 271 - tgg ccc gcc gac tac ggc cac tac ggg ccg
 301 - ctg ttt atc cgg atg gcg tgg cac gct gcc
 331 - ggc acc tac cgc atc cac gac ggc cgc ggc
 361 - ggc gcc ggg ggc ggc atg cag cgg ttc gcg
 391 - ccg ctt aac agc tgg ccc gac aac gcc agc
 421 - ttg gac aag gcg cgc cgg ctg ctg tgg ccg
 451 - gtc aag aag aag tac ggc aag aag ctc tca
 481 - tgg gcg gac ctg att gtt ttc gcc ggc aac
 511 - tgc gcg ctg gaa tcg atg ggc ttc aag acg
 541 - ttc ggg ttc ggc ttc ggc cgg gtc gac cag
 571 - tgg gag ccc gat gag gtc tat tgg ggc aag
 601 - gaa gcc acc tgg ctc ggc gat gag cgt tac
 631 - agc ggt aag cgg gat ctg gag aac ccg ctg
 661 - gcc gcg gtg cag atg ggg ctg atc tac gtg
 691 - aac ccg gag ggg ccg aac ggc aac ccg gac
 721 - ccc atg gcc gcg gcg gtc gac att cgc gag
 751 - acg ttt cgg cgc atg gcc atg aac gac gtc
 781 - gaa aca gcg gcg ctg atc gtc ggc ggt cac
```

1741 - gtg gaa tcc ttt gcc gtg ctg gag ccc aag

1771 - gca gat ggc ttc cga aac tac ctc gga aag

1801 - ggc aac ccg ttg ccg gcc gag tac atg ctg

1831 - ctc gac aag gcg aac ctg ctt acg ctc agt

1861 - gcc cct gag atg acg gtg ctg gta ggt ggc

1891 - ctg cgc gtc ctc ggc gca aac tac aag cgc

1921 - tta ccg ctg ggc gtg ttc acc gag gcc tcc

1951 - gag tca ctg acc aac gac ttc ttc gtg aac

1981 - ctg ctc gac atg ggt atc acc tgg gag ccc

2011 - tcg cca gca gat gac ggg acc tac cag ggc

2041 - aag gat ggc agt ggc aag gtg aag tgg acc

2071 - ggc agc cgc gtg gac ctg gtc ttc ggg tcc

2101 - aac tcg gag ttg cgg gcg ctt gtc gag gtc

2131 - tat ggc gcc gat gac gcg cag ccg aag ttc

2161 - gtg cag gac ttc gtc gct gcc tgg gac aag

2191 - gtg atg aac ctc gac agg ttc gac gtg cgc

2221 - tga

Appendix 11: Showing sequencing results

Appendix 11A 1: *katG* samples sent for DNA Sequencing

R376

CGGGGGAGCGCCCTGGCCCGCCCAACGGGTCCGGGATGGTGCCCGGCACCGGCGCCGTCC
TTGGCGGTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTAC
AGGATCTCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATG
CCGGTGGTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCC
ATCTGCTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCA
TGGGTCTTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATG
GCCATGCGCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCG
TTCGGCCCCTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCC
AGATCCCGCTTACCGCTGTAACGCTCATTCGGCGCCAA

R377

CGTGGGAGCGACCCTGGCCCGCCCTATACGGGTCCGGGATGGTGCCGGCACCGGCGCCGT
CCTTGGCGGTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGT
ACAGGATCTCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGA
TGCCGCTGGTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGC
CCATCTGCTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGC
CATGGGTCTTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCA
TGGCCATGCGCCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGGCCGGGTTG
CCGTTCGGCCCCTCCGGGTTCACGTAAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTT
CTCCAGATGCCGATTACCGCTGTAACTGTTATCTCAAAAAAGGTATTCTCTTGCTTTCTG
TTTTTTTTCGGTGTTTGTGTGTTTTTGTTTTATTTGTGTCTATTACGGGACGAAGAGCAT
ATGTTGTGTGTGTGGTGTGTGTGTGTTGTGTGTAGCGTGTGGGCGTCCTGCTAGAGTC
AGGAGCAACTTTTTTATTTTTAATTATTTTTTGTTATGTCACTTTTTTTTTGTTTGTGTT
GTTTTATTGCGTCCAGATTTTGCGCATCGATCCGTTTCTGTCTTCCTGTGTTTTGTGTGT
GGATTTTTTTGTTTTGAGGGATAAGTGTCTTCAGAACCGC

R380

GACGCCCTGGCCCGCCGAAACGGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCG
GTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATC
TCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTG
GTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGC
TCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTC
TTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATG
CGCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGC
CCCTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCC
CGCTTACCGCTGTAACGCTCATTCGCACGAAGCGGCGGCCTCCGTCGCTAGGCGGGGTGG
CGGCGGGCTCGTGGGTGGGTTCTTTCTCCCCGCCGCACGGCGTGGCGGGTCGGGGCACTG
GGGACGACGGGGCGAATAAAAAGATCGTGCCGGCCGTCGCTGTCGCTGCCTGATTGACTG
ATCCAATTTACCACACACCCGCGCTCCCCAGCGCCGCTGATGCGCTGCCTGCGTGGTGGC
GTCTGATTGTTGTGTGTGGTGCTGTCTCGGGACGCGCGCCGAAGCCGCCGGCGGCGCGGC
GTTTGATGTGTGTGTGTTGCGCGGCCAGTCA

R387

TCGTGGCAGCGACCCGTGGCCCGCCGCAACGGGTCCGGGATGGTGCCGGCACCGGCGCCG
TCCTTGGCGGTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCG
TACAGGATCTCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCG
ATGCCGGTGGTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAG
CCCATCTGCTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCG
CCATGGGTCTTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTC
ATGGCCATGCGCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTG
CCGTTCGGCCCCTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTC
TCCAGATCCCGCTTACCGCTGTAACGCTCATCCGCCGAA

KEH65688

GAGCGCCCTGGCCCGCCGCAACGGGTCCGGGGATGGTGCCGGCACCGGCGCCGTCCTTGG
CGGTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGA
TCTCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGG
TGGTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCT
GCTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGG
TCTTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCA
TGCGCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCG
GCCCCTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGAT
CCCGCTTACCGCTGTAACGCTCATCCGACGAACCCTGCCGGGGCGGCCGCTCCGGTTACG
CCTCGCCCCGTCGTGTTGGGGGTTGGCCGGTGCTTCGCGGGGCGGGCGGGAGGCGGCGCC
GCCACCCCGCCCGCACACACCCCCGCGCACTAGCGTATGTTTCTTGTTTTGTGTGGTGGT
GGTGGGGCGGGAGAGAGTGGGGAGGGCTCGTGTGTAATAGATGCTAGATGCCGCGCCCAG
GCCACGCGACGCTGACATTGTGCGCTGCCCTGTCCCCGCCCGCACCCCGTCCTGCTAGCC
TAC

KEH75311

AACGCCCTGGCCCGCCGAACGGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCGG
TGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCT
CGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGG
TGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCT
CCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCT
TACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGC
GCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCC
CCTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCC
GCTTACCGCTGTAACGCTCATCCGACGAA

KLM 05240

GACGCCCTGGCCCGCCGAAGGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCGGT
GTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTC
GAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGT
GATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTC
CAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTT
ACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCG
CCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCC
CTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCG
CTTACCGCTGTAACGCTCATCCGACGAA

MTN 053338

GGAAGCGCCCTGGCCCGCCGAAACGGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTG
GCGGTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGG
ATCTCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCT
CTGGTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATC
TGCTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGG
GTCTTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCC
ATGCGCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTC
GGCCCCTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGA
TCCCGCTTACCGCTGTAACGCTCATTCGACGAAACGCCGGGGTGTTGTTCTTCCTTCCGC
CCCCCCGCCCTTTCCCCGTTCTTTTCCTCCGGCGCGGCGCGCGCGGGGATGCCGCTGCTC
TTTCCAGGCCGTGCGCGCGTGGGGTGGGGGCGCACCCACCCCGCCGTGCCGCCGACCGCC
GCGGCGTCCGCCCCTACAGCCCCTCTTCACTTCGCGTTCTTGTATTATGTCGTGCCGACC
TGCGCCCCCCGCCCCTGCCACCGCCGCCACCACACAGACACGCCGTCGCGCGCCCCACGT
C

NOMI 052951

GCCCTGGCCCGCCGAAACGGGTCCGGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCGGT
GTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTC
GAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGT
GATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTC
CAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTT
ACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCG
CCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCC
CTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCG
CTTACCGCTGTAACGCTCATTAAACGAAGGGCTCCCGGCCGCCCCCCCCCCCCCCCCGC
CCCCGGTCCGTCGGCCCGCGCCGCGGCCCGCCTTCGTGCCCGTCCTGCTCCCCCTCTGCC
TGCTGCCGTTTCTCTCGGGCCCGTCCCCCCACCACCACTTCCCGGTCTGTCTCTCGACGC
CTGCGTGCGTCTTATGTGGACGCACGCGGCGCGCTCCCTCGCCGCCTCGCGCGCCTGGCT
GGTGCGTGGCGCGCGCGCGAGCGTTGCCTGCTGCTGTGTTTGGTGTTCTCTCTGTTTCAG
TTTTCCCTTCCACATC

RIM 052872

GTAGCGCCCTGGCCCGCCGAACGGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGC
GGTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGAT
CTCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGT
GGTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTG
CTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGT
CTTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCAT
GCGCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGG
CCCCTCCGGGTTCACGTAAATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATC
CCGCTTACCGCTGTAACGCTCATTCCGCCGA

SHN 053051

TAGCGCCCTGGACCGCACGAAACGTGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGG
CGGTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGA

TCTCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGC
TGGTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCT
GCTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGG
TCTTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCA
TGCGCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCG
GCCCCTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGAT
CCCGCTTACCGCTGTAACGCTCATCCAACCGAA

TBD 05128

GGAGCGCCCTGGCCCGCCGCAAGGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGC
GGTGTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGAT
CTCGAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGT
GGTGATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTG
CTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGT
CTTACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCAT
GCGCCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGG
CCCCTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATC
CCGCTTACCGCTGTAACGCTCATTCGACCGAA

WP 68137

CGCCCTGGCCCGCCGCAACGGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCGGT
GTATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTC
GAGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGT
GATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTC
CAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTT
ACCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCG
CCGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCC
CTCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCG
CTTACCGCTGTAACGCTCATCCGACGAACCTTTTTTTTGGGTCGCCCGCGCTGGCCGCGT
GTGTGTTGTTGTGGTGGGTCCCCCCGCCGCAGCCCCGCGCGCCGCGACCATGCCACGCCC
CGCCAGACCGAGCCCGCCCGCCGCGGGGTGGGGGCGTCGTAGGCACGGTCTGATGCAGTA
AAGACAAACACGCACAAACAAACAACATGGTCTTGTTCTTCTTTCTGCTGTGGGGGGGGG
CGGTGTGTCGGTCGGTTGTGTTGATGGGCTACAGCACCCCTGCCGCCCGCGCGCCCACT

R01

CCGCCAGAACTGTCGTTCCGGGATGGTGCCGGCTCACCGGCGCCGTCCTTGGCGGTGTAT
TGCCACAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAG
GAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGAT
CGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAG
CGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACC
GAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCG
AAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTC
CGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCGCTT
ACCGCTGTAACGCTCATTCGCCGAAAATTTTTTATTCCTCCTTTTATTTTGTCCGGCGCT
GTGGCCCCCCACCACTCACCTCCCTACACCCTCTCCCAACACTCACAACACATCATGCCG
CCGTGCGTTTGTCCTGATCTGATGTATTGTATTTTTATTGTATACATACTCTCTATACTC
TATTCTATTATACTATATATAAATTCCTAACAACCCACACCTCTCAGCTCTCTCTTCTCA
TTTATTCTCCTCACTCCAATCTCCCCTCTCCCTTTGTGCGTTACTGTTCTCTTTTACATC
TCATATACAACATCAGTCAATAATAGTATATACTTTCCTTTTTTCTACTCCATTTCTCCT
ACAACTGACATCTCTACCCACTACAGACTCACATCCAAACACTACCCTACATCACTACAT
CACTTAGTTTCAGC

R02

CGCCGTAACGCTGTCCGGGCATGGTGCCGGGCACCGGCGCCGTCCGTTGGCGGTGTATTG
CCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAA
ACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGC
GTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGG
AGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAA
AGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAA
CGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTCCGG
GTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCGCTTACC
GCTGTAACGCTCATTCGCCGAAAGGGTGTTTGGTGAGATGGGTTTGTTGGTGGGGGGGGG
GGGCGCTGTTGAGTATAGTCATACGCGAGATATCTAGAGTGTGTATGCGGGGTGTGTTCT
GTTTGGTTTGTGTTTGGGTAATGCTCTGCGCAAGCGACTCATGCCACACACGGAGAACGA
CGTACAGACGACGCATTCAGACGTACGTCAGGAACAGTACTACAAACATCAAAAGAAGCA
AAAAACAGAGAAAACAAAAAGCGACAGTGGGGCGTGCTTGTGACGTGACGTGGTCCGGTG
ACGGACTGAGGACCCCTAGACATACGATATTATAGACTGTGTGTTGTGGTGATGTGACAT
CGGTGTCGCCACTGCACGAGGGAAGACGAGCACACGCACCACGAGTGACGTCTGTTTGTG
TATGCTGCTCGCGCTGCGTGAGACGACGCCGCAGAGGCGGCTAG

R18

AATCATCGTGGTCCGGGAATGGTGCCCGGCACCGGCGCCGTCCGTTGGCGGTGTATTGCC
AAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAAAC
TGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGCGT
CCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGGAG
CAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAAAG
TGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAACG
TCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTCCGGGT
TCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAAGATCCCGCTTACCG
CTGTAACGCTCATTCCGCCGAACCCACCCCCCCCTTTCGGCCGTCGGGTTCTTGTTGGTT
TTCCCCTTACTGATAGTCACACGCGCACACTACCACCCGTATACGCTAGCACAGCACATC
GTCATAGTGATCTCCCGTGCTCGTCTTTAGCCGTGAGCTTGGTGTTTGGCTGTGTGCGCG
TGTCGTGTGTGGGAGCGGAGTTCCTGCTGGACACGCACTCGCACGACACACGCACACGCC
ACACACAACACGACGACGACCCGACGCGTCCTGTCGACCGACAGTGTGTCCGGCGTGCGG
CACGCTGCATCTGCGAGGTAAGGTGAGACCCACAGCAGCTAACACGCACACTGTCTCGTT
GCGTCGCTTGCGTGTGTTGTGTGTTAGTGTGCCGGAGACTCTGAGCGCCTCGAGATGTCT
CCGAGCGCGCCGCTCTGCTCGTGGCGAAGGTGCGAGCGGTTTAAGATGCAGGCCGCGAGT
GGTAAGCATGTTGGGGTCGTTGTTCGGGTGCTTGCTTGGCCCTGGGGGTTAGCTGGGGGT
TTGGTAAACTTGACCTAAACGTTTCCCTGTGTGCACCCGCCGCCAAATGTGGGGGGGGGC
AAAAAAACAAAAA

R23

ATCGAAGTACTCTCTTCCGGGGAGGCGCCGCGCACCGGCGCCGTCCTTGGCGGTGTATTG
CCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAA
ACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGC
GTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGG
AGCATGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGA
AAGTGTGACCGCCGACGATTAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAA
ACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTCCG
GTATCACGTAAATCAAGCCCCATCTGCACCGCGGCCAGCGGGATCTCCAGATCCCGCTTA
CCGCTGTAACGCTCATACACCGAAAAACCATATCATAAAAACAATTTAAATAAATAACAA
ATTTTGGTGTTATGATCTGCGTTTTTTATGATATTGCTGTTTCTATATGATATATAATAT

AAATACATCACAACTATCCAACACTACAGATCCCGCGGACATCGCCGTGCGCGCTGGACT
CCAGCACACATATCCTCATTATGTATTTGATATATATAATATTATTGACTATTAAGTATG
AAAGAGTCGAATACTGATCACGATATACCACATCGACCCGAACACGACATGATGTGGCGA
TCAAACCGACAACACTAATCAAACATCACTACAACACCACACAACTACATCACACACTAC
ATACATACATTTAATCACGTCATCGACCATGCACAACCACGACACACCAGCACACACCAA
CACACACAACATAACATCTAAACCCATCCAGCGACTCGCTCCAACCATCGC

R24

TGGCCCGCCGAAACGCGTTTCCGGGATGGTGCCGGGCACCGGCGCCGTCCGTTGGCGGTG
TATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCG
AGGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGCTGGTG
ATCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCC
AGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTA
CCGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGC
CGAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCC
TCCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCGC
TTACCGCTGTAACGCTCATTCGCCGAAA

R26

TCTCTGCCAGAATATCTTTTCCGGGAAAGGAGCCTGCGCACCGGCGACCGTCCGTTGGCG
GTGTATTGCCAAGCGCCAGCAGGGCTCGTTCGTCAGCTCCCACTCGTAGCCGTACAGGAT
CTCGAGGAAACTGTTGTCCCGGTTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGG
TGGTGATCGCGTCCTTACCGGTTCCGGTGCCATACCGAGCTCTTCCAGCCCAAGCCACAT
CTGACTCCAGCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGTCGGGCCGGCGCCAT
GGGGCTTACCGAAAGTGTGACCGCCGACGATCAAGCGCCGCTGTTTCGACGTCGTTCATG
GCCATGCGCCGAAACGTCTCGCGAATGTCGACCGCCGTCGGCCATGGGGTCCGGGTTGCC
GTTCGGCCCCTCCGGGTTCACGTAAGATCACACCCCATCTGCACAGCGGCCAGCGGGTTC
TCCAGATCCCGCTTACCGCTGTAACGCTCATTCGTCGAAATCCTTCCTACCCTCCCCACA
CCACCCTAAACTCACTCCACTCAGCACCAGACACACAAGTTAATACTACTGTGTTGCGGG
TGACGTGCTGACATGAGATAATTTTTTTAAAATATAAACATATATCACACTACTCCACAC
GCTCAGAGAGCGTGCTGTCTTCGATCTTTCATCTTTTTCCATTTATTCATTTTTATTTTG
TGATCTTGACTCGACTCGTGTCGCTCTATACGTATCATCATCAATTCACCTCCATTATCA
TCACTCTTACACACACCTGATATGCTATGCAGCTTAGAGTTATGCTTGTGTATTCCTGCT
CTGCTTCTCTGTCTGTCTGCTGCGTCTCTTGCGTTAGCTCTTATCTCTCTCATACTTCAC
CCTAACATTCTCATCATCATACTTTTTGTTCACTACATACACTGCTACGC

R32

GCACGAACATCGTTGTCCGGGCATGGTGCCGGCACCGGCGCCGTCCTTGGCGGTGTATTG
CCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAA
ACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGC
GTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGG
AGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAA
AGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAA
CGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTCCGG
GTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCGCTTACC
GCTGTAACGCTCATTCGCCGAAACGAGCCCAACCTAATTTTTTTATTTGTTTGTGTCTTT
TCAGGATTAACAAACAGGCACACTCACGAATAGTTGATAGTGATGCTGTGTGTGCAGAAG
TGAGGGGAGGCAGAGGGGCGAGTGAGCGTCGCGCGCTGTTTGCGGCAGCGTTGTGCGCGC
TCGCGCCGTCGTGAGGGCGCTTATGTAGATCTCATCAATACATCAGCACGCGACACTGTA

CATCACATGATCAACAAATGATATATAAGACTCAGAAGAGAAGGGAGGTAGCGCTGCGTA
CCCGTCTGCTTTTATGTGCGCGTCAGCGTCGCCCGCTGAGTGCGTACTATCTCTAATGAT
GTGTGATCATATCGATTCTGAATGTGTGTGATCGTGATGGGCTTGGGGAGTCCGCCGCCC
GAGCGCGTTGCAGTGCACGGCGGGCGACGGCCGATTAGGAAGGAGGTTAAAAAGCCAAGG
CGGGGCAAACGGGACAAAACCCGGGCAAATTTGGGTTTTGGGGAGATATAGGGGGGGGGG
GGGGGG

R43

AGTACTGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCGGTGTATTGCCAAGCGC
CAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAAACTGTTGT
CCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGCGTCCTTAC
CGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGGAGCAGCCT
CGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAAAGTGTGAC
CGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAACGTCTCGC
GAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTCCGGGTTCACGT
AAATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCGCTTACCGCTGTAAC
GCTCATTTGCCCGAAAAAAAAAATAAGGGGACTCGAAACAGTCGTGGGGGGGGTCTGGGGT
TAGTGTATGCCCAAACACGAAGCAACAGACGCAACGAGACGACAGAGATAGCATGCATAT
ATATATAACATATAGTGTTTGTGGGATGTGCTGCTGCGATTACTCAGCGCCGACTCCCTC
ACGCTGTGCGCCCGCAGAGACGGCCGCGCTCGCGGCGCGCGCGTCGAGAGGTGAGTTTGT
CGATGTAGTGTGCTGATCTGCGTCCACCTGGCGCCGTGCACGGCGCCAAGCACGGGGTAC
GAGTACTGCATATAGTGGTGATATGCGGCGACGAGAGTGACACGTGTACACGCGCACAGC
GCGCATATTTAAGTGTGCGAGCTCGCATGGGATCCGTCGACGGAGCGCGCACGACAGACA
AGAGGAAAGACGAGACTTTACGGAAAGGACGAGGGAGAACCGTTTGACCGGCCTAAAAAG
GAACTGTCTTGGGTAATGCCCAAATTCCCCGGAAGTGTGTCATTCCACAACCCCCCCAAA
CCGCCGGGCACCCCTTTCTTGGCGAGGGGGGGGCCCGGC

R47

GCCCGCCGAAACTGCGCGCTTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCGGTGT
ATTGCCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGA
GGAAACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGA
TCGCGTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCA
GCGGAGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTAC
CGAAAGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCC
GAAACGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCT
CCGGGTTCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCGGC
CTTTACCGCTGTAACGCTCATCCGCCGAAACCCCCCCCACCTTCTCTGACTTTTTTTATT
TTTTTTCCTCTTCTCCCCCCCCCACCCCCCCCACACTTGTGCTCCTCTCTTTCGTACTCT
CTTTCTTTTTTGCCTGCACTATCAGACAAACACAACATCGACGATATATTTTTATACCAC
CCCTCTCCACTCTTCTTTCGCCTTCACCCCACCCCCAACAAACACCTGCACTCTTCATCT
CTTCACTCTCCTAGCTTTGCTGTCGTGGCTTTGTTTCTTTCCCTTCCTCCTTCTG

R62

ACCGACACTGGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCGGTGTATTGCCAA
GCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAAACTG
TTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGCGTCC
TTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGGAGCA
GCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAAAGTG
TGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAACGTC

TCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTCCGGGTTC
ACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCGCTTACCGCTG
TAACGCTCATTCGCCGAAATGGGTTTGTTGTGGGGTGTCTGGGGGTGTTTGGGTGGTGGC
GCGTGTCGCGCCGCAGCCACGTAGAATATGTGTGTGGTGCGGAGCGTCGGGCGCTGCCGC
GACGGCGACGTGTGTGTACGGGGCGGGATGCTGTGGGGGCGCAGCCGAGTGTATATGTGT
GGTTTTGTTGGTGTTGGGTGTCTCGGCGGGCCCCCACCGCGCGCGCCGCGTGAGCGTGAC
GAGCTAGCTAGCCGATACACGAGAGTGACAGGTACTGCCTGTTGATGTCTCTGGTCCTGC
TCCGAATACAAGAAGTGCCGAGAGACGTGCTCGTTCGTGTGTGGTGTGCTGGAATGACGC
TCACGAGGCGGTCTACGTGTGCATATGGAAACTCGGGGGCGCAGCTACAGATGACGCGCG
CGCGCGGATACGGACCAGGCGGCTTGTCGGCGTCGGGCAGCTTGCGGCGGGGCGCGTGGC
TAACACCGGGAAAGGAAAGCGACGGGGGTTGGCCCCTTGGCCCACGGCGGCAGATTTTGG
GTGTGGGGGG

R226

AGACCGTAATGGGTCCGGGTATGGTGCCGGCACCGGCGCCGTCCTTGGCGGTGTATTGCC
AAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAAAC
TGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGCGT
CCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGGAG
CAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAAAG
TGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAACG
TCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTCCGGGT
TCACGTAGATCAGCCCCATCTGCACCGCGGCCAGCGGGTTCTCCAGATCCCGCTTACCGC
TGTAACGCTCATCCGCCGAATGTTGTGTTTGCGCTCTCTTCTTTTTCGTCGTGTTGTGGC
GCGCGACAGCGACAAACCAAGCCTGCGACTGTGCTTGTCTGCGCGTGTTCTTGAGCTGCT
GGTGTCGTGGGGATGTGCGCCGCTGCGCGGCGGGTGGGCGCGCGGCGCGCGGGCGGCGTG
ACTAATGCAGCATTAGTCGCGGTGGGTAATTGTCAGGTGCGCAGACCTGCTAGTGTTCTG
CTGACACCCATTGTTCTGTGTATCGTGTCGCGCGCAGACGCCACAGGCGCGCCGGCGATG
CTTGACATGGAGGGACTGATACGGTCATAAGCTAACTACTATATTATATACTATAATGTA
TCCTGCCTGTCGCTCGGCTGATGTGTCTGCGACGCGTGTGTGTGTGTGTTGTGGCTTTAT
TAAAGGGTCCCGAACGACATTATCACTTCTGGCCTCGTGCTTGCACACGCGGGCGTGTGC
ACGTCCGCGGACACCGGCCAAGGCTCTGGCCCGGCGCCCCGTGGCCCCCAACCGCCCGGC
GGGGGAAAAAAAAAAA

R300

CCGGGGATGGTGGCCGCGCACCGGCGTCCATTCCTTGAGCGGTGTATTGCCAAGCGCCAG
CAGGACTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAAACTGTTGTCCC
TTTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGCGTCCTTACCGG
TTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGGAGCAGCCTCGG
GTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAATAGTGTGACCG
CCGACGATCTAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAACGTCTCGCG
AATGCCGACCGCCGCGGCCATTGGGGTCCGGGTTTGCCGTTCGGCCCCTCCGGGTTCACG
TAGATCAAACCCCAGTCTGCAGCCGCGGGCAGGCGGGTTCTTCCAGATCCCGCTTACCGT
TGTAACGCTCATTCGCACGATAAGGGGGGGGCATGGCGGGGTGGAGTGGGGGTGGTCGGA
GGGGAGAAAGGGGTCGTGTGTGGTTGTGGTGTGTGTGGGGCGTGTGGGCGTTCGTGCGCG
CGGTGCTTGTGTTGTTTGAGGATATTAGTTATTTGACTGATGTTTTGTGCCTCGCGACGG
GGCTGCGCCGCGCTGTGCGGGCGCGCGTTGCGCTGGCCGAGGCGCGCCAGGACGGGGATT
GCAGAGCAATGTTCTGCGACGCAGCGCATCTCGCAGCAGGGCACGTGCGACCGCGTGTGC
TCGCGCGTGGATTTCTGCCGTCGCTGCCAGGGCGGCGTGATCGCGGCACAGCACAGGGCC
GCGACGCCGGCGCGCGTGTCGCGCGGTCGTGCGCGCAGAGGCTCGACAGAGTGGTCTCGT
GAGAGACTGATGCAGACTGCGATAATCGAGATGCACGCCGCAGGACGTTGACGCGCGCGA

TTCGCTGTTCCTCGGCTGGCTTGTTTCCGAGGGTGCCCGCCGTCGGGCAGGGCCGGGCCC
AAGGAGGGCTGGAGCTGGAAAGGCCCCCGCCACACCCTCGTTTCCCTTGTGCCTCCCGGC
CCGGGGCACTTCGGGGTTTTTCGGGTTTTGGAAACACCGGTGGGGCCCACACACCCTGGT
GTGGTGGGGGGGG

R375

TATGGTGCCCGGCACCGGCGCGCGATCCTTGGCGGTGTATTGCCAAGCGCCAGCAGGGCT
CTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAAACTGTTGCCCATTTCGTC
GGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGCGTCCTTACCGGTTCCGGTG
CCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGGAGCAGCCTCGGGTTCGGGG
CCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAAAGTGTGACCGCCGACGATC
AGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAACGTCTCGCGAATGTCGACC
AGCCGCGGCCATGGGGTCCGGGTTGCCGTTCCGGCCCCTCCGGGTTCACCGTAAGATCAG
CCCCAATCTGAACCAGCGGCCAAGCGGGTTCTCCCAGATCCCTGCTTACCTGCTGTAACG
GCTCATCCGGCAGAAAAGGGGTTGTGGGGTGTGGCACGGGTGGTTTGTTTTGGGGTGGGG
GGGGGGGTGAAGAGTAGATGCGACGTGTCGAGTGCGCGCAACGACGCGCAACAGGAAAAG
ACACAAAATACACCCATGCGTGTTCGCTCGCGGCGCGCTCGGCGCCGGACGCGCGGCTGC
GAGTGTGTTGTTGTTAGTGTTGTTGATTGTGGGGGTGTAGGTTTTTGTGCGCTGGTGCTC
GTCGGGTGGTGGAGGTGTAGGGAGTGTCACAATGTTGGTGTGGTGGGTGTGTGCGGCGCC
AGCGCTGCACGCCGACGGCGCCGCCACCCCAGACGCCCGCTCGTCCGAAATATATTGACA
GAAGCCACATTGACGAGTGCACTGTGTCTCGTTCTGTCGTGTGTTGCGTTGTCGGTGTGT
GGTGATGAGTGTGTGTTTTTTGCTATAGTGGTTTGTGTAGCAGTGTTGACGCGCTGTGTC
CTGGGGCGAAGACGCGGGAGACGCTCCGCGTTCAATTGAGCTTGGACAAGACTTAGGATG
ACAAACTCCAGAAGATGGAAGTTGGTTTGAAAAATTGGCGGCCGGAAAGTTAAGGGCAAA
TTGGTAAAATTTTATTTGGCACATTTGGGTTTTTCGCAATTTTTGGCACCAACCCCGTTT
TATTTTTTTTTTTTGGTGGGG

R443

AGGTCGCCCGGCCACCGCGCGCCCTGTCTCGTGTGGCCGGAGTTATTCGCCATAGCCGCC
AAGCAGGGGCCTCCTTCGGTCAGCGTCCCACCTCGTAGGCCGGTACAAGGATTCTCAGAG
GAGAACTGTTGGTCCCATATTCGGTCGGGGGTGGTTCGGTCCAATAACGACCTTCGATGG
CCGGGCGGTTGATCCGCGGTCCGTTACCGGGTTCCAGGTGCCATATCGAGGCTCGTTCCA
GGCCCAAGGCCCAGTCTGGCTCCAGGCCGGAGCAGGCCCTCCGGGTGTCGGGGGCCTGAC
CAAGATCGGGCCAGGGCCGGGCCGCCATTGGGTCCTGTACCGGAAAGGTGTAGACCCGCC
AGACTGATCACGCAGCCTGCTTGTTTCCGACGTTCGTTTCATCGGCCACTGCGGCCGAAA
ACCGTCTCCGCCGAATTGTCCGACCCGCCAGCGGCCATAGGGGGTCCGGGGTTGGCCCGT
TACGGCCGCCTTCCGGGGTTCAACGTAAGATCCACGCCCGCAATCTGGCAACCAGCAGGC
GCAGGCGGGGTTTCTTCCAGGATACCGCGGCTTAGCCGGCTTGTATACCGCTTCATTTTC
GGCCTAAAATTGGTGGTGCGCGGTGTATGGCATATAGGATGTTTTAATGAAGAGGGGGGT
TGGGTTGTGTGGGGTGTGTGGGGCGTGTGTGGTGGGGTGGTCTAGTGGGTGCTGCTGTGC
TTGGATTGCTGTAGCGCACAACGGCTCTGATCCTGAAGCTGACCCGAGGCAGACGATTGG
TGGACGTGTATCGCGGTGAGCGTGCGTGCGAGCTGTCGAGGGCACCGGCGCGAGTCGAAA
CAGAGGCAACAACAGAGAGCTACCACAGCTCCAGGTGTACGCGCGACAGATGCGTGATAG
TGCGGTGAGAGTATGGCTGGAGGTGATGTGGCGTGATGGTGTGATGTGTGTGTGACTGCT
CTGCAGGAAGACGCAGCACACGCGACAAACACATGAGAGAGTAGTGCAATGACAGCATGG
TGCTGTTCTGTATGACTGCCTGGTGATTGACTTGACTGAGCTGTGCGTGGCTAGATGTGT
GTGCGAGATGATGCGACAGTGCAGCGTGTGCGACTGATACTACTAGAGAAATGATATGCT
GACAGACAGCAGCCTCAGCGACGACTGACGACACGACTGGATGAGTCTGTAGTCCGCGTA
CTGTGGTATGCAGGATGACCGACGATGAGCTCGACAGGATGCGCCCTGTTCACAGACTTA

AATGCAACTGCGGGGCTTGAAGCCGCATGGAAGGACCAAGGACCGCCGCGGCATTCGGAC
AAAGGCCATGGCCCGACCGTAAACCCATCGGAACCATTGCCACGGCTTCGGGGCAAATGG
ATTGGAAAACATTTCGGAAACCGTACCGCGATTTGGGGAAAAGGAGAAACCCAAAAACCG
CGAAAAAACCAAATAGGGAACAAAAGGGCCCCGCAAAAAAGACCACACCACCCTATGAGT
GGGGTGTTTTCTGGTACCCCCCCCC

R503

CGCCGTACTATCGTGGTCCGGGATGGTGCCGGCACCGGCGCCGTCCTTGGCGGTGTATTG
CCAAGCGCCAGCAGGGCTCTTCGTCAGCTCCCACTCGTAGCCGTACAGGATCTCGAGGAA
ACTGTTGTCCCATTTCGTCGGGGTGTTCGTCCATACGACCTCGATGCCGGTGGTGATCGC
GTCCTTACCGGTTCCGGTGCCATACGAGCTCTTCCAGCCCAAGCCCATCTGCTCCAGCGG
AGCAGCCTCGGGTTCGGGGCCGACCAGATCGGCCGGGCCGGCGCCATGGGTCTTACCGAA
AGTGTGACCGCCGACGATCAGCGCCGCTGTTTCGACGTCGTTCATGGCCATGCGCCGAAA
CGTCTCGCGAATGTCGACCGCCGCGGCCATGGGGTCCGGGTTGCCGTTCGGCCCCTCCGG
GTTCACGTAAATCAAGCCCCATCGTGCACCGCGGCCAGCGGGTTCTCCAAGATCCCGCTT
ACCGCTGTAACGCTCATTCGGCCGAAAGGAGCTTGTTTTTTTTTTTTTGTTGGGGTGTGG
GTGGCGGGTTGGTGCTATTTGTGTCTATAGAGTGTGTTGTGGTTCTGTGTGCTGCTCTGT
CGTTTGTCACGCGCCGCGATACATGTAGACAGAACGCTAGAGACGAGCGTCACTTGTAGT
GATGGAACACAACACGGTAAACGTGGTATGCGAAACCTACTCGCGCCGGCGGCGCGTCGA
GCCCTGACTGTCGCCGTGCCTAGTCGCTGCTCTGCTGATGCTATTACTCATACACTCTAC
TCAACTACACTTAAGCGCAAACGCAACGGACACACGCGCAGAGAGCACGACGGCAGCACT
AGGTGTTGCTCATGTGCGCTTTCATGTAGTACATGGTCTATAATCCGTGTGAGATGAGTA
TATGTACTGATGCGCGGGACAGCGGTGTGGTCCGCGACAGCTGATCACTTGTCAATGCGT
GCACACGTTGGCGGATTGAATCCACTGGAATGAAAGTTTGCCCGGTAACGTTAATTTTAC
ACCCCCTTTGCGGGGCCTTTGTGTGTGCCACACACCGCCGGGGTGCGGG

Appendix 11B: *rpoB* samples sent for DNA sequencing

R377

CGTACTCCACCGTCGCCCGCCCTTGCGGCGGACAGCACGCGCGGCTCGACGAAGCGACCG
TCCGCATCGATCGGCGAATTGGCCTGTGCCACCACGTGGCGGTCCTCCTCGTCGGCGGTC
AGGTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGATG
AACCCGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGATCAGACCGATGTTGGGC
CCCTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACC
TCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCGGCGCCGACAGTCGGCGCTTG
TGGGTCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCG
AAGAACTCCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGATC
GCCTCCACGTCCTGGGTGGTCATCCGCTCCCGGACCACCCGCTCCATCCGCGACATGCCG
ACCCGGATCTGGTTTTGGATCACCCCCCGCCGAAA

R380

CGTACTCCACCTCGCCCGCCCTTGCGGCGGACAGCACGCGCGGCTCGACGAAGCGACCGT
CCGCATCGATCGGCGAATTGGCCTGTGCCACCACGTGGCGGTCCTCCTCGTCGGCGGTCA
GGTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGATGA
ACCCGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGAACAGACCGATGTTGGGCC
CCTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCT
CCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGT
GGGTCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGA
AGAACTCCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGATCG
CCTCCACGTCCTGGGTGGTCATCCGCTCCCGGACCACCCGCTCCATCCGCGACATGCCGA
CCCGGATCTGGTTTTGGATCGCTTCCCGCCGAA

R387

TTGTGCTCCACTCTGCGCACAGCCCTGTGCGGACCAGCACGCGCGGCTCGACGAAGCGAC
CGTCCGCATCGATCGGCGAATTGCCTGTGCCACCACGTGGCGGTCCTCCTCGTCGGCGGT
CAGGTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGAT
GAACCCGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGATCAGACCGATGTTGGG
CCCCTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGAC
CTCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCAACAGTCGGCGCTT
GTGGGTCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCC
GAAGAACTCCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGAT
CGCCTCCACGTCCTGGGTGGTCATCCGCTCCCGGACCACCCGCTCCATCCGCGACATGCC
GACCCGGATCTGGTTTTGGATCACCTCCCGCCGAACGTCCTTTTTTTTTTTTTTTTTTT
TCTCTCTTTCTTTTTTTCTTTTTCTCTACTCTCTTCCTTCTCTTCTTTTTTTTCTCCTTT
TTTTTTTTTTCTCTTATGTGTATGGGCGTCCCCCCCTCTCCTCCTTCCCTCTCACCAACT
TCATTCATCAAAATCACCACCATATTGCCTCTTTCATTTCTGTTGTCGCCCTCGCCCACC
CGCGCCTCCCGCGCCCTGTCTTCTGCTGCTGCTTCTCGCTATGAG

R389

CGTACTCCACCTCGCCCGCCCTTGCGGCGGACCAGCACGCGCGGCTCGACGAAGCGACCG
TCCGCATCGATCGGCGAATTGGCCTGTGCCACCACGTGGCGGTCCTCCTCGTCGGCGGTC
AGGTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGATG
AACCCGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGATCAGACCGATGTTGGGC
CCCTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACC
TCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCGGCGCCGACAGTCGGCGCTTG
TGGGTCAACCCCGACAGCGGGTTGTTCTGGCCCATGAATTGGCTCAGCTGGCTGGTGCCG
AAGAACTCCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGATC

GCCTCCACGTCCTGGGTGGTCATCCGCTCCCGGACCACCCGCTCCATCCGCGACATGCCG
ACCCGGATCTGGTTTTGGATCACTTCTGCCCCAAA

KLM 05240

TCCACCGTCGCCCGCCATTAGCGTGCGGTACAGCACGCGCGGATCGACGAAGCGACCGTC
CGCATCGATCGGCGAATTGGCCTGTGCCACCGCGTGGCGGTCCTCCTCGTCGGCGGTCAG
GTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGATGAA
CCCGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGATCAGACCGATGTTGGGCCC
CTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCTC
CAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCAACAGTCGGCGCTTGTG
GGTCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAA
GAACTCCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGATCGC
CTCCACGTCCTGGGTGGTCATCCGCTCCCGGACCACCCGCTCCATCCGCGACATGCCGAC
CCGGATCTGGTTTTGGATCAGCTCCGCCGAATTTGGGGGCCTTTCTGTTGTTTTTTTTTT
TGTTGTTTTGGGGGTGCTGGGTGCGGCGCGGCGGCTGCGGCGCGCCCGGCGCCGACGGTT
CGCTGGTCGTGAAACGGTGTGTTCTGCGTGTGAGCTGCCGCGGCACGCGGGTGAGCGATC
GCCGTCGCGAGTCAGACAATGGGCCGCTTCCGATGTCGATGCAGTCGCGACTCTGCCCCA
TGGCAGGTGCGCATGATGCAGGAGGATGATAGTCGGTGCAAATGTCACTGCGTTCCTGCG
CATGCTAGTACTGGAACACGCACACACTCAGACGCGCTGCGCTCTGACGGTGCGCTGGGG
AGAGGTTGTGGTGG

MTN 053338

CCACCGTCGCCCGCCATTGACGGCGGAACAGCACGCGCGGCTCGACGAAGCGACCGTCCG
CATCGATCGGCGAATTGGCCTGTGCCACCACGTGGCGGTCCTCCTCGTCGGCGGTCAGGT
ACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGATGAACC
CGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGATCAGACCGATGTTGGGCCCCT
CAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCTCCA
GCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCGGCGCCGACAGTCGGCGCTTGTGGG
TCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGA
ACTCCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGATCGCCT
CCACGTCCTGGGTGGTCATCCGCTCCCGGACCACCCGCTCCATCCGCGACATGCCGACCC
GGATCTGGTTTTGGATCAGCTCCCGCCGAAAGGGGGGTGCGTGGTGGCGGCTCTTTGTTT
GTTGGCGGGTGGTGCGCGGGGGGGGTGTTTGCGCTCTCCTATTTCGCCAAGTGAAATCTG
ACCTCGCCCCGAGACGGTGGTGTCGGCGTCGTGTCTGTGGTTGCCCCGTGTGGGGGCGCG
GTGAGCGTCGTTGTTGGGTGTCGTGGGATGTGCTTCTGCTCTTGCTCAGGGGGCGCAAGG
GACGCGAGCGGCGCGCCGCCGCGCTCGACGCGAGGCGGCGAGCGTGGCTGTTTTCAACTG
CTCTCTTCTGTCGTCTTATGTGAGCGTGCGTAGCTGGGACAGCGAGCGAGATGGTTACCA
GGCGTGAGCCCCCTGGG

NOMI 052951

GCTTCCGACCGTCGCCCGCCTCTGCGGCGGCACAGCACGCGCGGCTCGACAAAGCGACCG
TCCGCATCGATCGGCGGAATTGGCCTGTGCCACCGCGTGGCGGTCCTCCTCGTCGGCGGT
CAGGTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGAT
GAACCCGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGATCAGACCGATGTTGGG
CCCCTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCACGGAC
CTCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCAACAGTCGGCGCTT
GTGGGTCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTTCAGCTGGCTGGTGC
CAAAAGAAACTCCTTTGATCGGCGGGCGACCACCGGCCCGGTATGTTGATTCAAACGTCC
TGCGGGTGTGATTCGCCCTTCCACCGTTCCTAGGGTGGGTCCATTCCCGCCTCCCCGGGA
CCCACCCCGGCTTCCAATGCCGGCGTACCATTGCCCGGACCCGCGGGGATTCGTGGGTTG

TTTGGGATTCAGGCCTCCCGTCCCCGAAATCTCTCGTTCAGCGTGGGCGTTCAGCCAACG
GGGAGGCGCTGAGCGAGGCAGACTGGAGACGTGCCTTGCAGTGCATCTATCATACGTTGA
AGGTAGATCATGCAGAGCTACAGACGTCGGGTGCTGTGTCAGGTGATGCATGCGGCTGCC
TCGCGCTAACTATGACATATCTAGACTGCACTGAGAGCGACAGACTCACAGCTGTAGTCA
TGAATACCGTTCTCTGCATGCTACAACCACTTCCTGTGCATGGTTTCCCCCCACGTACTG
GATTAACGATGGCTCCGTCCCGGGCGGGCTTTGCCCGGCCCTGGGCCCGGGGCGCGCGGC
GCAACACCCGGTTTTTGGGGGGGGGGGGGGGCA

RIM 052872

CACGTCCACCGTCGCCCGCCTTAGCTGCGGTACAGCACGCGCGGCTCGACGAAGCGACCG
TCCGCATCGATCGGCGAATTGGCCTGTGCCACCACGTGGCGGTCCTCCTCGTCGGCGGTC
AGGTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGATG
AACCCGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGATCAGACCGATGTTGGGC
CCCTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACC
TCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCAACAGTCGGCGCTTG
TGGGTCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCG
AAGAACTCCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGATC
GCCTCCACGTCCTGGGTGGTCATCCGCTCCCGGACCACCCGCTCCATCCGCGACATGCCG
ACCCGGATCTGGTTTTGGATCAGCTCCAACGAACGACTTGTTTCTTTTCTCTTCCGCGCG
GTGTTGTTTGCCCCGCTGCCGTCATACGAGAGCTCTGGCCGTTATGCTGTGTGTGCTGGG
TGGTGTCTGTTACGGTGCTAAGTTATAAAAATACAATTCAGACAGTATACTCCTCCGCGT
CCGGCGGGGCTGCTGATGCTGCAGTGTTGGGTGTGAGAGTGGATGTAGGGCGTGAGTGTG
GGAGTGAAGAGTAAGCTCATATGTATATCATGACTCGCACTGCATTGAATCTGTCGCGCG
TGCGTGCGTGCGGCTGTGTGAGCTGTCGAGACGAAGTCGAACGATGCTCGACCGTGG

SHN 053051

TGTTCCAGTCGTCGCCACGACACATAGCTCTGTGGACCAGCACGCGCGTGTTCGACGAAG
CGACCGTCCGCATCGATCGGCGAATTGGCCTGTGCCACCACGTGGCGGTCCTCCTCGTCG
GCGGTCAGGTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTT
TCGATGAACCCGAACGGGTTGACCCGCGCGTACACCGACTGCGAGCCGATCAGACCGATG
TTGGGCCCCTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCG
CGGACCTCCTGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCAACTGTCGG
CGCTTGTGGGTCAACCTCGACAGCGGGTTGTTCTGGTCCATGAATCTGGCTCAACTGGCT
GGAGCCGAAAAACTCCTTGATCGCGGTGACCACCGGTCGGATGTTGATCAACGTCTGCAG
TGTGATCGCCTGCACCGTCCTGGGTGGTCATCCAGATATGTTTATTGATTTATGTGTTCA
GAGGAGCCACCCCCCACCCCCTCCGGGGGGGGGGGGGAGAAATCCCCTTTGCTTTTGCTTC
CCTCTACTCCCGTGGGGAGCTTCACCGATTCCGGCGGACCCCGCAAAAGTTTTGGGGCCC
GGAGCCCCCGCGATTCTTGGTCTTTTGGGGGGGGTAAATTCACGCCCCCATCTACCAAAA
TCGCGTTCTGCCCCCCTCGCGCGCGGCGTGGCCGGCCGACAGACAGCTGTGGTGTGCTGC
AGCGCGTGCACTGCGTCACGCGCGTGGGTTGCGCTGTTTAGATGATGATGGTGTGCGAGT
GCTGGGCAGAATAGAGAATAGATGGATTCGGGTAAATGTATAGGGTGGCTGCGGGTGCTG
AGGGGCTCGGTCTGTGGG

TBD 05128

ATCCATCGCGTCGCCCGCCATGACTGTGCGGCACAGCACGCGCTGGCTCGACGAAGCGAC
CGTCCGCATCGATCGGCGAATTGGCCTGTGCCACCACGTGGCGGTCCTCCTCGTCGGCGG
TCAGGTACACGATCTCGTCGCTAACCACGCCGTCGACCACCTTGCGGTACGGCGTTTCGA
TGAACCCGAACGGGTTGACCCGCGCGTACACCGACAGCGAGCCGATCAGACCGATGTTGG
GCCCCTCAGGGGTTTCGATCGGGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGA
CCTCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCAATCAGTCGGCGC
TTGTGGGTCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTCAACTGGCTGGTG
CCGAAGAACTCCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCTGTGTG
ATCGCCTCCACGTCCTGGGTGGTCATCCGCTCAAGAGACCAACCGATCCATCCGCGACAT
GCCGACCCGGATCTGGTTTTGGATCAACTTTAAACAAATTATCCTCCCCCCCCCCCCCCC
CCTCCCGTGCTTCCTTCTGGCCCTCTCTCTTGCTCCGACTAGAGATGGAGCTGAGACGTA
CGCAGTCCTGTGATGCCCTCGCGGTCTGCGCATGGCTTAGGGTATCTCTAGTACTAGCCA
TCATACTGTATAAAACTACTCTTCTCCATACTCACTCCTCTCTCCCTGCCACCGCCCCAG
CATGCGCAGCTTCAGCTGCGCTCCCCATGCACTAGTCTCATCTTGACTGGTTCTACTGTA
CTCTTTTTTCTGTATGTGCTATTATTGATGGATGGTCTGTGCGATGGGATGCGTGTGGGT
CGGCGCGCCCCTTGACTGGTGGCGGGCATATATCC