Innovative approaches to tuberculosis diagnosis with emphasis on nucleic acid amplification tests in a resource-constrained high-burden tuberculosis setting

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted by me in respect of a degree at any other University or tertiary institution.

Signed: _____this ___ day of _____ 2015

"The mind of the son of Adam will forever remain young when it is engaged in the pursuit of knowledge"

- Prophet Muhammad

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SUMMARY

The global control of tuberculosis (TB) is currently hindered by the low sensitivity of microscopy and the prolonged time-to-result of culture. Recent technical progress has improved both diagnostic accuracy and turnaround, namely, nucleic acid amplification tests (NAAT). The World Health Organization (WHO) has recently endorsed two NAATs, which South Africa has been in the forefront of adopting. Based on WHO recommendations, the Xpert MTB/RIF assay (Xpert) has replaced microscopy as the first-line test in the National Algorithm.

With current research and development primarily focused on rapid molecular tests, innovative methods of deployment are essential. In the work reported here, a contribution is offered towards fulfilling this need. This study aimed to show non-inferior diagnostic efficiency for the molecular detection of *Mycobacterium tuberculosis* from clinical sputum specimens in a novel specimen transport medium PrimeStore® - Molecular Transport Medium (PS-MTM).

Technical evaluations of the parameters offered by the transport medium when applied to *M. tuberculosis* were performed; its ability to inactivate the organism, stabilize its deoxyribonucleic acid (DNA) in specimen over time and show compatibility with silica and

magnetic bead-based DNA extraction systems for downstream molecular detection. Additionally, a novel and innovative sputum collection method, where a swab from sputum specimen placed into PS-MTM for the molecular detection of *M. tuberculosis*, is described. This collection system was evaluated in a routine clinical laboratory against mycobacterial culture, the reference standard. Collection method performance was further validated on sputum from suspected TB patients, at healthcare facilities in rural South Africa to a centralized laboratory for testing.

Complete inactivation of *M. tuberculosis* occurred by 30 minutes after exposure, with a 1:3 sputum to PS-MTM ratio. The specimen remained stable with no significant change over time by real-time polymerase chain reaction (PCR) detection (<5% on a mean starting value) for PS-MTM samples over 28 days at ambient temperature. PS-MTM showed compatibility with all extraction systems; however, the automated bead-based extraction systems displayed better performance, with an estimated 170 CFU/ml lower limit of detection.

Of 256 sputum specimens evaluated using the novel collection system, 10.2% were culture positive (routine specimen) and 11.0% positive by real-time PCR (PS-MTM swab from routine specimen). Against culture, detection of *M. tuberculosis* from swabbed sputum in PS-MTM had a sensitivity of 77% (CI 95%: 56-91%) and specificity of 96% (CI 95%: 93-98%).

Specimens obtained from 141 patients were included for the validation analysis, a subset of a larger cohort study. Concordance between the collection system under evaluation was 82% (McNemar, p=0.55) and 84% (McNemar, p=0.05) for culture and Xpert assay, respectively.

Our findings suggest that PS-MTM is capable of improving safety and is an ideal solution for collecting, transporting and stabilizing sputum at ambient temperatures for centralized molecular TB testing. This system provides opportunities for resource-limited settings to introduce or further scale-up molecular diagnostics.

PS-MTM samples are capable of bringing forward a significant number of positives, in addition to culture and Xpert testing, that could be regarded as real due to the system's lower limits of detection and not just false-positives. Application of this system provides quality samples allowing for better discrimination, which in turn could provide adequate management of low bacillary load patients prior to transmission of infection.

Keywords: Specimen transport medium, sputum, molecular, *Mycobacterium tuberculosis*, inactivation, stabilizing, compatibility, diagnosis, swab, ambient temperature

LIST OF ABBREVIATIONS

AFB	Acid-fast bacilli
AMTD	Amplified *M. tuberculosis* direct test
BSC	Biological safety cabinet
BSL	Biosafety Level
BCG	Bacille Calmette-Guérin
CFU	Colony-Forming Units
CI	Confidence Interval
CPC	Cetylpyridinium Chloride
CT or CAT	Computerized Tomography or Computerized Axial Tomography
CT	Cycle Threshold
DNA	Deoxyribonucleic acid
DNP	Deoxyribonucleoprotein
DST	Drug Susceptibility Testing
DTT	Dithiotreitol
dNTP	Deoxynucleotide triphosphates
E-AMTD	2nd Generation Amplified *M. tuberculosis* direct test
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration

FM	Fluorescent microscopy
Xpert	Xpert MTB/RIF assay
IFN-γ	Interferon gamma
IGRA	Interferon gamma release assays
IS6110	Insertion sequence 6110
LAM	Lipoarabinomannan
LAMP	Loop-mediated isothermal amplification
LED	Light emitting diodes
LJ	Lowenstein Jensen
MGIT	Mycobacterial Growth Indicator Tubes
MRI	Magnetic Resonance Imaging
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSDS	Materials and safety data sheet
MTB	*Mycobacterium tuberculosis*
PS-MTM	PrimeStore® - Molecular Transport Medium
NAAT	Nucleic Acid Amplification Test
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
NTM	Non-tuberculous mycobacteria
OADC	Oleic Albumin Dextrose Catalase

PCR	Polymerase Chain Reaction
POC	Point of Care
PPE	Personal protection equipment
RIF	Rifampin
RNA	Ribonucleic acid
RT	Real Time
SD	Standard Deviation
SR Buffer	Sample Reagent Buffer
TB	Tuberculosis
TST	Tuberculin skin test
USP	Universal Sample Processing
UV	Ultra Violet
WHO	World Health Organization
ZN	Ziehl-Neelsen

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Publications (2012-2015)

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Luke T Daum, Nazir A Ismail, Bernard Fourie, Anwar Hoosen, Shaheed V Omar, John D Rodriques, Sue A Worthy and Gerald W Fisher. **Genetic analysis of *Mycobacterium tuberculosis* drug-resistance genes from samples shipped in PrimeStore MTM and sequenced using the Next-Generation ion torrent.** Poster presented at the 30th Annual European Society for Paediatric Infectious Diseases Meeting (ESPIDS), Thessaloniki - Greece, May 8-12 2012.

CHAPTER 1

INTRODUCTION

Tuberculosis (TB), an infectious disease of major public health importance, accounts for close to two million deaths and nine million new infections annually. These estimates are the highest recorded in the history of the disease, with the majority of the worldwide burden (80%) of active disease carried by only 22 middle-to-low-income countries (WHO, 2010b). Factors contributing to the increasing TB burden include poverty and rapid urbanization, the impact of human immunodeficiency virus (HIV) co-infection with TB, poor health services infrastructure, and poor program management with inadequate case detection, diagnosis and cure.

South Africa ranks 5th amongst the 22 high-burden countries having the highest incidence and prevalence of the disease (WHO, 2014a). Sub-Saharan Africa has been the most affected where most of the world's HIV-associated TB is present (Lawn and Zumla, 2011). At best approximately 40% of the incident cases will be detected by smear microscopy, with the rest relying on more sophisticated procedures for a positive diagnosis. Most people infected with *Mycobacterium tuberculosis*, the pathogen responsible for the disease, remain asymptomatic, with only a small portion developing active disease. The lifetime risk of developing the disease from latent infection is about 10%, but in cases where an individual's immunity is compromised, such as by HIV, the risk changes to 10% per year, currently resulting in a large pool of HIV-driven reactivated TB cases (Young et al., 2008). It has been estimated that a third of the world's population is latently infected with *M. tuberculosis* which creates a huge reservoir for possible active disease (WHO, 2010b).

Given the upsurge in TB cases, especially in high-burden HIV settings, several international, governmental and non-governmental organizations have partnered to attempt to avert this situation and remove TB as a public health problem. In 2001, the Stop TB

Partnership launched the Global Plan to Stop TB 2001–2005 (WHO, 2008a). In 2006, a more progressive plan was issued giving direction to action on: the Global Plan to Stop TB 2006–2015, which aimed to reach the United Nations Millennium Development Goals (reversing the epidemic by 2015 and halving the prevalence and death rates by 2015 compared with levels in 1990). This plan has recently been further intensified with the Global Plan to Stop TB 2011-2015 (WHO, 2010a), which consists of two main parts. Firstly, the "implementation component" would be achieved through increasing access to existing interventions for the diagnosis and treatment of TB, and secondly, an intensified effort will be launched for "introducing new technologies", notably new diagnostic tests, with a strong emphasis on research and development towards new tools with the ability to revolutionize the prevention, diagnosis and treatment of TB, laying the foundation for elimination.

Current tools for diagnosing tuberculosis are inadequate, which creates a major obstacle to the global control of tuberculosis (Pai et al., 2010). The reference standard (culture) has a prolonged time-to-result and smear microscopy lacks sensitivity. However, several new tools and strategies have recently received WHO endorsement for implementation at country level, and are being promoted via the Global Laboratory Initiative and other organizations to middle-/low income countries, inclusive of skills and infrastructure development (WHO, 2008b, WHO, 2011). While microscopy and culture still form the mainstay for a laboratory diagnosis of TB, remarkable technical progress has been made in recent times, resulting in quicker and more accurate diagnostic services. The development of improved nucleic acid amplification tests (NAAT) has enabled the detection of *M. tuberculosis* deoxyribonucleic acids (DNA) as well as the determination of drug resistance patterns directly from clinical specimens.

Detecting genetic material rather than depending on the immune response can act as a direct marker to diagnose tuberculosis. The nucleic acids, deoxyribonucleic acids/ribonucleic acids (DNA/RNA) can be detected and quantified by means of the polymerase chain reaction (PCR). This occurs when a single of few pieces of a targeted DNA fragment gets amplified generating millions of copies of the DNA fragment.

Polymerase Chain Reaction (PCR) assays usually have high specificity and positive predictive values and excellent sensitivity on smear-positive specimens; on the other hand, they have relatively low (and highly variable) sensitivity and negative predictive values for smear-negative disease. Reasons for the wide range on sensitivity have been attributed to inhibition, but this must be regarded as a less than satisfactory explanation. Other possible factors may include the low number of bacilli present in the smear-negative specimens, or the method of extracting the nucleic acid (Cho, 2007, Trajman et al., 2008). Over the past decade several commercial and in-house molecular assays have been developed, each test varying in terms of its ability to detect or exclude disease, however, with gradual progress towards an adequate diagnostic (Beige et al., 1995, Cho, 2007, Haldar et al., 2007, Ling et al., 2008, Ani et al., 2009, Flores et al., 2009, Green et al., 2009, Aryan et al., 2010, Armand et al., 2011, Omar et al., 2011, de Assuncao et al., 2014).

We have moved into a molecular age for diagnosis. The Xpert MTB/RIF assay (Xpert) (Cepheid, Sunnyvale, CA, USA), based on real-time PCR and currently with the highest sensitivity for the detection of *M. tuberculosis*, has been introduced as a diagnostic for TB (Boehme et al., 2010, Boehme et al., 2011). The test is not only capable of detecting *M. tuberculosis* complex but rifampicin resistance as well. It is a fully automated system, which performs extraction to detection within a single cartridge in 120 minutes. Early data suggest a sensitivity of between 75% and 90% and specificity up to 97% for pulmonary TB (Bowles et al., 2011, Marlowe et al., 2011, Scott et al., 2011, Clouse et al., 2012, Steingart et al., 2014).

Despite these advances, several implementation challenges exist for the Xpert assay, which include refrigeration in countries with temperatures exceeding the 28°C reagent threshold, increased cost associated with reagents, staff and maintenance, and the availability of a stable, uninterrupted power supply (Carman and Patel, 2014, WHO, 2014b) These conditions require financial, operational and logistical support (Clouse et al., 2012), which may exist in urban and peri-urban locations (Boehme et al., 2011), however, difficult to achieve in rural settings in the same countries. Further to this, the Xpert requires samples to be processed within 3 days if kept at ambient temperature or stored at 2-8° C for a

maximum of 7 days if delays are expected, however, storage may not be possible in resource-poor countries (WHO, 2014b). In light of this, an urgent need exists to address these deficiencies in the diagnostic services for tuberculosis in high-burden settings.

Delays in specimen transport, poor infrastructure, lack of stable electrical supply including refrigeration and skills shortages all hamper implementation of these advanced tools. Rapid diagnosis and treatment of TB is needed, particularly in resource-constrained settings where HIV is highly prevalent, laboratories only offer smear-microscopy or first-line molecular testing and specimens need to be transported over long distances to reach referral labs for additional testing, such as in South Africa. With much of the current research and development effort directed at molecular methods for detecting TB from clinical specimens, the need for innovative approaches to employ current diagnostic test developments in high-burden and resource-constrained settings has become essential. Moreover, recent advances in optimization of specimens for processing, and the rational use of different tests combined in appropriate algorithms to ensure diagnostic efficiency and optimal patient care, offer attractive options for microbiologists and clinicians to combat tuberculosis effectively. In the work reported here, a contribution is offered towards achieving this goal, by ensuring an optimal specimen is captured, preserved and safely transported to a testing facility without the need for cold chain requirements.

HYPOTHESIS

Hypothesis: "A novel transport medium used for sputum specimens intended for downstream molecular detection of *M. tuberculosis* will achieve non-inferior diagnostic efficiency compared to mycobacterial culture, the reference-standard, and a similar concordance in the field to both mycobacterial culture and the Xpert MTB/RIF assay."

The objectives of the study were:

1. To determine inactivation of pure mycobacterial cultures and spiked sputum specimens using the transport medium.
2. To evaluate DNA stability in sputum specimens with application of the transport medium.
3. To evaluate the compatibility of the transport medium on silica and magnetic bead based nucleic acid extraction platforms for the isolation of *M. tuberculosis* DNA.
4. To pilot a novel sputum collection system using the transport medium for downstream real-time PCR detection of *M. tuberculosis*.
5. To determine the diagnostic sensitivity and specificity when using the novel sputum collection system for detection of *M. tuberculosis* by real-time PCR against the reference standard of mycobacterial culture.
6. Validation of the novel collection system for the detection of *M. tuberculosis* by real-time PCR in sputum specimens from TB suspects in the rural Mopani District of South Africa.

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CHAPTER 2

LITERATURE REVIEW

1. Introduction

Tuberculosis (TB) is a problem of global proportion that has killed more humans than any other infectious disease in history and over the past two centuries more than a billion lives were lost (Paulson, 2013). Despite rapid progress being made from the early 1900's to control the disease, the human immunodeficiency virus (HIV) pandemic and emergence of drug resistance allowed *Mycobacterium tuberculosis* to remain a significant threat. The organism is almost as old as human history with traces found at Neolithic burial sites dating back to 7000 years and in ancient Egyptian mummies (WHO, 2009). The World Health Organization (WHO) reports that in 2013, 9 million people contracted the disease of which an estimated 1.5 million died, more than 95% of these cases are found in the middle and low income countries and is the leading killer of HIV-infected people (WHO, 2014a).

The BRICS nations (Brazil, Russia, India, China and South Africa) collectively account for more than 50% of the global TB cases. In 2013, South Africa ranked amongst the top five countries with the highest incidence of TB (410 000 – 520 000), third highest incidence rate per 100 000 population and a prevalence rate of 715 (396 – 1126) per 100 000 population (Figure 1). These statistics may be attributed to missed cases, low level of treatment success and high default rates. Further to this, the high level of HIV co-infection has negatively affected control, with estimates showing a 62% co-infection rate (WHO, 2014a).

In 1882 German physician, Robert Koch, developed the staining technique to visualize the tubercle bacilli by microscopy (Koch, 1982). More than a century later, this tool remains the cornerstone of TB diagnosis despite its poor to moderate sensitivity (Perkins and Cunningham, 2007, Steingart et al., 2007c) and the technological advances of this century. In 2006, the Global Plan to Stop TB (2006-2014) was launched by the Stop TB Partnership, detailing a roadmap to halve TB related deaths to levels reported in 1990 by scaling up prevention and treatment strategies. This plan has recently been intensified with the Global Plan to Stop TB 2011-2015, which consists of two main objectives. Firstly, increasing access

to existing diagnostics and treatment, and secondly, driving research and development for revolutionary tools for prevention, diagnosis and treatment which will lay the foundation for TB elimination. At the time of launch, they estimated 27 medicines, 15 diagnostics and 8 vaccines were at various stages of development (WHO, 2010a).

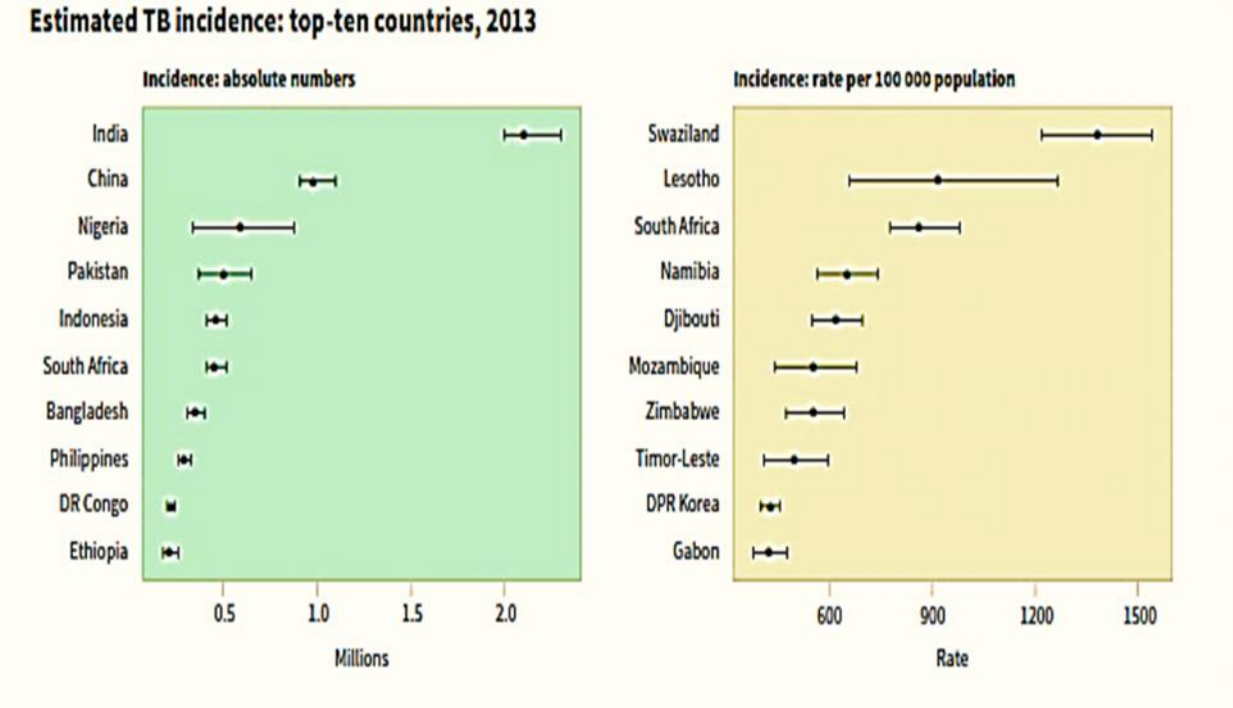

Figure 1. Estimated TB incidence 2013 - top 10 countries most affected (WHO, 2014a)

2. The Tubercle bacillus

The bacterium that Robert Koch discovered, dating back more than 100 years ago, and conclusively associated with the aetiology of tuberculosis belongs to the genus *Mycobacterium*. The Genus *Mycobacterium* is the only genus in the family *Mycobacteriaceae*, which itself is within the broad order *Actinomycetales* (Pratt, 2005). Mycobacteria are aerobic (some species have the ability to grow in reduced oxygen enviroments, non-spore-forming, non-motile, slightly curved or straight rods, measuring 0.2 to 0.6µm to 1.0 to 10µm, which may branch (Pfyffer et al., 2006).

The cell wall consists of two main layers; the peptidoglycolipid layer and the mycolate layer. The peptidoglycolipid component contains meso-diaminiopimelic acid, alanine, glutamic acid, glucosimine, muramic acid, arabnose and galactose and the mycolate layer consists of mycolic acids and together with free lipids provide a hydrophobic permeability barrier (Besra and Chatterjee, 1994, Brennan and Draper, 1994). Other important fatty acids are waxes, phospholipids, mycoserosic, and phthienoic acids (Pfyffer et al., 2006). Various patterns of cellular fatty acids are found as well, including tuberculostearic acid, a unique cell component for a number of *Actinomycetales* (Brennan and Draper, 1994). The high content of complex lipids of the cell wall prevents access by common aniline dyes. Once stained with special procedures, however, mycobacteria are not easily decolourised, even with acid-alcohol, i.e. they are acid fast (Pfyffer et al., 2006).

The cell membrane is similar to that found in all living cells and consists of a double layer of phosphate-containing lipid molecules. The cell membrane is closely associated with various enzymes involved in energy processes in the cell and it contains the pigments responsible for the orange and yellow color of some of the species of mycobacteria (Pfyffer et al., 2006).

The genetic material of mycobacterium is contained within a single, tightly wrapped, circular chromosome that forms the so-called nuclear body. This is not separated from the cytoplasm by a nuclear membrane (Pratt, 2005). *M. tuberculosis* has the second largest microbial genome, with an estimated 4 411 529 base pairs (bp). The genome is guanine and cytosine rich with a content of approximately 65% found uniformly across most of its length and contains close to 4200 protein-encoding genes (Portillo et al., 2007).

3. Tuberculosis

As TB is primarily spread by the respiratory route most cases of the disease affect the lung (pulmonary). In fewer cases, the disease spreads beyond the confines of the lung and the closely related pleurae to other tissues and organs (extra-pulmonary). The aetiological agent in most cases of TB belongs to *M. tuberculosis* complex, which includes *M. tuberculosis, M. bovis* (including *M. bovis* BCG), *M. africanum, M. microti,* and *M. canetti,* which form a tight, discrete group of organisms that display >95% DNA-DNA homology (Kritski and de Melo, 2007).

Airborne droplet nuclei are responsible for the transmission of this disease. These particles containing the *M. tuberculosis* complex have a 1–5 μm diameter. The small droplet size allows it to remain airborne for up to hours after being expectorated by infected individuals, usually by coughing, sneezing, singing, or talking. Inhalation of these infectious droplets allows the organism to enter and settle in the alveoli of the distal airways. Alveolar macrophages engulf the organism, which results in either suppression of the disease of advancement to disease (Kritski and de Melo, 2007). An overview of the infection and transmission cycle is shown in Figure 2.

Period of infection, age and host immunity contribute toward developing active disease. It is estimated that in a newly infected young child the lifetime risk for developing active disease is 10% and further to this, approximately 50% of this risk occurs within 2 – 4 years post-infection (Comstock et al., 1974). The organism continuously replicates at a slow rate after being ingested by alveolar macrophages and spreads through the lymphatic system to the hilar lymph nodes. Cell-mediated immunity usually occurs within 2–8 weeks after infection. Granuloma (caseating or cheese-like) formation occurs around the organism by activated T lymphocytes and macrophages minimizing the spread of the organism by limiting replication (Schluger and Rom, 1998). The infection is usually contained by this process, unless a defect in cell-mediated immunity is present, and active disease may never occur (Frieden et al., 2003b).

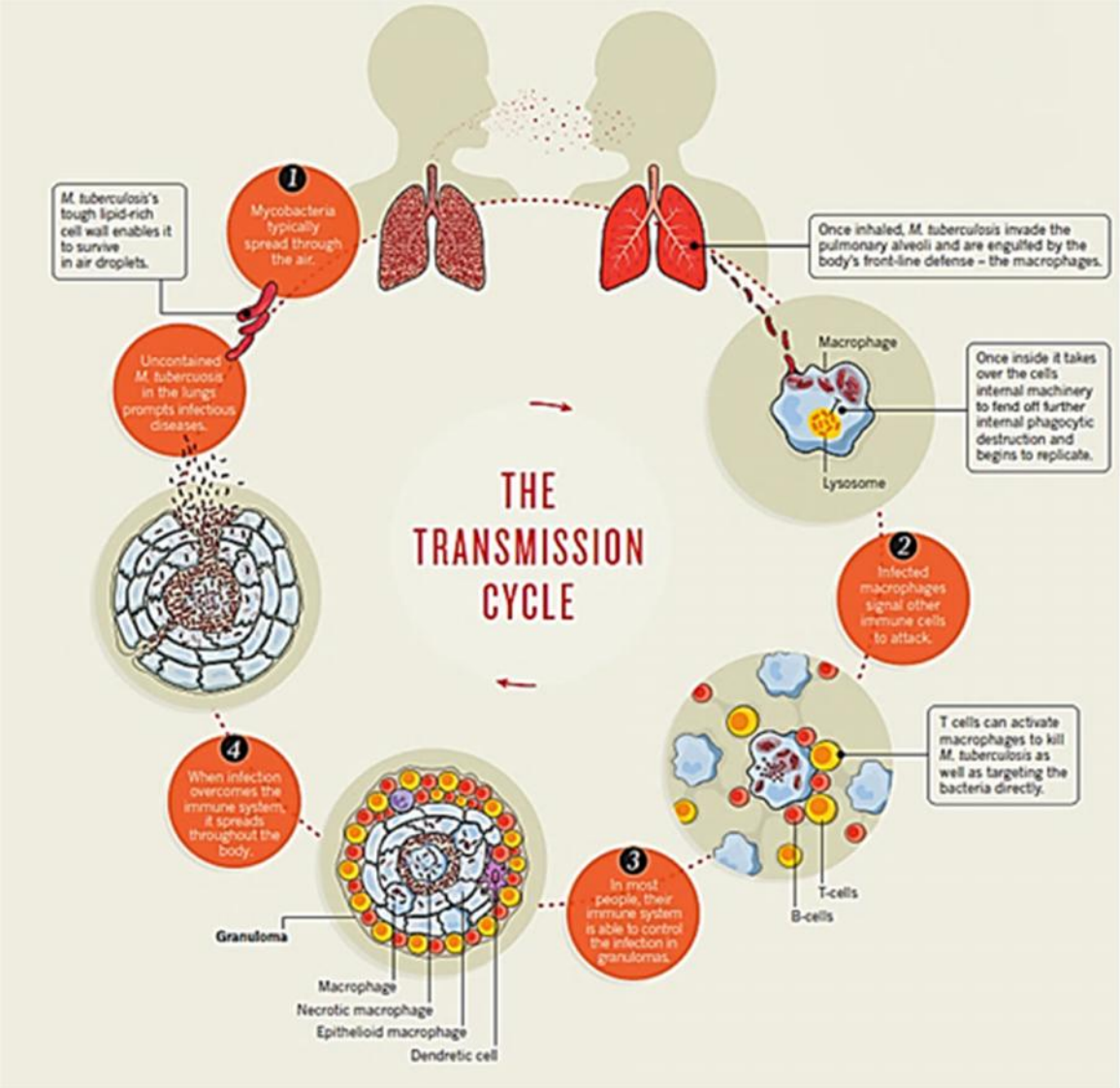

Figure 2. Transmission and infection with *Mycobacterium tuberculosis* (Paulson, 2013)

An efficiently functional immune system can contain *M. tuberculosis* infection; however, HIV co-infection is the greatest risk factor for progression of contained infection to active disease in adults. An HIV infected person's risk of developing active disease is more than 20 times greater than a non-infected person(WHO, 2007) and the risk increases if the person resides in a resource-limited TB-endemic setting (Fielder, 2010, WHO, 2014b). After infection and formation of stable granuloma, some *M. tuberculosis* bacilli can remain viable; this state is referred to as latent infection and may never result in active disease. In these cases the infection manifests itself through immunological testing such as the tuberculin skin test (TST) and interferon gamma assays (Pfyffer et al., 2006, Kunimoto et al., 2009, Pollock

et al., 2014). However, in individuals with compromised immunity these latent infection result in active disease which is known as reactivation TB (Pfyffer et al., 2006, CDC, 2013).

Of critical importance, an person with active TB left undiagnosed or untreated may infect 10-15 close contacts over a period of a year (WHO, 2014b). Three key areas have been identified to control the spread of TB; early diagnosis to prevent the transmission of disease to uninfected individuals, effective treatment with successful completion and prevention i.e. vaccines or prophylaxis (Young et al., 2008). The STOP TB strategy aims to dramatically reduce the global burden of TB by 2015 in-line with its target by achieving universal access to high quality care, reduce socioeconomic burden associated with TB, protect vulnerable populations from TB, HIV and drug-resistant TB, support the development of new tools to improve TB outcomes and promote human rights in TB prevention, care and control (WHO, 2010b).

The cornerstone of these objectives is sensitive and accurate TB diagnostics; early detection of disease not only allows favorable outcomes for those infected but reduces further transmission to uninfected individuals. Perkins and Kritski (2002) described the rate-limiting step in TB control being the lack of accurate case detection (Perkins and Kritski, 2002). Currently, one of the key challenges that healthcare workers face is ensuring that patients are accurately diagnosed. In low-to-middle income countries with the highest concentration of the global burden, access to basic TB diagnostics are limited due to the lack of resources and expertise even more so with current global recession.

Tuberculosis can be diagnosed clinically by, microbiological, radiological, immunological and the use of molecular methods. Despite all the tools available, each has significant limitations and most have to be used in combination for an accurate diagnosis resulting in a slower turnaround time. This therefore impedes on effective TB control (Weyer et al., 2011). The poor sensitivity of microscopy with additional pressure from HIV co-infection (Fielder, 2010, Weyer et al., 2011) and the current gold standard of culturing the organism has a unfavorable turnaround time with a diagnosis being made within 1 to 6 weeks (Frieden et al., 2003a). Rapid molecular assays have been released in recent years that offer sensitivity close to the gold standard (Boehme et al., 2010). Implementation of these assays require huge investment in terms of laboratory infrastructure, biosafety in the case of *M. tuberculosis* requiring a Biosafety Level (BSL) 3 and skilled staff, which is beyond the means of most resource-constrained settings, where majority of the cases reside (Evans, 2011).

The increased attention in the development of new diagnostics tools over the past decade provides hope for addressing one of the main obstacles for adequate TB control. However, any diagnostic test is only as good as its access to use and will fail if access to health services are not improved in parallel (Weyer et al., 2011).

The critical role played by diagnostic tests for TB control necessitates improving on their current accuracy, speed and effectiveness (WHO, 2009). Current tools for diagnosing tuberculosis are inadequate in turn creating a major obstacle to the global control (Pai et al., 2010) . However, new tools and strategies have recently received WHO endorsement for implementation at country level, and are being promoted via the Global Laboratory Initiative and other organizations to middle/low income countries, inclusive of skills and infrastructure development. While microscopy and culture still form the basis for a laboratory diagnosis of TB, remarkable technical progress has been made in recent times, resulting in quicker and more accurate diagnostic services (Ramsay et al., 2010, van Kampen et al., 2010, Ghanashyam, 2011, Weyer et al., 2013).

Failure to accurately diagnose can lead to two possible scenarios. Firstly, a “false positive” results in treatment initiation - this causes toxicity and prevents possible diagnoses of possibly more severe illnesses such as lung cancer or other pulmonary diseases (Hughes et al., 2012). Secondly, a “false negative” results in non-treatment of an infectious individual who has the ability to infect at least 10 to 15 other people in his lifetime (Amo-Adjei and Awusabo-Asare, 2013, WHO, 2014b). Clearly, both consequences have a negative effect on the success of TB control programs, highlighting the dire need for an effective, sensitive, affordable and rapid diagnostic.

4. Pulmonary specimens

Sputum, a sign of chronic inflammatory airway disease, is the most commonly collected respiratory specimen for the diagnosis of TB. It could be collected as an expectorated sample or induced sample by nebulization of individuals having difficulty producing sputum. In cases where expectorated or induced sputum is unlikely a gastric aspirate is obtained, collection of the aspirate is invasive and has to be performed by a trained health professional within a hospital.

During infection and disease of the respiratory system, cilia are damaged and are no longer able to clear mucus from the airways (Mossberg and Camner, 1980). This results in mucus being trapped in the airways and the formation of a mucus plug that requires clearing (Voynow and Rubin, 2009). The resultant expectoration is referred to as sputum, a complex substance having both viscous and elastic properties therefore being described as a viscoelastic (VA) solid (Nielsen et al., 2004, Kater et al., 2007).

Sputum is made up of a primary network of mucin monomers linked together by disulfide bonds and a secondary network of DNA and filamentous actin. In addition, it contains other components such as bacteria, cell debris, proteoglycans, leukocytes, inflammatory mediators, DNA/deoxyribonucleo-protein (DNP) and elastin fibers (Gupta and Jentoft, 1992, Sheils et al., 1996, Voynow and Rubin, 2009). These all contribute to the color (opaque white to green) and viscosity (purulent to mucoid) of the sputum (Stockley et al., 2000, Tsang et al., 2000). The proportion of each of the components varies between samples giving rise to the heterogeneity of the specimen and the varying viscosity from a fluid-like to highly viscous consistency (Broughton-Head et al., 2007).

Novel diagnostics for TB are becoming increasingly available; however, their cost and sophistication hampers the implementation where it is most required. The New Diagnostics Working Group of the Stop TB partnership has outlined key objectives for these diagnostics. These include, simplify and improve TB detection, create and distribute simple, accurate, safe and inexpensive tests that could ideally be performed at the point of care (POC) with results available the same day. In addition, enable effective monitoring of treatment, identify drug resistance and reliably identify latent TB to predict risk of progression to active disease. (WHO, 2009)

The development of a diagnostic tool that satisfies these criteria is by no means impossible. TB diagnostics have come a long way from their inception but not at the pace required. Huge strides have been made in the identification of TB, its mechanism of action and its treatment; however, reliance is still placed on methods developed more than a century ago. The discovery of the groundbreaking technique, i.e. culturing of *M. tuberculosis* bacteria on media containing potato and agar, by Robert Koch in 1882 is still used today, albeit with a modernized approach (Koch, 1982). Culturing plays an integral role in TB diagnosis and is constantly used as a gold standard to compare novel diagnostics. Despite its popularity, culturing of *M. tuberculosis* bacteria has many drawbacks, the most important being the length of time *M. tuberculosis* bacteria require to grow. Novel diagnostics have been continuously introduced for TB but have been unable to surpass the specificity and sensitivity that culturing provides. Below, we detail multiple diagnostics available for TB, their evolution to their current form including specimen collection and transportation, specimen processing and safety requirements with particular focus on sputum.

5. Sputum collection and processing

5.1. Transport

Laboratory guidelines require sputum to be collected and rapidly be transported, according to national biosafety guidelines, to the laboratory for processing. At present the WHO recommends testing of one morning specimen and one spot sputum for laboratory testing (WHO, 2008) when using microscopy. However, using the Xpert as the primary diagnostic a single sputum is recommended despite multiple samples may improve the sensitivity this may be limited by resource availability (WHO, 2014c). Early morning samples have the highest yield of acid-fast bacilli (AFB), however it has been proven that a good specimen collected at any time would give an equivalent diagnostic yield (European Centre for Disease Prevention and Control, 2011, Parsons et al., 2011) .

The process of sputum collection involves the production of droplets that are highly infectious from an individual that has untreated TB. It is recommended that the collection takes place at a distance from other people in a well-ventilated area or, if unavailable, outside the building (WHO, 2008, European Centre for Disease Prevention and Control, 2011). Ideally, these samples should be transported immediately to the laboratory, however, in cases

where this is not possible WHO guidelines recommend no longer than 48 hours and the specimen should be kept refrigerated or on ice till received by the laboratory to prevent overgrowth of contaminant bacteria (Global Laboratory Initiative, 2014).

5.1.1.Transport media

It is important to note that at all times a TB specimen should be treated as highly infectious as it may contain viable *M. tuberculosis* bacilli and poses an infection risk to laboratory staff involved in its processing.

Cetylpyridinium chloride (CPC): If storing or transporting specimen at refrigerated temperatures is not possible cetylpyridinium chloride (CPC) could be used as it eliminates contaminant bacteria associated with sputum, however, further processing of the sputum should not be performed prior to culture (Smithwick et al., 1975). The recovery of *Mycobacteria* using CPC has shown no difference when compared to N-acetyl-L-cysteine-sodium hydroxide method (NALC-NaOH), however, after a 7 day incubation, specimen treated with CPC had a recovery double that of the comparator (Pal et al., 2009). Drawbacks associated with CPC; it reduces the performance of Ziehl-Neelsen (ZN) staining (Selvakumar et al., 2006) and inhibits mycobacterial growth in Middlebrook 7H9 and 7H10 as they are unable to sufficiently neutralize the quanternary ammonium compound (Smithwick et al., 1975).

FTA® Cards for Molecular testing: The FTA® card system (GE Healthcare biosciences, Pittsburg, USA), allows for the collection, transport, preservation and purification of biological samples for downstream molecular testing. The system is based on a dry chemistry on paper at room temperature, binding the sample, lysing the cells and releasing DNA for testing. The card is impregnated with a chaotropic agent that inactivates infectious agent rendering it safe. A portion of the card can be added directly to the molecular test. Studies have shown the usefulness of this application for transporting at ambient temperature and the molecular detection of *M. tuberculosis* (Guio et al., 2006, Scott et al., 2011a). It has also been shown that *M. tuberculosis* could be stored on the card at ambient temperature for up to six months without affecting the reproducibility of the molecular test (Guio et al., 2006).

6. Biosafety requirements of a tuberculosis laboratory

Since the infectious dose of *M. tuberculosis* is estimated at 10 bacilli by inhalation, for humans, biosafety is of utmost importance when working with materials containing the organism. The WHO has designed a manual detailing the biosafety requirements for a TB laboratory (WHO, 2012).

The biosafety level requirement of a laboratory is determined based on a risk assessment. It considers; the bacterial load of the materials (i.e. sample type or isolated *Mycobacteria*); route of transmission; the handling of the material that is likely to cause aerosols and the number of steps in a procedure that is likely to cause aerosols; the work load of a laboratory, its location, epidemiology and patient population served; competency and experience of staff and the health of laboratory staff (i.e. HIV status) (WHO, 2012).

Tuberculosis laboratories can be classified into three levels based on the worked performed and associated risks, in particular, the production of aerosols. Below is a summary of the minimum requirement for each level based of the Tuberculosis laboratory biosafety manual (WHO, 2012);

Low TB Risk (WHO, 2012): performance of direct smear microscopy or Xpert following guidance and recommendations from the WHO. The minimum requirements include, an open bench specially designated for preparation in an area with adequate ventilation when appropriate microbiological techniques are used. Personal protection equipment (PPE) is recommended; however, in the case of smear preparation a respirator may not be required

Moderate TB Risk (WHO, 2012): preparation of specimen for inoculation on solid-media and preparation of specimen for direct drug susceptibility testing (DST). The minimum requirements include, two levels of containment; i) the use of a biological safety cabinet (BSC) as the primary containment and ii) the laboratory as the secondary containment. PPE is mandatory at this level.

High TB Risk (WHO, 2012): Also known as a TB-containment laboratory, these high risk facilities have the design features allowing safe manipulation of TB culture with minimal risk and may not need to meet the requirement of a BSL 3 laboratory. These laboratories manipulate TB cultures for identification purposes and indirect DST from both solid and liquid media containing the TB bacilli. The minimum requirements include those of the Moderate TB risk level with the addition of a double door entry creating an anteroom that

provides a physical barrier between the containment laboratory and the outer laboratory area. An autoclave must be available in the TB-containment laboratory to allow sterilization of all waste containing TB bacilli prior to removal. PPE is mandatory at this level as well. It is further recommended that the TB-containment laboratory be sealed off to allow for decontamination by fumigation.

7. Processing sputum for direct sputum - smear microscopy

Sputum specimens are directly applied to a microscopy slide and stained for microscopy (International Union Against Tuberculosis and Lung Disease, 2000). However, due to the viscous nature of sputum, it requires digestion (liquefaction) allowing release of the *M. tuberculosis* bacteria from the matrix and making the specimen more manageable for diagnostic processing. An approach commonly use to achieve this is the addition of sodium hypochlorite (bleach) for sedimentation. It is inexpensive, initially reported to have improved detection by digesting mucus and cell debris resulting in a clearer microscopic field of view (Bonnet et al., 2008) and its disinfective properties may have a positive effect on infection control (Cattamanchi et al., 2010). Although, a meta-analysis on the use of bleach concluded that no real improvement could be detected when compared to direct microscopy (Cattamanchi et al., 2010).

7.1. Processing sputum for concentrated sputum-smear microscopy, culture and molecular assays

Sputum specimens, intended for smear microscopy and culture, are processed to achieve two objectives. These objectives are; decontamination of bacteria other than mycobacterium and liquefaction of the mucous and organic debris in the specimen. As mycobacteria have a long generation time (20-22 hours) compared to fast-growing bacteria (40-60 minutes), overgrowth by the contaminant bacteria will occur if they are not inhibited. Further to this, the accumulation of the contaminant bacteria's waste products may accumulate in the growth medium inhibiting mycobacterial growth (Roberts, 1988). Several methods are available to achieve these objectives; however, they all vary in terms of selectively decontaminating the contaminating bacteria and the level to which they are capable of liquefying the sputum. It is recommend that all sputum specimens be processed in this manner for preparation of

microscopy and culture (Global Laboratory Initiative, 2014). In addition to CPC described above (Section 7.1.1.), below are the commonly employed decontamination procedures. Despite the several options available, the most widely used is the NALC or DTT-NaOH method.

Sodium hydroxide (NaOH)- modified Petroff (European Centre for Disease Prevention and Control, 2011): This method utilizes NaOH at concentrations between 2 – 4%. It liquefies and decontaminates the sputum simultaneously. Timing is critical as the increasing incubation time may kill-off the mycobacteria even at the lower concentrations.

N-acetyl-L-cysteine (NALC) or Dithiothreitol (DTT) and NaOH (Macdonald, 1972): This method utilizes the mycolytics NALC or DTT to rapidly liquefy the sputum in combination with NaOH (2 - 4%) to eliminate the contaminating bacteria. NALC-NaOH is the most widely used system.

Oxalic acid (de Waard and Robledo, 2007): This method is recommended for pulmonary specimens obtained from patients that may have *Pseudomonas aeruginosa*. This methods use is restricted to inhibit *P. aeruginosa*.

Ogawa-Kudoh (de Waard and Robledo, 2007): This method was developed for culturing mycobacterium in the field or without the use of a centrifugation step prior to inoculation. The sample is liquefied and decontaminated using NaOH (2 – 4%) and inoculated into the Ogawa media. However, this method does have a higher contamination rate.

Universal Sample Processing (USP) (Chakravorty and Tyagi, 2005): This methodology was developed for downstream microscopy, culture and molecular methods. The solution contains non-toxic gaunidinium hydrochloride as the principle component, a mucolytic agent and detergent. This methodology has shown to improve detection by smear microscopy in comparison to the direct and concentrated methods, equivalence to the NALC-NaOH procedure for detection by culture and compatibility for PCR testing (Chakravorty et al., 2005, Haldar et al., 2007).

7.1.1.Processing sputum for direct Xpert MTB/RIF assay detection

The Xpert is the sputum-smear microscopy replacement test in South Africa. It is a WHO endorsed (WHO, 2011) molecular based test detecting *M. tuberculosis* and rifampicin

resistance using five molecular probes directly from sputum (Blakemore et al., 2010, Boehme et al., 2010, Helb et al., 2010).

The assays contain a sample reagent buffer, which is added prior to testing. The solution is propriety; however, based on the materials safety data sheet (MSDS) contains NaOH and isopropanol. The solution is reported to liquefy sputum and reduce the viability of *M. tuberculosis* by 8 logs (Helb et al., 2010). The sputum specimen is added to the sample reagent buffer at a ratio of 1:2. This should result in a mandatory 2 ml specimen solution, which could only be used exclusively for molecular testing as the bacteria are non-viable.

8. Diagnostics

8.1. Imaging technologies

The introduction of the chest X-ray in 1895 was thought to revolutionize the diagnosis of pulmonary TB (Kritski and de Melo, 2007), which enabled progressive stages of the disease to be diagnosed while also tracking disease severity. These chest X-rays identified key radiological features such as tubercles or nodules seen in the lungs of infected patients. However, it did little to assist patients with active TB who lacked the radiographic features at disease onset (Wasserburg, 1951). Performance in HIV-TB co-infected patients with low CD4 counts (<300 cells/µl) is poor with nearly 22% showing normal radiographs in certain studies (Carman and Patel, 2014). The poor performance in this population is concerning as they are the most vulnerable to TB infection. Radiographer inter-variability also compromises the reliability of chest X-rays and is an important limitation to take into account (Carman and Patel, 2014). Chest X-rays are limited to pulmonary TB and may not be as useful when considering extrapulmonary sites.

Similar to X-rays, computerized tomography (CT) also tracks disease progression and severity in the lungs and in other organs by a 3D determination of legion/nodule size. Although, a huge improvement, this procedure is highly dependent on trained physicians to accurately distinguish between TB positive and negative cases (Dorman, 2010). Additional imaging technologies that may be applied for extrapulmonary TB include magnetic resonance imaging (MRI), ultrasound and echocardiograms (Sharma and Mohan, 2004, Lammie et al., 2009, Porcel, 2009). Imaging techniques are advantageous in that they are not invasive and

have rapid time-to-results. However, due to their low sensitivities , particularly in the era of HIV infection, their utility is limited for a TB diagnosis (Dorman, 2010).

8.2. Sputum - smear microscopy

Microscopy still remains the cornerstone of TB diagnosis due to its cost-effectiveness, rapid analysis and minimal requirements for infrastructure or equipment. The first observation of the tubercle bacilli was performed by Robert Koch in 1882. Using methylene blue staining he described the “beautifully blue” aetiological agent, known today as *M. tuberculosis,* as being responsible for TB infection (Koch, 1982). A few weeks after this, Paul Ehrlich discovered the acid-fastness of mycobacteria, a characteristic which allows only mycobacteria stained with arylmethane dyes to retain its color after decolourization with acid alcohol solutions (Barrera, 2007). ZN staining is an adaptation of Ehrlich’s staining method and became the widely adopted technique for detection of *M. tuberculosis* (Titford, 2010). The staining, together with the distinct cording morphology of a select few mycobacteria allows smear microscopy to be highly specific in identifying TB positive cases.

ZN-stained smears of sputum specimens examined by conventional light microscopy remains the primary tool for diagnosing pulmonary TB in disease endemic countries. It is rapid and inexpensive to perform, with high specificity for the detection of infectious cases of TB in high prevalence areas (Steingart et al., 2006) While the technique is highly specific, its sensitivities range from anywhere between 35-70 % making it a poor diagnostic. In general, approximately 50% of culture-positive cases are detected by microscopy (Perkins, 2000, Kivihya-Ndugga et al., 2003, Steingart et al., 2006). Diagnostic sensitivity has been associated with the skill of the microscopist, with training taking at least 2 weeks or longer prior to a confident diagnoses being made (Boehme et al., 2011). Furthermore, the inadequate production of sputum in HIV positive patients have resulted in a lower proportion of smear-positive TB cases detected (Carman and Patel, 2014).

Auramine-O staining for fluorescence microscopy (FM) offers a fast and simple staining procedure, requiring lower power magnification (40x versus 100x for ZN) allowing more of the smear area to be observed in a shorter period of time. Because of the higher contrast between the bright green bacilli and the dark background, a 10% higher diagnostic sensitivity and reduced microscopist fatigue compared to ZN microscopy have been reported (Steingart

et al., 2006, Hanscheid, 2008) Conversely, its widespread implementation has been hampered by the relatively high initial cost of the fluorescence microscope, and the significant replacement cost of the high pressure mercury vapor or xenon arc lamp. The lamp has a short lifetime (<300 hours), requires a stable electricity supply (Hanscheid, 2008, Torrea et al., 2008) and supply in low-income countries is often unreliable, leading to under-utilization or even abandonment of the equipment.

In an attempt to reduce running costs on fluorescence microscopes, mercury lamps have been replaced with light-emitting diodes (LEDs), saving up to 90% on the lamp cost. LEDs have a lifetime in excess of 50 000 hours and can be run on batteries or low voltage power supplies (Hanscheid et al., 2007). Initial studies have demonstrated the potential of these light sources in combination with fluorescence microscopes to produce brighter images and provide comparable results to mercury vapor lamps in the diagnosis of TB (Anthony et al., 2006, Hung et al., 2007, Marais et al., 2008). Thus, enabling TB diagnostic laboratories, in resource-poor countries, to adopt the more sensitive FM.

8.3. Culture methods

8.3.1.Solid media

Robert Koch was a pioneer in the investigation of infectious diseases. This is the premise for culture of *M. tuberculosis* bacteria from a patient's biological sample. Initially, Koch used a heated, solidified serum to culture the bacteria (Koch, 1982). From the early 1900's, egg-based media replaced Koch's serum slants and these egg-based media were formulated and adapted by many scientists including Dorset, Lubenau, Petroff and Petragnani (de Waard and Robledo, 2007). In 1931, Ernst Lowenstein made vital modifications to the egg-based media by adding in potato starch, asparagine and glycerine (de Waard and Robledo, 2007). Following this, Jensen performed a thorough investigation on the cultivation of *M. tuberculosis* bacteria, which would allow for simple and effective culturing (without animal inoculation) and species differentiation (de Waard and Robledo, 2007). Presently, egg-based media with its enhanced formulation, commonly known as Lowenstein-Jensen (LJ) medium is routinely used in the culturing of *M. tuberculosis* bacteria. Despite the efficiency and accuracy of culturing the organism on this media, a major limitation is the time to detection, which could extend from 14 days to 8 weeks (Naveen and Peerapur, 2012). An

advantage of this slow-growing characteristic of *M. tuberculosis* bacteria allows for species differentiation. Fast growth (<1 week) is generally indicative of saprophytic mycobacteria whereas slow growth (7 days or more) is a characteristic of pathogenic mycobacteria (Hett and Rubin, 2008).

An alternative to the egg-based medium was developed by Dubos and Middlebrook, which contained oleic acid and albumin after investigation of several formulations. The medium was suitable for *M. tuberculosis* growth and protected the organism from toxic compounds (Dubos and Middlebrook, 1947). Middlebrook and Cohn further refined this formulation and named it 7H10 which allowed for faster and excessive growth (Middlebrook and Cohn, 1958). A reported advantage of this media in comparison to the egg-based media is its ability to reduce the growth of contaminants (Kubica and Dye, 1967) . Middlebrook is one of the most commonly used media as it can be prepared as both solid and liquid and is the preferred medium for automated systems.

8.3.2.Liquid media

Liquid media for culturing *M. tuberculosis* bacteria were available at the same time as solid egg-based media. However, liquid media were overlooked due to their susceptibility to contamination (de Waard and Robledo, 2007). The determination of antibiotic combinations, which were selective for the growth of *M. tuberculosis*, re-introduced the use liquid media for the isolation of pure culture of mycobacteria. This advancement allowed the automation of culturing *M. tuberculosis*.

The radiometric BACTEC system was introduced in 1983 and utilized Middlebrook 7H12 liquid media together with ^{14}C-labelled palmitic acid to detect growth of mycobacteria (Tortoli and Palomino, 2007). It significantly reduced the average time to positive culture detection from approximately 13 days to 9 days (Morgan et al., 1983). The BACTEC-460 radiometric method used in laboratories was later phased out with the introduction of fluorescent technology and the health and safety concerns surrounding use of radio-labeled material. The fluorescent-based BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system utilizes a modified Middlebrook 7H9 liquid media favoring the growth of *M. tuberculosis* and utilizing fluorescence of an oxygen sensor, present at the bottom of the culture medium tube (Hanna et al., 1999). Fluorescence emission indicates oxygen

consumption, therefore aerobic respiration of the growing organism with the added advantage of improved culturing time and early detection (Siddiqi and Rusch-Gerdes, 2006). Other instrumentation with similar technologies to the BACTEC MGIT 960 include the VersaTREK and BacT/ALERT 3D. New formulations of liquid media are constantly being evaluated to improve growth times, yield better recovery and improve selectivity for *M. tuberculosis* (Essawy et al., 2014).

8.4. Immunological methods

8.4.1.Tuberculin skin test

Koch discovered in 1882 that the protein derivative tuberculin from *M. tuberculosis* bacteria was curative of TB in guinea pigs (Dubovsky, 1973, Koch, 1982). However, this was unsuccessful as a treatment in humans. This spurred investigation to use tuberculin for the development of a diagnostic skin test by Clemens Freiherr von Pirquet (Daniel, 2006). The tuberculin skin test was developed in 1891 and subjected to various methods of manipulation, such as the intradermal injection described by Mantoux in 1903 (Singh and Espitia, 2007), which provided reproducibility. Further developments made by Florence Seibert in 1934 (Daniel, 2006), by purifying the tuberculin used for TST, allowed standardization of this diagnostic assay. The reaction patch has a certain cut-off size (~5mm), which must be measured by a trained health care worker to provide an appropriate diagnosis and patients must return the following day for their reaction patches to be measured. There are a number of drawbacks experienced with this technique and due to the lack of a better performing diagnostic, quality is sacrificed. Prior to the development of interferon gamma (IFN-γ) assays, the TST was the only biological assay capable of determining whether TB infections were present in an individual. Despite its widespread use, this century-old test, lacks the ability to produce accurate results (Luetkemeyer et al., 2007), mostly as it is believed that one third of the world's population is latently infected with TB (WHO, 2014b). Individuals with latent TB infections; those who use steroids; HIV infected individuals; Bacille Calmette-Guérin (BCG)-vaccinated individuals; individuals who often undergo TST (such as healthcare workers) and individuals infected with non-tuberculous mycobacteria all show false positive TST results for TB (Lalvani, 2007). This wide discrepancy makes the use of the TST unfeasible. In addition to this, infections must progress sufficiently for an effective immune response to have been created with a resultant true positive TST result (Dannenberg and Collins, 2001).

Like the TST, other serological assays are based on detecting the immune response of an individual to TB antigens. The advantage with these assays (detailed below) is that immunological detection is performed *in vitro* and the antigen does not need to be injected into the patient. The disadvantage with these assays is that a low level of bacteria in the patient (paucibacillary TB) results in the production of low antibody numbers, which in turn

affects the serological diagnosis of the disease. An additional disadvantage is the need for drawing blood for these assays, which is a much more invasive procedure than obtaining a sputum sample (Madariaga et al., 2007).

8.4.2.Enzyme-linked immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is commonly used for the detection of immune response and has been adapted for the detection of an immune response to a few TB antigens. Commercial ELISAs are available for TB, making access to these assays convenient (Steingart et al., 2007b). The general format of an ELISA has an immobilized antigen on a microtiter plate, patient serum containing antibodies is applied to the plate and a conjugate with a detector is used for detection of the binding reaction. These ELISA assays are reproducible, require basic training to perform and are rapid (Islam et al., 2014). Despite this, these assays have not been widely introduced at POC facilities. This is probably due to the lack of specificity (protein-protein interactions), inconsistent sensitivities and the requirement for a high bacterial load (which would facilitate higher antibody production) (Steingart et al., 2007a, Ivanyi, 2012, Islam et al., 2014).

8.4.3.Rapid tests

More amenable to POC facilities and field research are rapid tests based on latex agglutination. These are immunochromatographic tests, which are inexpensive, easy-to-use and compact. Results are obtained from the use of a suspected patient's serum and can be returned in as little as 20 minutes (Singh and Espitia, 2007). Newer lateral stick assays include the detection of lipoarabinomannan (LAM) antigen in the urine of patients suspected for TB; this is an adaptation of the ELISA assay format. The sensitivity of this assay is dependent on the immune status of the patient and increased sensitivity is found in HIV-TB co-infected patients (Pai et al., 2010, Minion et al., 2011). The specificity of this assay is a point of contention due to inter-study variability (Minion et al., 2011).

8.4.4.Interferon gamma release assays (IGRA)

Repeated exposure of T-cells to antigens of *M. tuberculosis* bacteria results in the production of IFN-γ and can then be detected. IGRA assays are based on the release of IFN-γ after exposure of either whole blood or peripheral blood mononuclear cells to a *M. tuberculosis*-specific antigen (Cattamanchi et al., 2011). The use of an *M. tuberculosis*-specific antigen rules out the effect of prior BCG vaccination and of non-tuberculous mycobacteria. Two blood tests are currently available based on in-vitro stimulation of T-lymphocyte cells, using antigens unique to *M. tuberculosis*, to release IFN-γ. One assay, the enzyme-linked immunospot (ELISpot) [T-SPOT.TB; Oxford Immunotec; Oxford, UK] estimates T-lymphocyte cells secreting IFN-γ, while the other assay uses an enzyme-linked immunosorbent assay (ELISA) to measure secreted IFN-γ concentrations [QuantiFERON-TB Gold; Cellestis; Carnegie, Australia].

Several studies confirm the higher specificity of these assays compared to the TST, due to the uniqueness of the antigens which are absent from BCG and other non-tuberculous mycobacteria and are therefore not confounded by previous exposure to the BCG vaccine and environmental mycobacteria (Kariminia et al., 2009, Katsenos et al., 2010). A drawback of these tests is their inability to distinguish active disease from latent infection, keeping the specificity for active disease low especially in high prevalence areas (Pai and Menzies, 2007). For both active TB disease and latent tuberculosis infection, evidence suggests the ELISA has similar sensitivity to the TST skin test, while the ELISpot is appears more sensitive (Kang et al., 2007, Pai et al., 2007, Pai and Menzies, 2007, Dyrhol-Riise et al., 2010, Cattamanchi et al., 2011, Pai et al., 2014).The United States of America Food and Drug Administration (FDA) has approved both QuantiFERON-Gold-In-Tube and T-Spot as *in vitro* diagnostic for the indirect detection of *M. tuberculosis* infection when used together with risk assessment, radiography, and other medical and diagnostic suggestions (Mazurek et al., 2010). Of note, is the high incidence of latent TB or sensitization of T-cells to TB antigens due to continuous exposure of individuals to TB sufferers, which affects the performance of IGRAs in TB burdened countries (Rangaka et al., 2007, Zwerling et al., 2012, Zwerling et al., 2013).

8.5. Genetic identification methods

8.5.1.Nucleic acid amplifications tests - Polymerase Chain Reaction (PCR)

In the early 90's, the application of PCR to the identification of mycobacteria was employed (Palomino et al., 2007). PCR enabled the detection of *M. tuberculosis* genetic material as opposed to a possible immune response (as in serological methods), leading to the development of direct markers for TB diagnosis. Nucleic acids, i.e. DNA or RNA, can be detected and quantified by means of PCR. This occurs when a few strands of targeted DNA/RNA fragments are amplified, generating millions of fragment copies, detected both post- or during PCR (Real-Time PCR). The amplification of *M. tuberculosis* organism-specific DNA fragments enables PCR detection on a variety of specimen types.

8.5.2.Specimen processing and DNA extraction for NAATs

The value of specimen processing and extraction systems for detection of *M. tuberculosis* DNA in sputum is critical and has been underestimated (Young et al., 2008, Niemz et al., 2011). The amplification efficiency is directly related to the extraction methodology (De Almeida et al., 2013). The neglect could be attributed to the oversight by funding bodies on the importance of this step with focus largely placed on the performance of the molecular assays itself (Young et al., 2008).

A challenge to the accuracy of molecular methods is the processing of specimen. Theoretically, PCR tests require small quantities of DNA from approximately 5 (Helb et al., 2010) to 28 genomes (Omar et al., 2011), however, once in the matrix of specimen this performance cannot be reproduced. Studies have shown that the methodology applied for nucleic acid extraction has a direct effect on the performance of the PCR test (Aldous et al., 2005, Santos et al., 2010, De Almeida et al., 2013). Therefore, specimens processing and nucleic acid extraction should be efficient to allow maximal detection by PCR assays.

The increased use of NAATs in clinical laboratories, due to their high sensitivities and specificities have warranted the use on nucleic extraction systems that are safe, and provide a short turnaround time without affecting quality of the isolated material. In addition to this, these methods, when employed in a high-volume clinical laboratory environment need to be robust, require minimal skills, efficient, highly reproducible and reduce labor intensiveness.

The majority of commercially available products for these purposes are based on solid phase extraction methods where the analyte of interest, i.e. nucleic acids, is separated from other components of the material based on their chemical and physical properties in solution.

Classical chemical-based nucleic acid purification includes cell lysis, creating a lysate after cellular disruption, inactivation of nucleases, and separating nucleic acid within the lysate. This separation is achieved using organic solvents. This methodology is not ideal for clinical routine studies due to related toxicity issues as well as complex handling; requiring centrifugation and removal of specific phases (Rudi and Jakobsen, 2006). The principles of solid phase extraction is applied to many formats such as cartridges, columns and magnetic beads (Tan and Yiap, 2009). Application of magnetic beads allows sample manipulation by magnets therefore, avoiding the use of centrifugation and enabling automation. In the presence of alcohol, high salt or chaotropic agents DNA binds to several surfaces including glass and silica and the binding reversed in the presence of low-salt buffers (Boom et al., 1990, Rudi and Jakobsen, 2006). Today, the most commonly used commercial extraction systems are based on this principal, which includes both column-based and magnetic bead-based systems.

In brief, using silica particles as an example, the extraction methodology consists of four steps (Boom et al., 1990);

1. Lysing the starting material using detergents in the presence of protein degrading enzymes
2. Suspension of the lysed material with a chaotropic agent (i.e. gaunidium thiocyanate) and silica particles results in an instantaneous binding of nucleic acids to the silica particle surface
3. Washing the silica beads several times for effective removal of unwanted substrates, particularly PCR inhibitors
4. Separation of DNA bound to silica particles occurs by eluting into a low-salt buffer (i.e. Tris-EDTA buffer) thereby decreasing the chaotropic agent concentration.

The sample output from this process is purified nucleic acids, which are compatible with downstream molecular applications. Several adaptations of these principles have given rise to both spin-column based systems such as the QiaAMP DNA mini kit (Qiagen) and silica-coated magnetic bead systems (NucliSENS EasyMAG/Roche MagNA PURE) with comparable outcomes (Tan and Yiap, 2009). For routine purposes, particularly involving

infectious pathogens, automation is the best-suited methodology as it protects the user from biological hazards, has a low turnaround time, reduces human resources and produces reproducible results by minimizing human error (Taylor et al., 1990, Knepp et al., 2003, Dundas et al., 2008, Dauphin et al., 2009).

8.6. Nucleic acid amplification test (NAAT) methods

Traditionally, NAATs/PCR was carried out using conventional thermocyclers and the resulting products were detected using DNA gel electrophoresis together with a UV fluorescent DNA-intercalating dye. Real-time PCR has replaced these methods where the PCR reaction as well as detection occurs simultaneously. The use of a single reaction vessel for this concurrent reaction therefore reduces the risk of contamination and decreases the overall work load (Espy et al., 2006).

Two types of PCR assays are commonly found; “in-house” or commercial standardized assays. The former is based on protocols developed in a non-commercial environment. Commercial assays, which undergo a standardized manufacture process show better reproducibility compared to “in-house” assays with both making use of the most common genetic targets such as the *IS6110, MBP64, rpoB and hsp65*. Several commercial PCR assays are available, where each differs to the genomic region it targets of the *M. tuberculosis* complex. These kits include: the GenProbe Amplified *M. tuberculosis* Direct test (AMTD) (Gen-Probe Inc., San Diego, CA, USA), the Roche Amplicor MTB test (Roche Diagnostics Inc., Indianapolis, USA), the Cobas Amplicor test (Roche Diagnostics Inc., Indianapolis, USA), the Abbott LCx test(Abbott Laboratories, Chicago, USA), LightCycler Mycobacterium detection kit (Roche Diagnostics, Mannheim, Germany), the BD-ProbeTec (SDA) test (Becton Dickinson, Madison, USA). In a meta-analysis of commercially available NAATs including Amplicor-MTB , Cobas Amplicor MTB, Becton Dickinson ProbeTecET, E-MTD and LCx, the overall pooled sensitivity was 85% and specificity 96% for acid fast bacilli smear-positive specimens (Ling et al., 2008). The meta-analysis showed a sensitivity of 66% and specificity of 98% for AFB smear-negative specimens (Ling et al., 2008). Commercially available PCR assays are widespread, limited only by their need for specialized equipment. This drawback explains the reliance that many diagnostic laboratories place on “in-house” assays. All of these amplification techniques, whether “in house” or

commercial require sample preparation, amplification and detection to arrive at a result (Niemz et al., 2011).

The majority of the above-mentioned PCR techniques exploit the presence of a common repetitive DNA insertion sequence, such as *IS6110*, due to a multiple copy occurrence of this element in the *M. tuberculosis* genome (Kent et al., 1995, Kremer et al., 1999, Ani et al., 2009, Aryan et al., 2010, Armand et al., 2011, Miller et al., 2011). In addition to *IS6110*, other target genes include *MBP64* (coding for the immunogenic *M. tuberculosis* complex-specific protein MPT64) (Aziz et al., 2004, Baba et al., 2008), *rpoB* (portion of the *M. tuberculosis* gene encoding the beta subunit of the RNA polymerase) (Blakemore et al., 2010, Helb et al., 2010) and *hsp65* (gene encoding the heat shock protein 65) (Varma-Basil et al., 2013).

With pulmonary TB diagnosis, two types of specimens must be taken into account; i.e. smear-positive and smear-negative (low to negligible bacterial load) specimens. From the above commercial PCR assays, only a select few have FDA approval for respiratory specimens. The AMTD and Amplicor tests are licensed for testing smear-positive specimens, while the FDA recently approved a 2nd-generation AMTD (E-AMTD) (Gen-Probe Inc., San Diego, CA, USA) test for smear-negative specimens (Ling et al., 2008).

Other PCR formats are the recently developed—the Loop-mediated Isothermal Amplification (LAMP) test (with limited research experience) and the Xpert. The Xpert is not only capable of detecting *M. tuberculosis* complex but rifampin resistance as well making it a highly valuable system for TB diagnostics. It is a fully-automated system, which performs extraction to detection within a single cartridge in 120 minutes. Initially, due to the simplicity of the assay it was thought to be placed as a point of care diagnostic (Boehme et al., 2010, Boehme et al., 2011). However, due to concerns of handling infectious material without the required biosafety resulted in its positioning at near patient facilities (primarily microscopy centers). The assay requires only two steps prior to loading the cartridge. Sputum samples, which are added to a decontamination /liquefying solution (SR buffer; Cepheid, Sunnyvale, CA, USA), are transferred to the cartridge as the starting point for this procedure. This is followed by automated DNA extraction, hemi-nested PCR and detection of the *rpoB* gene.

Early data suggest a sensitivity of between 75 - 90% and specificity ranging between 97% for pulmonary TB (Blakemore et al., 2010, Boehme et al., 2010, Helb et al., 2010, Armand et al., 2011, Blakemore et al., 2011, Bowles et al., 2011, Evans, 2011, Marlowe et al., 2011, Miller

et al., 2011, Scott et al., 2011b, Chang et al., 2012, Friedrich et al., 2013, Lawn et al., 2013, Sudarsanam and Tharyan, 2013). The primary concern, however, voiced by the WHO as well as other TB experts is the adaptation of this to resource-poor settings (Kirwan et al., 2012, WHO, 2013). This is due to the fact that the instrumentation is dependent on adequate infrastructure including; a stable electrical supply, temperature-controlled storage space for cartridges (with a maximum storage temperature of 28°C) and associated cost of equipment and consumables (Clouse et al., 2012, Carman and Patel, 2014, WHO, 2014c). The impact on morbidity would not be realized without the assay being placed at its intended position i.e. point of care (Lawn et al., 2012).

8.6.1.Technology pipeline for molecular methods

Although no new technology has been endorsed by the WHO after the Xpert, several molecular technologies have been developed and approved for use in certain countries or regions. Majority of these assays are based on the design of the Xpert (all-in-one extraction, amplification and detection) and are known as the "fast followers" (Niemz and Boyle, 2012, UNITAID, 2013). Most of them are as yet not ready for WHO policy review due to limited performance data. Larger powered studies are required to determine the diagnostic accuracy of these technologies and their feasibility for implementation as these assays are performed on their specific equipment.

Epistem's (Manchester, UK) Genedrive Mycobacterium ID test-kit is real-time PCR based and designed for poor resource settings. It is a portable, light, benchtop real-time PCR instrument able to perform a single test within 45 minutes. The sample processing is independent of the instrument using the concept of dry chemistry (as in the case of FTA cards) and can be completed within 10 minutes. The cartridge contains three tubes containing lyophilized regent, one for the detection of *M. tuberculosis*, the second for rifampicin resistance and the final tube an internal control. Punched segments from the extraction cards are inserted into the tube with molecular grade water and inserted into the Genedrive. Limited data is currently available on this assay; with published data on simulated sputum specimen showing a sensitivity of 90.8% and specificity of 100%. In addition, this assay showed better detection for *M. tuberculosis* in the scanty/negative smear grading compared to the Xpert (Castan et al., 2014).

Molbio Diagnostic's (BigTech Private Ltd. And Tulip Group, India) Truelab *Mycobacterium tuberculosis* detection system is a semi-automated extraction to detection system. The extraction system is based on solid phase extraction using silica coated magnetic beads. The extract is then transferred to a novel chip (on which thermo-cycling conditions are individually controlled in comparison to the traditional PCR systems which have thermo-cycler block control). Within this chip, amplification and detection occurs using a PCR analyzer. The real advantage of this system is that it can operate using battery power and can even incorporate Wi-Fi technology (wireless internet), which further allows transfer of information (including wireless printing) from the instrument to any location. According to manufacturer's protocols, the entire process from sample extraction to detection can be completed within an hour. Early evidence on the performance of this technology showed the assay to have a sensitivity of 91.1% and 100% specificity in a cohort of 226 suspected TB patients (Nikam et al., 2013).

NATeasy TB diagnostic system, developed by Ustar Biotechnologies (China), is based on DNA extraction, isothermal amplification targeting the *IS6110* element of *M. tuberculosis* and nucleic acid lateral flow end point detection (Niemz and Boyle, 2012, UNITAID, 2013). Several ancillary pieces of equipment found commonly in most laboratories are required for processing. Reagents could be store at ambient temperatures for a maximum of two months; however, longer storage would require amplification reagents to be stored at -20°C and 2 – 8°C for extraction materials. Due to these requirements and infrastructural gaps in TB burdened countries, the company is working on developing thermo-stable reagents. In a study evaluating this technology, without the use of the system's current extraction process, the assay had a sensitivity of 92.2% and specificity of 98.8% (Fang et al., 2009).

PCR assays show poor performance in smear-negative and extra-pulmonary disease (Ling et al., 2008). Possible factors may include the low bacilli number present or the method of extracting the nucleic acid (Pai, 2004, Pai et al., 2004, Trajman et al., 2008). Over time, as detailed above, limitations in PCR and PCR-based assays have been addressed by i) combining amplification and detection to a single reaction vessel (Real Time PCR); ii) using polymerases with higher proof reading capacity and iii) enhanced PCR parameters, which assist in the amplification of low DNA quantities and iv) optimization of nucleic acid extraction.

9. Summary

With the continuous advancement of TB diagnostics, resource fatigue is a key issue that needs to be addressed. Novel diagnostics requiring validation cannot continuously be purchased and the likelihood at present of a diagnostic surpassing the reference standard is low. What is actually needed is an all-in-one solution, bypassing the implementation of several technologies based on similar principles for diagnosis infectious diseases. With current molecular infrastructure in place, adaption and improvements of specimen collection is necessary to allow for centralized testing on shared platforms. Technologies placed at point of care may improve outcomes in infected patients; however, does not necessarily impact on long-term changes in morbidity (Theron et al., 2014). This particularly applies to high infectious disease burdened, resource-poor countries. As poor infrastructure is a limiting step for implementation of advanced diagnostics; centralized facilities have the capacity to perform these tests. Telecommunications in most of these countries are highly advanced, which could be utilized for transferring results to facilities. Innovative methods are currently required to address limitations in poor-resourced countries to address escalating burdens of disease. The present study was performed to address the issues discussed above by providing a supportive framework, which assists a specific component in the diagnosis of TB in resource-poor setting. This includes the application of a transport medium for the collection of sputum specimens to the diagnostic facilities without compromising the specimen's integrity during transport.

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CHAPTER 3

Laboratory evaluation of a specimen transport medium for downstream molecular processing of sputum samples to detect *Mycobacterium tuberculosis*

Editorial Style of the Journal of Microbiological Methods was followed for this Experiment

1. ABSTRACT

Background: Modern molecular-based approaches for the detection of *M. tuberculosis* in sputum samples promise quicker and more accurate detection of cases. However, processing sputum samples at central diagnostic facilities provides a diagnostic approach, but requires a safe and efficient system that is not affected by transport delays and ambient temperature to be feasible. We evaluated the technical properties of PrimeStore® - Molecular Transport Medium (PS-MTM) for its ability to inactivate mycobacteria, ensuring stability of DNA over time at ambient temperatures and to assess the compatibility of the transport medium with DNA extraction systems.

Methods: Assessment of the transport medium for application of sputum samples processed for the detection of *M. tuberculosis* included; the inactivation of *M. tuberculosis* in spiked sputum samples, compatibility of the medium with three commercial nucleic extraction systems and stability of DNA in the medium at ambient temperature over 28 days. We further performed a clinical laboratory evaluation on 256 sputum specimen sent for tuberculosis investigation.

Results: Complete inactivation of *M. tuberculosis* occurred within 30 minute of exposure at ratio of 1:3 for sputum to PS-MTM. Sputum specimen in PS-MTM showed very good compatibility with automated bead-based extraction systems, producing high DNA output (estimated lower limits of detection: ~170 CFU/mL). Furthermore, PS-MTM samples remained stable over 28 days at ambient temperature displaying no significant change over time in Ct-values (<5% on a mean starting value of 22.47). Of 256 clinical sputum specimens, 10.2% were culture positive and 11.0% positive by real-time PCR of PS-MTM samples.

Conclusions: Collecting and transporting sputum from TB suspects in PS-MTM offers safe transport at ambient temperature, DNA stability for extended periods without cooling and

specimens directly suitable for molecular testing. This novel approach may support introduction and further scale-up of molecular diagnostics for TB in resource-limited settings.

Keywords

Mycobacterium tuberculosis; specimen transport medium; molecular detection; DNA stability

2. INTRODUCTION

Current tuberculosis (TB) infection rates are estimated to be the highest in the history of the disease, where 22 middle-to-low income countries are burdened with 80% of active disease (WHO, 2010, Paulson, 2013). To improve diagnosis of TB, the World Health Organization (WHO) promotes the early introduction of rapid molecular testing globally (WHO, 2013). In light of that, South Africa has replaced smear microscopy with the molecular Xpert MTB/RIF assay (Xpert, Cepheid, Sunnyvale, CA, USA) in its National algorithm. A similar scenario may not be possible in other countries due to relatively high associated cost and poor infrastructure. This warrants exploration of alternative approaches to introduce molecular diagnostics in high-burden countries, for example by transporting sputum specimens to a centralized molecular diagnostic facility. In that regard, safe (inactivated for purpose of infection control) and efficient (preservation and stability of DNA) transportation of sputum specimens is of paramount importance.

Several methods have been described for inactivation of viable *Mycobacterium tuberculosis* from cultures by either heat or a combination of heat and the use of chemicals (Doig et al., 2002, Djelouagji and Drancourt, 2006, Inoue et al., 2014). However, the only relevant published data on the effect of reagents added directly to sputum on the viability of TB bacilli is the sample reagent buffer (SR) of the Xpert assay. The SR has demonstrated capability of producing an 8 log reduction, within 15 minutes, in *M. tuberculosis* viability in sputum spiked with this organism (Helb et al., 2010). In *M. tuberculosis*-positive sputum specimens, with bacillary loads of less than 10^7 colony forming units/milliliter (CFU/ml), total inactivation was achieved within 15 minutes (Banada et al., 2010). In practice, the SR buffer is only added to the sample when processed at the laboratory and not intended for specimen transport.

The effect of ambient transportation and subsequent delays to laboratory testing on sputum *M. tuberculosis* bacillary and DNA loads for molecular testing is not known, unlike the documented negative effects of delay on culture yield of sputum specimens (Paramasivan et al., 1983, Banda et al., 2000). However, studies on other pathogens in different clinical specimens have shown a negative effect on molecular testing when kept at ambient temperature for extended periods of time (Ingersoll et al., 2008, Hasan et al., 2012). In that context, the manufacturer of the Xpert assay recommends that sputum samples be kept for no longer than 3 days at ambient temperatures up to 35° C until processing (Banada et al., 2010). Delays in transport and testing could negatively affect the diagnostic yield when using sputum specimens.

A liquid transport medium, PrimeStore® - Molecular Transport Medium (PS-MTM); Longhorn Vaccines & Diagnostics, San Antonio, TX, USA) is a commercial specimen collection and transport solution, specifically formulated for downstream molecular diagnostic testing. The proposed use of this chemically-defined medium, composed of chaotrophic, chelating, reducing and defoaming agents, detergents and electrolytes to buffer to an optimal pH, is for inactivation and lysis of biological pathogens as well as stabilizing/preserving the released nucleic acids (DNA and RNA) for prolonged periods. PS-MTM has been reported to reduce viability of microorganisms almost completely (99.9%) using a standard panel of variety of viruses, bacteria and fungi, but not including *M. tuberculosis* (Daum et al., 2011b). Furthermore, complete viral inactivation of enveloped viruses occurred within seconds after being placed in PS-MTM and viral RNA was preserved for up to 30 days at ambient temperature (Daum et al., 2011b).

Expanding potential use of PS-MTM to TB diagnostics, a recent study of PS-MTM showed inactivation within 30 min of *M. tuberculosis* at a bacillary load of 10^6 CFU/ml (Daum et al., 2014). Based on the relatively scant information on the use of PS-MTM for TB diagnosis and research, as indicated above, insight into its potential application in routine diagnostics of TB is warranted. In this study, we aim to determine further the performance and safety potential use of PS-MTM as a collection vehicle and transport preservation method of sputum specimens for molecular detection and characterization of *M. tuberculosis*.

3. MATERIALS AND METHODS

3.1. Design and steps of evaluation

The value of PS-MTM was determined in several steps: (a) potential for inactivation of *M. tuberculosis* at high bacterial loads with both cultured bacilli as well as spiked sputum samples, (b) effect on preservation of *M. tuberculosis* DNA over time, (c) compatibility with various downstream nucleic acid extractions systems, (d) use of swabs to inoculate sputum into PS-MTM tubes, (e) laboratory diagnostic performance of this system on clinical sputum specimens. Ethics approval for this study was obtained from the University of Pretoria's Research Ethics Committee of the Faculty of Health Sciences (129/2010)[1].

3.2. Preparation of *Mycobacterium tuberculosis* suspensions for laboratory evaluations

Standard *M. tuberculosis* suspensions were prepared by growing the *M. tuberculosis* H37Rv laboratory strain (ATCC 27294) as per routine protocol[2] to the turbidity of a 0.5 McFarland standard; the equivalent concentration of ~1.5 x 10^8CFU/ml (Bollela et al., 1999, Murray and Baron, 2007, Global Laboratory Initiative, 2014). The optical turbidity was measured using the PhoenixSpec Nephelometer (BD Diagnostics, Franklin lakes, NJ, USA) against a standard curve. A series of ten-fold dilutions was prepared from the McFarland standard, producing concentrations ranging from 10^6 to 10^1 CFU/ml. Manual colony counts to confirm the dilution series plated on Middlebrook 7H11 agar medium were performed for the concentrations that could be enumerated (10^1-10^3 CFU/ml).

3.3. Inactivation of *Mycobacterium tuberculosis* in PrimeStore® - Molecular Transport Medium

The ability of PS-MTM to inactivate *M. tuberculosis* was assessed for purified culture isolates and spiked sputum samples. A 0.5 ml suspension of *M. tuberculosis* H37Rv strain (~1.5 x 10^8cfu/ml) was inoculated into 1.5 ml of PS-MTM, briefly vortexed and sampled after ambient temperature incubation for 5, 10, 20, 40, 80 and 160 seconds. Sample aliquots of 0.2 ml were directly plated on Middlebrook 7H11 agar[3] and 0.5 ml inoculated into liquid culture and

[1] APPENDIX A #13
[2] APPENDIX A #7
[3] APPENDIX A #5

processed using the Bactec Mycobacterial Growth Indicator Tubes (MGIT) 960™ (Becton Dickinson Diagnostics, Sparks, MD, USA) as previously described[4] (Siddiqi and Rusch-Gerdes, 2006). A positive control was included (suspension in saline without PS-MTM) and the experiment was performed in triplicate. Sputum samples not submitted for TB investigation were obtained from the routine diagnostic laboratory at the University of Pretoria and assessed for the presence of acid-fast bacilli (AFB) by smear microscopy, cultured by MGIT to confirm the absence of *M. tuberculosis* followed by quality assessment using the Bartlett Scoring System (Winn Jr et al., 2005). Good quality purulent sputum specimens (Bartlett test score of 2+) were included for use in the spiking matrix experiments. These sputa were split and spiked with *M. tuberculosis* H37Rv strain with concentrations of 1.5 $x10^6$ and 1.5 x 10^8 CFU/ml followed by inoculation into PS-MTM (without decontamination or other pre-culture steps). A matrix assessment to determine effect of concentration was performed by adding to 1ml of spiked sputum 3, 2, 1, 0.5 and 0.2 ml of PS-MTM. Samples were incubated in triplicate at ambient temperature for 1, 5, 10, 30, 60 and 180 minutes, including two positive and negative controls, and analyzed using the MGIT 960 system. Effective inactivation at certain concentration and time point was defined as no growth in all samples after 42 days.

3.4. Evaluation of DNA stability in sputum inoculated in PrimeStore® - Molecular Transport Medium

Three sputum specimens, positive for *M. tuberculosis* on microscopy for AFB, with a smear microscopy grading of 3+ were included in this evaluation. The sputum specimens were split into two equal aliquots with one added to PS-MTM at 1:2, and one refrigerated, with sterile water added to the latter aliquots at the same ratio as PS-MTM. Nucleic acid extraction using NucliSENS easyMAG was performed on all test samples at baseline and weekly intervals over a 4 week period. This time period would be sufficient for the primary diagnosis, extended delays due to logistic challenges in countries with poor infrastructure and if needed specimen availability for additional reflex molecular testing if required. Preservation of DNA was measured by real-time PCR on each of the extracts where an absence of significant decrease in cycle-threshold (CT) scores over tested time points indicates stability**.**

[4] APPENDIX A #6

3.5. Compatibility with three DNA extraction systems

Ten-fold dilutions of the *M. tuberculosis* stock solutions were prepared to a range of 10^1 to10^6 CFU/ml and spiked into 20 remnant clinical culture-negative sputum samples (as described in 2.2 and 2.3). These sputum samples were inoculated into PS-MTM at a volume ratio of 1:2. The final concentration of *M. tuberculosis* in the spiked sputum ranged from an estimated 3 to 250 000 CFU/ml. Aliquots of sputum in PS-MTM were prepared and processed using the following DNA extraction systems as per manufacturers' instructions: the QiaAMP DNA mini kit[5] (Qiagen, Hilden, Germany), the MagNA Pure 96 System (Roche Diagnostics, Mannheim, Germany) using the DNA Bacterial/Viral small volume kit[6], the NucliSENS easyMAG (Biomerieux, Marcy I'Etoile, France) using the generic protocol[7]. The all-in-one extraction and detection system, the Xpert MTB/Rif assay was performed as a control for routine detection; an aliquot of sputum sample was tested by Xpert before inoculating in PS-MTM, as instructed by the manufacturer[8] (**Fig. 1**). An input volume of 200µl PS-MTM sample was used with 50µl output volume except in the case of MagNA Pure 96 (Roche, Germany) where the final volume was 100µl. Real-time PCR (Daum et al., 2011a) targeting the *M. tuberculosis* specific insertion sequence element 6110 (*IS6110*)[9] was used on the LightCycler 480II platform (Roche Diagnostics, Mannheim, Germany) to detect *M. tuberculosis* from DNA extracts.

3.6. Evaluation of using swabs to inoculate sputum into PrimeStore® - Molecular Transport Medium for molecular testing

Flocculated cotton swabs (Copan Diagnostics Inc., Brescia, Italy) were evaluated for capacity to inoculate sputum into PS-MTM for downstream molecular testing. This swab-for-inoculation procedure was further evaluated in fourteen sputum specimens that were culture positive for *M. tuberculosis* and with a known smear microscopy grading for AFB. The volume captured on swab was determined by the difference in swab weight pre- and post- collection using a calibrated laboratory scale (Adam Equipment Co. Ltd, Milton Keynes, UK). As a control the remaining volume was pipetted into an equal volume of PS-MTM. After inoculation, PS-MTM

[5] APPENDIX A #8
[6] APPENDIX A #9
[7] APPENDIX A #10
[8] APPENDIX A #12
[9] APPENDIX A #11

tubes were kept overnight at ambient temperature followed by DNA extraction on the MagNA Pure 96 System and real-time PCR by LightCycler 480II.

3.7. Evaluation of detecting *Mycobacterium tuberculosis* in clinical samples stored in PrimeStore® - Molecular Transport Medium

A total of 297 sputum specimens sent for TB investigation were prospectively collected at the Tshwane Academic Division (University of Pretoria) of the National Health Laboratory Service, South Africa. Specimens were included irrespective of quality; however, a minimum volume of 2 ml was required to prevent compromising the routine testing. From each specimen, an aliquot was transferred into PS-MTM using the swab procedure. Routine diagnostic testing of specimens was performed using fluorescent microscopy[10] (Auramine-O), mycobacterial culture on the MGIT 960 system with confirmation of *M. tuberculosis* in positive cultures by the TBcID rapid antigen test (BD, Sparks, MD, USA). DNA was extracted from the specimens stored in PS-MTM using the MagNA Pure 96 System followed by real-time PCR for *M. tuberculosis* detection by LightCycler 480II.

[10] APPENDIX A #2 & 4

4. RESULTS

4.1. Inactivation of *Mycobacterium tuberculosis* in PrimeStore® - Molecular Transport Medium[11]

In order to assess the inactivation ability of PS-MTM, the high concentration suspension (of the reference strain was inoculated in triplicate into PS-MTM, stored at ambient temperature and followed by sampling after different interaction times (min. 5 to max. 60 seconds). There was no growth of *M. tuberculosis* after 42-day incubation period in the MGIT 960 liquid culture system and on solid media compared to the positive control where growth was observed after 11 and 14 days respectively in the different culture systems.

Smear-negative sputa with a positive Bartlett score were spiked with *M. tuberculosis* concentration of 1.5 x 10^6 CFU/ml showed no growth after 42 days at the ratios of 1:1, 1:2 and 1:3 for sputum vs. PS-MTM, except for one specimen at the one minute interval (**Tab.1**). The latter showed growth in liquid culture after 24 hours, but a sub-culture of this same sample (on Middlebrook 7H10 agar) showed no mycobacterial growth. Full growth of *M. tuberculosis* was observed when the volume of sputum exceeds the volume of PS-MTM (2:1 and 5:1). Erratic growth was observed at each time point at the higher inoculation concentration (1.5 x 10^8 CFU/ml) with a 1:1 ratio. An exposure time of 60 minutes was required for inactivation of *M. tuberculosis* with a ratio of at least 1:2 for sputum vs. PS-MTM.

4.2. Evaluation of DNA stability in PrimeStore® - Molecular Transport Medium

Three microscopy smear-positive samples were split into two aliquots and stored in either PS-MTM at ambient temperature or in a normal sputum container, without processing, at refrigeration temperature (4° C). PCR detection of *M. tuberculosis* DNA from these containers was done on weekly basis for a period of four weeks. There was no significant change in Ct-value (<5%) over time observed for samples stored in PS-MTM or for those stored at 4° C in a normal sputum container (**Tab. 2**).

[11] APPENDIX B Table 1 & 2

4.3. Compatibility with DNA extraction systems[12]

The automated magnetic bead-based extraction systems (NucliSENS EasyMAG and MagNA Pure 96 System) had estimated lower limits of detection of 169 and 173 CFU/ml respectively producing the highest DNA yield from specimens stored in PS-MTM (**Fig.2 & Tab.3**). The yield was higher than for the silica-based extraction system (QiaAMP) which detected all replicates up to the 25000 CFU/ml concentration, but missed an increasing proportion of replicates at subsequent (lower) concentrations (2,500-25,000 CFU/ml) that were not missed by the bead-based methods (**Fig.2 & Tab.3**). A significant difference was seen between the MagNA Pure and positive control (Xpert system) in their ability to detect positives a various concentration, with MagNA Pure maintaining its ability to detect positives at the lower CFU/ml concentration (Chi-square =15.23 ;p=0.009). None of the other systems produced significantly different results from the Xpert control.

4.4. Swab procedure for inoculation of sputum into PrimeStore® - Molecular Transport Medium tubes

Using clinical culture-positive specimens, the estimated median volume of sputum captured by the swabbing procedure was 50µl and ranged from 50 µl – 250µl where samples with a higher viscosity (purulent) retained a lower volume of sample compared to those less viscous (salivary) (**Tab.4**).*M. tuberculosis* DNA was detected by real-time PCR in all samples using the swabbing procedure and in all except one of the pipetted samples. Cycle threshold values were generally lower for pipetted specimens, but the swab to PS-MTM ratio was relatively higher for those samples (1:1). DNA concentrations after extraction ranged between 231 and 281 ng/µl (data not shown). These concentrations comprise DNA of *M. tuberculosis* as well as DNA from host cells and commensal organisms present in sputum samples.

4.5. Detection of *Mycobacterium tuberculosis* in clinical samples stored in PrimeStore® - Molecular Transport Medium[13]

Of the 297 specimens prospectively collected; 41 were excluded from routine diagnostics due to poor sputum quality in 8 (2.7%) or culture contamination in 33 (11.1%) of which *M. tuberculosis*

[12] APPENDIX B Table 3a, b & c
[13] APPENDIX B Table 4

was detected in 5. Thus, from the specimens analyzed (n=256), 26 (10.2%) were culture positive including 13 (5.1%) that were smear-positive. Real-time PCR of sputum in PS-MTM was positive in all smear-positive specimens (13/13) and 7/13 (54%) of smear-negative culture-positive sputum specimens (**Tab. 5**). PCR was positive in another 8 specimens (3.1%) that were negative by both microscopy and culture.

5. DISCUSSION

This laboratory evaluation demonstrates that PS-MTM rapidly inactivates *M. tuberculosis* in sputum specimens thereby rendering the specimen safe, compatible with various nucleic acid extraction systems and suitable for downstream molecular processing in a routine diagnostic setting. These observations are in line with two studies describing evaluation of this transport medium for molecular processing of sputum specimens for respiratory viruses (Daum et al., 2011b, Schlaudecker et al., 2014). Our observations are of importance as concentrations of viral DNA or RNA are generally higher than of *M. tuberculosis* DNA in sputum samples.

Inactivation of the organism was measured by the ability to grow in an internationally standardized culture medium; the current gold standard to viability testing (van Zyl-Smit et al., 2011). Maximum bacillary load of *M. tuberculosis* in a clinical sputum specimen is approximately 10^6 organisms/ml (Rieder et al., 2007); therefore we performed most laboratory experiments at this concentration and a 100-fold higher. With regard to mycobacterial inactivation, *M. tuberculosis* in pure culture, grown to a concentration of 10^8 CFU/ml, was inactivated within 5 seconds of exposure to the PS-MTM transport medium. However, time to inactivation of *M. tuberculosis* was considerably longer in the proteinaceous matrix of sputum. The sputum to PS-MTM ratio is important and should be at least 1:2 to achieve complete inactivation in a reasonable period of time (60 minutes; 30 minutes at 1:3 ratio) at *M. tuberculosis* concentration of 1.5 x 10^8 CFU/ml. The rapid inactivation is in line with findings from Daum *et al.* (2014), which showed that PS-MTM is an effective medium to inactivate a pure culture of *M. tuberculosis* at ~10^6 CFU/ml at an exposure time of 30 minutes. This is a similar effect as achieved by the Xpert SR buffer where an 8 and 9 log reduction was demonstrated at 15 minutes and 2 hours respectively (Helb et al., 2010). A minimum time of 60 minutes from collection to specimen processing is likely to be routine and will provide a safe specimen for downstream testing, particularly in resource-constrained settings.

Stability of *M. tuberculosis* DNA in PS-MTM at ambient temperature was demonstrated for a period of 4 weeks. This period is more than sufficient to use PS-MTM in a diagnostic approach. However, additional follow-up testing is warranted to determine the value of PS-MTM for long-term bio-banking. A limitation of this evaluation was not directly comparing the effect on DNA stability in sputum at ambient temperature in comparison to the PS-MTM sample.

PS-MTM was compatible with the most commonly used DNA extraction systems and in most cases did not differ in their ability to detect positives. Thus it could be used in most laboratories, regardless of their set-up, and for a variety of downstream molecular applications. The two automated magnetic bead-based extraction systems (NucliSENS easyMAG and MagNA Pure system), ideal for routine services due to their robustness, performed best and demonstrated similar high performance, efficiency and reproducibility (Dundas et al., 2008). The observed difference in performance between magnetic bead and silica extraction systems may be due to loss of DNA released by PS-MTM, particularly during processing prior to silica binding.

Evaluation of almost 300 clinical sputum specimens using PS-MTM as pre-step demonstrated good sensitivity and specificity compared to smear microscopy and liquid culture. Performance was in line with generally accepted characteristics of molecular testing for *M. tuberculosis*: 90-100% sensitivity in microscopy smear-positive sputum specimens and 55-75% among smear-negative cases (Dinnes et al., 2007, Armand et al., 2011, Miller et al., 2011, Steingart et al., 2014). There was no apparent effect of chemical composition of PS-MTM on detection of lower bacillary loads as diagnostic performance among smear-negative cases was adequate.We did not test amplification suppression, but think that the effect, if present, was minimal as we were able to successfully amplify *M. tuberculosis* at relatively low CFU/ml as well as detect smear-negative TB cases in the clinical laboratory evaluation. This was achieved by transferring a very small quantity of the primary sputum specimen (0.2 ml) into PS-MTM. Although the volume captured on a swab is only an estimated fifth of the volume required for the Xpert assay, this small volume was sufficient to adequately detect *M. tuberculosis* at sensitivities similar to that of the Xpert assay.

The method of using swabs to inoculate sputum into PS-MTM provides an adequate yield for molecular processing. Moreover, it provides a practical approach that allows healthcare workers to directly inoculate sputum into PS-MTM for rapid inactivation and stabilization after the specimen is produced. Such an alternative method is warranted as pipetting sputum at healthcare facilities would not be feasible.

6. CONCLUSIONS

PS-MTM may have several applications in addition to the diagnosis of *M. tuberculosis* including: safe transport and preservation of specimen for molecular testing, its inactivating capability of pure cultures could allow the convenient ambient temperature transport of the organism for surveillance activities (i.e. molecular typing and sequencing) and ambient temperature molecular bio-banking which could remove the current storage costs associated with the conventional method. Continuous improvements in molecular diagnostics require innovative methods for transporting specimens safely, efficiently and without compromising integrity that may influence diagnosis. With regard to *M. tuberculosis* detection, PS-MTM provides a promising tool for transport between clinical and centralized diagnostic facilities for molecular testing which could expand molecular diagnosis of tuberculosis in resource-poor settings.

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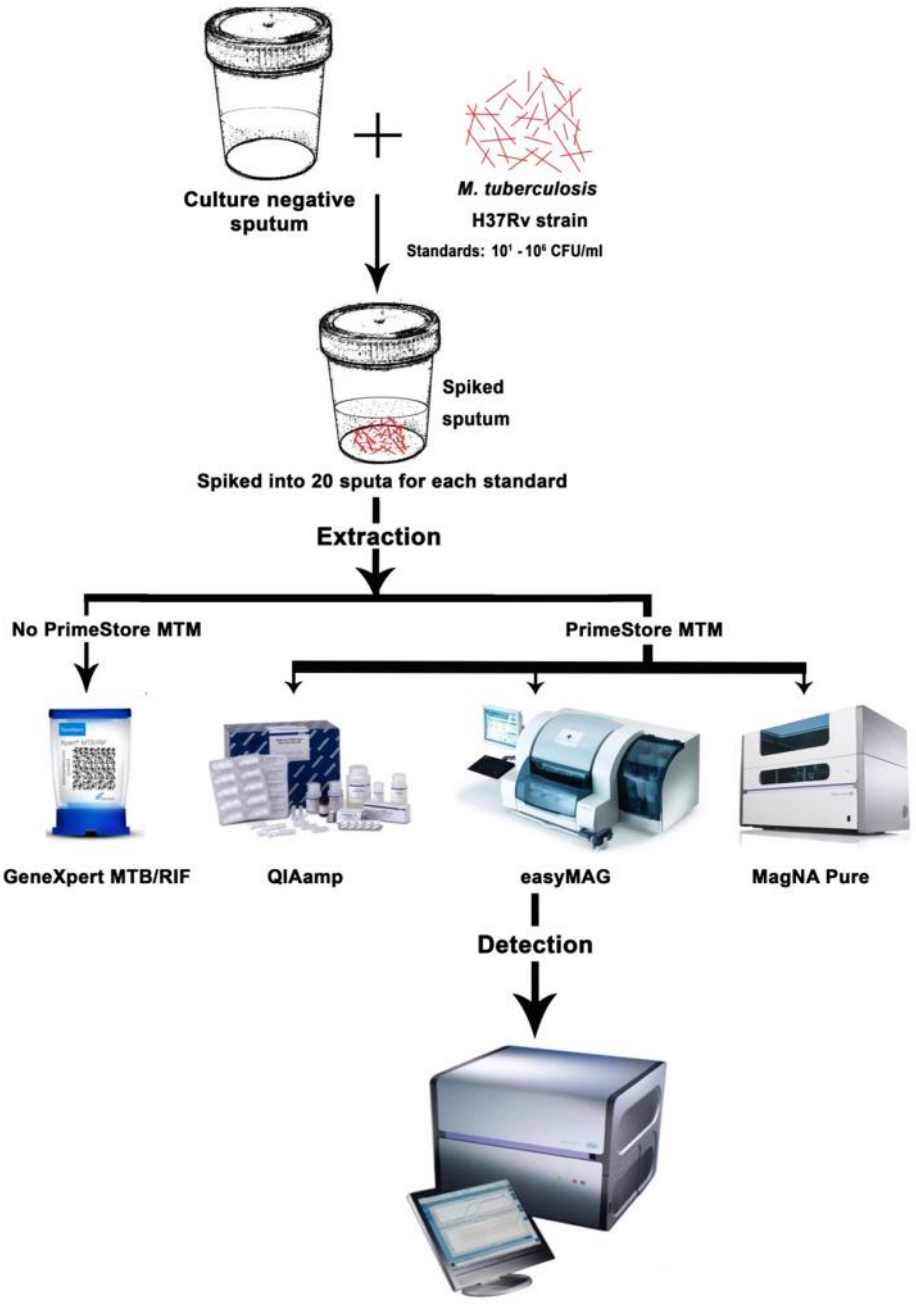

Figure 1. Experimental design for assessment of compatibility of PrimeStore® - Molecular Transport Medium with different molecular platforms

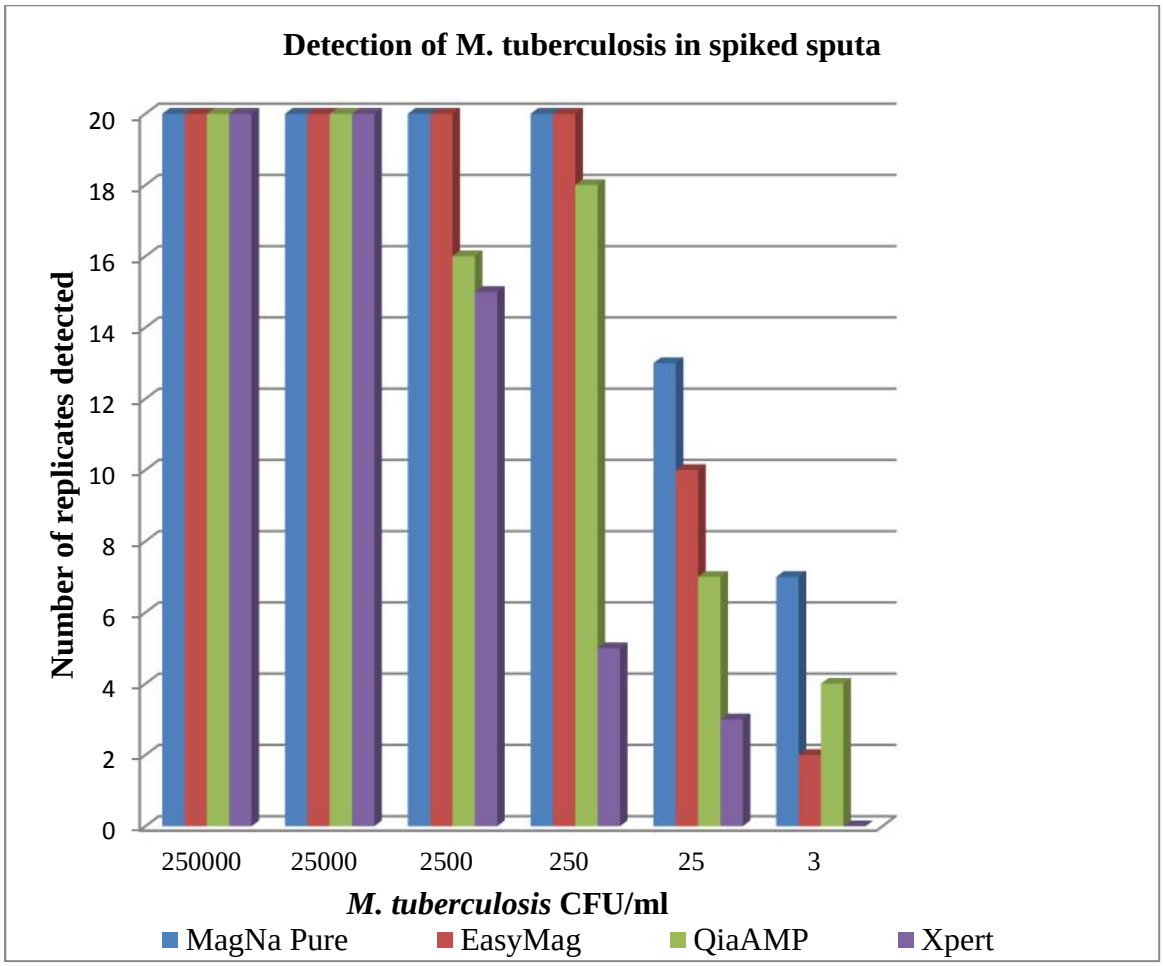

Figure 2. Detection of *M. tuberculosis* spiked in sputum, by real-time PCR of twenty replicates per concentration, processed by each of the nucleic extraction systems evaluated (MagNA PURE, easyMAG & QiaAMP) and Xpert MTB/RIF assay as the reference method

Table1: Culture detection of *M. tuberculosis* from sputum samples spiked into PrimeStore® - Molecular Transport Medium at varying concentration, volume ratio and exposure time.

Concentration of *M. tuberculosis* spiked in sputum	Ratio of spiked sputum to PS-MTM	Exposure time (minutes)						
		1	5	10	15	30	60	180
1.5 x 10^6 CFU/ml	0.33:1	0	0	0	0	0	0	0
	0.5:1	1/3	0	0	0	0	0	0
	01:01	0	0	0	0	0	0	0
	02:01	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	05:01	3/3	3/3	3/3	3/3	3/3	3/3	3/3
1.5 x 10^8 CFU/ml	0.33:1	0	1/3	0	1/3	0	0	0
	0.5:1	3/3	0	0	0	2/3	0	0
	01:01	3/3	1/3	2/3	0	0	1/3	2/3
	02:01	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	05:01	3/3	3/3	3/3	3/3	3/3	3/3	3/3

Table 2: Cycle threshold values of real-time PCR of *M. tuberculosis* DNA from sputum samples kept over a 28-day period in PrimeStore® - Molecular Transport Medium (PS-MTM) at ambient temperature compared to storage in a normal sputum container at refrigeration temperature (4°C).

	Sputum in PS-MTM Ambient temperature			**Sputum only4° C**		
Day(s)	***Sample1***	***Sample2***	***Sample3***	***Sample1***	***Sample2***	***Sample3***
0	22.95	23.48	20.97	22.84	23.09	21.80
7	22.54	23.07	20.58	22.50	22.96	20.64
14	22.58	23.23	20.41	22.85	23.38	20.97
21	22.87	23.47	20.97	23.39	23.60	21.72
28	22.49	22.99	20.57	22.65	23.56	20.97

Note. Ambient temperature in this setting is generally 25-30° C.

Table 3: Performance of DNA extraction systems by real-time PCR detection ofspiked sputum samples with varying concentrations of*M. tuberculosis* stored in PrimeStore® - Molecular Transport Medium (PS-MTM). The number of replicates detected and mean CT scores for those detected are indicated

		***Mycobacterium tuberculosis* CFU/ml in sputum**											
		250,000		**25,000**		**2,500**		**250**		**25**		**3**	
System*	**Lower limit of detection (CFU/ml) [CI 95%]**	**No. of positive replicates**	**Mean CT score (SD)**	**No. of positive replicates**	**Mean CT score (SD)**	**No. of positive replicates**	**Mean CT score (SD)**	**No. of positive replicates**	**Mean CT score (SD)**	**No. of positive replicates**	**Mean CT score (SD)**	**No. of positive replicates**	**Mean CT score (SD)**
		(n=20)		**(n=20)**		**(n=20)**		**(n=20)**		**(n=20)**		**(n=20)**	
MagNA Pure†	169 [62-1,698]	**20**	26.33 (0.97)	**20**	29.96 (1.62)	**20**	33.19 (0.75)	**20**	33.45 (2.63)	**13**	34.71 (0.46)	**7**	34.68 (0.68)
EasyMAG	173 [78-891]	**20**	25.13 (0.59)	**20**	29.32 (2.13)	**20**	32.19 (0.99)	**20**	34.09 (2.98)	**10**	35 (0)	**2**	35 (0)
QiaAMP	6397 [3,162-53,703] [1,660-74,131]	**20**	25.64 (2.62)	**20**	29.40 (2.03)	**16**	32.20 (1.36)	**18**	34.22 (2.81)	**7**	37.00 (1.57)	**4**	35.7 (1.40)
Positive control (Xpert MTB/RIF assay)†	8749 [1,660-74,131]	**20**	23.51 (1.50)	**20**	26.71 (2.23)	**15**	29.20 (3.53)	**5**	29.66 (5.24)	**3**	27.83 (4.51)	**0**	-

* all replicate testing performed on each system used the same spiked sputum samples and were processed by sampling into PS-MTM †The positive control was prepared without PS-MTM

Table 4: Performance of swabbing procedure for transferring sputum into PrimeStore® - Molecular Transport Medium (PS-MTM) for the detection of *M. tuberculosis* in fourteen culture-positive samples with range of smear microscopy grades

	Sample number	Viscosity	Smear Microscopy grade*	Captured sputum Volume (ml)	Volume ratio of Swabbed sputum in PS-MTM	Ct-value of PCR after swab inoculation¥	Ct-value of PCR after pipette inoculation**¥
Smear-positive	1	Purulent	+++	0.05	1 : 30	32.74	28.96
	2	Purulent	+++	0.05	1 : 30	26.56	23.97
	3	Salivary	++	0.15	1 : 10	28.96	26.30
	4	Salivary	+	0.25	1 : 6	31.67	35.00
	5	Purulent	scanty 9	0.05	1 : 30	33.75	31.76
	6	Purulent	scanty 7	0.05	1 : 30	31.85	30.20
	7	Purulent	scanty 1	0.05	1 : 30	34.21	33.59
Smear-negative	8	Purulent	Negative	0.05	1 : 30	33.89	35.00
	9	Purulent	Negative	0.05	1 : 30	32.72	32.68
	10	Salivary	Negative	0.1	1 : 15	29.19	26.16
	11	Purulent	Negative	0.05	1 : 30	34.47	Negative
	12	Purulent	Negative	0.05	1 : 30	33.59	30.05
	13	Salivary	Negative	0.1	1 : 15	33.11	35.00
	14	Salivary	Negative	0.1	1 : 15	Negative	Negative

*WHO smear microscopy scoring system: For 3 scanty samples, numbers 9, 7, 1 indicate AFB/100 FOV

**Sputum was inoculated at 1:1 ratio in PrimeStore (equal volume)

¥No statistically significant differences across all results (smear-positive and negative) between swab and pipette inoculation (Paired T = 0.70, DF=13, p=0.50).

Table 5: Laboratory performance of real-time PCR detection of *M. tuberculosis* from sputum specimens (n=256) inoculated in PrimeStore® - Molecular Transport Medium (PS-MTM) using the swabbing procedure compared to routine smear microscopy and culture

	Culture positive (n=26)		**Culture negative (n=230)**
	Smear-positive (n=13)	**Smear-negative (n=13)**	
PCR positive	13 (100%)	7 (54%)	8 (3.5%)
PCR negative	0	6 (46%)	222 (96.5%)

CHAPTER 4

Field evaluation of a novel preservation medium to transport sputum specimens for molecular detection of *Mycobacterium tuberculosis* in an African setting

Editorial Style of the Journal Tropical Medicine and International Health was followed for this Experiment

1. ABSTRACT

Objectives: We assessed the performance of an innovative method of transporting sputum to centralized facilities for molecular detection of *M. tuberculosis*: using a swab to inoculate sputum in a transport medium, PrimeStore® Molecular Transport Medium (PS-MTM).

Methods: Two sputum specimens were obtained from suspected tuberculosis (TB) patients at rural healthcare facilities in South Africa. A swab taken from each specimen and placed into PS-MTM, prior to it being processed by either liquid culture or Xpert MTB/Rif assay (Xpert).

Results: A selection of 141 patients (including 47 with laboratory-confirmed TB) was included in this analysis. . *M. tuberculosis* was detected at 29% by culture and 29% by Xpert whereas 31% and 36% tested positive by *IS6110* real-time PCR of PS-MTM from the culture- and Xpert-paired specimen respectively. Concordance between the method under evaluation with culture was 82% (McNemar, p=0.55) and 84% (McNemar, p=0.05) for Xpert. Stratified by culture result, detection rate by *IS6110* real-time PCR of PS-MTM was similar to Xpert for patients with positive culture (p=0.32), but significantly higher if culture was negative (p=0.008).

Conclusions: These results suggest that the method of swab collection of sputum into PS-MTM for transport provides a promising application for diagnosis of TB in rural healthcare settings thereby potentially improving the options available for molecular diagnosis of TB in countries incapable of applying decentralized high-tech molecular testing.

2. INTRODUCTION

Tuberculosis (TB) remains one of the greatest eradication challenges this century, with most of the disease burden occurring in resource-constrained settings (WHO, 2014a). Important factors that hamper the eradication of TB include the ineffective vaccine protection (Colditz et al., 1995, Andersen and Doherty, 2005, Roy et al., 2014), delayed turnaround time and suboptimal sensitivity of current diagnostic tests, poor uptake of new diagnostic tools, operational and logistic delays, undiagnosed cases and treatment default (WHO, 2014a). Mycobacterial culture remains the diagnostic gold standard, despite its known delay to report a negative result (Palomino et al., 2007, Lawn et al., 2013, Carman and Patel, 2014). In countries with the highest burdens of TB, microscopy remains the cornerstone for a microbiological diagnosis, especially in settings where culture methods are not readily accessible such as rural health care facilities. The use of molecular methods for detecting *M. tuberculosis* provides an alternative approach for the rapid diagnosis of TB. Several studies highlight the benefits of molecular testing for the detection of *M. tuberculosis* (Haldar et al., 2007, Neonakis et al., 2008). The endorsement by the World Health Organization (WHO) of the Xpert MTB/Rif assay (Xpert) (WHO, 2011) for screening of TB suspects has revolutionized testing for TB globally. In South Africa, Xpert has been implemented as the baseline diagnostic test for pulmonary tuberculosis at previous smear-microscopy laboratories (Meyer-Rath et al., 2012). A review by Steingart *et al.* (Steingart et al., 2014) has shown that, using pooled sensitivity estimates, Xpert could diagnose 88% of cases compared to 65% by smear microscopy.

Despite the successful implementation of molecular diagnostics in South Africa, including the Xpert, South Africa, several challenges exist. Xpert requires samples to be processed within 3 days if kept at ambient temperature or can be stored at 2-8° C for a maximum of 7

days if delays are expected, both of which may not be realistic in many poor-resourced countries (WHO, 2014b). On average a specimen may take up to 3-5 days before testing in a laboratory mainly due to delays in transport of specimens from outlying areas with poor infrastructure (Mundy et al., 2002) and laboratory capacity (Cohen et al., 2014). Furthermore, Xpert generally uses the complete sputum volume necessitating collection of an additional specimen should further tests be required; this may be complex in rural settings. Other challenges include the requirement of refrigeration in countries with temperatures exceeding the 28°C reagent threshold, increased cost associated with reagents, staff and maintenance, and the availability of stable uninterrupted power supply (Carman and Patel, 2014, WHO, 2014b). These conditions require financial, operational and logistical support (Clouse et al., 2012) which may exist in urban and peri-urban locations (Boehme et al., 2011), but are difficult to achieve in rural settings in the same countries.

An alternative approach to Xpert testing may be provided by sputum specimen collection and transport to centralized facilities for molecular processing. PrimeStore® - Molecular Transport Medium (PS-MTM, Longhorn Vaccines & Diagnostics, San Antonio, TX, USA) was developed to collect respiratory specimens and to allow for transport of these under extreme environmental conditions by stabilizing nucleic acids over time until processing. The medium effectively inactivates pathogens, including *M. tuberculosis* by 30 minutes, inhibits nuclease activity and preserves nucleotides at ambient temperature for at least 4 weeks (Daum et al., 2011b, Daum et al., 2014, Omar et al., in press). PS-MTM has been successfully evaluated for detection of respiratory viruses, but may also provide a useful method for molecular detection of *Mycobacterium tuberculosis*, particularly in low-resource settings where operational and logistic challenges are frequent, the climate is hot, and considerable delays between specimen production and testing may occur (Omar et al., in press). In this study, we evaluate a system of centralized molecular testing of swabbed

sputum specimens inoculated in PS-MTM, as an alternative to Xpert or mycobacterial culture, for the detection of *M. tuberculosis* in a rural African setting.

3. METHODS

3.1. Study design and sputum specimens

Individuals were recruited from a larger cohort study in which individuals with a cough for more than two weeks were recruited at primary healthcare facilities in rural Mopani District, South Africa (Peters *et al.* submitted). Two sputum specimens, produced at least 1.5 hours apart, were obtained from each patient: one for Xpert testing (at point-of-care or in the NHLS laboratory) and one for mycobacterial culture at the University of Pretoria. The order of specimens from each patient was randomized for the two tests. For this evaluation, paired PS-MTM specimens were selected from 141 patients: those that had a positive result for Xpert and/or MGIT (n=47) and combined with randomly selected patients with negative specimen result (n=94) at a 1:2 ratio.

At the study site, shortly after production by the patient, a swabbed sample was collected from each sputum specimen by rotating the flocculated cotton swab (Copan Diagnostics Inc., Brescia, Italy) a minimum of five times within each specimen container and placing it into PS-MTM collection tubes (Fig. 2). The PS-MTM specimens were batched and sent for molecular processing bi-weekly at ambient temperature across ~500 km from the study sites to the National TB Reference Laboratory, Johannesburg, South Africa. The time-lag between sample collection and molecular processing was sufficient to ensure total inactivation by the transport medium of mycobacteria that may have been present in the sample.

3.2 Routine microbiological tests

IS6110 real-time PCR results of sputum collected in PS-MTM were compared to those of routine Xpert, smear-microscopy[14] and liquid culture. Of the two collected sputum specimens, one was tested using the Xpert MTB/Rif assay (version G4 cartridges) (Cepheid, Sunnyvale, CA, USA) according to the manufacturer's instructions[15] and the other decontaminated and concentrated using the NaLC-NaOH method[16] (Kent, 1985); sediments were used to prepare smears for light microscopy by Ziehl-Neelsen staining (WHO, 2008b) and 0.5 ml was inoculated for culture using the Mycobacterial Growth Indicator Tubes (MGIT) 960 system (BD, Sparks, Maryland, USA) as described by the MGIT procedure manual (Siddiqi and Rusch-Gerdes, 2006). Smears were graded according to WHO recommendations (WHO, 2008b) and a culture was considered negative by the MGIT 960 system after 42 days of incubations.

3.2 Detection of *Mycobacterium tuberculosis* in specimens collected in PS-MTM

The sputum specimen in PS-MTM, was vortexed using the Vortex Genie (Scientific Industries Inc. USA) at maximum speed for one minute and a 200µl aliquot was processed using the generic protocol on the NucliSENS EasyMAG (Biomerieux, Marcy I'Etoile, France) to extract DNA. A Real-time PCR targeting the *M. tuberculosis* complex specific insertion sequence element (IS) 6110 for detection of DNA was performed on the StepOne Plus (ThermoFisher Scientific, Waltham, MA USA) following manufacturers' instructions (Daum et al., 2011a). The PCR comprised of 40 cycles and threshold of 0.1 was applied for analysis. Specimens were either considered positive, indeterminate or negative based on the following predefined Cycle-Threshold (CT) values; ≤ 38 positive, 38.01 – 39.99 indeterminate and ≥ 40 (no amplification signal) as negative. In general, real-time PCR cycle

[14] APPENDIX A #2 & 3
[15] APPENDIX A #12
[16] APPENDIX A #1

threshold is indicative of the level of target template DNA, reactions with a CT below 37 have moderate to high levels of template whereas 38 to 40 may indicate a low level of template or an environmental contamination (and usually requires another clinical sample for confirmation).

3.3 Statistical analysis

Descriptive statistics are provided including frequency measurements. Results of *IS6110* real-time PCR of sputum collected in PS-MTM were compared to Xpert and MGIT culture as standards through cross-table comparison (using concordance rate and McNemar test instead of sensitivity/specificity due to the selection of specimens). Furthermore, a composite reference standard was used with the following definition for TB positivity: specimen positive in either MGIT or Xpert. For purposes of comparison an indeterminate result of PCR on PS-MTM was excluded from analysis as an additional patient sample could not be requested to confirm the presence or absence of *M. tuberculosis*. Statistical analyses were performed using Statistix v.7.0 (Analytical Software, Tallahassee, FL, USA).

3.4 Ethics approval

Ethics approval for this study was obtained the Human Research Ethics Committee at the University of Witwatersrand, Johannesburg, South Africa (Ref: M120226).

4 RESULTS

4.1 Detection of *Mycobacterium tuberculosis* in sputum specimens[17]

A total of 282 paired sputum specimens from 141 patients were evaluated. At most 2.8% of sputum specimens were not available for testing within each test set. Overall, a diagnosis of TB was made for 25 (18%) cases by microscopy, 41 (29%) by culture and 40 (29%) by Xpert (Fig. 1). MGIT culture and Xpert were both positive for *M. tuberculosis* for 34 samples with an additional 7 for culture only and 6 for Xpert only. As such, the detection of *M. tuberculosis* was similar for both specimens and methods. Further, *M. tuberculosis* DNA was detected in specimens collected in PS-MTM for 43 patients (31%) paired to culture specimens and 49 patients (36%) paired to Xpert specimens. There was no difference in detection of *M. tuberculosis* DNA between first (32%) or second (36%) sputum specimen stored in PS-MTM (McNemar, p=0.21).

4.2 Concordance between IS6110 real-time PCR of sputum in PS-MTM and liquid culture

M. tuberculosis DNA was detected from sputum in PS-MTM in 21/25 sputum specimens that were smear-microscopy positive and 29/41 specimens that were positive by culture (71%) (Tab. 1). An indeterminate result was observed for one culture-positive specimen and for two culture-negative specimens. Agreement, excluding indeterminate results, was 73% for culture-positive and 85% for culture-negative samples resulting in an overall agreement of 82% (McNemar, p=0.55). *IS6110* real-time PCR of DNA from PS-MTM samples showed discordance for 11 positive and 14 negative specimens by culture (Tab. 2). When comparing this discordance to the same patient's Xpert specimen result; the Xpert result concurred with 6 of the 11 culture positives and 11 of the 13 negative cultures (1 Xpert not done) (Tab. 2).

[17] APPENDIX B TABLE 5

4.3 Concordance between IS6110 real-time PCR from sputum in PS-MTM and Xpert

Of the Xpert-positive samples, *IS6110* real-time PCR of DNA from sputum in PS-MTM was positive for 32/39 (82%) (Tab. 1). One Xpert positive and two Xpert negative sputum specimens had indeterminate results of PCR from sputum in PS-MTM. Agreement, excluding indeterminate results, between *IS6110* real-time PCR from PS-MTM and Xpert was 84% for both negative Xpert results resulting in an overall agreement of 84% (McNemar, p=0.05). The relatively low agreement for negative specimens is the result of higher detection rate by *IS6110* real-time PCR of PS-MTM: 15 Xpert-negative specimens had *M. tuberculosis* DNA detected from samples in PS-MTM. When stratifying Xpert results by the result of liquid culture, there was good concordance between *IS6110* real-time PCR of DNA in PS-MTM and Xpert (Tab. 2).

A significant difference was observed for the culture negative specimens (McNemar, p= 0.008) in which *M. tuberculosis* was detected exclusively in 14 PS-MTM samples in the negative group compared to three by the Xpert (Tab. 2).

4.4 Sputum *Mycobacterium tuberculosis* DNA load by result of routine test [18]

In cases of positive *IS6110* real-time PCR result of sputum in PS-MTM, the median CT value was significantly higher for specimens with a positive results paired to culture than a negative (28.47 vs. 34.47; p<0.001). This was also observed when comparing CT-value between specimens with positive and negative paired Xpert result (28.34 vs. 34.81; p<0.001).

[18] APPENDIX B TABLE 6

5 DISCUSSION

This field evaluation in a rural setting with poor infrastructure shows that PS-MTM provides a promising tool that could support centralized molecular testing for *M. tuberculosis* as an alternative to the Xpert assay and mycobacterial culture. Sputum specimens were transported ~500km from health care facilities to the National TB Reference Laboratory in Johannesburg. Previous research has shown that this is a safe way of transporting sputum specimens as PS-MTM inactivates the bacilli, thereby making it safe from an infection control perspective, and that stability of DNA is achieved over time at ambient temperature (Daum et al., 2014). In a laboratory evaluation, we recently demonstrated good sensitivity and specificity of *IS6110* real-time PCR detection of *M. tuberculosis* from clinical samples inoculated into PS-MTM (Omar et al., in press). This study confirms those results in the field setting of rural healthcare facilities whereby sputum specimens were, using a swab, inoculated into PS-MTM by the research nurse shortly after they were produced by the patient.

In this study, *IS6110* real-time PCR detection of *M. tuberculosis* from PS-MTM specimens showed a slightly higher positivity rate than culture and Xpert. A few samples had an indeterminate result of *IS6110* real-time PCR from PS-MTM as low concentration of DNA was detected (CT-value >38 cycles). In these cases, the origin of *M. tuberculosis* DNA is not clear and, since background contamination cannot be ruled out, a repeat specimen would normally be requested in clinical practice for further interpretation. It is of note that one of the specimens with indeterminate result was culture positive and another Xpert positive.

Concordance of *IS6110* real-time PCR from PS-MTM with culture and Xpert was good for both positive and negative results, except for the concordance with Xpert negative specimens: a considerable number of samples were *IS6110* real-time PCR from PS-MTM positive and Xpert negative (n=15). When stratified by culture result, the detection rate of *IS6110* real-

time PCR from PS-MTM was equal to that of Xpert among culture-positive patients, but significantly higher than Xpert in specimens from patients with negative paired culture result. A potential explanation for the latter observation is that the genetic target of the real-time PCR used for the PS-MTM (*IS6110*) occurs multiple times throughout the *M. tuberculosis* genome compared to the Xpert's single copy target; this would in theory allow for more sensitive detection by the PS-MTM approach used in this study (Armand et al., 2011).

M. tuberculosis DNA load (reflected by higher CT-value) of sputum collected in PS-MTM was significantly lower in negative paired culture or Xpert results. This observation could possibly be due to the low organism load in a specimen that may be killed during the harsh decontamination procedure of sputum prior to culture (European Centre for Disease Prevention and Control, 2011), which resulted in lack of growth in culture, the presence of non-viable organism (up to six months) in patients that may have previously been treated for *M. tuberculosis* infection or latently infected patients (Levee et al., 1994, Beige et al., 1995, Hernandez-Pando et al., 2000, Kaul, 2001).

6 CONCLUSION

Since culture is the reference standard for the diagnosis of pulmonary TB (Abebe et al., 2011) and Xpert has a known high sensitivity for the detection of *M. tuberculosis* (Boehme et al., 2010, Armand et al., 2011, Bowles et al., 2011), the good level of agreement between these diagnostic methods and *IS6110* real-time PCR from PS-MTM makes the latter a promising approach to enhance availability of molecular diagnostic systems in resource-poor settings with often challenging logistics and climate. An additional advantage is that only an aliquot of the sputum specimen in PS-MTM is tested by *IS6110* real-time PCR allowing for further molecular or phenotypic characterization and repeat testing of the remaining sputum volume (which is not possible in Xpert where the entire sputum volume is consumed). Improving control of TB is reliant on improving diagnosis and treatment outcomes (WHO, 2008a). In an effort to contain the disease, South Africa is the only high-burden country to have implemented two molecular methods in routine diagnostic approach: the Xpert for first-line diagnosis and the line probe assay for detection of drug resistance. Many countries are unable to afford the costs associated with this implementation to appreciate true benefit, in particular, the infrastructural and operational costs (Lawn et al., 2012, Carman and Patel, 2014).

High quality sputum specimen that is stabilized and transported at ambient temperature to a central diagnostic facility may provide a safe alternative approach for molecular detection of *M. tuberculosis* in resource-constrained settings and offers a unique opportunity for detection of additional positives in low load specimens.

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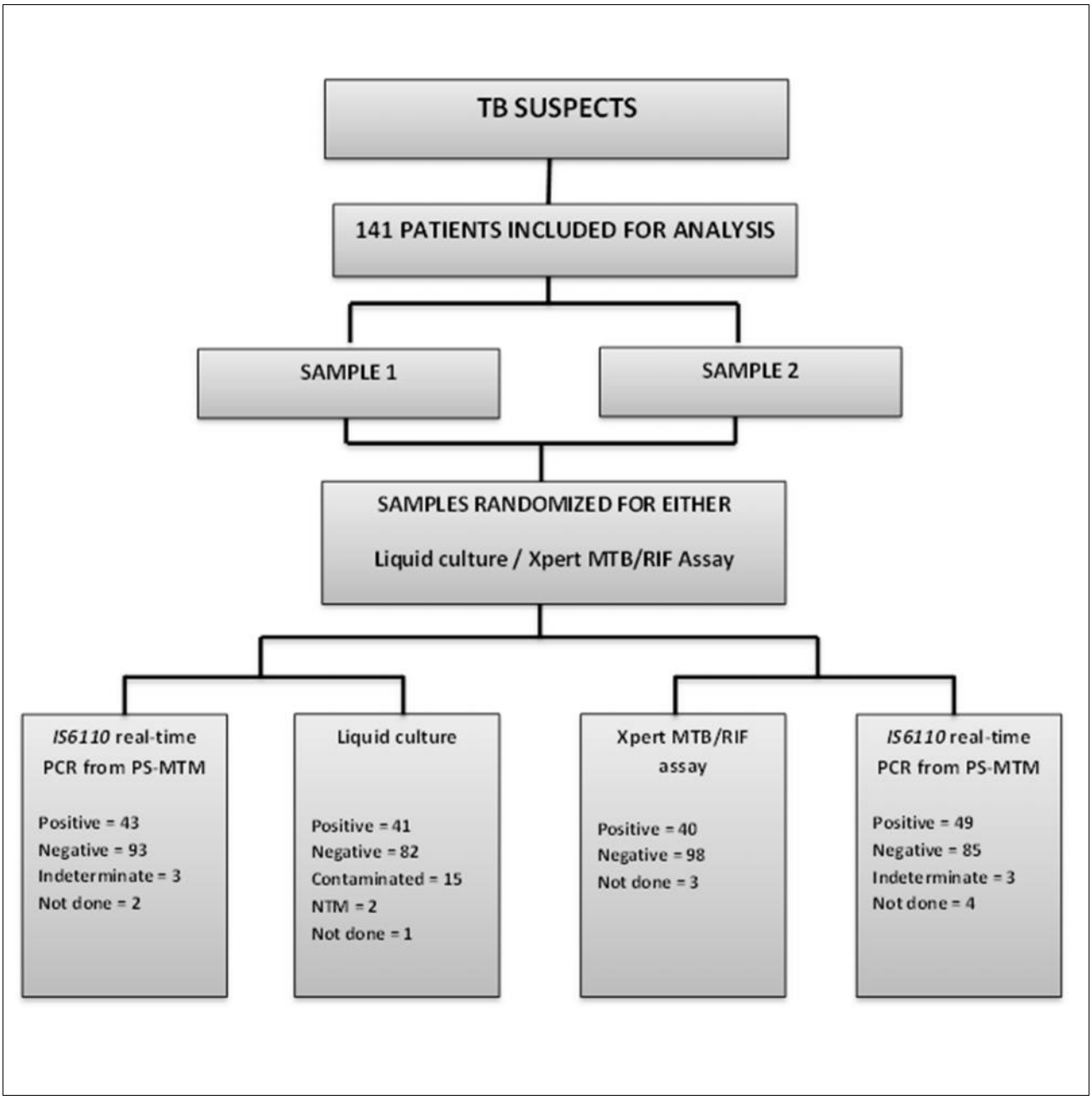

Figure 1. Study design and results of testing sputum specimens for the presence of *M. tuberculosis* by liquid culture (MGIT 960 system), Xpert MTB/RIF assay and IS6110 real-time PCR of sputum in PrimeStore® - Molecular Transport Medium (PS-MTM)

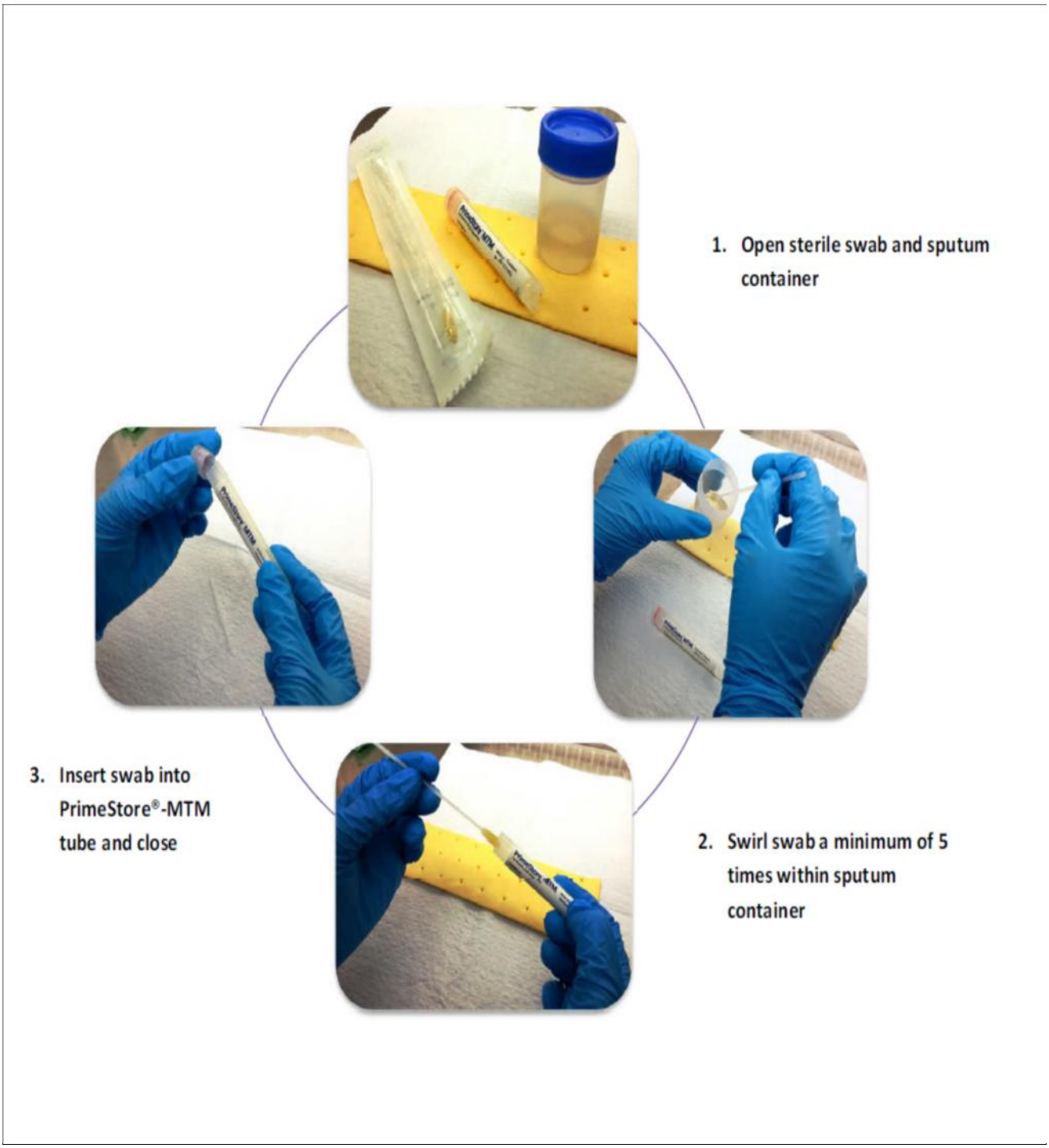

Figure 2. Collection of sputum using a flocculated cotton swab inserted in the PrimeStore® - Molecular Transport Medium for downstream molecular processing

Table 1: Concordance of IS6110 real-time PCR detection of *M. tuberculosis* DNA from sputum in PS-MTM compared to liquid culture and Xpert.

	Liquid culture		**Xpert**	
***IS6110* real-time PCR from PS-MTM**	**Positive (n=41)**	**Negative (n=98)**	**Positive (n=39)**	**Negative (n=95)**
No. (%*) PCR positive	29 (71)	14 (14)	32 (82)	15 (16)
No. (%*) PCR negative	11 (27)	82 (84)	6 (15)	78 (82)
No. (%) PCR indeterminate	1 (2)	2 (2)	1 (3)	2 (2)
Concordance* (McNemar)	82% (p=0.55)		84% (p=0.05)	

*Excluding specimens with indeterminate PCR result from the denominator.

Table 2: Detection of *M. tuberculosis* DNA by IS6110 real-time PCR from PS-MTM samples compared to Xpert result stratified by the MGIT culture result.

IS6110* real-time PCR from PS-MTM	**Culture Positive**		**Culture Negative**	
	Xpert Positive (n=32)	**Xpert Negative (n=5)**	**Xpert Positive (n=6)**	**Xpert Negative (n=87)**
No. (%) PCR positive	29 (90.6)	1 (20.0)	3 (50.0)	14 (16.1)
No. (%) PCR negative	3 (9.4)	4 (80.0)	3 (50.0)	73 (83.9)
Concordance* (McNemar)	89% (p=0.32)		82% (p=0.008)	

*Excluding specimens with indeterminate PCR result.

CHAPTER 5

CONCLUDING REMARKS

Diagnostic technologies for TB are evolving with the promise of improved detection at closer proximity to the patient, ideally, point of care. These improvements have as yet not been translated into a rapid reduction on morbidity associated with the disease. WHO estimates have shown no major decline in disease burden and in certain countries an increase was noted (WHO, 2014). An apparent obstacle is the costs associated with these technologies, where the highest disease burdened countries are usually resource-poor and unable to afford them. Despite philanthropic efforts from developed countries, these sophisticated technologies still cannot be adopted due to; poor infrastructure, lack of skilled personnel and costs associated with increased detection. The question remains: Is the adoption of new technologies closer to patients a viable solution for the current crisis or could leveraging on current infrastructure provide the same impact?

Theme 1: Inactivation of *M. tuberculosis* and stabilization of the bacilli's DNA in clinical specimens using a novel transport medium designed for downstream molecular application

Over the past decade, developments in molecular diagnostics have moved at a rapid pace and several commercial assays have been developed specifically for the diagnosis of *M. tuberculosis*. The most prominent include a series of assays from Hain Lifescience (MTBDR plus, CM and AS), the Lightcycler Mycobacterium detection assay (Roche, Mannheimm, Germany) and the Xpert (Cepheid, Sunnyvale, CA, USA). Of these, the Hain MTBDR plus assay and the Xpert have received WHO endorsement (WHO, 2008b, WHO, 2011) with the latter being approved for both smear-negative and positive specimens and having the ability to detect up to 90% of TB cases.

Molecular assays have proved to be very promising for rapid diagnosis of TB; however, implementation of these tests carries considerable cost in terms of capital and human resources (Carman and Patel, 2014). The delays in specimen transfer from site of collection to the testing facility could impair test performance (Paramasivan et al., 1983). In resource-poor settings, the average time from sample collection to processing is estimated to be 5 days (Mundy et al., 2002).

Sputum is the most commonly collected respiratory specimen for the diagnosis of TB (Daniel, 2009). Microscopy and culture for TB diagnosis require the sputum specimen to be liquefied using effective mucolytic agents prior to processing. Usage of NALC-NaOH and DTT are the most common, however, their function as mucolytics and decontaminants precedes culturing and therefore, they are not traditionally used for the inactivation of *M. tuberculosis*. Their inactivation activities are therefore inadequate to allow for sufficient operator protection for further processing by molecular applications. Thus, a key requirement of sputum processing is the use of specialized BSL 3 facilities or biological safety cabinets as viable *M. tuberculosis* organism may be present.

Safety when dealing with infectious agents is a priority in a high-burdened setting. It is known that an infectious dose as low as 10 bacilli is sufficient to establish disease based on animal models (WHO, 2012). PrimeStore® – Molecular Transport Medium (PS-MTM) is novel, with the benefit of inactivating the organism (this includes organisms such as *Bacillus subtilis* spores), lysing it during exposure and preserving nucleic acids at ambient temperatures for downstream molecular applications (Daum et al., 2011). In our study, we show that PS-MTM applied to *M. tuberculosis* has the ability to inactivate pure cultures rapidly. When the bacilli are spiked into a matrix of sputum, at concentrations expected in an infectious person, the transport medium is capable of inactivating the bacilli. This inactivation is dependent on specimen to medium ratios as well as the exposure time. At a concentration of 10^8 bacilli per milliliter in sputum, the organism was inactivated by 1 hour using a ratio of 1:2 sputum to the transport medium, increasing the ratio to 1:3 permitted inactivation by 30 minutes, however, at ratios at or lower than 1:1 the organism remained viable at the maximum exposure time tested (3 hours). Using sputum spiked with a concentration of 10^6 bacilli per milliliter, an equal volume of the transport medium was able to effectively inactivate the organism at 5 minutes. These results are consistent with a study by Daum *et al.* showing similar activity by the transport medium (Daum et al.,

2014). Another medium known to inactivate *M. tuberculosis*, the Xpert's SR buffer, has shown to reduce the viability of the bacilli by 6 - 8 logs within 15 minutes (Banada et al., 2010, Helb et al., 2010).

PS-MTM, in addition to its inactivating capability, stabilizes and preserves nucleic acids. This study confirmed the stability of *M. tuberculosis* DNA in clinical sputum specimens stored in PS-MTM at ambient temperature over a 28 day period, where no significant change on real-time PCR CT scores occurred at the 5 time points tested. In comparison, published data shows that the Xpert's SR buffer negatively impacted on the sensitivity of the assay when exposed for 3 days or longer (Banada et al., 2010).

Our findings suggest that PS-MTM is capable of inactivating high concentrations of *M. tuberculosis* as well as stabilizing organism DNA in the specimen at ambient temperatures, thereby, limiting sample degradation and the need for a cold chain. The required inactivation exposure time is less than what would be expected for the transfer of specimen from a health care facility to a testing laboratory. Therefore, a specimen could safely be transported without the compromising sample integrity and the risk of infection even in the event of extended delays. This would allow resource-poor settings an opportunity to introduce or scale-up molecular testing at centralized facilities and avoid diagnostic delays impacting on patient care (Meintjes et al., 2008, Sreeramareddy et al., 2009). PS-MTM may have several applications in addition to the diagnosis of *M. tuberculosis* including: safe transport and preservation of specimen for molecular testing; its inactivating capability on pure cultures could allow for the convenient ambient temperature transport of the organism for surveillance activities (i.e. molecular typing and sequencing) and ambient temperature molecular bio-banking, which could remove the current storage costs associated with the conventional method.

Theme 2: Compatibility of a novel transport medium for the detection of *Mycobacterium. tuberculosis* by real-time PCR using established nucleic acid extractions methods

While current research and development effort is directed at molecular methods for detecting TB from clinical specimens; innovative approaches to employing these developments in high-

burdened and resource-constrained settings has become essential. The primary objective of this work is to introduce a technique, which eliminates multiple limitations faced by current diagnostics. Moreover, recent advances in optimization of specimens for processing and the rational use of different tests combined in appropriate algorithms to ensure diagnostic efficiency and optimal patient care offer attractive options for microbiologists and clinicians to combat TB effectively.

In this set of experimental procedures, we sought to determine the compatibility of the PS-MTM with established extraction methodologies (QiaAMP DNA mini kit, NucliSEN easyMAG and MagNA Pure 96), which could easily be applied to routine clinical laboratories. This was determined by measuring the lower limit of detection using sputum spiked with a log-fold series dilution of *M. tuberculosis*. Magnetic bead-based extraction systems yielded higher DNA concentrations at the lower range of colony forming units per milliliter (CFU/ml) tested in comparison to the silica-based system. This may be due to the reduced handling error by the operator or the loss of nucleic acids released into the transport medium during exposure. Overall, no statistical difference was observed between extraction systems for the detection of *M. tuberculosis* by real-time PCR. PS-MTM was incompatible with the Xpert; a possible explanation may be the lysing activity of the transport medium, which may release the nucleic acids in solution, therefore, not meeting the assay's requirement of an intact bacillus (Blakemore et al., 2010, Miller et al., 2011). Determining the lower limits of detection for each of the extraction systems evaluated by Probit regression analysis showed a significant difference in favor of the magnetic bead extraction systems compared to the control arm (Xpert). This possibly alludes to the fact that the transport medium may be enhancing the extraction performance; however, further evidence is required.

The compatibility of the transport medium with these DNA extraction systems is promising, as they are well suited for clinical laboratories because of their reproducibility and efficiency (Taylor et al., 1990, Knepp et al., 2003, Dundas et al., 2008, Dauphin et al., 2009, Lee et al., 2010). In addition, PS-MTM's inactivating capability makes it an ideal complement to improve safety for molecular testing, particularly, clinical diagnostics.

Theme 3: Laboratory and field evaluation of a novel sputum collection system in PS-MTM for the detection of *Mycobacterium. tuberculosis* by real-time PCR

The United Nations Millennium Development Goals of halting TB, beginning reversal of the epidemic and halving disease prevalence and death rates by 2015, to levels comparable to those of 1990, are currently being implemented by Global Plan to Stop TB 2006-2015. The strategies considered to achieve this were the scaling-up of existing diagnostic and treatment interventions, introduction of new technologies (notably new diagnostics) and developing new tools to revolutionize prevention, diagnosis and treatment (WHO, 2008a).

Diagnosis of TB today still relies on microscopy and culture, however, limited they may be in sensitivity and turnaround time (Lalvani, 2007, Lemaire and Casenghi, 2010). Molecular testing for TB is well established and holds great promise for the future of TB diagnosis. Nucleic acid amplification tests (NAATs) have been applied successfully for the diagnosis of several microbial infections and were introduced to TB diagnostics in the early 1990s. Technical advances have been made in the development of NAATs that enable the detection of *M. tuberculosis*-specific DNA as well as the determination of drug resistance profiles directly from clinical specimen, thereby, improving the speed and accuracy of the diagnostic services (Nahid et al., 2006, Cho and Brennan, 2007, Boehme et al., 2010, Armand et al., 2011). The development and improvement of diagnostic assays have therefore been and remain a major priority.

A pilot evaluation of the novel sputum collection method, using a flocculated cotton swab to capture a minute volume from the primary specimen and inserting it into PS-MTM for downstream molecular detection, displayed excellent capability. In the prospective clinical laboratory evaluation, this method showed an adequate sensitivity (76.9%) and specificity (96.5%) using a real-time PCR method targeting *IS6110* to detect *M. tuberculosis*. Sensitivities were in range of other molecular detection methodologies (Dinnes et al., 2007, Armand et al., 2011, Miller et al., 2011, Steingart et al., 2014); however, our data was generated using a minimal sampling from the primary specimen.

These clinical laboratory results were validated by a field evaluation in a rural region in South Africa. Samples were transported at ambient temperature across 500km for testing at a

centralized clinical laboratory and compared to culture (the reference standard) and Xpert. In this study, detection of *M. tuberculosis* from PS-MTM samples showed similar positivity rates to that of MGIT culture and Xpert. Agreement with mycobacterial culture was 82% and 84% for the Xpert; both these methods (culture and Xpert) are known to have a high sensitivity for the detection of *M. tuberculosis* (Boehme et al., 2010, Armand et al., 2011, Bowles et al., 2011). This high level of agreement between these diagnostic methods, therefore, makes the PS-MTM sample collection an attractive pre-step to molecular TB diagnosis. Additional positives, negative by both mycobacterial culture and Xpert, were detected by PS-MTM collected specimens; however, further investigation and interpretation of these results is required.

PS-MTM offers an ideal solution where samples can be batched, stored and transported at ambient temperature for centralized TB testing. The swab procedure disregards a minimum volume requirement as in the case of the Xpert, which is advantageous in cases where insufficient material is available for testing. Further to this, the collection system is an innovative approach where the primary specimen could still be used for further confirmatory testing without the need for a patient returning to submit an additional specimen as in the case of the Xpert. The system has an advantage of being an open platform therefore allowing its application on existing laboratory methodology and infrastructure. To conclude, sputum specimens transported using the PS-MTM collection method provide a high quality and safe starting point for the centralized molecular detection of *M. tuberculosis*, which may be a viable option for resource-constrained settings.

Future Research

The availability of novel diagnostics, even with global policy recommendations, does not guarantee their implementation nor their impact on public health (UNITAID, 2012). Resource-poor settings struggle to adopt these new technologies due to infrastructure limitations. An ideal diagnostic that would overcome the current inadequacies presented for use, one that is simple, safe, accurate, rapid, and inexpensive without the need for specialized infrastructure. Innovative approaches such as PS-MTM are a useful adjunct to current molecular diagnostics enhancing coverage of sophisticated technologies by providing a safe, uncompromised specimen for testing with the opportunity of detecting disease in low load specimen.

The use of PS-MTM in both, the clinical laboratory evaluation and field validation, detected the presence of *M. tuberculosis* in several additional specimen negative by both mycobacterial culture and Xpert. These unique positives could be a result low bacillary load detected in PS-MTM specimen only. Further investigation on the clinical relevance of these specimens should be the area of focus for future research.

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APPENDIX A

DETAILED METHODOLOGY

1.Sputum Digestion and Decontamination (NALC/NaOH)

Reagents

4% Sodium Hydroxide (NaOH) (TB Diagnostic Services, Kromdraai, South Africa)

2.9% Trisodium Citrate (TB Diagnostic Services, Kromdraai, South Africa)

Phosphate Buffered Saline pH 6.8 (TB Diagnostic Services, Kromdraai, South Africa)

N-Acetyl-L-Cysteine (TB Diagnostic Services, Kromdraai, South Africa)

AFB fixative

1.1. Transfer sputum to a 50 ml conical screw cap centrifuge tube.

1.2. Add an equal volume of NaOH/sodium Citrate/ NALC to the sputum specimen and tighten the cap.

1.3. Vortex (Vortex-Genie 2, Scientific Industries) the tubes for 30 seconds, invert the tube to ensure the entire specimen is exposed to the solution.

1.4. Incubate the tube at ambient temperature for 20 minutes. Invert the tubes every 10 minutes during the incubation.

1.5. Ensure that the entire specimen has been liquefied.

1.6. Thereafter fill phosphate buffer saline (pH 6.8) to the 50ml marking. Vortex lightly for 30 seconds.

1.7. Centrifuge the specimen at 3000 x g for 20 minutes

1.8. After centrifugation allow tubes to stand for 5 minutes to allow any aerosols to settle. Then carefully decant the supernatant into a suitable container containing a mycobacterial disinfectant.

1.9. Add 1-2 ml of phosphate buffer saline to the sediment and re-suspend.

1.10. Add approximately 100µl to the culture medium i.e. MGIT tube, Middlebrook 7H10 agar plate and/or onto a slide.

2.Smear Preparation

2.1. Working in a biological safety cabinet, vortex the decontaminated sediment to mix thoroughly.

2.2. Use a transfer pipette to place ~100 µl (2 drops) of well-mixed resuspended pellet from the digested-decontaminated specimen onto the slide, spreading over an area approximately 1 x 2 cm containing a drop of AFB fixative if required.

2.3. Air-dry the smear for approximately 15 minutes.

2.4. Place the slides on a hot plate or slide warmer at a temperature between 65°C to 75°C for at least 2 hours, to heat-fix.

3.Ziehl-Neelsen Staining

Stains

Ziehl-Neelsen carbol fuschin (Diagnostic Media Products, Johannesburg, South Africa)

3% acid-alcohol (Diagnostic Media Products, Johannesburg, South Africa)

Methylene blue (Diagnostic Media Products, Johannesburg, South Africa)

3.1. Place slides on staining rack so they are at least 1 cm apart, and flood with carbol fuchsin.

3.2. Heat the slide with the flame from a Bunsen burner till steam arises.

3.3. Apply only enough additional heat to keep the slide steaming for 5 minutes. Do not let the stain boil or dry.

3.4. Wash off the stain with distilled water.

3.5. Flood slides with 3% acid-alcohol.

3.6. Allow to stand for 2-3 min

3.7. Wash off the acid-alcohol with distilled water and tilt the slides to drain.

3.8. Flood the slides with methylene blue and let stand for 1-2 minutes.

3.9. Wash off the methylene blue with distilled water. and tilt the slides to drain.

4.Fluorescent Staining (Auramine-O)

Stains

Auramine O (Diagnostic Media Products, Johannesburg, South Africa)

Potassium permanganate ($KMnO_4$) (Diagnostic Media Products, Johannesburg, South Africa)

0.5% Acid Alcohol (Diagnostic Media Products, Johannesburg, South Africa)

4.1. Place slides on staining rack so they are at least 1 cm apart, and flood with Auramine O and allow to stand for 20 min.

4.2. Rinse the stain away with distilled water and tilt slide to drain.

4.3. Flood the slide with 0.5% acid alcohol and let stand for 2 min.

4.4. Wash off the acid alcohol with distilled water.

4.5. Flood slides with potassium permanganate for 1-2 min. Do not allow potassium permanganate to act over 2 min, or it might quench the fluorescence of acid-fast bacilli.

4.6. Wash off the stain with distilled water.

4.7. Allow slides to air dry in the slide rack.

4.8. Protect smears from light and examine immediately using the fluorescent microscope. If unable to read right away, place slides in covered box.

5.Middlebrook 7H10 culture (Diagnostic Media Products, Johannesburg, South Africa)

5.1. Work inside a biological safety cabinet for specimen inoculation.

5.2. Pipette 100µl of the liquefied/decontaminated sputum (described above) onto the surface of the solid medium.

5.3. Rotate the plate to allow the solution to spread evenly across the surface of the medium.

5.4. Seal the plate with parafilm and incubate in at incubator at 37°C.

5.5. Review plates on a weekly basis until confluent growth is observed, once sufficient growth is visible the plate can be removed from the incubator.

5.6. Plate on which no growth has been observed after a period of 8 weeks can be removed and regarded as negative.

6.Mycobacterial Growth Indicator Tubes culture (Becton Dickinson Diagnostics, Sparks, MD, USA)

Work inside the biological safety cabinet for specimen inoculation.

6.1.Reconstituting PANTA (Becton Dickinson Diagnostics, Sparks, MD, USA)

Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement. Mix until completely dissolved. Add 0.8 ml of this enrichment to each MGIT tube. The enrichment with reconstituted PANTA should be added to the MGIT medium prior to inoculation of specimen in MGIT tube. Do not add PANTA/enrichment after the inoculation of specimen. Do not store MGIT tube after the addition of enrichment/ PANTA.

6.2.Inoculation of MGIT medium

a.Label MGIT tubes with specimen number.

b.Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube. Use of an adjustable pipettor is recommended.

c.Using a sterile pipette or a transfer pipette, add up to 0.5 ml of a well-mixed processed/concentrated specimen to the appropriately labeled MGIT tube. Use separate pipette or pipette tip for each specimen.

d.Immediately recap the tube tightly and mix by inverting the tube several times.

e.Wipe tubes and caps with a mycobactericidal disinfectant and leave inoculated tubes at room temperature for 30 minutes.

6.3.Loading the MGIT 960 instrument

All inoculated MGIT (7mL) tubes should be entered in the BACTEC MGIT 960 instrument (Becton Dickinson Diagnostics, Sparks, MD, USA) after scanning each tube (refer to the BACTEC MGIT 960 Instrument Manual for details). It is important to keep the cap tightly closed and not to shake the tube during the incubation. This helps in maintaining the oxygen gradient in the medium. The instrument maintains 37ºC + 1ºC temperature. Since the optimum temperature for growth of *M. tuberculosis* is 37ºC, make sure the temperature is close to 37ºC.

7.Preparation of mycobacterial standard suspensions

7.1. Standard *M. tuberculosis* suspensions are prepared by growing the *M. tuberculosis* H37Rv laboratory strain (ATCC 27294) to a McFarland turbidity standard of 0.5 (the equivalent concentration of ~1.5 x 108 colony forming units (CFU)/ml).

7.2. The bacilli are grown in Middlebrook 7H9 broth supplemented with PANTA and OADC (BD Diagnostics, Franklin lakes, NJ, USA) using a shaking incubator at 37°C for bio-aeration and to minimize clumping.

7.3. Once culture vials show mycobacterial growth, they are pooled in a 50 ml conical vial and bacilli concentrated by centrifugation at 3000 x g for 10 min.

7.4. Thereafter, the supernatant is discarded and the pellet resuspended in 2 ml phosphate buffered saline (PBS).

7.5. Three to five glass beads with a diameter of 3 mm (Merck, Darmstadt, Germany) are added and the vial vortexed vigorously for one min to disperse clumped bacilli resulting in a homogenous suspension.

7.6. The optical turbidity is measured using the PhoenixSpec Nephelometer (BD Diagnostics, Franklin lakes, NJ, USA) against a standard curve.

7.7.PBS is gradually added until a 0.5 McFarland density reading is attained.

7.8.A series of ten-fold dilutions is then prepared from the McFarland standard using PBS, producing concentrations ranging from 106 to 101 CFU/ml.

7.9. The dilution series is confirmed by colony counts on Middlebrook 7H11 agar medium.

8.QiaAMP DNA mini kit (Qiagen, Hilden, Germany)

8.1. Pipette a 100µl sample into a 1.5 ml microcentrifuge tube and centrifuged at 5000 x g for 10 minutes.

8.2. Discard the supernatant and add 180µl of Buffer ATL to the pellet.

8.3.A 20µl volume of Proteinase K is then added to the suspension and vortexed briefly at maximum speed.

8.4. The suspension is then incubated at 56°C for one hour.

8.5. The tubes are then centrifuged at maximum speed for one minute to collect condensate.

8.6. Thereafter, 200µl of Buffer AL is added to each sample, briefly vortexed at maximum speed and incubated for 10 minutes at 70°C.

8.7. Tubes are then centrifuged at maximum speed for one minute to collect condensate.

8.8.A volume of 200µl absolute ethanol is added to the suspension and mixed by pulse vortexing for 15 seconds.

8.9. The tubes are then centrifuged at maximum speed for one minute to collect condensate.

8.10. The suspensions is then transferred to the QiaAMP Mini spin column coupled to a microcentrifuge tube and centrifuged at 6000 x g for one minute.

8.11. The collected filtrate is discarded and the QiaAMP Mini spin column transferred to a clean microcentrifuge tube.

8.12.A volume of 500µl Buffer AW1 was added to the spin column and centrifuged at 6000 x g for one minute and the filtrate discarded.

8.13. The spin column is then transferred to a clean microcentrifuge tube and 500µl of Buffer AW2 added to the spin column.

8.14. The spin column is then centrifuged for three minutes at maximum speed and the filtrate discarded.

8.15. Finally, the spin column is added to a clean microcentrifuge tube and 200µl of Buffer AE added to the column.

8.16. The spin column is then incubated at room temperature for one minute with Buffer AE and thereafter centrifuged at 6000 x g for one minute.

8.17. The filtrate containing the nucleic acids is then used immediately or stored at -20°C until further use.

9.MagNA PURE 96 DNA Bacterial/Viral small volume kit (Roche Diagnostics, Mannheim, Germany)

Instrument reagents and consumables is provided by the manufacturer

9.1. The extraction procedure was performed according to the manufacturer's instruction using an input sample volume of 200µl with isolated nucleic acids eluted in a volume of 100µl.

9.2. Nucleic acid isolation is done using the MagNA Pure 96 System and the DNA and Viral NA Small Volume.

9.3. Pipette 200µl of the sample into the MagNA Pure 96 Processing Cartridge and thereafter load onto the instrument.

9.4. Place all the consumables provided on the deck of the platform.

9.5. The software is opened, a new run selected and identification numbers of the samples assigned.

9.6. The instrument is initialized, confirms the presence of all the required consumables and reagents.

9.7. The extraction procedure is started on the software and once completed the eluted DNA is pre-loaded into a 96 well plate.

9.8. The plate is removed from the instrument and sealed.

9.9. The isolated nucleic acids is used immediately or stored at -20°C until further use.

10.NucliSENS easyMAG DNA extraction protocol (Biomerieux, Marcy I'Etoile, France)

Instrument reagents and consumables provided by the manufacturer

10.1. Open the NucliSENS easyMAG software.

10.2. Prepare a series: select the sample type (sputum), the extraction protocol (Generic), the sample volume (200µl), the elution volume of each sample (50µl) and the type of lysis (primary).

10.3. Enter identification of samples to be processed on the software.

10.4. Select incubation lysis must be performed on-board by the instrument NucliSENS easyMAG and the same for the incubation with silica.

10.5. Select all samples and click 'Add selected samples to run'

10.6. Enter the barcode of the reagents with the reader, first scan on the barcode of the machine and then on the bottle.

10.7. Install 3 disposables and their suction combs on the instrument, first scan the bar code of the position, then the bar code of the disposable.

10.8. Remove the disposable and distribute 200µl of inactivated sample per well of disposable in the biological safety cabinet. Open the tubes one by one to avoid as much as possible contamination and mix sample by pipetting up and down.

10.9. Initiate delivery of lysis buffer and on board incubation occurs for 10 minutes.

10.10. Once complete remove the disposable and add 50µl of silica per well, mix with a multichannel pipette and install disposables on the instrument.

10.11. Re-enter the barcode of disposables and launch the instrument

10.12. Once the run is complete, remove the disposables and transfer the eluted nucleic acids to a sterile 1.5ml microcentrifuge tube. Use immediately or store at -20°C until further use.

11.Real-time PCR

The procedures followed for the use of the LightCycler 480 II (Roche Diagnostics, Mannheim, Germany) and StepOne Plus (ThermoFisher Scientific, Waltham, MA USA) is as described in the operators manual [1, 2], with the following cycling conditions (Table 1) and using the primers and probes described below (Table 2); a final reaction volume of 20µl was used.

Table 1: Cycling condition for real-time PCR

Step	Target Temperature	Hold time (sec)	Acquisition mode	Cycles
Hot-start	95°C	600	None	1
Denaturation	95°C	10	None	40
Annealing and Extension	60°C	32	Single	40
Store	4°C	∞		

Table 2: Primer and Probes utilized for real-time PCR detection of *M. tuberculosis*

IS6110 target	Oligonucleotide sequence
Forward Primer	5'CTCGTCCAGCGCCGCTTC 3'
Reverse Primer	5'ACAAAGGCCACGTAGGCGA 3'
Probe	(6FAM) - 5'ACCAGCACCTAACCGGCTGTGGGTA3'– (MGBNFQ)

1. LightCycler 480 II operators manual
2. StepOne Plus operators manual

12.GeneXpert: Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA)

12.1. Using a plastic disposable pipette, measure and note the sputum volume and transfer to a 15 ml conical vial

12.2. Carefully discard the pipette.

12.3. Using separate plastic disposable pipette, add the sample reagent at 2:1 ratio to the sputum sample.

12.4. Vortex the vial vigorously for 30 seconds

12.5. Incubate the sample in the sputum cup for 15 minutes at room temperature.

12.6. After 10 minutes of the incubation period, vortex the vial, as described above.

12.7. The sputum sample should be liquefied with no visible clumps of sputum after incubation.

12.8. Label each Xpert MTB/RIF cartridge with the sample identification number.

12.9. Start the test within 30 minutes of adding the sample to the cartridge.

12.10. Using the sterile transfer pipette provided in the Xpert/Rif kit, draw the liquefied sample into the transfer pipette until the meniscus of pipette is above the minimum mark. Do not process the sample further if there is insufficient volume.

12.11. Avoid touching the sterile transfer pipette. Open the front of the pipette wrapper at the bulb-end of pipette. Take the pipette out carefully. Retain the paper cover.

12.12. The minimum required amount to be loaded into the cartridge is 2 ml (as marked on the Pasteur pipette).

12.13. Open the cartridge lid. Transfer sample into the open port of the Xpert MTB/RIF cartridge. Dispense slowly to minimize the risk of aerosol formation.

12.14. Carefully put the pipette back into the paper/plastic cover. Discard the transfer pipette into bio-hazard waste bin.

12.15. Close the cartridge lid. Make sure the lid snaps firmly into place. Remaining liquefied sample may be kept for up to 12 hours at 2-8°C (for repeat testing).

12.16. Be sure to load the cartridge into the GeneXpert instrument and start the test within 30 minutes of preparing the cartridge.

12.17. Turn on the computer, the GeneXpert instrument and open the GeneXpert software.

12.18. In the GeneXpert system window, click “Create Test”. The “Scan Cartridge Barcode” dialog box appears.

12.19. In the Sample ID box, type the sample identification number.

12.20. Click Start Test.

12.21. Open the instrument module door which displays the blinking green light, and load the cartridge

12.22. Close the door of module firmly (an audible click sound should be heard).

12.23. The test starts and the green light stops blinking. When the test is finished, the light turns off.

12.24. Continue with loading next cartridge following the steps described above.

12.25. Once the run is completed- results are printed automatically. It takes around 1 hour 55 minutes to complete run.

12.26. Once run ends, the result print-out is generated automatically.

12.27. Wait until the system releases the door lock at the end of run, then open the module door and remove the cartridge.

12.28. Dispose the used cartridge in the biohazard waste container.

13. Ethics clearance

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* FWA 00002567. Approved dd 22 May 2002 and Expires 13 Jan 2012.
* IRB 0000 2235 IORG0001762 Approved dd Jan 2006 and Expires 13 Aug 2011.

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Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 10/08/2010

PROTOCOL NO.	**129/2010**
PROTOCOL TITLE	New methods for the molecular and immunological diagnosis of tuberculosis in patients presenting with tuberculous pleural effusion and meningitis.
INVESTIGATOR	**Principal Investigator:** Mr S V Omar
SUPERVISOR	Prof P B Fourie **E-Mail:** bernard.fourie@up.ac.za
DEPARTMENT	**Dept:** Medical Microbiology **Phone:** 012-3742763 **E-Mail:** shaheed.vally@tuks.co.za **Cell:** 0824786678
STUDY DEGREE	PhD Medical Microbiology
SPONSOR	None
MEETING DATE	**28/07/2010**

The Protocol was approved on 28/07/2010 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for 4 years period, and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Prof VOL Karusseit	MBChB; MFGP(SA); MMed(Chir); FCS(SA) - Surgeon
Prof JA Ker	MBChB; MMed(Int); MD – Vice-Dean (ex officio)
Dr NK Likibi	MBBCh – Representing Gauteng Department of Health)
Prof TS Marcus	(female) BSc(LSE), PhD (University of Lodz, Poland) – Social scientist
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM(UP); PhD; Dipl.Datametrics (UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MBChB(Natal); FCS(SA)
Snr Sr J Phatoli	(female) BCur(Bet.A); BTec(Oncology Nursing Science) – Nursing representative
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Dr L Schoeman	(female) B.Pharm, BA(Hons)(Psych), PhD – Chairperson: Subcommittee for students' research
Mr Y Sikweyiya	MPH; SARETI Fellowship in Research Ethics; SARETI ERCTP; BSc(Health Promotion) Postgraduate Dip (Health Promotion) – Community representative

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Dr R Sommers	(female) MBChB; MMed(Int); MPharmMed – **Deputy Chairperson**
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE – School of Dentistry repeesentative
Prof C W van Staden	MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM - **Chairperson**

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

APPENDIX B

DATA AND DETAILED RESULTS

Table 1: Temporal measurements of the inactivation of pure cultured *M. tuberculosis* by PS-MTM at a concentration of 10^8 CFU/ml

Exposure time (sec)	*M. tuberculosis* 1.5 x 10^8		
5	-	-	-
10	-	-	-
20	-	-	-
40	-	-	-
80	-	-	-
160	-	-	-

- no growth

Table 2: Temporal measurements of the inactivation of *M. tuberculosis* by PS-MTM using sputum spike at concentrations of 10^8 and 10^6 CFU/ml against

	spiked Sputum (*M. tuberculosis* 10^8) : PS-MTM														
Exposure time (min)	***0.33 : 1 (triplicate)***			***0.5 : 1 (triplicate)***			***1 : 1 (triplicate)***			***2 : 1 (triplicate)***			***3 : 1 (triplicate)***		
1	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
5	+	-	-	+	-	-	+	-	-	+	+	+	+	+	+
10	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+
15	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
30	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+
60	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+
180	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+

	spiked Sputum (*M. tuberculosis* 10^6) : PS-MTM														
Exposure time (min)	***0.33 : 1 (triplicate)***			***0.5 : 1 (triplicate)***			***1 : 1 (triplicate)***			***2 : 1 (triplicate)***			***3 : 1 (triplicate)***		
1	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+
5	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
10	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
15	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
30	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
60	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
180	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+

+ growth / - no growth

Table 3a: Compatibility of PrimeStore® - MTM with the qiaAMP DNA mini kit (Qiagen), NucliSENS easyMAG (easyMAG) and MagNA PURE 96 (MagNA Pure) for the detection of *M. tuberculosis* spiked in sputum at various concentrations with the Xpert as a control (n=120)

M. tuberculosis		**Real-time PCR CT score**			**Xpert MTB/RIF Assay**					
CFU/ml		***qiaAMP***	***easyMAG***	***MagNA Pure***	***Probe D***	***Probe C***	***Probe E***	***Probe B***	***SPC***	***Probe A***
250000.0	1	27.99	25.67	26.77	23.5	22.0	23.4	23.0	24.8	21.8
250000.0	2	24.88	25.41	25.55	25.3	23.8	24.9	25.4	25.1	23.6
250000.0	3	28.95	25.68	27.54	27.1	25.6	26.9	26.7	27.0	25.4
250000.0	4	26.03	25.11	25.32	25.3	23.7	25.1	25.1	26.4	23.5
250000.0	5	24.79	25.23	25.52	27.5	26.1	27.4	27.4	28.5	26.0
250000.0	6	25.84	25.23	25.85	25.6	24.3	25.7	25.4	26.8	24.1
250000.0	7	25.55	25.69	26.93	24.1	22.4	23.5	23.9	24.7	22.4
250000.0	8	24.93	25.42	26.38	25.3	23.8	25.1	25.4	27.2	23.8
250000.0	9	25.24	25.33	26.69	27.9	26.6	27.8	28.0	31.4	26.5
250000.0	10	25.68	25.74	27.84	22.8	21.3	22.7	22.5	25.3	21.2
250000.0	11	23.68	24.57	25.72	24.3	22.7	24.1	24.3	24.9	22.7
250000.0	12	24.75	26.68	26.36	23.9	22.4	23.7	23.6	25.1	22.2
250000.0	13	40.00	24.90	26.82	25.6	24.5	25.7	25.7	26.6	24.4
250000.0	14	23.39	24.64	25.18	22.6	21.1	22.5	22.4	25.8	21.1
250000.0	15	23.36	24.60	25.65	25.1	23.6	25.0	25.1	27.3	23.5
250000.0	16	25.26	24.63	26.52	25.5	24.0	25.3	25.5	24.8	23.9
250000.0	17	23.60	24.30	24.50	24.3	23.0	24.1	24.1	25.4	22.7
250000.0	18	25.11	24.69	28.62	25.0	23.4	24.6	24.9	25.8	23.3
250000.0	19	25.39	24.60	26.55	27.0	25.4	26.9	27.1	28.2	25.4
250000.0	20	35.00	24.52	26.25	24.3	22.8	24.2	24.2	25.9	22.7
25000.0	1	29.66	28.49	29.72	29.3	27.7	29.0	29.5	27.1	27.9
25000.0	2	28.70	29.41	30.26	23.1	21.5	22.6	22.9	24.6	21.3
25000.0	3	29.10	28.66	29.68	27.5	26.1	27.4	27.2	26.3	26.1
25000.0	4	29.66	28.81	30.37	27.5	26.2	27.6	27.3	25.7	26.1
25000.0	5	28.52	28.79	29.41	23.0	21.4	22.6	22.5	24.5	21.2
25000.0	6	30.95	28.57	29.26	31.0	29.1	29.7	31.2	29.6	29.0
25000.0	7	28.16	28.80	29.59	28.3	26.9	28.4	27.9	26.2	26.8
25000.0	8	28.74	28.65	29.91	27.8	26.3	27.3	28.1	27.7	26.1
25000.0	9	29.98	28.99	30.04	27.5	26.0	27.4	27.2	26.3	26.0
25000.0	10	34.91	35.00	35.00	27.7	26.4	27.8	27.6	26.4	26.2
25000.0	11	27.28	28.07	29.24	27.8	26.4	27.6	27.6	26.9	26.2
25000.0	12	28.20	31.50	31.27	30.9	29.8	31.1	30.7	28.4	29.7
25000.0	13	40.00	30.20	30.00	31.6	30.2	31.6	31.1	28.7	30.0
25000.0	14	27.88	29.92	30.09	28.5	27.1	28.8	28.2	25.8	27.1
25000.0	15	29.63	29.34	29.43	30.1	28.5	29.9	30.2	30.1	28.5
25000.0	16	30.89	31.10	29.26	28.4	27.0	28.4	28.0	26.6	26.8
25000.0	17	32.34	23.64	27.53	29.3	26.4	30.1	27.7	26.4	27.0
25000.0	18	29.94	31.72	31.80	28.4	29.2	28.8	26.9	27.0	27.5
25000.0	19	40.00	28.19	30.56	29.9	30.4	27.3	31.6	28.3	26.6
25000.0	20	25.16	28.48	26.75	26.9	27.9	29.3	29.0	27.5	28.1
2500.0	1	40.00	32.30	33.06	31.6	30.4	31.8	31.4	30.9	30.2
2500.0	2	32.60	32.47	33.78	32.1	30.9	32.9	32.0	27.6	30.7
2500.0	3	33.61	32.28	33.20	31.1	29.9	31.4	30.8	28.4	29.7

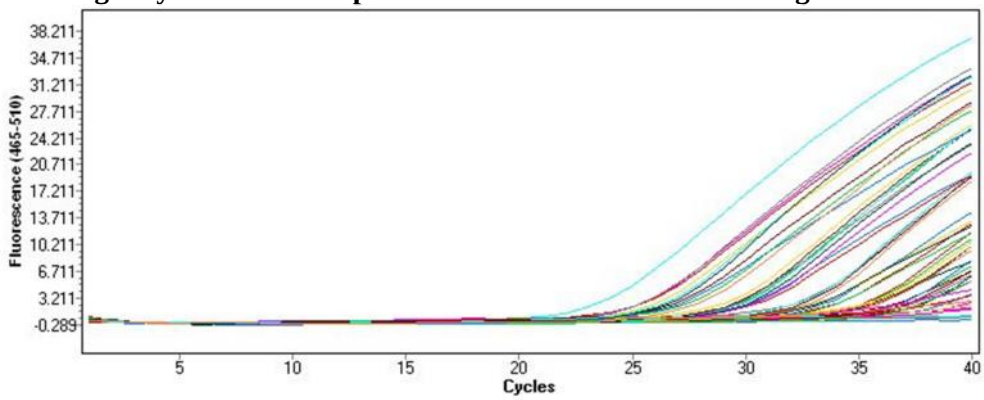
M. tuberculosis		Real-time PCR CT score				Xpert MTB/RIF Assay				
CFU/ml		*qiaAMP*	*easyMAG*	*MagNA Pure*	*Probe D*	*Probe C*	*Probe E*	*Probe B*	*SPC*	*Probe A*
2500.0	4	40.00	34.32	35.00	32.9	31.5	33.3	32.2	26.5	31.5
2500.0	5	33.75	32.40	32.66	30.0	28.6	29.7	30.3	27.8	28.4
2500.0	6	35.65	32.73	32.76	31.9	30.8	32.3	35.8	26.6	32.1
2500.0	7	32.66	32.05	32.51	33.3	32.0	34.5	32.5	26.9	32.5
2500.0	8	32.15	32.01	32.52	32.4	31.3	33.1	31.6	25.1	31.3
2500.0	9	31.14	31.75	32.26	22.8	21.3	22.7	22.6	25.4	21.2
2500.0	10	31.93	32.15	32.83	30.4	28.8	30.5	29.8	25.6	28.5
2500.0	11	30.67	34.23	35.00	40.0	40.0	40.0	40.0	29.2	40.0
2500.0	12	31.25	31.74	33.35	40.0	40.0	40.0	40.0	27.7	40.0
2500.0	13	40.00	31.35	32.68	29.6	28.3	29.9	29.0	24.7	29.2
2500.0	14	32.18	30.33	32.97	40.0	40.0	40.0	40.0	24.8	40.0
2500.0	15	33.25	30.48	33.44	30.3	29.0	30.6	30.0	26.3	28.9
2500.0	16	30.98	31.22	32.64	23.2	21.6	22.7	22.9	24.4	21.4
2500.0	17	40.00	32.03	33.41	-	-	-	-	-	-
2500.0	18	30.93	32.74	33.48	-	-	-	-	-	-
2500.0	19	30.77	32.05	32.63	-	-	-	-	-	-
2500.0	20	31.77	33.10	33.56	30.3	29.3	30.7	30.2	28.3	29.2
250.0	1	35.73	35.00	35.00	40.0	40.0	40.0	40.0	26.0	40.0
250.0	2	34.77	35.00	35.00	35.1	34.0	36.1	34.6	28.1	34.2
250.0	3	36.04	35.00	35.00	40.0	40.0	40.0	40.0	26.3	40.0
250.0	4	34.62	35.00	35.00	40.0	40.0	40.0	40.0	27.3	40.0
250.0	5	40.00	35.00	35.00	40.0	40.0	40.0	40.0	28.2	40.0
250.0	6	34.86	35.00	35.00	40.0	40.0	40.0	40.0	25.3	40.0
250.0	7	35.77	35.00	35.00	33.7	32.5	34.1	33.2	29.6	32.3
250.0	8	23.33	22.11	23.67	22.8	21.3	22.7	22.6	25.4	21.2
250.0	9	35.52	35.00	35.00	40.0	40.0	40.0	40.0	28.1	40.0
250.0	10	34.50	33.88	32.48	40.0	40.0	40.0	40.0	25.6	40.0
250.0	11	35.00	35.00	34.11	40.0	40.0	40.0	40.0	27.5	40.0
250.0	12	35.00	35.00	31.62	40.0	40.0	40.0	40.0	27.4	40.0
250.0	13	34.31	35.00	34.40	29.3	28.2	29.6	29.1	26.1	28.1
250.0	14	40.00	35.00	32.68	40.0	40.0	40.0	40.0	36.2	40.0
250.0	15	34.22	35.00	34.49	40.0	40.0	40.0	40.0	27.9	40.0
250.0	16	35.00	35.00	31.68	33.5	32.4	34.4	32.6	25.1	32.5
250.0	17	35.00	30.84	32.43	40.0	40.0	40.0	40.0	27.0	40.0
250.0	18	34.26	35.00	32.03	40.0	40.0	40.0	40.0	26.1	40.0
250.0	19	35.00	35.00	34.83	40.0	40.0	40.0	40.0	30.0	40.0
250.0	20	32.94	35.00	34.52	40.0	40.0	40.0	40.0	31.8	40.0
25.0	1	37.25	35.00	35.00	40.0	40.0	40.0	40.0	26.9	40.0
25.0	2	37.97	40.00	35.00	40.0	40.0	40.0	40.0	26.8	40.0
25.0	3	40.00	40.00	35.00	25.4	24.1	25.5	25.3	25.9	24.0
25.0	4	40.00	35.00	35.00	33.6	32.4	34.6	32.8	26.0	32.8
25.0	5	40.00	40.00	40.00	40.0	40.0	40.0	40.0	28.2	40.0
25.0	6	36.82	35.00	35.00	40.0	40.0	40.0	40.0	26.8	40.0
25.0	7	40.00	40.00	35.00	40.0	40.0	40.0	40.0	25.7	40.0
25.0	8	40.00	40.00	40.00	40.0	40.0	40.0	40.0	26.5	40.0
25.0	9	39.29	35.00	35.00	40.0	40.0	40.0	40.0	26.7	40.0
25.0	10	37.70	35.00	35.00	40.0	40.0	40.0	40.0	26.6	40.0
25.0	11	40.00	35.00	40.00	40.0	40.0	40.0	40.0	29.0	40.0

M. tuberculosis		Real-time PCR CT score				Xpert MTB/RIF Assay				
CFU/ml		*qiaAMP*	*easyMAG*	*MagNA Pure*	*Probe D*	*Probe C*	*Probe E*	*Probe B*	*SPC*	*Probe A*
25.0	12	40.00	35.00	34.03	-	-	-	-	-	-
25.0	13	40.00	40.00	40.00	40.0	40.0	40.0	40.0	24.3	40.0
25.0	14	35.00	35.00	40.00	40.0	40.0	40.0	40.0	24.6	40.0
25.0	15	40.00	35.00	33.98	-	-	-	-	-	-
25.0	16	40.00	40.00	35.00	29.2	27.0	27.4	30.9	27.4	26.7
25.0	17	40.00	35.00	40.00	40.0	40.0	40.0	40.0	31.1	40.0
25.0	18	35.00	40.00	34.32	40.0	40.0	40.0	40.0	28.0	40.0
25.0	19	40.00	40.00	40.00	40.0	40.0	40.0	40.0	28.0	40.0
25.0	20	40.00	40.00	33.93	40.0	40.0	40.0	40.0	25.6	40.0
2.5	1	40.00	40.00	40.00	40.0	40.0	40.0	40.0	25.6	40.0
2.5	2	37.80	40.00	40.00	40.0	40.0	40.0	40.0	25.8	40.0
2.5	3	40.00	40.00	35.00	40.0	40.0	40.0	40.0	27.2	40.0
2.5	4	40.00	40.00	33.19	40.0	40.0	40.0	40.0	27.8	40.0
2.5	5	40.00	40.00	35.00	40.0	40.0	40.0	40.0	27.7	40.0
2.5	6	40.00	40.00	40.00	40.0	40.0	40.0	40.0	27.8	40.0
2.5	7	40.00	40.00	40.00	40.0	40.0	40.0	40.0	28.3	40.0
2.5	8	40.00	40.00	40.00	40.0	40.0	40.0	40.0	24.7	40.0
2.5	9	40.00	40.00	34.58	40.0	40.0	40.0	40.0	27.7	40.0
2.5	10	40.00	40.00	40.00	40.0	40.0	40.0	40.0	27.2	40.0
2.5	11	35.00	40.00	40.00	40.0	40.0	40.0	40.0	25.2	40.0
2.5	12	35.00	40.00	35.00	40.0	40.0	40.0	40.0	27.2	40.0
2.5	13	40.00	40.00	40.00	40.0	40.0	40.0	40.0	29.1	40.0
2.5	14	35.00	40.00	40.00	40.0	40.0	40.0	40.0	30.2	40.0
2.5	15	40.00	40.00	40.00	40.0	40.0	40.0	40.0	24.6	40.0
2.5	16	40.00	35.00	40.00	40.0	40.0	40.0	40.0	26.4	40.0
2.5	17	40.00	40.00	35.00	40.0	40.0	40.0	40.0	27.3	40.0
2.5	18	40.00	35.00	35.00	40.0	40.0	40.0	40.0	27.1	40.0
2.5	19	40.00	40.00	40.00	40.0	40.0	40.0	40.0	25.3	40.0
2.5	20	40.00	40.00	40.00	40.0	40.0	40.0	40.0	28.7	40.0

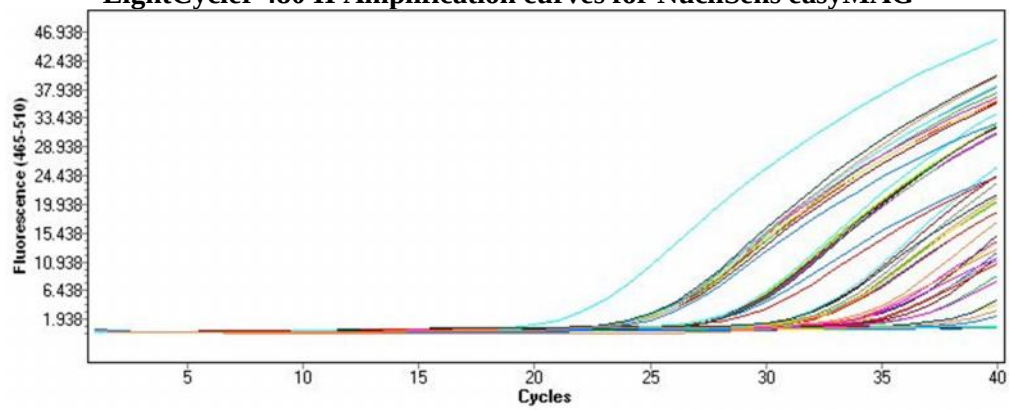
- ***Reaction failed***

Table 3b: Amplification curves generated on the LightCycler 480 II to determine the compatibility of PrimeStore® - MTM with the qiaAMP DNA mini kit (Qiagen), NucliSENS easyMAG (easyMAG) and MagNA PURE 96 (MagNA Pure) for the detection of *M. tuberculosis* spiked in sputum at various concentrations with the Xpert as a control (n=120)

LightCycler 480 II Amplification curves for the Roche MagNA Pure



LightCycler 480 II Amplification curves for NucliSens easyMAG



LightCycler 480 II Amplification curves for Qiagen QiaAMP

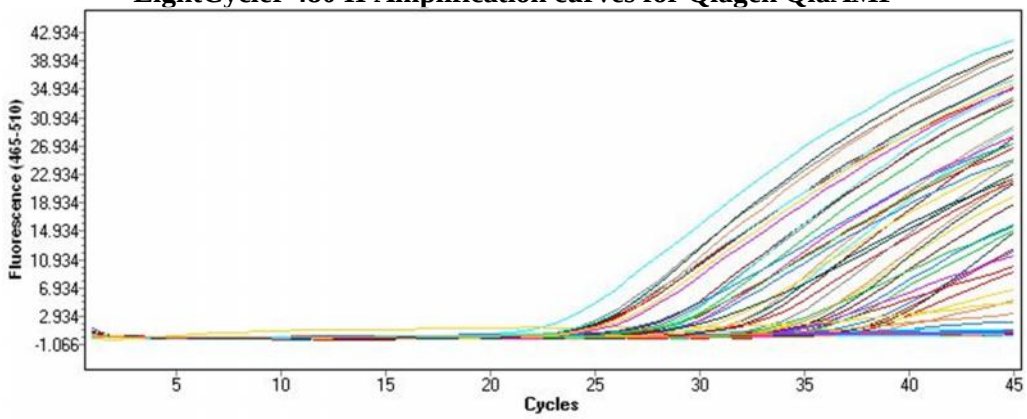


Table 3c: Probit regression analysis to determine the lower limits of detection of *M. tuberculosis* by real-time PCR for PrimeStore® - MTM with the qiaAMP DNA mini kit (Qiagen), NucliSENS easyMAG (easyMAG) and MagNA PURE 96 (MagNA Pure) for the detection of *M. tuberculosis* spiked in sputum at various concentrations with the Xpert as a control (n=120)

MagNA PURE: Probit analysis - probit sigmoid curve

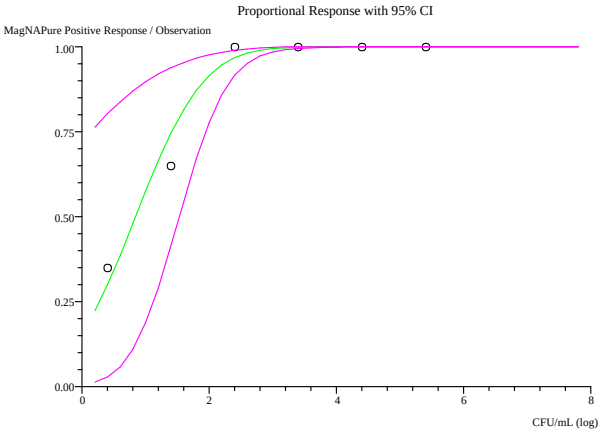
Constant = -0.999253
Slope = 1.186068

Median * Dose = 0.842492
Confidence interval (No Heterogeneity) = 0.385945 to 1.167861

* Dose for centile 95 = 2.229305 **(169 CFU/mL)**
Confidence interval (No Heterogeneity) = 1.78576 to 3.229744

Chi² (heterogeneity of deviations from model) = 1.900231 (4 df) P = 0.7541

t for slope = 4.396178 (4 df) P = 0.0117



Probit analysis - further statistics

Iterations = 11

Sxx = 13.738256
Sxy = 16.294499
Syy = 21.226608

Variance of B = 0.034579
Standard error of B without heterogeneity = 0.269795

Index	Subjects	Responses	Expected	Deviation
1	20	7	5.997043	1.002957
2	20	13	14.915427	-1.915427
3	20	20	19.352977	0.647023
4	20	20	19.975817	0.024183
5	20	20	19.999755	0.000245
6	20	20	19.999999	6.46E-07

easyMAG: Probit analysis - probit sigmoid curve

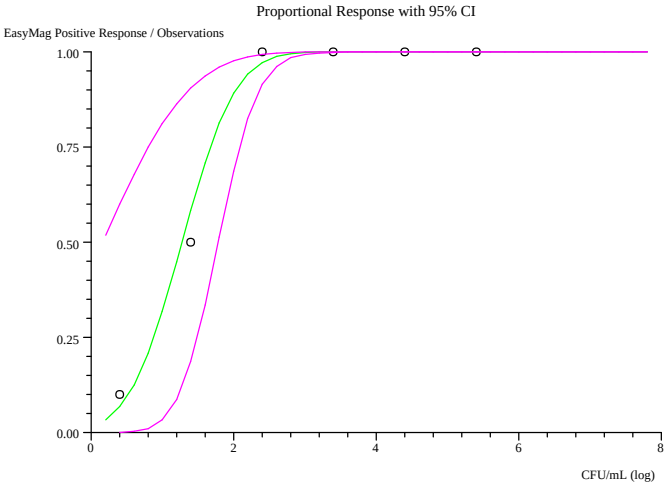
constant = -2.174522
slope = 1.703131

Median * Dose = 1.276779
Confidence interval (No Heterogeneity) = 1.000668 to 1.547903

* Dose for centile 95 = 2.242562 **(173 CFU/mL)**
Confidence interval (No Heterogeneity) = 1.894917 to 2.948104

Chi² (heterogeneity of deviations from model) = 1.475787 (4 df) P = 0.8309

t for slope = 4.928362 (4 df) P = 0.0079



Probit analysis - further statistics

Iterations = 12

Sxx = 8.373543
Sxy = 14.261238
Syy = 25.764541

Variance of B = 0.044061
Standard error of B without heterogeneity = 0.345577

Index	Subjects	Responses	Expected	Deviation
1	20	2	1.353666	0.646334
2	20	10	11.662238	-1.662238
3	20	20	19.442509	0.557491
4	20	20	19.99701	0.00299
5	20	20	19.999999	0.000001
6	20	20	20	2.18E-11

QiaAMP: Probit analysis - probit sigmoid curve

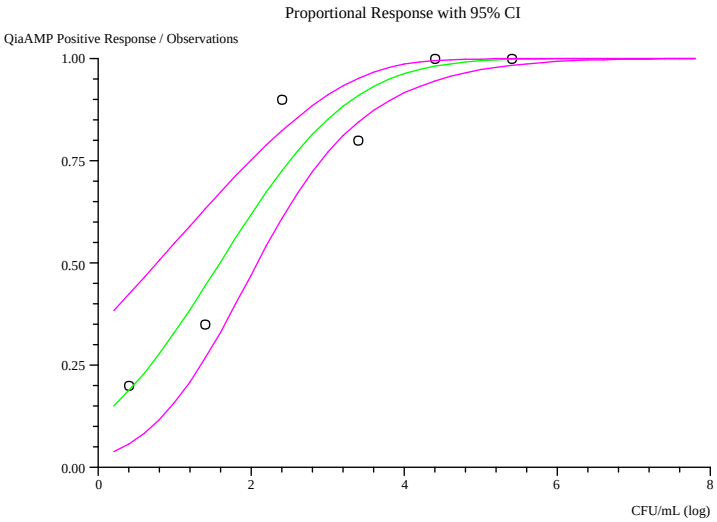
constant = -1.184233
slope = 0.743214

Median * Dose = 1.593393
Confidence interval (No Heterogeneity) = 1.098667 to 1.998542

* Dose for centile 95 = 3.806555 **(6397 CFU/mL)**
Confidence interval (No Heterogeneity) = 3.217333 to 4.871016

Chi² (heterogeneity of deviations from model) = 7.179959 (4 df) P = 0.1267

t for slope = 5.826322 (4 df) P = 0.0043



Probit analysis - further statistics

Iterations = 6

Sxx = 61.455494
Sxy = 45.674606
Syy = 41.125982

Variance of B = 0.029208
Standard error of B without heterogeneity = 0.127562

Index	Subjects	Responses	Expected	Deviation
1	20	4	3.751075	0.248925
2	20	7	8.857118	-1.857118
3	20	18	14.511484	3.488516
4	20	16	18.206297	-2.206297
5	20	20	19.630133	0.369867
6	20	20	19.953325	0.046675

Xpert MTB/RIF assay: Probit analysis - probit sigmoid curve

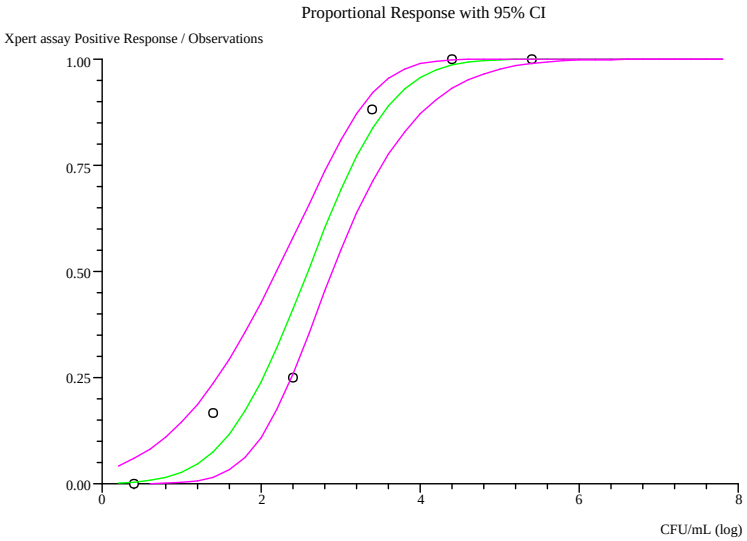
Constant = -3.131798
Slope = 1.211767

Median * Dose = 2.584488
Confidence interval (No Heterogeneity) = 2.260574 to 2.911534

* Dose for centile 95 = 3.941888 **(8749 CFU/ml)**
Confidence interval (No Heterogeneity) = 3.50286 to 4.734255

Chi² (heterogeneity of deviations from model) = 4.903221 (4 df) P = 0.2974

t for slope = 5.798316 (4 df) P = 0.0044



Probit analysis - further statistics

Iterations = 9

Sxx = 22.896297
Sxy = 27.744986
Syy = 38.523691

Variance of B = 0.053537
Standard error of B without heterogeneity = 0.208986

Index	Subjects	Responses	Expected	Deviation
1	20	0	0.081187	-0.081187
2	18	3	1.360754	1.639246
3	20	5	8.231026	-3.231026
4	17	15	14.254082	0.745918
5	20	20	19.721916	0.278084
6	20	20	19.993545	0.006455

Table 4: Summary of all patients included for the prospective routine laboratory evaluation using a novel collection method for the detection of *M. tuberculosis* from sputum (n=297)

ID	Fluorescent Microscopy	MGIT culture	Ziehl-Neelsen Microscopy	Identification	*IS6110* real-time PCR
1	negative	negative	negative	negative	40.00
2	negative	negative	negative	negative	40.00
3	positive	positive	negative	*Mycobacterium tuberculosis complex*	28.00
4	negative	negative	negative	negative	40.00
5	negative	negative	negative	negative	40.00
6	negative	negative	negative	negative	40.00
7	negative	negative	negative	negative	40.00
8	negative	negative	negative	negative	40.00
9	negative	negative	negative	negative	40.00
10	negative	negative	negative	negative	40.00
11	negative	negative	negative	negative	40.00
12	negative	negative	negative	negative	40.00
13	negative	positive	Positive for acid-fast bacilli	*Mycobacterium gordonae*	40.00
14	negative	negative	negative	negative	40.00
15	negative	negative	negative	negative	40.00
16	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	35.00
17	negative	negative	negative	negative	40.00
18	negative	negative	negative	negative	40.00
19	negative	negative	negative	negative	40.00
20	negative	negative	negative	negative	40.00
21	negative	negative	negative	negative	40.00
22	negative	negative	negative	negative	40.00
23	negative	negative	negative	negative	40.00
24	negative	negative	negative	negative	35.00
25	negative	negative	negative	negative	40.00
26	negative	negative	negative	negative	40.00
27	excluded	negative	excluded	negative	40.00
28	Negative	contaminated	not done	negative	40.00
29	excluded	negative	excluded	negative	40.00
30	Negative	contaminated	not done	negative	40.00
31	excluded	negative	excluded	negative	40.00
32	negative	negative	negative	negative	40.00
33	negative	negative	negative	negative	40.00
34	positive	positive	negative	*Mycobacterium tuberculosis complex*	28.00
35	negative	negative	negative	negative	40.00
36	negative	negative	negative	negative	40.00

ID	Fluorescent Microscopy	MGIT culture	Ziehl-Neelsen Microscopy	Identification	*IS6110* real-time PCR
37	negative	negative	negative	negative	40.00
38	negative	negative	negative	negative	40.00
39	Negative	contaminated	not done	negative	35.00
40	negative	negative	negative	negative	40.00
41	negative	negative	negative	negative	40.00
42	Negative	contaminated	not done	negative	40.00
43	positive	positive	negative	*Mycobacterium tuberculosis complex*	30.50
44	negative	negative	negative	negative	40.00
45	positive	positive	negative	*Mycobacterium tuberculosis complex*	22.90
46	negative	negative	negative	negative	40.00
47	negative	negative	negative	negative	35.00
48	negative	negative	negative	negative	40.00
49	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	40.00
50	negative	negative	negative	negative	40.00
51	Negative	contaminated	not done	negative	40.00
52	negative	negative	negative	negative	40.00
53	Negative	contaminated	not done	negative	40.00
54	Negative	contaminated	not done	negative	35.00
55	negative	negative	negative	negative	40.00
56	negative	negative	negative	negative	40.00
57	negative	negative	negative	negative	40.00
58	negative	negative	negative	negative	40.00
59	negative	negative	negative	negative	40.00
60	Negative	contaminated	not done	negative	40.00
61	negative	negative	negative	negative	40.00
62	negative	negative	negative	negative	40.00
63	negative	negative	negative	negative	40.00
64	negative	negative	negative	negative	40.00
65	Negative	contaminated	not done	negative	40.00
66	Negative	contaminated	not done	negative	40.00
67	negative	negative	negative	negative	35.00
68	Negative	contaminated	not done	negative	40.00
69	negative	negative	negative	negative	35.00
70	negative	negative	negative	negative	40.00
71	excluded	negative	excluded	negative	35.00
72	negative	negative	negative	negative	40.00
73	negative	negative	negative	negative	40.00
74	negative	negative	negative	negative	35.00
75	negative	negative	negative	negative	40.00
76	negative	negative	negative	negative	40.00
77	negative	positive	Positive for acid-fast bacilli	*Mycobacterium scrofulaceum*	40.00

ID	Fluorescent Microscopy	MGIT culture	Ziehl-Neelsen Microscopy	Identification	*IS6110* real-time PCR
78	positive	positive	negative	*Mycobacterium tuberculosis complex*	25.50
79	negative	negative	negative	negative	40.00
80	negative	negative	negative	negative	40.00
81	negative	negative	negative	negative	40.00
82	negative	negative	negative	negative	40.00
83	negative	negative	negative	negative	40.00
84	negative	negative	negative	negative	40.00
85	negative	negative	negative	negative	40.00
86	negative	negative	negative	negative	40.00
87	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	40.00
88	negative	negative	negative	negative	40.00
89	negative	negative	negative	negative	40.00
90	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	40.00
91	negative	negative	negative	negative	40.00
92	negative	negative	negative	negative	40.00
93	negative	negative	negative	negative	40.00
94	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	35.00
95	negative	negative	negative	negative	40.00
96	Negative	contaminated	not done	negative	40.00
97	negative	negative	negative	negative	40.00
98	excluded	negative	excluded	negative	40.00
99	negative	negative	negative	negative	40.00
100	negative	negative	negative	negative	40.00
101	negative	negative	negative	negative	40.00
102	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	40.00
103	negative	negative	negative	negative	40.00
104	negative	positive	Positive for acid-fast bacilli	*Mycobacterium intracellulare*	40.00
105	negative	negative	negative	negative	40.00
106	negative	negative	negative	negative	40.00
107	negative	negative	negative	negative	40.00
108	negative	negative	negative	negative	40.00
109	negative	negative	negative	negative	40.00
110	negative	positive	Positive for acid-fast bacilli	*Mycobacterium intracellulare*	40.00
111	negative	negative	negative	negative	40.00
112	negative	negative	negative	negative	40.00
113	negative	negative	negative	negative	40.00
114	negative	negative	negative	negative	40.00
115	negative	negative	negative	negative	40.00
116	negative	negative	negative	negative	40.00
117	negative	positive	Positive for acid-fast bacilli	*Mycobacterium intracellulare*	40.00
118	negative	negative	negative	negative	40.00

ID	Fluorescent Microscopy	MGIT culture	Ziehl-Neelsen Microscopy	Identification	*IS6110* real-time PCR
119	negative	negative	negative	negative	40.00
120	negative	negative	negative	negative	40.00
121	negative	negative	negative	negative	40.00
122	negative	negative	negative	negative	40.00
123	negative	negative	negative	negative	40.00
124	negative	negative	negative	negative	40.00
125	negative	negative	negative	negative	40.00
126	Negative	contaminated	not done	negative	40.00
127	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	40.00
128	positive	positive	negative	*Mycobacterium tuberculosis complex*	28.90
129	negative	negative	negative	negative	40.00
130	negative	negative	negative	negative	40.00
131	negative	negative	negative	negative	40.00
132	positive	positive	negative	*Mycobacterium tuberculosis complex*	32.70
133	negative	negative	negative	negative	40.00
134	Negative	contaminated	not done	negative	40.00
135	negative	negative	negative	negative	40.00
136	negative	negative	negative	negative	40.00
137	negative	negative	negative	negative	40.00
138	negative	negative	negative	negative	40.00
139	negative	negative	negative	negative	40.00
140	negative	negative	negative	negative	40.00
141	negative	negative	negative	negative	40.00
142	negative	negative	negative	negative	40.00
143	negative	negative	negative	negative	40.00
144	negative	negative	negative	negative	40.00
145	negative	negative	negative	negative	40.00
146	Negative	contaminated	not done	negative	40.00
147	negative	negative	negative	negative	40.00
148	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	40.00
149	negative	negative	negative	negative	40.00
150	negative	negative	negative	negative	40.00
151	negative	negative	negative	negative	40.00
152	Negative	contaminated	not done	negative	40.00
153	negative	negative	negative	negative	40.00
154	negative	negative	negative	negative	40.00
155	negative	negative	negative	negative	40.00
156	negative	negative	negative	negative	40.00
157	negative	negative	negative	negative	40.00
158	positive	positive	negative	*Mycobacterium tuberculosis complex*	26.40
159	Negative	contaminated	not done	negative	40.00

ID	Fluorescent Microscopy	MGIT culture	Ziehl-Neelsen Microscopy	Identification	*IS6110* real-time PCR
160	negative	negative	negative	negative	40.00
161	Negative	contaminated	not done	negative	40.00
162	negative	negative	negative	negative	40.00
163	negative	negative	negative	negative	40.00
164	negative	negative	negative	negative	40.00
165	negative	negative	negative	negative	40.00
166	negative	negative	negative	negative	40.00
167	negative	negative	negative	negative	40.00
168	negative	negative	negative	negative	40.00
169	negative	negative	negative	negative	40.00
170	negative	negative	negative	negative	40.00
171	negative	negative	negative	negative	40.00
172	negative	negative	negative	negative	40.00
173	negative	negative	negative	negative	40.00
174	negative	negative	negative	negative	40.00
175	excluded	negative	excluded	negative	40.00
176	Negative	contaminated	not done	negative	40.00
177	negative	negative	negative	negative	40.00
178	negative	negative	negative	negative	40.00
179	negative	negative	negative	negative	40.00
180	negative	negative	negative	negative	40.00
181	negative	negative	negative	negative	40.00
182	negative	negative	negative	negative	40.00
183	negative	negative	negative	negative	40.00
184	negative	negative	negative	negative	40.00
185	negative	negative	negative	negative	40.00
186	negative	negative	negative	negative	40.00
187	negative	negative	negative	negative	40.00
188	negative	negative	negative	negative	40.00
189	negative	negative	negative	negative	40.00
190	Negative	contaminated	not done	negative	40.00
191	Negative	contaminated	not done	negative	40.00
192	negative	negative	negative	negative	40.00
193	positive	positive	negative	*Mycobacterium tuberculosis complex*	35.00
194	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	35.00
195	positive	positive	negative	*Mycobacterium tuberculosis complex*	28.70
196	negative	negative	negative	negative	40.00
197	positive	positive	negative	*Mycobacterium tuberculosis complex*	28.00
198	Negative	contaminated	not done	negative	40.00
199	negative	negative	negative	negative	40.00
200	negative	negative	negative	negative	40.00

ID	Fluorescent Microscopy	MGIT culture	Ziehl-Neelsen Microscopy	Identification	*IS6110* real-time PCR
201	negative	negative	negative	negative	40.00
202	negative	negative	negative	negative	40.00
203	negative	negative	negative	negative	40.00
204	negative	negative	negative	negative	40.00
205	negative	negative	negative	negative	40.00
206	Negative	contaminated	not done	negative	35.00
207	Negative	contaminated	not done	negative	40.00
208	Negative	contaminated	not done	negative	40.00
209	negative	negative	negative	negative	40.00
210	negative	negative	negative	negative	40.00
211	negative	negative	negative	negative	40.00
212	negative	negative	negative	negative	40.00
213	negative	negative	negative	negative	40.00
214	negative	negative	negative	negative	40.00
215	negative	negative	negative	negative	40.00
216	negative	negative	negative	negative	40.00
217	negative	negative	negative	negative	40.00
218	negative	negative	negative	negative	40.00
219	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	35.00
220	negative	negative	negative	negative	40.00
221	negative	negative	negative	negative	40.00
222	negative	negative	negative	negative	40.00
223	negative	negative	negative	negative	40.00
224	negative	negative	negative	negative	40.00
225	negative	negative	negative	negative	40.00
226	negative	negative	negative	negative	40.00
227	negative	negative	negative	negative	40.00
228	negative	negative	negative	negative	40.00
229	negative	negative	negative	negative	35.00
230	excluded	negative	excluded	negative	40.00
231	negative	negative	negative	negative	40.00
232	negative	negative	negative	negative	40.00
233	negative	negative	negative	negative	35.00
234	negative	negative	negative	negative	40.00
235	positive	positive	negative	*Mycobacterium tuberculosis complex*	33.30
236	negative	negative	negative	negative	40.00
237	negative	negative	negative	negative	40.00
238	negative	negative	negative	negative	40.00
239	Negative	contaminated	not done	negative	35.00
240	negative	negative	negative	negative	40.00
241	negative	negative	negative	negative	40.00

ID	Fluorescent Microscopy	MGIT culture	Ziehl-Neelsen Microscopy	Identification	*IS6110* real-time PCR
242	negative	negative	negative	negative	40.00
243	negative	negative	negative	negative	40.00
244	negative	positive	Positive for acid-fast bacilli	*Mycobacterium intracellulare*	40.00
245	Negative	contaminated	not done	negative	40.00
246	Negative	contaminated	not done	negative	40.00
247	excluded	negative	excluded	negative	29.80
248	negative	negative	negative	negative	40.00
249	negative	positive	Positive for acid-fast bacilli	*Mycobacterium intracellulare*	40.00
250	Negative	contaminated	not done	negative	27.10
251	negative	negative	negative	negative	40.00
252	negative	negative	negative	negative	40.00
253	negative	negative	negative	negative	40.00
254	negative	negative	negative	negative	40.00
255	negative	positive	Positive for acid-fast bacilli	*Mycobacterium avium*	40.00
256	negative	negative	negative	negative	40.00
257	negative	negative	negative	negative	40.00
258	negative	negative	negative	negative	40.00
259	negative	negative	negative	negative	40.00
260	negative	negative	negative	negative	35.00
261	negative	negative	negative	negative	40.00
262	Negative	contaminated	not done	negative	40.00
263	negative	negative	negative	negative	40.00
264	negative	negative	negative	negative	40.00
265	negative	negative	negative	negative	40.00
266	negative	negative	negative	negative	40.00
267	negative	negative	negative	negative	40.00
268	Negative	contaminated	not done	negative	40.00
269	Negative	contaminated	not done	negative	40.00
270	Negative	contaminated	not done	negative	40.00
271	negative	negative	negative	negative	40.00
272	negative	negative	negative	negative	40.00
273	negative	negative	negative	negative	40.00
274	negative	negative	negative	negative	40.00
275	negative	negative	negative	negative	40.00
276	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	35.00
277	negative	negative	negative	negative	40.00
278	negative	negative	negative	negative	40.00
279	negative	negative	negative	negative	40.00
280	negative	negative	negative	negative	40.00
281	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	34.52
282	negative	negative	negative	negative	40.00

ID	Fluorescent Microscopy	MGIT culture	Ziehl-Neelsen Microscopy	Identification	*IS6110* real-time PCR
283	negative	negative	negative	negative	40.00
284	negative	negative	negative	negative	40.00
285	negative	negative	negative	negative	40.00
286	negative	negative	negative	negative	40.00
287	negative	positive	Positive for acid-fast bacilli	*Mycobacterium tuberculosis complex*	35.00
288	negative	negative	negative	negative	40.00
289	negative	negative	negative	negative	40.00
290	negative	negative	negative	negative	40.00
291	negative	negative	negative	negative	40.00
292	negative	negative	negative	negative	40.00
293	positive	positive	negative	*Mycobacterium tuberculosis complex*	35.00
294	negative	negative	negative	negative	40.00
295	negative	negative	negative	negative	40.00
296	negative	negative	negative	negative	40.00
297	negative	negative	negative	negative	40.00

Table 5: Summary of the field evaluation for the detection of *M. tuberculosis* by real-time PCR using a novel sputum collection method (n=141)

Study Number	Sample ID		First sample test process	*IS6110* real-time PCR (sample process method)				Xpert MTB/RIF assay	MGIT 960 liquid culture	Ziehl-Neelsen Microscopy
	MGIT	Xpert		Cycle Threshold (MGIT)	Cycle Threshold (Xpert)	Result (MGIT)	Result (Xpert)			
SHO2012080083	-	25	Xpert	not done	40	not done	Negative	Negative	not done	not done
BAS2012091110	270	281	Xpert	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
BAS2012091164	289	296	Xpert	38.24	40.00	Indeterminate	Negative	Negative	Contaminated	Negative
BAS2013041176	303	315	Xpert	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
BAS2013051187	308	320	MGIT	38.13	40.00	Indeterminate	Negative	Negative	Contaminated	Negative
KRT2012092092	161	173	Xpert	34.65	34.65	Positive	Positive	Negative	Contaminated	Negative
KRT2012092132	183	188	Xpert	40.00	37.58	Negative	Positive	Negative	Contaminated	Negative
MHL2012115001	312	324	Xpert	28.05	40.00	Positive	Negative	Negative	Contaminated	Negative
SHO2012106010	43	53	MGIT	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
SHO2012106042	84	96	Xpert	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
SHO2012091120	18	28	Xpert	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
SHO2012091124	22	32	Xpert	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
MOD2012107027	123	135	MGIT	40.00	40.00	Negative	Negative	Positive	Contaminated	Negative
MOD2013027040	143	149	MGIT	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
MOD2013047053	144	150	Xpert	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
MOD2013047055	146	152	Xpert	40.00	40.00	Negative	Negative	Negative	Contaminated	Negative
KRT2012092090	159	171	MGIT	40.00	40.00	Negative	Negative	Negative	NTM isolated	Negative
MOD2012107012	119	131	MGIT	40.00	40.00	Negative	Negative	Negative	NTM isolated	Negative
BAS2012091109	269	280	Xpert	29.63	31.34	Positive	Positive	Positive	Positive	Negative
BAS2013051188	309	321	Xpert	40.00	33.41	Negative	Positive	Positive	Positive	Negative
KRT2012082072	157	169	Xpert	26.59	38.46	Positive	Indeterminate	Positive	Positive	Positive
KRT2012092122	166	178	Xpert	21.15	22.52	Positive	Positive	Positive	Positive	Positive

Study Number	Sample ID		First sample test process	*IS6110* real-time PCR (sample process method)				Xpert MTB/RIF assay	MGIT 960 liquid culture	Ziehl-Neelsen Microscopy
	MGIT	Xpert		Cycle Threshold (MGIT)	Cycle Threshold (Xpert)	Result (MGIT)	Result (Xpert)			
KRT2012092134	190	203	MGIT	25.32	25.38	Positive	Positive	not done	Positive	Positive
KRT2012092136	192	-	Xpert	21.42	not done	Positive	not done	Positive	Positive	Positive
KRT2013042167	217	223	Xpert	40.00	23.27	Negative	Positive	Positive	Positive	Positive
MHL2013035022	327	333	MGIT	28.47	27.09	Positive	Positive	Positive	Positive	Positive
MHL2013045024	329	335	MGIT	22.20	22.87	Positive	Positive	Positive	Positive	Positive
SHO2012106011	44	54	Xpert	20.58	20.45	Positive	Positive	Positive	Positive	Positive
SHO2012106035	68	78	Xpert	29.09	28.22	Positive	Positive	Positive	Positive	Positive
SHO2012106038	71	81	Xpert	27.18	40.00	Positive	Negative	Positive	Positive	Positive
SHO2012106050	90	102	Xpert	31.81	31.83	Positive	Positive	Positive	Positive	Positive
SHO2012080079	6	13	MGIT	23.44	25.74	Positive	Positive	Positive	Positive	Positive
SHO2012080081	7	14	MGIT	40.00	23.15	Negative	Positive	Positive	Positive	Positive
MOD2012107026	122	134	Xpert	24.41	26.48	Positive	Positive	Positive	Positive	Positive
MOD2012107035	127	139	MGIT	22.29	20.34	Positive	Positive	Positive	Positive	Positive
MOD2013027039	130	142	Xpert	25.66	24.62	Positive	Positive	Positive	Positive	Positive
BAS2012091175	302	314	MGIT	29.07	30.40	Positive	Positive	Positive	Positive	Positive
KRT2012092131	182	187	Xpert	28.52	27.60	Positive	Positive	Positive	Positive	Positive
SHO2012106036	69	79	MGIT	22.80	23.76	Positive	Positive	Positive	Positive	Positive
SHO2012106062	94	106	Xpert	30.80	33.03	Positive	Positive	Positive	Positive	Positive
BAS2012091145	275	286	MGIT	38.04	30.01	Indeterminate	Positive	Positive	Positive	Positive
KRT2012092155	201	214	MGIT	28.92	31.66	Positive	Positive	Positive	Positive	Positive
SHO2012106021	47	57	Xpert	26.77	27.64	Positive	Positive	Positive	Positive	Positive
SHO2012091121	19	29	MGIT	28.98	28.14	Positive	Positive	Positive	Positive	Positive
BAS2012091073	232	242	Xpert	30.72	40.00	Positive	Negative	Positive	Positive	Negative
BAS2012091107	267	278	Xpert	31.92	29.52	Positive	Positive	Positive	Positive	Negative
BAS2012091165	290	297	MGIT	40.00	40.00	Negative	Negative	Negative	Positive	Negative
BAS2012091167	292	299	MGIT	32.50	34.46	Positive	Positive	Positive	Positive	Negative
KRT2012080029	148	154	MGIT	40.00	40.00	Negative	Negative	Negative	Positive	Negative

Study Number	Sample ID		First sample test process	*IS6110* real-time PCR (sample process method)				Xpert MTB/RIF assay	MGIT 960 liquid culture	Ziehl-Neelsen Microscopy
	MGIT	Xpert		Cycle Threshold (MGIT)	Cycle Threshold (Xpert)	Result (MGIT)	Result (Xpert)			
KRT2012092091	160	172	Xpert	40.00	40.00	Negative	Negative	Negative	Positive	Negative
KRT2012092148	198	211	MGIT	30.30	32.79	Positive	Positive	Positive	Positive	Negative
SHO2012106032	65	75	MGIT	40.00	40.00	Negative	Negative	Negative	Positive	Negative
SHO2012106033	66	76	Xpert	40.00	40.00	Negative	Negative	Positive	Positive	Negative
SHO2012106037	70	80	Xpert	35.48	37.01	Positive	Positive	Positive	Positive	Negative
SHO2012106049	89	101	MGIT	40.00	33.88	Negative	Positive	Positive	Positive	Negative
SHO2012106073	109	115	Xpert	30.29	34.81	Positive	Positive	Negative	Positive	Negative
SHO2012091122	20	-	Xpert	40.00	not done	Negative	not done	Negative	Positive	Negative
SHO2012091138	24	34	MGIT	40.00	30.94	Negative	Positive	Positive	Positive	Negative
MOD2012107011	112	118	Xpert	24.74	28.45	Positive	Positive	Positive	Positive	Negative
KRT2012082071	156	168	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Positive
BAS2012080033	219	225	Xpert	32.76	40.00	Positive	Negative	Negative	Negative	Negative
BAS2012091072	231	241	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091075	233	243	Xpert	40.00	40.00	Negative	Negative	not done	Negative	Negative
BAS2012091080	234	244	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012090081	235	245	MGIT	35.86	34.39	Positive	Positive	not done	Negative	Negative
BAS2012090082	236	246	Xpert	36.61	36.07	Positive	Positive	Negative	Negative	Negative
BAS2012090085	249	258	MGIT	40.00	29.17	Negative	Positive	Positive	Negative	Negative
BAS2012091105	265	276	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091106	266	277	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091108	268	279	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091140	273	284	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091144	274	285	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091146	287	294	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091163	288	295	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091166	291	298	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2012091168	293	300	Xpert	40.00	29.60	Negative	Positive	Negative	Negative	Negative
BAS2012091174	301	313	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative

Study Number	Sample ID		First sample test process	*IS6110* real-time PCR (sample process method)				Xpert MTB/RIF assay	MGIT 960 liquid culture	Ziehl-Neelsen Microscopy
	MGIT	Xpert		Cycle Threshold (MGIT)	Cycle Threshold (Xpert)	Result (MGIT)	Result (Xpert)			
BAS2013041182	304	316	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2013041183	305	317	Xpert	32.89	40.00	Positive	Negative	Positive	Negative	Negative
BAS2013041184	306	318	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2013051186	307	319	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2013051189	310	322	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
BAS2013051190	311	323	MGIT	40.00	33.34	Negative	Positive	Positive	Negative	Negative
KRT2012080028	147	153	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2012080030	155	167	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2012082073	158	170	MGIT	40.00	38.03	Negative	Indeterminate	Negative	Negative	Negative
KRT2012092114	162	174	MGIT	40.00	37.61	Negative	Positive	Negative	Negative	Negative
KRT2012092121	165	177	Xpert	40.00	34.04	Negative	Positive	Negative	Negative	Negative
KRT2012092123	179	-	MGIT	40.00	not done	Negative	not done	Negative	Negative	Negative
KRT2012092129	180	185	MGIT	37.66	40.00	Positive	Negative	Negative	Negative	Negative
KRT2012092130	181	186	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2012092133	189	202	MGIT	36.50	40.00	Positive	Negative	Negative	Negative	Negative
KRT2012092135	191	204	MGIT	40.00	22.09	Negative	Positive	Negative	Negative	Negative
KRT2012092137	193	206	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2012092143	196	209	MGIT	40.00	34.46	Negative	Positive	Negative	Negative	Negative
KRT2012092147	197	210	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2012092149	199	212	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2012092154	200	213	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2012092156	215	221	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2013042166	216	222	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
KRT2013042168	218	224	MGIT	40.00	36.53	Negative	Positive	Negative	Negative	Negative
MHL2013025020	325	331	Xpert	40.00	33.05	Negative	Positive	Negative	Negative	Negative
MHL2013025021	326	332	Xpert	40.00	29.35	Negative	Positive	Negative	Negative	Negative
MHL2013045023	328	334	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative

Study Number	Sample ID		First sample test process	*IS6110* real-time PCR (sample process method)				Xpert MTB/RIF assay	MGIT 960 liquid culture	Ziehl-Neelsen Microscopy
	MGIT	Xpert		Cycle Threshold (MGIT)	Cycle Threshold (Xpert)	Result (MGIT)	Result (Xpert)			
MHL2013055025	330	336	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012096003	36	40	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012096004	37	41	Xpert	40.00	40.00	Negative	Negative	Positive	Negative	Negative
SHO2012096005	38	42	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106020	46	56	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106022	48	58	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106025	49	59	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106026	50	60	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106028	51	61	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106029	-	62	MGIT	not done	40.00	not done	Negative	Negative	Negative	Negative
SHO2012106030	63	73	Xpert	26.06	40.00	Positive	Negative	Negative	Negative	Negative
SHO2012106031	64	74	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106034	67	77	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106039	72	82	MGIT	34.29	38.83	Positive	Indeterminate	Negative	Negative	Negative
SHO2012106040	83	95	Xpert	33.71	40.00	Positive	Negative	Negative	Negative	Negative
SHO2012106043	85	97	MGIT	34.69	36.35	Positive	Positive	Negative	Negative	Negative
SHO2012106044	86	98	Xpert	40.00	35.79	Negative	Positive	Negative	Negative	Negative
SHO2012106046	87	99	Xpert	40.00	35.66	Negative	Positive	Negative	Negative	Negative
SHO2012106048	88	100	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106051	91	103	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106052	92	104	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106061	93	105	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106072	108	114	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012106074	110	116	MGIT	37.24	40.00	Positive	Negative	Negative	Negative	Negative
SHO2012091119	17	27	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012091123	21	31	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012091137	23	33	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative

Study Number	Sample ID		First sample test process	*IS6110* real-time PCR (sample process method)				Xpert MTB/RIF assay	MGIT 960 liquid culture	Ziehl-Neelsen Microscopy
	MGIT	Xpert		Cycle Threshold (MGIT)	Cycle Threshold (Xpert)	Result (MGIT)	Result (Xpert)			
SHO2012091139	35	39	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012080073	3	10	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
SHO2012080085	16	26	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
MOD2012107010	111	117	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
MOD2012107024	120	132	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
MOD2012107025	121	133	MGIT	40.00	40.00	Negative	Negative	Negative	Negative	Negative
MOD2012107034	126	138	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
MOD2012107036	128	140	Xpert	40.00	40.00	Negative	Negative	Negative	Negative	Negative
MOD2013027038	129	-	MGIT	40.00	not done	Negative	not done	Negative	Negative	Negative
MOD2013047054	145	151	MGIT	28.81	26.76	Positive	Positive	Positive	Negative	Negative

Table 6: Raw data followed by the amplification plot generated on the StepOne plus for the field evaluation of the novel collection system using PrimeStore® - MTM detecting *M. tuberculosis* by real-time PCR (n=141)

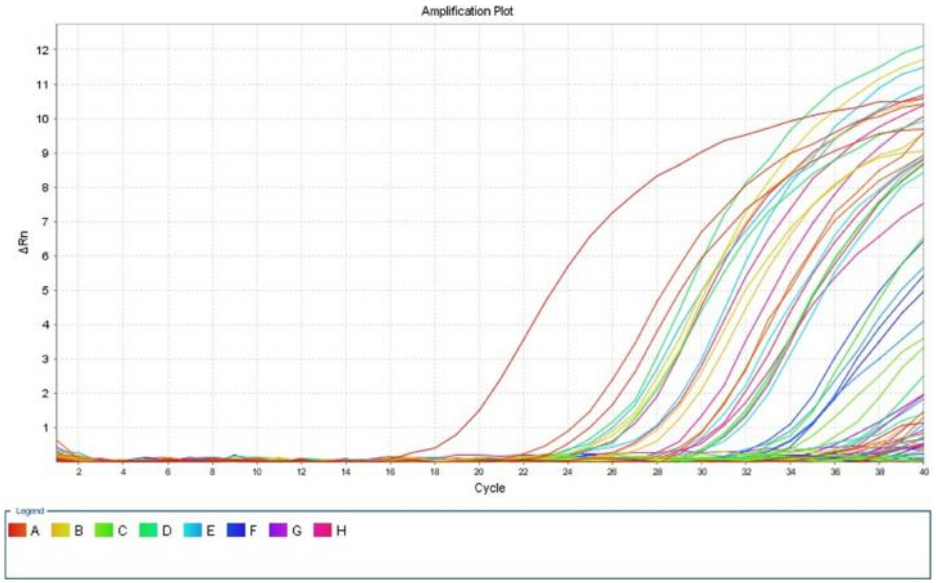
Block Type	96well
Chemistry	TAQMAN
Experiment File Name	D:\Applied Biosystems\StepOne Software v2.2.2\experiments\Anova PS Study\ANOVA SUBSET RUN1 06012014 treshold 01.eds
Experiment Run End Time	2014-01-09 15:05:34 PM PST
Instrument Type	steponeplus
Passive Reference	ROX

Well	Sample ID*	Study Number	Target Name	Reporter	Quencher	Cт	Ct Threshold	Baseline Start	Baseline End	BADROX	CTFAIL
A1	Negative		Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A2	Positive		Target 1	FAM	NFQ-MGB	16.22542763	0.1	3	15	Y	N
A3	177	KRT2012092121	Target 1	FAM	NFQ-MGB	34.04155731	0.1	3	15	N	N
A4	165	KRT2012092121	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A5	178	KRT2012092122	Target 1	FAM	NFQ-MGB	22.52157784	0.1	3	15	N	N
A6	166	KRT2012092122	Target 1	FAM	NFQ-MGB	21.15174675	0.1	3	15	Y	N
A7	180	KRT2012092129	Target 1	FAM	NFQ-MGB	37.65538025	0.1	3	15	N	N
A8	186	KRT2012092130	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A9	181	KRT2012092130	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A10	187	KRT2012092131	Target 1	FAM	NFQ-MGB	27.60429001	0.1	3	15	N	N
A11	182	KRT2012092131	Target 1	FAM	NFQ-MGB	28.52414894	0.1	3	15	N	N
A12	188	KRT2012092132	Target 1	FAM	NFQ-MGB	37.58263016	0.1	3	15	N	N
B1	183	KRT2012092132	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B2	202	KRT2012092133	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B3	189	KRT2012092133	Target 1	FAM	NFQ-MGB	36.50348282	0.1	3	15	N	N
B4	203	KRT2012092134	Target 1	FAM	NFQ-MGB	25.37902832	0.1	3	15	N	N
B5	190	KRT2012092134	Target 1	FAM	NFQ-MGB	25.32406425	0.1	3	15	N	N
B6	204	KRT2012092135	Target 1	FAM	NFQ-MGB	22.0938282	0.1	3	15	Y	N
B7	191	KRT2012092135	Target 1	FAM	NFQ-MGB	24.99560356	0.1	3	15	N	N
B8	192	KRT2012092136	Target 1	FAM	NFQ-MGB	21.42495155	0.1	3	15	N	N
B9	206	KRT2012092137	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B10	209	KRT2012092143	Target 1	FAM	NFQ-MGB	34.45597839	0.1	3	15	N	N
B11	196	KRT2012092143	Target 1	FAM	NFQ-MGB	21.79323959	0.1	3	15	N	N
B12	193	KRT2012092137	Target 1	FAM	NFQ-MGB	23.9287529	0.1	3	15	N	N
C1	210	KRT2012092147	Target 1	FAM	NFQ-MGB	25.72839928	0.1	3	15	N	N
C2	197	KRT2012092147	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N

C3	211	KRT2012092148	Target 1	FAM	NFQ-MGB	32.78787994	0.1	3	15	N	N
C4	198	KRT2012092148	Target 1	FAM	NFQ-MGB	30.30061913	0.1	3	15	N	N
C5	212	KRT2012092149	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	Y
C6	199	KRT2012092149	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C7	213	KRT2012092154	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C8	200	KRT2012092154	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C9	222	KRT2013042166	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C10	216	KRT2013042166	Target 1	FAM	NFQ-MGB	23.97019005	0.1	3	15	N	N
C11	214	KRT2012092155	Target 1	FAM	NFQ-MGB	31.65860748	0.1	3	15	N	N
C12	201	KRT2012092155	Target 1	FAM	NFQ-MGB	28.92263985	0.1	3	15	N	N
D1	221	KRT2012092156	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	Y
D2	215	KRT2012092156	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D3	223	KRT2013042167	Target 1	FAM	NFQ-MGB	23.26810837	0.1	3	15	N	N
D4	331	MHL2013025020	Target 1	FAM	NFQ-MGB	33.04520035	0.1	3	15	N	N
D5	217	KRT2013042167	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	Y
D6	325	MHL2013025020	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D7	328	MHL2013045023	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D8	332	MHL2013025021	Target 1	FAM	NFQ-MGB	29.35029411	0.1	3	15	N	N
D9	224	KRT2013042168	Target 1	FAM	NFQ-MGB	36.53406906	0.1	3	15	N	N
D10	218	KRT2013042168	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D11	324	MHL2012115001	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D12	312	MHL2012115001	Target 1	FAM	NFQ-MGB	28.05423546	0.1	3	15	N	N
E1	326	MHL2013025021	Target 1	FAM	NFQ-MGB	31.67507553	0.1	3	15	N	N
E2	333	MHL2013035022	Target 1	FAM	NFQ-MGB	27.09027481	0.1	3	15	N	N
E3	327	MHL2013035022	Target 1	FAM	NFQ-MGB	28.46723175	0.1	3	15	N	N
E4	334	MHL2013045023	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E5	335	MHL2013045024	Target 1	FAM	NFQ-MGB	22.86555672	0.1	3	15	N	N
E6	329	MHL2013045024	Target 1	FAM	NFQ-MGB	22.20428658	0.1	3	15	N	N
E7	336	MHL2013055025	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E8	110	SHO2012106074	Target 1	FAM	NFQ-MGB	37.24374008	0.1	3	15	N	N
E9	116	SHO2012106074	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E10	109	SHO2012106073	Target 1	FAM	NFQ-MGB	30.29249763	0.1	3	15	N	N
E11	115	SHO2012106073	Target 1	FAM	NFQ-MGB	34.81002045	0.1	3	15	N	N
E12	90	SHO2012106050	Target 1	FAM	NFQ-MGB	31.81121445	0.1	3	15	N	N
F1	108	SHO2012106072	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F2	114	SHO2012106072	Target 1	FAM	NFQ-MGB	24.53095245	0.1	3	15	N	N
F3	94	SHO2012106062	Target 1	FAM	NFQ-MGB	30.80118179	0.1	3	15	N	N

F4	106	SHO2012106062	Target 1	FAM	NFQ-MGB	33.03367615	0.1	3	15	N	N
F5	93	SHO2012106061	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F6	105	SHO2012106061	Target 1	FAM	NFQ-MGB	30.38479996	0.1	3	15	N	N
F7	92	SHO2012106052	Target 1	FAM	NFQ-MGB	4.884222507	0.1	3	15	N	N
F8	104	SHO2012106052	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F9	91	SHO2012106051	Target 1	FAM	NFQ-MGB	15.80223274	0.1	3	15	N	N
F10	103	SHO2012106051	Target 1	FAM	NFQ-MGB	19.71814919	0.1	3	15	N	N
F11	102	SHO2012106050	Target 1	FAM	NFQ-MGB	31.82706642	0.1	3	15	N	N
F12	89	SHO2012106049	Target 1	FAM	NFQ-MGB	7.460934162	0.1	3	15	N	N
G1	101	SHO2012106049	Target 1	FAM	NFQ-MGB	33.87815475	0.1	3	15	N	N
G2	88	SHO2012106048	Target 1	FAM	NFQ-MGB	35.16324997	0.1	3	15	N	N
G3	87	SHO2012106046	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G4	100	SHO2012106048	Target 1	FAM	NFQ-MGB	36.32299042	0.1	3	15	N	N
G5	99	SHO2012106046	Target 1	FAM	NFQ-MGB	35.66370392	0.1	3	15	N	N
G6	86	SHO2012106044	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G7	98	SHO2012106044	Target 1	FAM	NFQ-MGB	35.78660583	0.1	3	15	N	N
G8	85	SHO2012106043	Target 1	FAM	NFQ-MGB	34.68870163	0.1	3	15	N	N
G9	97	SHO2012106043	Target 1	FAM	NFQ-MGB	36.34910202	0.1	3	15	N	N
G10	84	SHO2012106042	Target 1	FAM	NFQ-MGB	32.7866745	0.1	3	15	N	N
G11	96	SHO2012106042	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G12	83	SHO2012106040	Target 1	FAM	NFQ-MGB	33.71409225	0.1	3	15	N	N
H1	95	SHO2012106040	Target 1	FAM	NFQ-MGB	15.23451614	0.1	3	15	N	N
H2	72	SHO2012106039	Target 1	FAM	NFQ-MGB	34.29183578	0.1	3	15	N	N
H3	82	SHO2012106039	Target 1	FAM	NFQ-MGB	38.83275604	0.1	3	15	N	N
H4	71	SHO2012106038	Target 1	FAM	NFQ-MGB	27.18260384	0.1	3	15	N	N
H5	81	SHO2012106038	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	Y
H6	70	SHO2012106037	Target 1	FAM	NFQ-MGB	35.47990036	0.1	3	15	N	N
H7	80	SHO2012106037	Target 1	FAM	NFQ-MGB	37.00905609	0.1	3	15	N	N
H8	69	SHO2012106036	Target 1	FAM	NFQ-MGB	22.79857445	0.1	3	15	N	N
H9	79	SHO2012106036	Target 1	FAM	NFQ-MGB	23.75571442	0.1	3	15	Y	N
H10	68	SHO2012106035	Target 1	FAM	NFQ-MGB	29.09397125	0.1	3	15	N	N
H11	78	SHO2012106035	Target 1	FAM	NFQ-MGB	28.22037506	0.1	3	15	N	N
H12	67	SHO2012106034	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N

* for detailed information on the sample refer to APPENDIX B Table 6



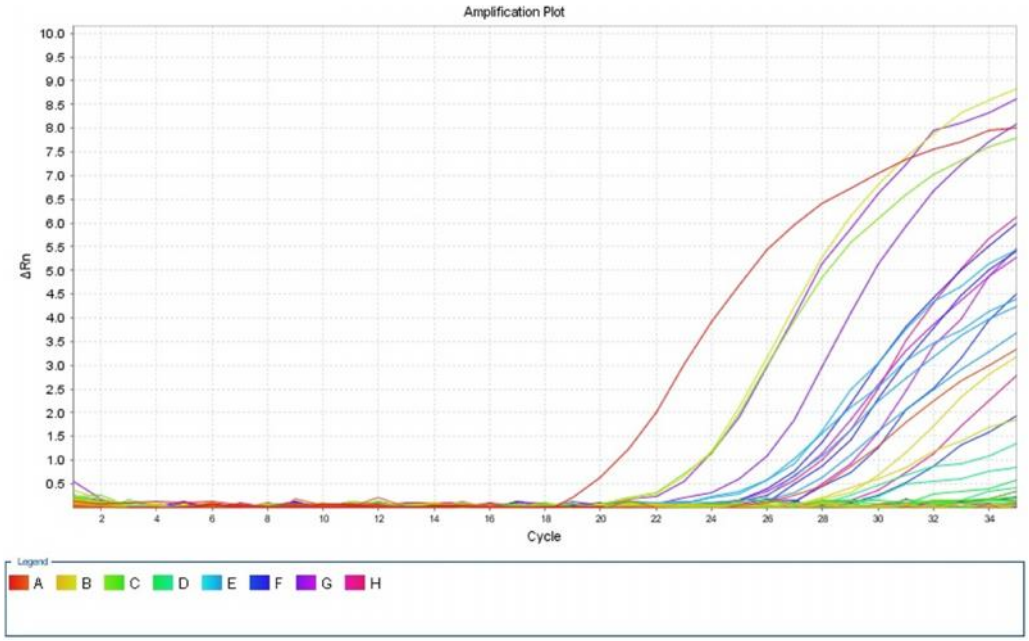
Block Type	96well
Chemistry	TAQMAN
Experiment File Name	D:\Applied Biosystems\StepOne Software v2.2.2\experiments\Anova PS Study\ANOVA SUBSET RUN2 10012014 treshold 01.eds
Experiment Run End Time	2014-01-10 14:32:11 PM PST
Instrument Type	steponeplus
Passive Reference	ROX

Well	Sample ID*	Study Number	Target Name	Reporter	Quencher	Cт	Ct Threshold	Baseline Start	Baseline End	BADROX	CTFAIL
A1	Negative		Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A2	Positive		Target 1	FAM	NFQ-MGB	18.67899323	0.1	3	15	Y	N
A3	76	SHO2012106033	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A4	65	SHO2012106032	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A5	75	SHO2012106032	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A6	64	SHO2012106031	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A7	74	SHO2012106031	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A8	63	SHO2012106030	Target 1	FAM	NFQ-MGB	26.05816841	0.1	3	15	N	N
A9	73	SHO2012106030	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A10	52	not available	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
A11	62	SHO2012106029	Target 1	FAM	NFQ-MGB	11.39928246	0.1	3	15	N	N
A12	51	SHO2012106028	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B1	61	SHO2012106028	Target 1	FAM	NFQ-MGB	24.90525436	0.1	3	15	N	N
B2	50	SHO2012106026	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B3	60	SHO2012106026	Target 1	FAM	NFQ-MGB	14.86735249	0.1	3	15	N	N
B4	49	SHO2012106025	Target 1	FAM	NFQ-MGB	13.99548531	0.1	3	15	N	N
B5	59	SHO2012106025	Target 1	FAM	NFQ-MGB	5.940559387	0.1	3	15	N	N
B6	48	SHO2012106022	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B7	58	SHO2012106022	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B8	47	SHO2012106021	Target 1	FAM	NFQ-MGB	26.77157784	0.1	3	15	N	N
B9	57	SHO2012106021	Target 1	FAM	NFQ-MGB	27.64020348	0.1	3	15	N	N
B10	46	SHO2012106020	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B11	56	SHO2012106020	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
B12	44	SHO2012106011	Target 1	FAM	NFQ-MGB	20.57607269	0.1	3	15	N	N
C1	54	SHO2012106011	Target 1	FAM	NFQ-MGB	20.44835472	0.1	3	15	N	N
C2	43	SHO2012106010	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C3	53	SHO2012106010	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C4	38	SHO2012096005	Target 1	FAM	NFQ-MGB	31.4912281	0.1	3	15	N	N
C5	42	SHO2012096005	Target 1	FAM	NFQ-MGB	11.78656864	0.1	3	15	N	N

C6	37	SHO2012096004	Target 1	FAM	NFQ-MGB	31.73222542	0.1	3	15	N	N
C7	41	SHO2012096004	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C8	36	SHO2012096003	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C9	40	SHO2012096003	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C10	35	SHO2012091139	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
C11	39	SHO2012091139	Target 1	FAM	NFQ-MGB	20.75268173	0.1	3	15	N	N
C12	24	SHO2012091138	Target 1	FAM	NFQ-MGB	33.2641983	0.1	3	15	N	N
D1	34	SHO2012091138	Target 1	FAM	NFQ-MGB	30.94244385	0.1	3	15	N	N
D2	23	SHO2012091137	Target 1	FAM	NFQ-MGB	34.95143127	0.1	3	15	N	N
D3	33	SHO2012091137	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D4	22	SHO2012091124	Target 1	FAM	NFQ-MGB	24.46552086	0.1	3	15	N	N
D5	32	SHO2012091124	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D6	21	SHO2012091123	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D7	31	SHO2012091123	Target 1	FAM	NFQ-MGB	32.35656357	0.1	3	15	N	N
D8	20	SHO2012091122	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D9	30	not available	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
D10	19	SHO2012091121	Target 1	FAM	NFQ-MGB	28.9781208	0.1	3	15	N	N
D11	29	SHO2012091121	Target 1	FAM	NFQ-MGB	28.14431763	0.1	3	15	N	N
D12	18	SHO2012091120	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E1	28	SHO2012091120	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E2	17	SHO2012091119	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E3	27	SHO2012091119	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E4	16	SHO2012080085	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E5	26	SHO2012080085	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E6	15	not available	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E7	25	SHO2012080083	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
E8	7	SHO2012080081	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	Y
E9	14	SHO2012080081	Target 1	FAM	NFQ-MGB	23.15410042	0.1	3	15	N	N
E10	6	SHO2012080079	Target 1	FAM	NFQ-MGB	23.44121361	0.1	3	15	N	N
E11	13	SHO2012080079	Target 1	FAM	NFQ-MGB	25.73655891	0.1	3	15	N	N
E12	3	SHO2012080073	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F1	10	SHO2012080073	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F2	146	MOD2013047055	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F3	152	MOD2013047055	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F4	145	MOD2013047054	Target 1	FAM	NFQ-MGB	28.81046867	0.1	3	15	N	N
F5	151	MOD2013047054	Target 1	FAM	NFQ-MGB	26.76038742	0.1	3	15	N	N
F6	144	MOD2013047053	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N

F7	150	MOD2013047053	Target 1	FAM	NFQ-MGB	32.89796829	0.1	3	15	N	N
F8	143	MOD2013027040	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F9	149	MOD2013027040	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
F10	130	MOD2013027039	Target 1	FAM	NFQ-MGB	25.65528488	0.1	3	15	N	N
F11	142	MOD2013027039	Target 1	FAM	NFQ-MGB	24.62432289	0.1	3	15	N	N
F12	129	MOD2013027038	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G1	128	MOD2012107036	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G2	140	MOD2012107036	Target 1	FAM	NFQ-MGB	4.709994316	0.1	3	15	N	N
G3	127	MOD2012107035	Target 1	FAM	NFQ-MGB	22.29228973	0.1	3	15	N	N
G4	139	MOD2012107035	Target 1	FAM	NFQ-MGB	20.33523369	0.1	3	15	N	N
G5	126	MOD2012107034	Target 1	FAM	NFQ-MGB	31.67858696	0.1	3	15	N	N
G6	138	MOD2012107034	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G7	123	MOD2012107027	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G8	135	MOD2012107027	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G9	122	MOD2012107026	Target 1	FAM	NFQ-MGB	24.40513611	0.1	3	15	N	N
G10	134	MOD2012107026	Target 1	FAM	NFQ-MGB	26.48463631	0.1	3	15	N	N
G11	121	MOD2012107025	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
G12	133	MOD2012107025	Target 1	FAM	NFQ-MGB	32.85749435	0.1	3	15	N	N
H1	120	MOD2012107024	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H2	132	MOD2012107024	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H3	119	MOD2012107012	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H4	131	MOD2012107012	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H5	112	MOD2012107011	Target 1	FAM	NFQ-MGB	24.73799133	0.1	3	15	N	N
H6	118	MOD2012107011	Target 1	FAM	NFQ-MGB	28.44670486	0.1	3	15	N	N
H7	111	MOD2012107010	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H8	117	MOD2012107010	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H9	330	MHL2013055025	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H10	77	SHO2012106034	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H11	66	SHO2012106033	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N
H12	185	KRT2012092129	Target 1	FAM	NFQ-MGB	Undetermined	0.1	3	15	N	N

* for detailed information on the sample refer to APPENDIX B Table 6



Block Type	96well
Chemistry	TAQMAN
Experiment File Name	D:\Applied Biosystems\StepOne Software v2.2.2\experiments\Anova PS Study\ANOVA SUBSET RUN3 10012014 trshold 01.eds
Experiment Run End Time	2014-01-10 15:49:36 PM PST
Instrument Type	steponeplus
Passive Reference	ROX

Well	Sample ID*	Study number	Target Name	Reporter	Quencher	Cт	Ct Threshold	Baseline Start	Baseline End	BADROX	CTFAIL
A1	Negative		Target 1	FAM	None	Undetermined	0.1	3	15	N	N
A2	Positive		Target 1	FAM	None	19.46019363	0.1	3	15	Y	N
A3	179	KRT2012092123	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
A4	162	KRT2012092114	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
A5	174	KRT2012092114	Target 1	FAM	None	37.61253357	0.1	3	15	N	N
A6	161	KRT2012092092	Target 1	FAM	None	37.61275482	0.1	3	15	N	N
A7	171	KRT2012092090	Target 1	FAM	None	34.64521027	0.1	3	15	N	N
A8	160	KRT2012092091	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
A9	172	KRT2012092091	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
A10	159	KRT2012092090	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
A11	171	KRT2012092090	Target 1	FAM	None	11.99212456	0.1	3	15	N	N
A12	170	KRT2012082073	Target 1	FAM	None	38.0303421	0.1	3	15	N	N
B1	248	not available	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
B2	257	not available	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
B3	236	BAS2012090082	Target 1	FAM	None	36.60742569	0.1	3	15	N	N
B4	246	BAS2012090082	Target 1	FAM	None	36.07131958	0.1	3	15	N	N
B5	245	BAS2012090081	Target 1	FAM	None	34.39445877	0.1	3	15	N	N
B6	158	KRT2012082073	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
B7	235	BAS2012090081	Target 1	FAM	None	35.86291885	0.1	3	15	N	N
B8	219	BAS2012080033	Target 1	FAM	None	32.76418686	0.1	3	15	N	N
B9	225	BAS2012080033	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
B10	234	BAS2012091080	Target 1	FAM	None	36.22570419	0.1	3	15	N	N
B11	244	BAS2012091080	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
B12	157	KRT2012082072	Target 1	FAM	None	26.5860424	0.1	3	15	N	N
C1	233	BAS2012091075	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
C2	243	BAS2012091075	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
C3	232	BAS2012091073	Target 1	FAM	None	30.72310257	0.1	3	15	N	N
C4	242	BAS2012091073	Target 1	FAM	None	Undetermined	0.1	3	15	N	Y

C5	231	BAS2012091072	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
C6	241	BAS2012091072	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
C7	249	BAS2012090085	Target 1	FAM	None	21.61459923	0.1	3	15	N	N
C8	280	BAS2012091109	Target 1	FAM	None	31.33592987	0.1	3	15	N	N
C9	268	BAS2012091108	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
C10	279	BAS2012091108	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
C11	278	BAS2012091107	Target 1	FAM	None	29.51679993	0.1	3	15	N	N
C12	258	BAS2012090085	Target 1	FAM	None	29.17499542	0.1	3	15	N	N
D1	267	BAS2012091107	Target 1	FAM	None	31.91991234	0.1	3	15	N	N
D2	266	BAS2012091106	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
D3	277	BAS2012091106	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
D4	265	BAS2012091105	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
D5	276	BAS2012091105	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
D6	275	BAS2012091145	Target 1	FAM	None	38.04206848	0.1	3	15	N	N
D7	286	BAS2012091145	Target 1	FAM	None	30.0106411	0.1	3	15	N	N
D8	274	BAS2012091144	Target 1	FAM	None	20.28178024	0.1	3	15	N	N
D9	285	BAS2012091144	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
D10	273	BAS2012091140	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
D11	284	BAS2012091140	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
D12	270	BAS2012091110	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
E1	281	BAS2012091110	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
E2	269	BAS2012091109	Target 1	FAM	None	29.62574577	0.1	3	15	N	N
E3	298	BAS2012091166	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
E4	290	BAS2012091165	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
E5	297	BAS2012091165	Target 1	FAM	None	36.74507523	0.1	3	15	N	N
E6	289	BAS2012091164	Target 1	FAM	None	38.2425766	0.1	3	15	N	N
E7	296	BAS2012091164	Target 1	FAM	None	24.05189896	0.1	3	15	N	N
E8	288	BAS2012091163	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
E9	295	BAS2012091163	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
E10	287	BAS2012091146	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
E11	294	BAS2012091146	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
E12	302	BAS2012091175	Target 1	FAM	None	29.07076836	0.1	3	15	N	N
F1	314	BAS2012091175	Target 1	FAM	None	30.39703941	0.1	3	15	N	N
F2	301	BAS2012091174	Target 1	FAM	None	22.49476624	0.1	3	15	N	N
F3	313	BAS2012091174	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
F4	293	BAS2012091168	Target 1	FAM	None	31.94607544	0.1	3	15	N	N
F5	300	BAS2012091168	Target 1	FAM	None	29.59727669	0.1	3	15	N	N

F6	292	BAS2012091167	Target 1	FAM	None	32.49721146	0.1	3	15	N	N
F7	299	BAS2012091167	Target 1	FAM	None	34.46057129	0.1	3	15	N	N
F8	291	BAS2012091166	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
F9	319	BAS2013051186	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
F10	306	BAS2013041184	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
F11	318	BAS2013041184	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
F12	323	BAS2013051190	Target 1	FAM	None	33.34452438	0.1	3	15	N	N
G1	303	BAS2013041176	Target 1	FAM	None	34.41697311	0.1	3	15	N	N
G2	315	BAS2013041176	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
G3	311	BAS2013051190	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
G4	305	BAS2013041183	Target 1	FAM	None	32.88952637	0.1	3	15	N	N
G5	317	BAS2013041183	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
G6	304	BAS2013041182	Target 1	FAM	None	28.6992054	0.1	3	15	N	N
G7	153	KRT2012080028	Target 1	FAM	None	15.88409424	0.1	3	15	N	N
G8	316	BAS2013041182	Target 1	FAM	None	32.92887878	0.1	3	15	N	N
G9	320	BAS2013051187	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
G10	310	BAS2013051189	Target 1	FAM	None	7.835472107	0.1	3	15	N	N
G11	321	BAS2013051188	Target 1	FAM	None	33.40740967	0.1	3	15	N	N
G12	309	BAS2013051188	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
H1	322	BAS2013051189	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
H2	308	BAS2013051187	Target 1	FAM	None	38.13114166	0.1	3	15	N	N
H3	307	BAS2013051186	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
H4	169	KRT2012082072	Target 1	FAM	None	38.46376038	0.1	3	15	N	N
H5	156	KRT2012082071	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
H6	168	KRT2012082071	Target 1	FAM	None	37.79994965	0.1	3	15	N	N
H7	155	KRT2012080030	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
H8	167	KRT2012080030	Target 1	FAM	None	16.68369675	0.1	3	15	N	N
H9	148	KRT2012080029	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
H10	154	KRT2012080029	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
H11	147	KRT2012080028	Target 1	FAM	None	Undetermined	0.1	3	15	N	N
H12	blank		Target 1	FAM	None	Undetermined	0.1	3	15	N	N

* for detailed information on the sample refer to APPENDIX B Table 6