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Development and Evaluation of Urine based Rapid Molecular Diagnostic Test for Pulmonary Tuberculosis with Potential for Point of Care: Cape Town Cohort

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ABSTRACT

Background: Current sputum-based diagnostic tests for tuberculosis (TB) do not serve all target populations, making optimal diagnosis difficult. TB diagnosis among scarce sputum patients is challenging for timely diagnosis and treatment initiation. Thus, an alternative non-sputum-based diagnostic test is urgently needed. *Mycobacterium tuberculosis* (MTB)-specific transrenal (Tr) DNA from urine is a potential target for molecular testing to close this gap in TB diagnosis. The objective of this cross-sectional study was to evaluate the accuracy of a novel, molecular test using a portable fully-automatic analyser with the potential for usage in limited resource settings at the point of care.

Materials/Methods: Spot urine, blood and sputum samples from 428 adults with suspected pulmonary TB (164 HIV positive, 263 HIV-negative) were collected at three clinical sites in Cape Town, South Africa. Tr-DNA was isolated from 4 ml of EDTA urine using an in-house method optimised for DNA fragments larger than 38 bp. A rapid double-stranded primer-based real-time PCR method targeting an MTB-specific direct repeat region was applied for Tr-DNA identification. The eluate was tested in triplicate using an automated molecular analyser with assay process controls included in each test run. The Tr-DNA-based test had a short time to result of 45 minutes including DNA isolation.

Results: Liquid cultures were performed on 412 of the 428 TB suspects. Of these, 175/412 (42.5%) were microbiologically positive. Using liquid culture as gold standard, the Tr-DNA test showed sensitivity of 42.86% (95% CI: 35.42 – 50.54) and specificity of 86.61% (95% CI: 83.86 – 92.36). The Tr-DNA test had positive predictive value of 73.53% and negative predictive value of 67.74%. Among HIV-infected TB patients, the sensitivity and specificity were 45.24% and 89.04%, respectively. Combination of smear microscopy and Tr-DNA increased the sensitivity to 83.82% (smear microscopy alone was 75.14%); the specificity remained unchanged (96.61%).

Conclusions: This multi-centre proof of concept study indicates that Tr-DNA has a high specificity and modest sensitivity. Furthermore, future study should be performed on larger cohort not restricted to pulmonary TB using fresh urine samples with patient follow up. Although unsuitable for implementation as a stand-alone test, it may have the potential, in combination with smear microscopy, to aid TB diagnosis in HIV-endemic regions where sputum scarce and extra-pulmonary TB are common.

Keywords: Pulmonary tuberculosis, urine, transrenal DNA, diagnosis, PCR, HIV

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List of Abbreviations

AIDS	Acquired immune deficiency Syndrome
BCG	Bacille Calmette-Guerin
BHQ	Black Hole Quencher
BMGF	Bill and Melinda Gates foundation
BMI	Body mass index
bp	Base pairs
BSC	Biosafety cabinet
CI	Confidence interval
CMA	Competitive reporter monitored amplification
CRF	Case reporting form
Ct	Cycle threshold
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DSP	Double-stranded primer
DST	Drug susceptibility testing
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
FIND	Foundation for innovative new diagnositcs
gDNA	Genomic DNA (H37Rv)
HIV	Human immunodeficiency virus
IGRA	Interferon gamma release assay
IPC	Internal process control

IS	Insertion sequence
KCL	Potassium chloride
LAM	Lipoarabinomannan
LJ	Löwenstein-Jensen medium
$MgCl_2$	Magnesium chloride
MGIT	Mycobacterium grown indicator tube
ml	Millilitre
mM	Millimolar
MTB	*Mycobacterium tuberculosis*
N/A	Not applicable
NALC	N-acetyl-L-cysteine
NB	Nota bene
NaOH	Sodium hydroxide
NAAT	Nucleic acid amplification test
NHLS	National Health Laboratory Services
NLR	Negative likelihood ratio
NPV	Negative predictive value
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PLR	Positive likelihood ratio
POC	Point of care
PPV	Positive predictive value
PTB	Pulmonary tuberculosis
RFU	Relative fluorescence unit
ROC	Receiver operating characteristic

RNA	Ribonucleic acid
RR	Risk ratio
RT	Real time
SD	Standard deviation
TAT	Turnaround time
TB	Tuberculosis
Tm	Melting temperature
Tr	Transrenal
TST	Tuberculin skin test
WHO	World Health Organization

1. Introduction

1.1 Tuberculosis

Tuberculosis (TB) is not a new disease; archaeological evidence shows that TB has existed for more than 9000 years [1]. Robert Koch discovered the causative pathogen for TB, *Mycobacterium tuberculosis* (MTB), in 1882 [2, 3]. MTB is a Gram-positive and highly aerobic bacterium [4]. Furthermore, MTB is such a well-adapted infectious agent that exposure to only a few bacilli are sufficient to cause infection [5]. TB is transmitted by droplets produced during coughing and sneezing. This airborne disease primarily infects the lungs, and other organs can also later be affected. Furthermore, risk factors such as human immunodeficiency virus (HIV) infection, malnutrition, diabetes mellitus, alcoholism and malignancy are associated with TB disease development after primary infection [6, 7]. In addition, HIV infection also supports the spread of MTB bacilli to other body parts [8, 9].

1.2 Epidemiology of tuberculosis

TB may have killed more people than any other infectious disease. Between 1990 and 2014, mortality due to TB had declined by 47% [10]. However, TB received widespread media attention in 2015 when it killed more people than HIV and became the leading infectious cause of death [11]. An estimated 10.4 million new TB cases were reported in 2015, and an estimated 1.4 million people died from TB-related illness in that year (1 million HIV-negative and 0.4 million HIV-positive individuals) [12]. At the geographic level, the prevalence of TB is high in regions with limited access to resources, such as sub-Saharan Africa and Asia [13]. Among other high burden countries, South Africa has the highest TB incidence rate, of 834 per 100,000 people [12]. Furthermore, HIV infection reduces the CD4 T cell count and promotes the growth of MTB [14]. Figure 1.1 shows the global trends in TB incidence (1990-2014), prevalence, and mortality rates (1990-2015). Figure 1.2 shows the estimate of 2014 TB incidence rates/100,000 people.

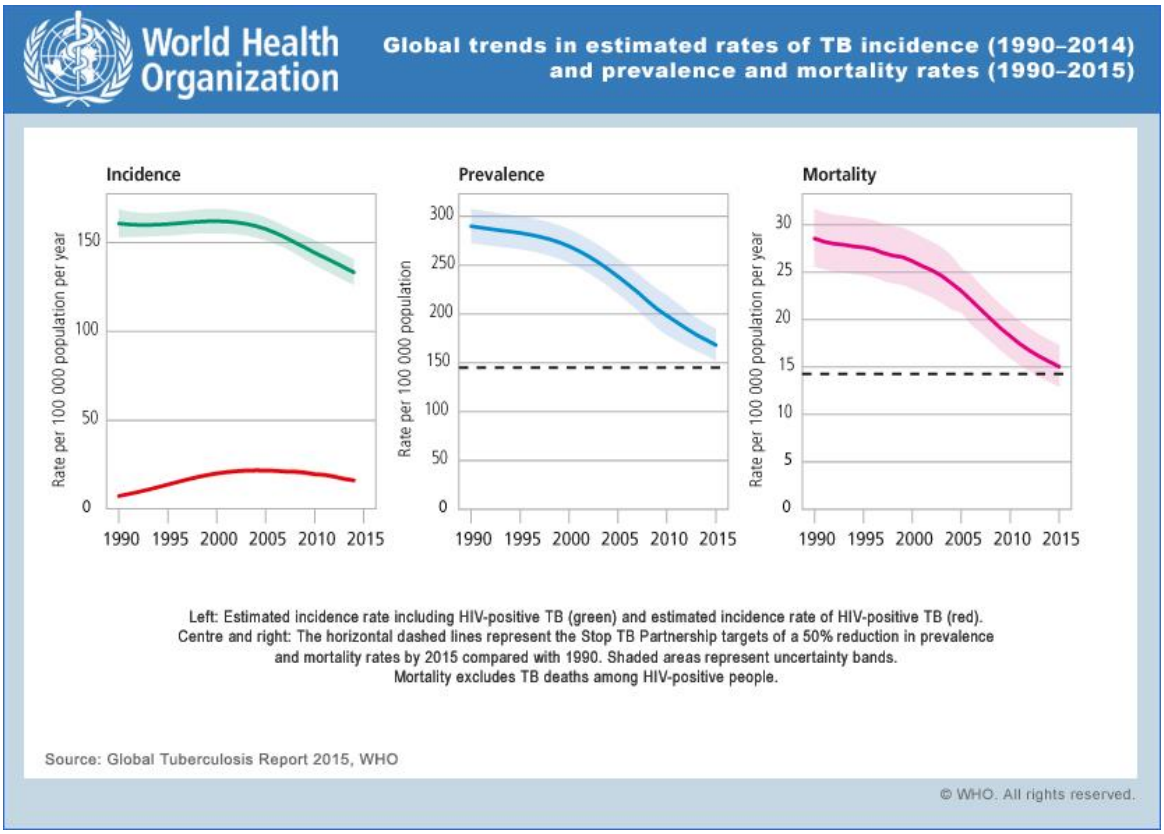


Figure 1.1: Global trends of TB incidence, prevalence, and mortality between 1990 and 2014

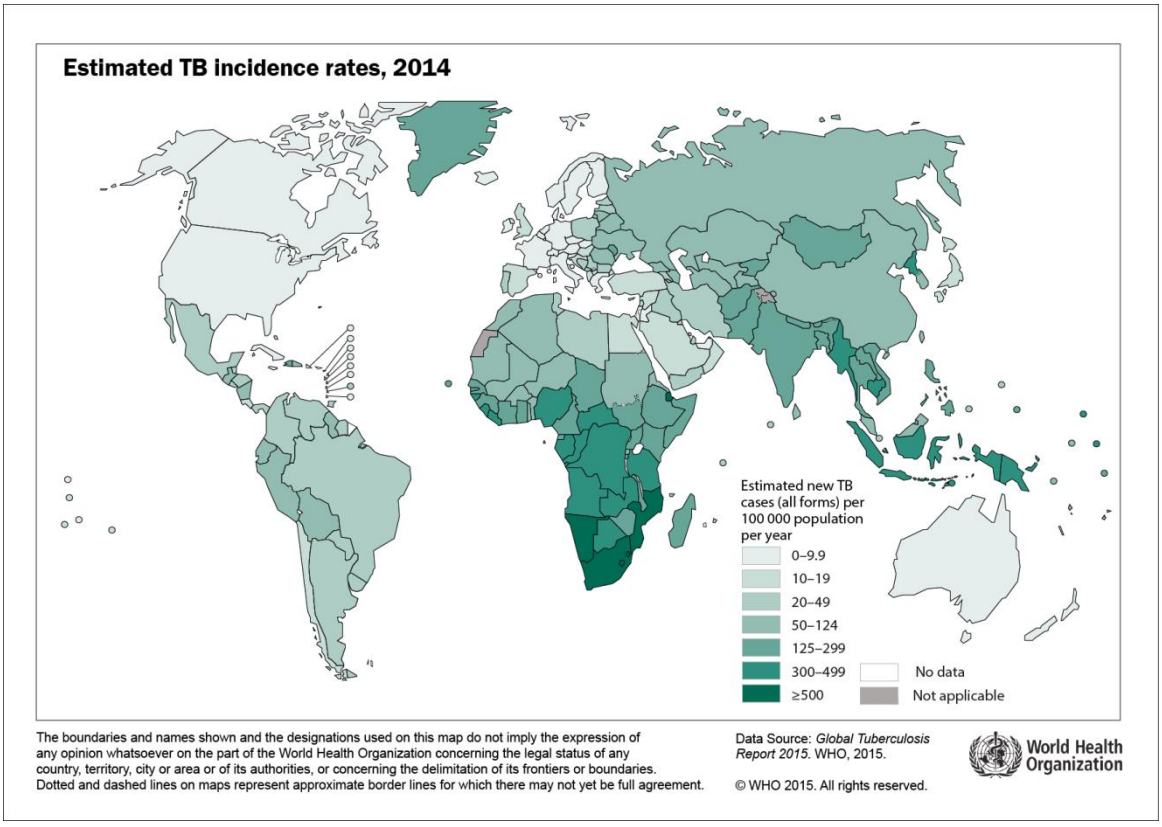


Figure 1.2: Estimated TB incidence rates in 2014

1.3 Tuberculosis vaccine

A TB vaccine could potentially prevent disease transmission by reducing the risk of MTB infection and progression to disease from infection. Between 1908 and 1919, Albert Calmette and Camille Guerin developed a live attenuated vaccine from *Mycobacterium bovis;* the so-called Bacille Calmette-Guerin (BCG) vaccine [15]. Almost a century later, the BCG vaccine remains the only TB vaccine available, providing protection for children by decreasing MTB infection progression. Although the BCG vaccine protects children against disseminated TB and TB-related meningitis during the early childhood, it does not protect against pulmonary TB in adults [16].

1.4 Tuberculosis disease diagnosis

Primary screening tools

TB suspects are currently screened using clinical symptoms, such as prolonged cough (more than two weeks), night sweats, fever, weight loss, and dyspnea, which are common among pulmonary TB patients, although not specific [17]. Furthermore, chest X-ray (CXR) is a cost-effective method of detecting early signs of disease [18]. CXR is also easy to perform and results are quickly available [19]. However, the abnormalities found on CXR in TB are not pathognomic, as other respiratory diseases can show similar abnormalities [20, 21].

Smear microscopy

Smear microscopy is by far the most commonly used TB diagnostic test. It is based on the staining of acid-fast bacilli present in sputum. The Ziehl-Neelsen-based method is widely used and is a relatively time-consuming procedure. Although inexpensive, the method has a low sensitivity (approximately 50%) among culture confirmed TB cases [22]. Moreover, smear microscopy has even lower sensitivity among HIV-infected patients [23]. This low sensitivity further support the TB transmission unnoticed [24]. Therefore, sputum smear negative TB cases have an impact on the diagnostic algorithm in resource constrain settings [25].

Sputum culture

To date, sputum culture remians the gold standard for TB diagnosis [26]. However, implementation of culture diagnostics in limited resource settings remains challenging [27]. MTB present in sputum is cultivated on Löwenstein-Jensen (LJ)/Middlebrook medium or in liquid mycobacterium growth indicator tubes (MGIT) at 37°C for several weeks. MTB grows slowly, requiring up to eight weeks for a solid culture and up to six weeks for a liquid culture to make a conclusive diagnosis [28]. Furthermore, culture contamination is relatively common despite appropriate lab quality management in place [29].

Nucleic acid amplification tests (NAAT)

Development of rapid molecular tests using sputum has been a breakthrough in TB diagnosis [30]. NAAT-based tests such as Xper® MTB/RIF (Cepheid Inc., USA) have achieved a sensitivity of 90.4% (95% CI: 89.2 – 91.4) and specificity of 98.4% (95% CI: 98.0 – 98.7) and can be implemented in resource scarce settings or at the point of care (POC) [31-33]. The availability of NAAT-based analysers has made it possible to diagnose TB using various sample types, such as biopsies, urine, pus and cerebral spinal fluid [34].

Immune response-based diagnostic tests

The tuberculin skin test (TST) is the oldest and most commonly used immune diagnostic test [35]. Nevertheless, among high-prevalence countries, TST results can be false positive due to sensitization with MTB [36]. Interferon-gamma release assays (IGRA) such as T-SPOT.TB (Oxford Immunotec, UK), and QuantiFERON®-TB Gold in-tube test (Quest Diagnostics, USA) are novel immune-based assays. IGRAs are blood-based tests and mainly used for diagnosis of latent TB infection but can also be used to detect active disease [37]. However, among high TB-prevalent low and middle-income countries, IGRA use has not been shown to be advantagous over the TST [38]. Additionally, TB diagnosis made using IGRA do not show any benefit among HIV-infected patients [39], or among children [40]. Therefore, routine use of IGRA remians limited [37].

Antigen-based rapid test

The low cost, urine-based, lateral flow diagnostic test detects the presence of lipoarabinomannan (LAM) in urine [41]. Although it can be performed at POC in resource-constrained settings [42], A meta-analysis of the TB-LAM test showed a sensitivity of 13 – 93% and specificity of 87 – 99% [43]. This wide range of assay performance is mainly due to the factors such as CD4 cell count and level of immunosuppression [44]. Test performance is limited to HIV-positive patients with a CD4 count below 100 cells/mm^3 [45]. In other words, TB screening is possible among advanced immunosuppressed HIV patients using this rapid test [46]. The world health organization (WHO) recommends the TB-LAM lateral flow test (Alere, USA) for TB diagnosis and screening among people living with HIV [47, 48].

1.5 Biomarker for tuberculosis diagnosis

Efforts to develop a novel TB diagnostic test have accelerated in recent years, due to involvement from the WHO and non-governmental organisations such as the Foundation for Innovative New Diagnostics (FIND) and the Bill and Melinda Gates Foundation (BMGF). Blood and urine-based biomarkers are the outcomes of recent advances in research, and have shown potential for TB diagnosis [49, 50]. However, low concentrations of target biomarkers may require larger sample volumes, which is only possible when using urine. Moreover, biomarkers present in blood may also be present in urine when they can pass the kidney barrier, e.g., cell-free nucleic acid and transrenal DNA (Tr-DNA) [51, 52]. Cell-free nucleic acids present in blood and other bodily fluids have shown potential for use in the diagnosis of infectious diseases and cancers [53, 54]. In addition, a urine-based POC test could be used in combination with smear microscopy or with liquid culture for pulmonary and extra-pulmonary TB suspects [50, 55].

1.6 Problem statement

Smear microscopy and liquid culture remain the tests of choice in high-prevalence settings [56]. Due to the nature of TB infection, children and HIV-positive patients are often unable to produce sufficient sputum on demand and may require sputum induction to provide enough volume for testing [57, 58]. Due to this limitation and the lack of a single

test for diagnosis, we currently rely on second and even third diagnostic tests to identify all cases of TB [59]. Delay in TB diagnosis and treatment in turn hampers disease control and increases the public health burden [60, 61]. Therefore, a rapid and easy to perform POC test is needed to cover all TB suspects in HIV and TB-endemic areas such as South Africa [12]. A urine-based diagnostic test could complete the current diagnostic algorithm and cover all groups of patients regardless of their co-infection and age.

Urine-based molecular TB diagnostic assays have achieved varying sensitivities and specificities for both pulmonary and extrapulmonary TB among HIV-positive and negative cohorts. Meta-analyses of Tr-DNA based tests have shown a sensitivity of 55% and specificity of 94% [62].This rather lower assay performance might be due to the use of non-standardised Tr-DNA isolation method and MTB target that is amplified in the PCR method for TB diagnosis. A Tr-DNA-based diagnostic test is a combination of two steps: 1) Isolation of Tr-DNA from urine, and 2) amplification of the target-specific DNA. Many critical factors are involved that play a role in the development of a robust and reproducible test, such as reagent quality, isolation method, cost, and the availability of a PCR machine for DNA analysis. The Tr-DNA isolation step is essential for targeting and detecting the MTB-specific DNA fragments. There are currently a select few nucleic acid isolation kits available for various bodily fluids that require the use of different reagents and additional instruments [63]. Therefore, a Tr-DNA method primarily developed and optimised for urine could help to isolate target DNA fragments smaller than 200 base pairs (bp) in size [64, 65].

Moreover, the availability of molecular tests remains limited to resource-rich settings due to the requirements for infrastructure and a skilled lab technician to perform the tests. Implementation of a novel molecular assay for diagnosis in limited resource settings could improve disease control [66].

1.7 Rationale, goals, and objectives

1.7.1 Rationale

Recent advances in diagnostic test development and their availability in limited resource settings have aided in the control of TB disease [35, 67]. A test using a sample type other than sputum could further improve diagnostic ability and control TB in highly endemic countries. The ability to collect a larger urine volume, in comparison to other bodi-

ly fluids, is advantageous. The urine sample collection procedure is non-invasive, and sample collection is also possible from sputum-scarce HIV-infected individuals and children.

Advancement in the fields of medical device technology and molecular biology have made it possible to bring lab-based molecular diagnostic tests to the POC [68]. Furthermore, molecular tests provide more conclusive results in comparison to immune-based assays, allowing clinical decisions to be made based on test results [69]. In addition, urine-based molecular assays targeting the MTB-specific DNA fragments can be useful for TB diagnosis and therapy monitoring [70]. The availability of a TB molecular test at smear microscopy centres or HIV clinics could further improve TB control [71].

1.7.2 Goals

To make available a urine-based rapid molecular test for the diagnosis of pulmonary and extra-pulmonary TB with potential for implementation at POC settings in areas of high TB prevalence.

1.7.3 Objective

Main objective

The main objective of this study is to evaluate a urine-based rapid molecular diagnostic test for pulmonary TB using an automated molecular analyser in a patient cohort from Cape Town, using a cross-sectional study design

Secondary objectives

i) Optimisation and standardisation of an in-house developed Tr-DNA isolation method.
ii) Validation of a rapid molecular assay based on POC Alere q by comparing this with standard lab-based PCR methods.
iii) Definition of the target population where sputum-based diagnostic tools fail, and determining whether the Tr-DNA based assay brings an advantage.
iv) Comparison of the assay performance among HIV-positive and negative TB suspects.
v) Determine the effects of urine characteristics on assay performance.

The materials and methods used in this study will be described in chapters 2 and 3, respectively. The results obtained will be presented in chapter 4.

2 Materials

2.1 Instruments, commercial kits, reagents and consumables

During the study different sets of instruments, commercial kits, reagents and consumables were used. Detailed information about particular material is mentioned in appropriate tables below.

Table 2.1: List of instruments used for the Tr-DNA isolation method optimisation and evaluation of novel Tr-DNA assay.

Instrument name	Supplier/Manufacturer
7500 Real-Time PCR System	Thermo Fischer Scientific, Waltham, USA
NanoDrop 3300	Thermo Fischer Scientific GmbH, Dreieich Germany
Centrifuge	Eppendorf, Wesseling-Berzdorf, Germany
DNA electrophoresis system	VWR International, Dresden, Germany
Gel imager	Bio-Rad Laboratories GmbH, Munich, Germany
Biosafety cabinet	Thermo Fischer Scientific GmbH, Dreieich Germany
Syringe pump: PHD ultra infuse	Harvard Apparatus GmbH, Hugstetten, Germany
Thermomixer	Eppendorf, Wesseling-Berzdorf, Germany
Pipettes	Eppendorf, Wesseling-Berzdorf, Germany
Water bath	Memmert, Schwabach, Germany
Refrigerator	Liebherr, Biberach an der Riss, Germany
Alere q Analyser	Alere Technologies, Jens, Germany
GeneXpert XVI	Cepheid, Sunnyvale, USA
BAC- TEC MGIT 960 system	BD Diagnostics, Franklin lakes, NJ, USA

Table 2.2: List of commercial kits used for the Tr-DNA isolation method optimisation and evaluation of novel Tr-DNA assay.

All commercial kits were within their shelf life and analysis performed according to manufacturer instruction.

Kit name	Supplier/Manufacturer
PicoGreen® dsDNA Assay Kit	Thermo Fischer Scientific GmbH, Dreieich Germany
Combur-Test® 10 dipstick test	Roche Diagnostics GmbH, Grenzach-Wyhlen, Germany
QuantiTect SYBR Green PCR Kit	Qiagen GmbH, Hilden, Germany
QIAamp Circulating Nucleic Acid Kit	Qiagen GmbH, Hilden, Germany
***E. coli* plasmid (pEX vector)**	Eurofins, Jena, Germany
GeneXpert MTB/RIF	Cepheid, Sunnyvale, USA
MTBDR plus line probe assay	Hain Lifescience, Nehren, Germany

Table 2.3: List of reagents and consumables used for the Tr-DNA isolation method optimisation and evaluation of novel Tr-DNA assay.

All reagents were within their shelf life and to avoid contamination, the reagents were aliquoted into smaller portion and stored according to manufacturer instruction till further use.

Reagent/Consumable name	Supplier/Manufacturer
BD Falcon tubes (15 ml, 50 ml)	VWR International, Dresden, Germany
Centrifuge tubes (1.5 ml, 2 ml)	VWR International, Dresden, Germany
Pipette tips	VWR International, Dresden, Germany
Syringe with Luer taper	VWR International, Dresden, Germany
Mobicol classic with screw cap without filter	Mobitec GmbH, Göttingen, Germany
Mobitec filter (50 µm)	Mobitec GmbH, Göttingen, Germany
H37Rv genome bacterial qDNA	Tebu-bio GmbH, Offenbach, Germany
PBS w/o calcium, w/o magnesium	Thermo Fischer Scientific GmbH, Dreieich, Germany
Lambda DNA	Thermo Fischer Scientific GmbH, Dreieich, Germany
Agarose for gel electrophoresis	Thermo Fischer Scientific GmbH, Dreieich, Germany

Reagent/Consumable name	Supplier/Manufacturer
96-well plates	Thermo Fischer Scientific GmbH, Dreieich, Germany
Primers, DNA fragments	Eurogentec GmbH, Cologne, Germany
Sarstedt Monovette (10 ml)	Sarstedt, Nümbrecht, Germany
Isopropanol	Carl Roth GmbH, Karlsruhe, Germany
Nuclease-free water (molecular grade)	Carl Roth GmbH, Karlsruhe, Germany
Binding buffer	Alere Technologies, Jena, Germany
Capture matrix	Alere Technologies, Jena, Germany
Washing buffer	Alere Technologies, Jena, Germany
DNA-Exitus	VWR Darmstadt, Germany

2.2 DNA fragments

DNA fragments of varying lengths (38 bp, 50 bp, 75 bp, 100 bp, 150 bp, and 200 bp) and MTB reference standard H37Rv genomic DNA (gDNA) were used for experiments. The DNA fragments were specific for the *MTB* gene IS6110 and contained target specific primer binding sites at the 5’ and 3’ ends. Spacers of varying lengths were used to achieve the desirable synthetic DNA fragment length. The ultra-short DNA fragments were ordered as an oligonucleotide (single-stranded DNA of 38 bp and 50 bp), and corresponding pairs annealed to form double-stranded DNA. The longer DNA fragments (75 bp, 100 bp, 150 bp, and 200 bp) were cloned in *E. coli* plasmids (pEX-A vector, Eurofins) and were replicated using clone culture. The final concentration of DNA fragments was determined using the PicoGreen® method on a NanoDrop analyser (Thermo Scientific, Germany) according to manufacturer instructions. The synthesised fragments were diluted, aliquoted, and stored for further use at -80°C. Two different sets of forward and reverse primers were designed for IS6110 to avoid cross contamination when testing with clinical samples.

2.3 Urine samples from healthy control cohort

Five voluntary co-workers (healthy) at Alere Technologies GmbH, Jena, Germany, provided up to 1000 ml of control urine. The urine samples were aliquoted into 50 mL falcon tubes and stored between -70°C and -80°C for further use. No data regarding the donors were collected. The in-house Tr-DNA isolation method was developed and optimised using this control urine.

3 Methods

3.1 Study design

3.1.1 Study cohort

Patients with suspected pulmonary tuberculosis (PTB) were consecutively recruited from July – November 2013 at three different clinical sites (Gugulethu, Langa, and Vanguard) in Cape Town, South Africa. Demographic and clinical characteristics were recorded for each participant using the case reporting form (CRF). Body weight and height was also recorded for body mass index (BMI) calculation. For the detailed CRF, please refer to Appendix 1.

3.1.1.1 Inclusion/exclusion criteria

The inclusion criteria for the study were as follows.

- Adult pulmonary TB suspects ($\geqslant$ 18 years of age);
- Presence of clinical symptoms:
    - for HIV-negative participants: cough for more than two weeks, night sweats, weight loss, malaise (general feeling of being unwell), fever $\geqslant$ two weeks, temperature $\geqslant$ 38°C, chest pain, or haemoptysis (coughing up of blood);
    - for HIV-positive participants: at least one of the abovementioned symptoms;
- Providing a signed informed consent form;
- Participants did not plan to leave the municipal area during the following two months;
- Willingness to undergo HIV testing; and
- Able to provide at least two sputum samples (volume $\geqslant$ 1.5 ml per sample) and 60 ml of spot urine.

The exclusion criteria for the study are outlined below.

- Pregnancy;
- TB therapy more than one week in duration; and
- Presence of non-TB mycobacterial infection.

3.1.2 Study period and sites

For the evaluation of the novel molecular assay, a cross-sectional study was designed. Urine samples were processed for Tr-DNA isolation in May – August 2015 at the Research Centre Borstel, Germany, and Alere Technologies, Jena, Germany. All PCR experiments were performed during October 2015 – November 2016 at Alere Technologies, Jena, Germany.

3.1.3 Ethical approval

Ethical approval was received from the Health Sciences Faculty Research Ethics Committee at University of Cape Town and approved under number IRB# IRB00001938. Participants were insured through Federal Wide Assurance # FWA00001637. Written informed consent was collected from each participant.

3.1.4 Sample collection, storage and processing

The participants provided two spot sputum samples (volume $\geqslant$ 1.5 ml per sample) in sterile container during their initial clinic visit, with a one-hour interval between samples. When possible, a third sputum sample was also collected for the biobank and stored at -80°C. Eight ml EDTA blood was also collected. 50 ml spot urine was collected in a sterile container holding 25 mM EDTA to prevent DNA degradation, and the sample was finally stored at -80°C. Urine samples were shipped on dry ice to the Research Centre Borstel, Germany, for further processing. Before use, the frozen samples were brought to room temperature using a thermal heater. The flow diagram (Figure 3.1) below provides an overview of the patient enrolment process and the samples collected.

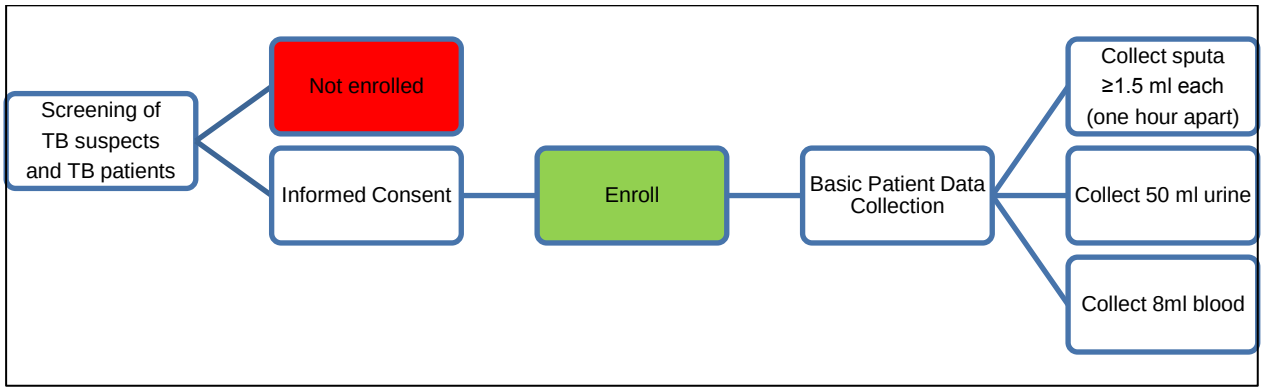


Figure 3.1: Overview of patient study enrolment and sample collection (blood, urine, and sputum)

3.2 Sputum microbiology

The initial spot sputum was labelled and sent to the National Health Laboratory Service (NHLS), a centralised and accredited microbiology lab in Cape Town, South Africa. The second sputum sample was stored raw at -80°C for future testing (as a precaution against errors or contamination issues). All samples were processed using standardised and quality assurance procedures. The sputum samples were decontaminated using a N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method, according to the Mycobacteriology Laboratory Manual [72]. Decontaminated and centrifuged deposits of sputum samples were re-suspended in phosphate buffer (pH 6.8) and processed samples were divided into three equal portions for liquid culture, Xpert MTB/RIF, and smear microscopy tests. Liquid culture was performed using mycobacterial growth indicator tubes (MGITs) in the BACTEC MGIT 960 System (BD Diagnostics, USA) and incubated at 37°C for six weeks/42 days. Liquid culture positive samples underwent then Genotype MTBDR plus line probe assay (Hain Lifescience, Nehren, Germany). Xpert MTB/RIF assay was performed according to the procedure previously described [73]. If possible, same day sputum smear microscopy was performed and semi-quantitative grading (scanty, 1+, 2+, and 3+) was assigned to observed bacilli, according to the WHO/International Union Against Tuberculosis and Lung Disease procedures [74]. The sputum smear microscopy results were not considered to define TB status.

The final results from MGIT and Xpert were taken into consideration for TB diagnosis. However, Tr-DNA assay outcomes were compared to the gold standard liquid MGIT culture only. Discordant results (liquid culture negative and Xpert positive) were separately taken into consideration for sensitivity and specificity determination. Figure 3.2, shows the algorithm schematic used for sputum microbiology.

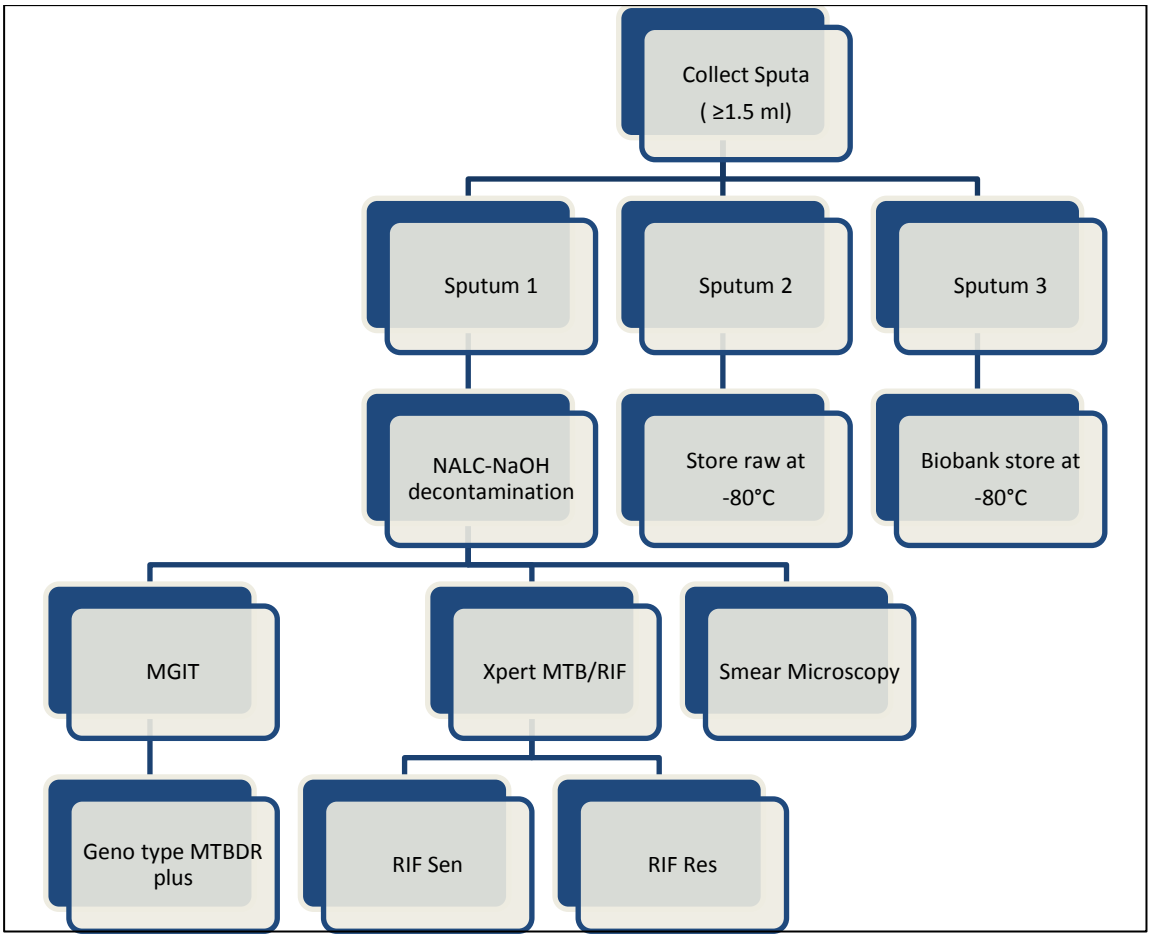


Figure 3.2: Schematic of diagnostic algorithm used for microbiology testing using sputum

Liquid culture and Xpert MTB/RIF were performed on the day of sputum collection, and if possible smear microscopy was also performed on the same day. However, smear microscopy results were not considered for TB diagnosis.

3.3 HIV screening and CD4 cell count

Participants without known HIV status were tested using serological test at local clinics after appropriate counselling. The ABON HIV1/2/O Tri-Line Human Immunodeficiency Virus Rapid Test (ABON Biopharm (Hangzhou) Co. Ltd, China) and First Response HIV 1-2.O Rapid Whole Blood Test (Premier Medical Corporation Ltd, Nani Daman, India) were used to determine HIV status. The CD4 cell count (measured during the last six months) was obtained from the clinical records for HIV-positive patients.

3.4 Dipstick test for urine characterisation

Frozen urine samples were thawed using a thermal heater and homogenised by inverting the tube several times. All urine samples underwent visual examination for colour and clarity. Furthermore, each urine specimen was characterised using the Combur-Test® 10 dipstick test (Roche Diagnostics GmbH, Grenzach-Wyhlen, Germany). This test is a lateral flow test and determines parameters such as density (specific gravity), pH, leukocyte presence, nitrite, protein, glucose, ketone, urobilinogen, bilirubin, and erythrocyte presence in semi-quantitative or qualitative manners. The tests were consecutively performed in a biosafety cabinet to avoid cross contamination and for safety reasons.

3.5 DNA concentration measurement using the PicoGreen method

Total DNA content was measured for the urine eluate using the PicoGreen® ds DNA Assay Kit (Thermo Scientific, USA) on the NanoDrop 3300 analyser (Thermo Scientific, USA) according to the manufacturer's instructions for DNA quantification. The DNA eluate was diluted 1:5 using the supplied buffer. The DNA concentration value (ng/ml) was calculated using a standard curve generated with lambda DNA (supplied by the manufacturer in the following concentrations: 5 ng/ml, 10 ng/ml, 25 ng/ml, 100 ng/ml, 500 ng/ml, and 1000 ng/ml). For the setup of the standard curve, all DNA concentrations were measured in five replicates. Each DNA eluate measured in three replicates together with a negative control (molecular grade water). DNA concentration of the eluate was calculated using the standard curve.

3.6 Polymerase chain reaction using the standard PCR cycler approach

The classical three step RT-PCR method using SYBR® Green reagents on the 7500 real-time (RT) PCR analyser (Thermo Fischer Scientific, USA) was performed for Tr-DNA analysis. Table 3.1, below, shows the pipetting scheme used for the PCR master mix. The PCR steps consisted of an initial denaturation phase for 15 min at 95 °C followed by cyclic denaturation for 15 sec at 95 °C, annealing for 30 sec at 62 °C and elongation for 30 sec at 72 °C. The forward primer (5'-GACGCGATCGAGCAAGCC-3') and reverse primer (5'-TCTTGTTGGCGGGTCCAG-3') were designed for the highly conservative MTB-specific insertion sequence (IS) 6110, and were the same length (18

nucleotides) [65]. The Ct-value for the PCR reactions were calculated using the baseline of cycle 3-15 and a threshold of 50,000 relative fluorescence units (RFU).

Table 3.1: Reaction mix required for PCR targeting the IS6110 region of MTB

Reagent	Volume in µl (eluate: 20 µl)	Volume in µl (eluate: 40 µl)	Volume in µl (eluate: 10 µl)
Nuclease-free water	24.0	4.0	34.0
SYBR Green master mix	50.0	50.0	50.0
Forward primer (0.3 µM)	3.0	3.0	3.0
Reverse primer (0.3 µM)	3.0	3.0	3.0
DNA fragments/ H37rv/nuclease-free water	20.0	40.0	10.0
Final volume	100.0	100.0	100.0

To calculate PCR efficiency, the Ct-values were plotted against the log concentration of the amplified copies. PCR efficiency (E) was calculated according the following formula:

$$\mathrm{E} = 10^{(-1/\mathrm{b})} - 1$$

where “b” represent slope obtained from standard curve by plotting the log of the starting quantity of DNA fragments or genomic DNA (gDNA) against the cycle threshold (ct) value obtained during amplification [75]. All qPCR experiments were evaluated using the Applied Biosystems 7500 software version 2.0.6.

The delta Ct-value is a good indicator of the efficiency of the nucleic acid isolation method. The delta Ct-value is the Ct difference between the values measured for inhibition control and the urine eluate. The larger delta Ct-values indicates a higher level of PCR inhibition, and the lower values indicate higher PCR efficiency. Delta Ct values were calculated for all DNA fragments for both methods using following formula [76]:

$$\Delta\, ct = ct\ (TS) - ct\ (RS)$$

Where: TS = Test sequence, RS = Reference sequence (e.g., positive control or inhibition control)

3.7 PCR analysis of synthetic DNA fragments

The functionality of MTB-specific IS6110 synthesised DNA fragments (38 bp, 50 bp, 75 bp, 150 bp, and 200 bp) was confirmed by target-specific PCR using the SYBR Green method on an RT-PCR AB cycler. For quality and stability, the DNA fragments were analysed in a logarithmic concentration series (10 cp, 10^2 cp, 10^3 cp, 10^4 cp, 10^5 cp, 10^6 cp, and 10^7 cp) by dilution into nuclease-free water, and then measured in triplicates. Genomic DNA from the MTB reference strain H37Rv was run as the PCR positive control; a negative control was also run in all PCR experiments.

3.8 Gel electrophoresis

Gel electrophoresis is a classic tool used to confirm the specificity of generated PCR products by measuring their particular lengths. The PCR products were loaded into a 2.5% w/v agarose gel prepared according to the manufacturer's instructions (Thermo Fisher Scientific, USA). 1.5 µl of 50 bp ladder was added to 5 µl bromophenol blue and run as a base pairs marker in the gel. Furthermore, 25 µl of PCR product was mixed with 5 µl of bromophenol blue, and 15 µl of sample was applied into the gel for PCR product analysis. Electrophoresis was run at 130 volts for 45 minutes using the DNA electrophoresis system (VWR International, Germany); the gel was then analysed using a gel imager (Bio-Rad, Germany).

3.9 QIAamp circulating nucleic acid method

The QIAamp circulating nucleic acid kit was used as a reference method to compare and optimise the in-house Tr-DNA isolation method. This commercially available kit can isolate circulating cell-free nucleic acids from various bodily fluids, including blood, plasma, serum, and urine. The isolation method is comprised of four steps: lysis, binding, washing, and elution. The kit provides selective binding to a silica-based membrane; nucleic acids specifically bind to the QIAamp mini column, while impurities such as PCR inhibitors, divalent cations and proteins can be completely removed in three washing steps. The pure nucleic acids are the eluted using an elution buffer. The purified concentrated circulating DNA and RNA can be used for downstream RT-PCR analysis. A sample volume of up to 5 ml can be isolated using the Qiagen method, with a flexible elution volume between 20 and 150 µl.

Figure 3.3, below, shows the material required for circulating nucleic acid isolation using the Qiagen method. In order to set up the filtration unit, the Leur taper syringe (B) with screw cap must be attached to the mobicol column. The QIAamp mini column can now be inserted into the mobicol column (A). The syringe plunger is then removed and the binding suspension is transferred in the syringe. The plunger is inserted into the syringe and the suspension is filtered by applying pressure to the plunger. The waste can be collected in 15 ml falcon tubes.

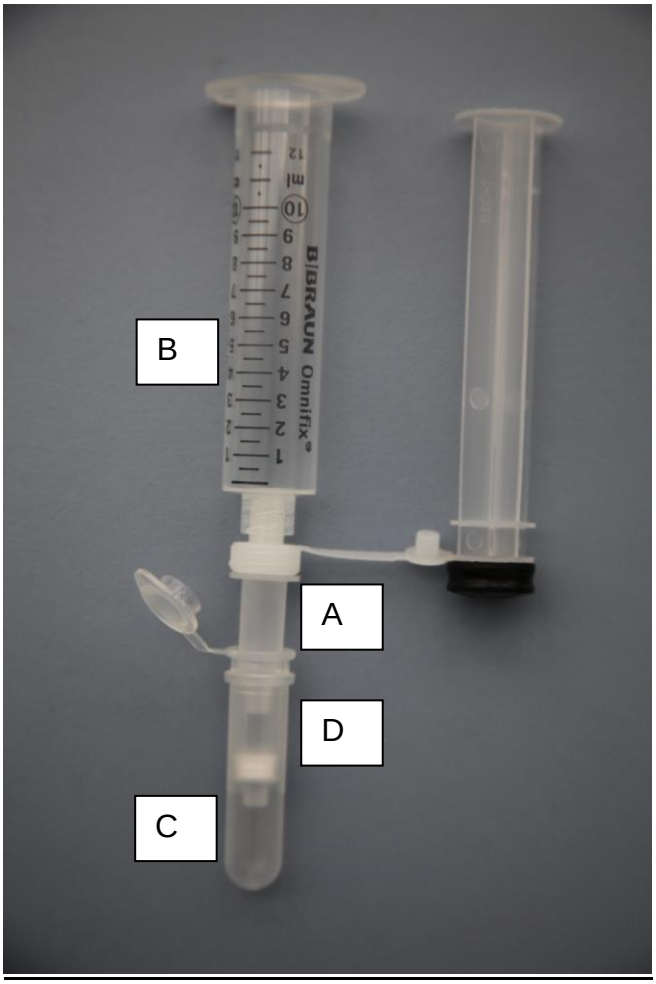


Figure 3.3: Material required to perform nucleic acid isolation using the Qiagen method

Mobicol system (A), 10 ml syringe with Luer taper (B), micro centrifuge tube (C), and QIAamp mini column (D)

The standard QIAamp protocol was modified for use with 4.0 ml urine, as shown in the flow diagram, below (Figure 3.4).

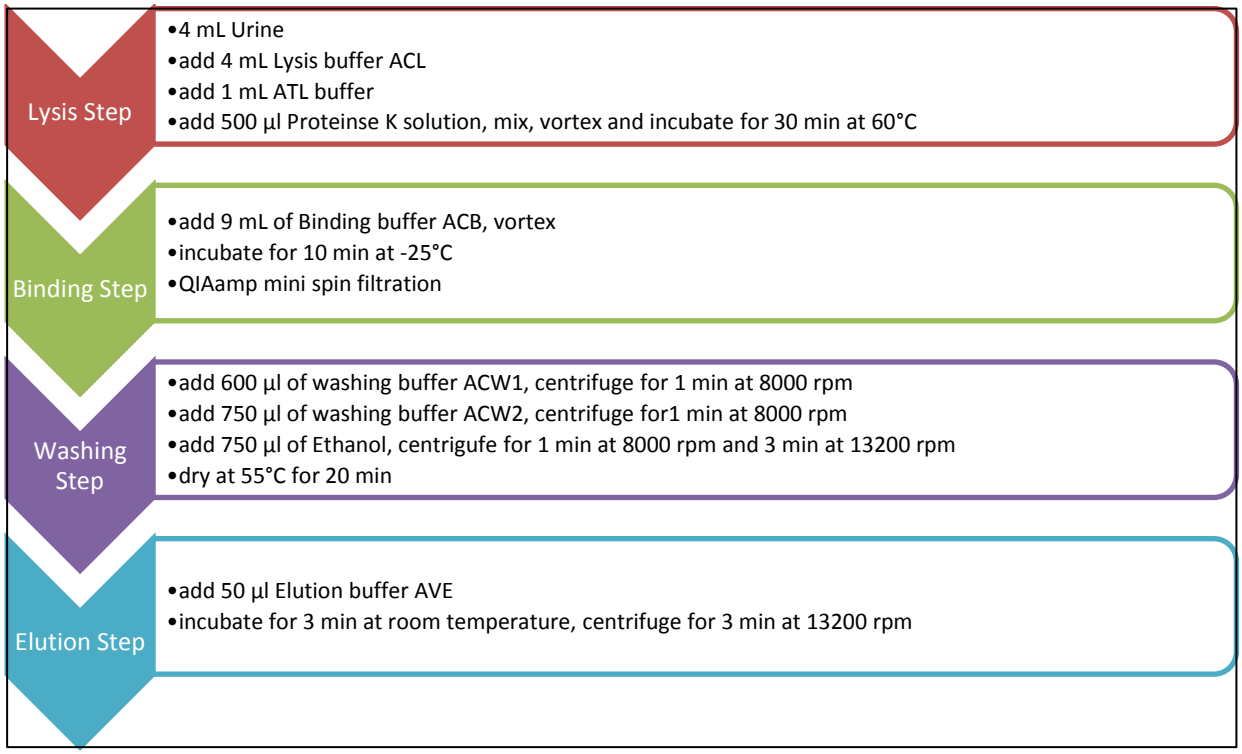


Figure 3.4: Overview of nucleic acid isolation protocol using the Qiagen method (QIAamp)

3.10 In-house Tr-DNA isolation method

In-house developed Tr-DNA isolation method was developed and optimised to enrich ultrashort DNA fragments ($\geqslant$ 38 bp) from large urine volumes (up to 4 ml) including human and pathogen-specific DNA. To amplify pathogen-specific DNA fragments from the isolated total DNA, the development of a target specific PCR method was essential. The in-house Tr-DNA isolation method was designed and developed to enable i) enrichment of ultrashort DNA fragments from large urine volumes, and ii) simplification of DNA isolation and amplification/detection for implementation in limited-resource settings. The detailed steps required for the manual isolation, including the materials and solutions used, are shown in Figure 3.5.

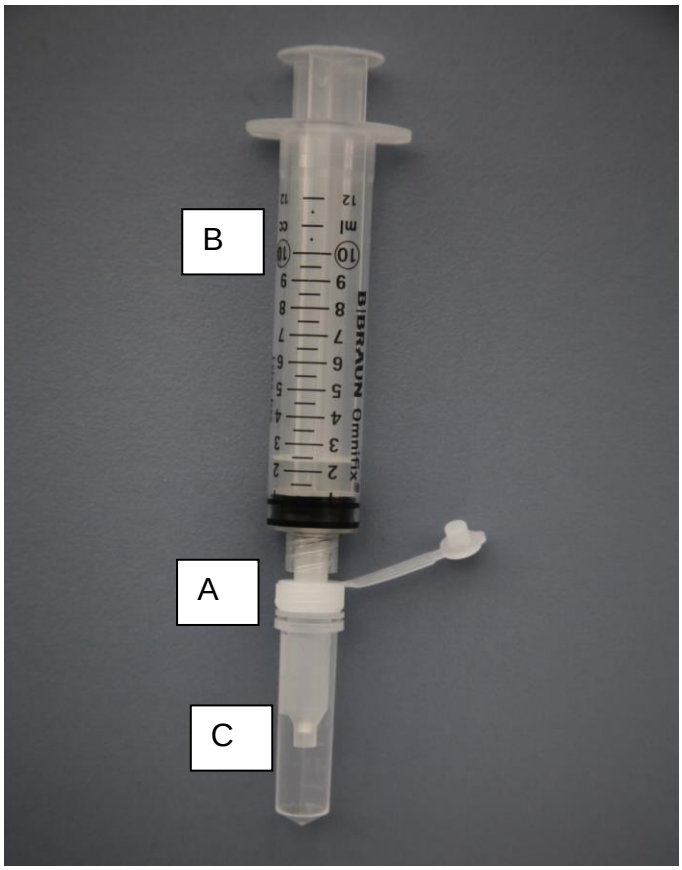


Figure 3.5: Materials required to perform manual nucleic acid isolation using the in-house method

Mobicol system (A), 10 ml syringe with Luer taper (B), and 2.0 ml microcentrifuge tube (C)

The syringe pump for the isolation process was implemented for ease of handling and to increase the sample throughput. Process atomisation enabled the isolation of up to 10 samples in parallel (10 syringes). The total turn-around time (TAT) was reduced for the whole assay. The Figure 3.6 shows the syringe pump with 10 different urines being isolated in parallel, which required a total of approximately three minutes.

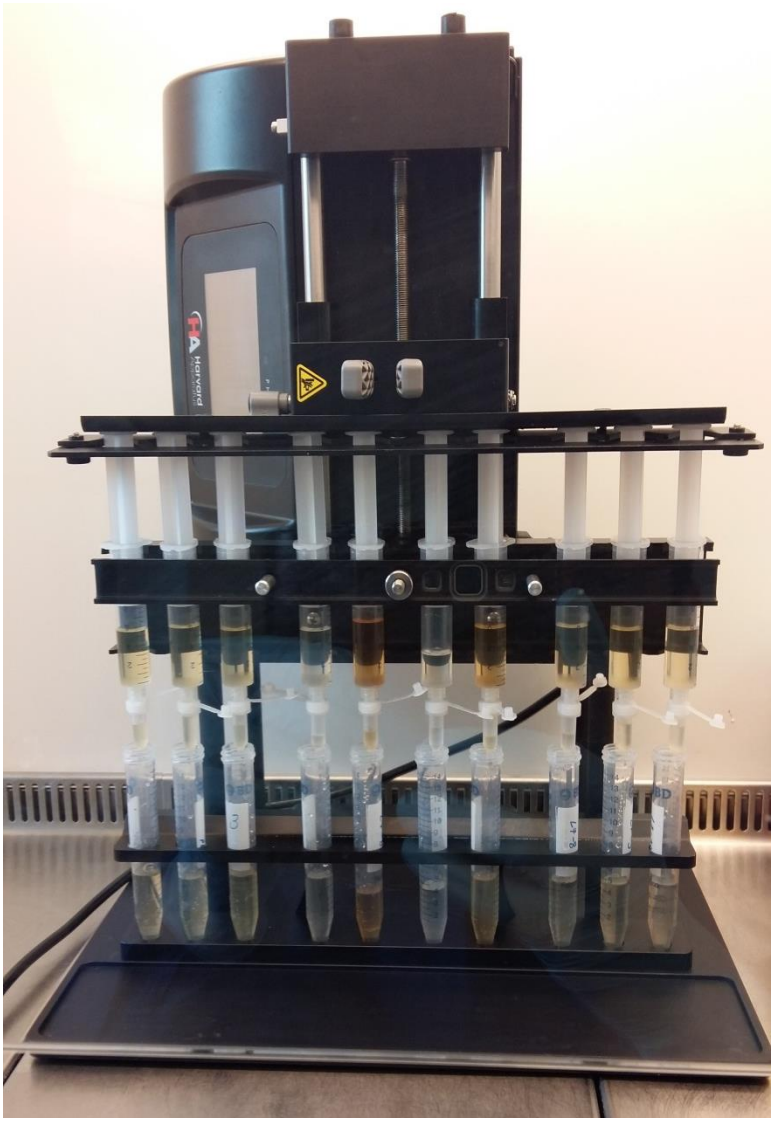


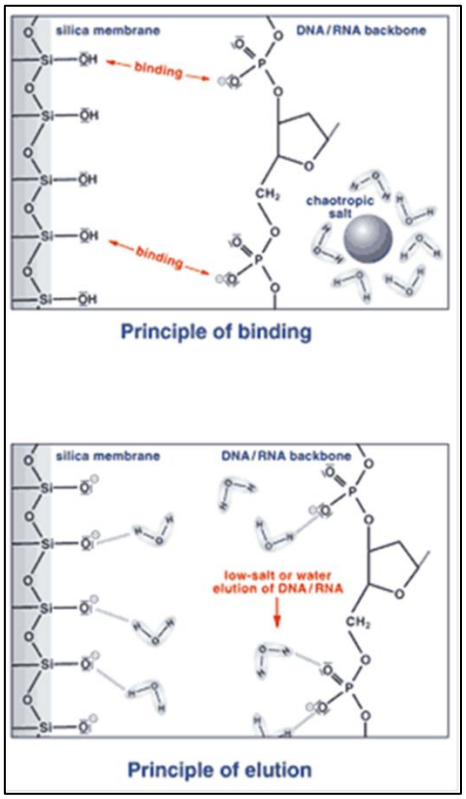
Figure 3.6: Tr-DNA isolation using a syringe pump with a modified stand for vertical positioning to accommodate 10 samples

The 10 mL Leur lock syringe was used for isolation. The syringe pump was set at a force level of 50% and a constant rate of 3 ml/min. The isolation was performed under a class III biosafety cabinet.

3.10.1 Binding and elution principle of the in-house method

The in-house method uses capture matrix and binding buffer. This method, unlike the Qiagen kit, does not contain a lysis step. The standard capture matrix usually consists of silicates ($Si(OH)_4$), polysaccharides (dextran, Sepharose a cross-linked agarose, or cellulose) or synthetic polymers (polyvinyl alcohol). These matrices have free hydroxyl (OH) groups that are modified and bind DNA molecules using their functional groups. The chaotropic agent present in the binding matrix desaturates biomolecules by inter-

rupting the hydration shell around the DNA/RNA. Furthermore, positively-charged ions enable a cation bridge to form between the negatively-charged capture matrix and the negatively-charged DNA backbone in high salt concentrations. The DNA backbone and the surface of the capture matrix build an electrostatic bond. The washing buffer (80 mM potassium acetate, 8.3 mM Tris-HCl (pH 7.5), 40 µM EDTA and 70% ethanol) removes the residual inhibitors from the sample without losing the DNA bound to the capture matrix. The ethanol in the wash buffer acts as a precipitant and helps the DNA backbone adhere to the capture matrix. In the final step, nuclease-free water breaks the bond between the capture matrix and the DNA. The free DNA can now be eluted for downstream analysis. Figure 3.7 shows the DNA binding principle for the in-house method.



(Source: Promega)

Figure 3.7: Representation of DNA binding principle for the in-house method using the capture matrix

3.10.2 In-house Tr-DNA isolation method optimisation

The Tr-DNA isolation method, capable of capturing shorter DNA fragments (> 75 bp) was optimised for ultrashort fragments (⩾ 38 bp) using the previously described method [70]. The in-house developed Tr-DNA isolation method was compared with the commercially available QIAamp Circulating Nucleic Acid Kit. The performance characteristics of both approaches (Qiagen and in-house) were analysed using different sets of experiments to optimise Tr-DNA isolation.

Inhibition of the PCR reaction is the main concern for molecular assays, and removing inhibitors in pre-PCR steps is needed to keep PCR performance high. DNA capture efficiency and the effect of total DNA content on PCR performance were taken into consideration for in-house method optimisation. The entire optimisation effort was divided into three major groups: i) reagent optimisation, ii) process optimisation, and iii) elution optimisation. The optimised method should increase PCR efficiency, stablise the isolation process, and reduced the degree of PCR inhibition.

3.10.2.1 Optimisation of urine and reagent volume

The urine and reagent volume required for Tr-DNA isolation were then optimised for the in-house method. Urine volumes of 4.0, 2.0, 1.0, and 0.5 ml were used for optimisation. The assay reagents for the in-house developed method were also reduced according to the urine volume used. 25 µl of (100 cp/µl) 50 bp DNA fragment was spiked into each volume using two different urine samples. Each urine sample was isolated in duplicates, and each eluate was further measured in duplicates using the SYBR Green-based PCR method. From 50 µl of eluate, 10 µl of eluate was added to 90 µl of master mix solution for the PCR process. For the positive control, 10 µl of (100 cp/µl) 50 bp DNA fragments was spiked into nuclease free water. Table 3.2 summarises the reagent volumes required for different urine volume.

Table 3.2: Reagent volumes required for different urine volume isolation using the in-house method

Urine volume (ml)	Binding buffer (ml)	Capture matrix (ml)	DNA fragments (50 bp, 100 cp/µl)
4	6	1	25 µl
2	3	0.5	25 µl
1	1.5	0.25	25 µl
0.5	0.75	0.125	25 µl

3.10.2.2 Isolation method ability to recover DNA from spiked urine

Up to five urine samples were spiked with three different concentrations (100 cp/µl, 1000 cp/µl, and 10,000 cp/µl) of synthetic DNA fragments (38 bp, 50 bp, 75 bp, 100 bp, 150 bp, 200 bp), and H37Rv gDNA to assess the isolation performance of the in-house method. Briefly, 25 µl of DNA fragments or H37Rv gDNA was added to 4 ml of urine. DNA isolation was performed on the same spiked samples for the in-house and the Qiagen methods. DNA was recovered in 50 µl of eluate. 20 µl of eluate was used for the SYBR Green PCR analysis to compare method performance regarding DNA yield and reproducibility. In total 100 µl of final PCR volume was analysed in duplicates. Inhibition control was performed in parallel to monitor the PCR process and reagent quality.

3.10.2.3 Influence of inhibitors and urine samples on PCR efficiency

The DNA eluate isolated using the Tr-DNA method may have carried unknown PCR inhibitors, which could have originated from the isolation method or from the sample itself. Thus, the potential effect of the inhibitors on PCR efficiency was evaluated from six different urine samples. Control urine eluates were spiked with DNA fragments of different sizes (38 bp, 50 bp, 75 bp, 100 bp, 150 bp, 200 bp), and H37Rv gDNA. 1 µl of DNA fragments was added to form a concentration of 1000 cp for inhibition control. A negative control was run in each PCR experiment. In total, 100 µl of final reaction volume was analysed using the SYBR Green-based PCR method. Each urine eluate was measured in duplicate.

3.10.2.4 Influence of eluate volume on PCR efficiency

The level of PCR inhibition was further investigated by analysing eluate volumes of 10, 20 and 40 µl from three urine samples, which were processed using the in-house method. 25 µl of 75 bp of DNA fragment (1000 cp/µl) was spiked directly into the PCR solution. The SYBR Green-based PCR method was used for analysis. All samples were measured in triplicates. Table 3.3 shows the pipetting scheme for PCR samples.

Table 3.3: Pipetting scheme for different eluate volumes

Sample	Master mix (µl)	Urine eluate (µl)	Molecular water (µl)	DNA fragments (µl)	Total volume (µl)
10 µ eluate	60	10	20	10 µl (10 cp/µl)	100
20 µl eluate	60	20	10	10 µl (10 cp/µl)	100
40 µl eluate	60	40	N/A	1 µl (1000 cp/µl)*	101
Positive control	60	N/A	30	10 µl (10 cp/µl)	100
Negative control	60	N/A	40	N/A	100

*A higher concentration of the DNA solution was used in order to achieve the final PCR volume of 100 µl for 40 µl of urine eluate, N/A = not applicable (not performed)

3.10.2.5 Influence of eluate dilution on PCR efficiency

Together with a reduction in eluate volume, the eluate dilutions could aid in reducing the effect of PCR inhibitors. Therefore, five different urine samples spiked with 250 copies of H37Rv gDNA were isolated using in-house method to analyse the effect of eluate dilution on PCR efficiency. 20 µl from the total 50 µl eluate volume was diluted in series of 1:2, 1:4, 1:8, and 1:16 using molecular grade water. Urine was isolated in duplicates, and each isolate was twice measured to allow analysis of reproducibility and method reliability. The PCR positive control H37Rv gDNA was diluted in same range as the urine eluate in order to monitor the effect of diluation. In total, 100 µl of sample volume was used for the PCR reaction (80 µl master mix and 20 µl eluate).

3.10.3 Tr-DNA isolation using an optimised in-house developed method

Tr-DNA isolation was then performed using the optimised in-house developed method. 6 ml of lysis/binding buffer and 1 ml of binding matrix were added to 4 ml of urine, the solution was mixed gently and transferred to the filter column (pore size 10 µm). The pellet was twice washed using 80 mM potassium acetate, 8.3 mM Tris-HCl (pH 7.5), 40 µM EDTA, and 70% ethanol solution, to eliminate residual inhibitors from urine and buffer. The DNA was eluted in two steps in a final volume of 75 µl of molecular grade water. Each urine sample was isolated in duplicates and the DNA eluate pooled and stored at -80°C until final analysis. Urine from healthy volunteers was isolated together with clinical samples to check for cross contamination and purity of reagents at the beginning and end of the day. As a positive control, urine was spiked with 35 µl of H37Rv gDNA. As a negative control, normal urine was isolated. All control samples were isolated in duplicate. The in-house method protocol using 4 ml of urine is shown in the flow diagram, below (Figure 3.8).

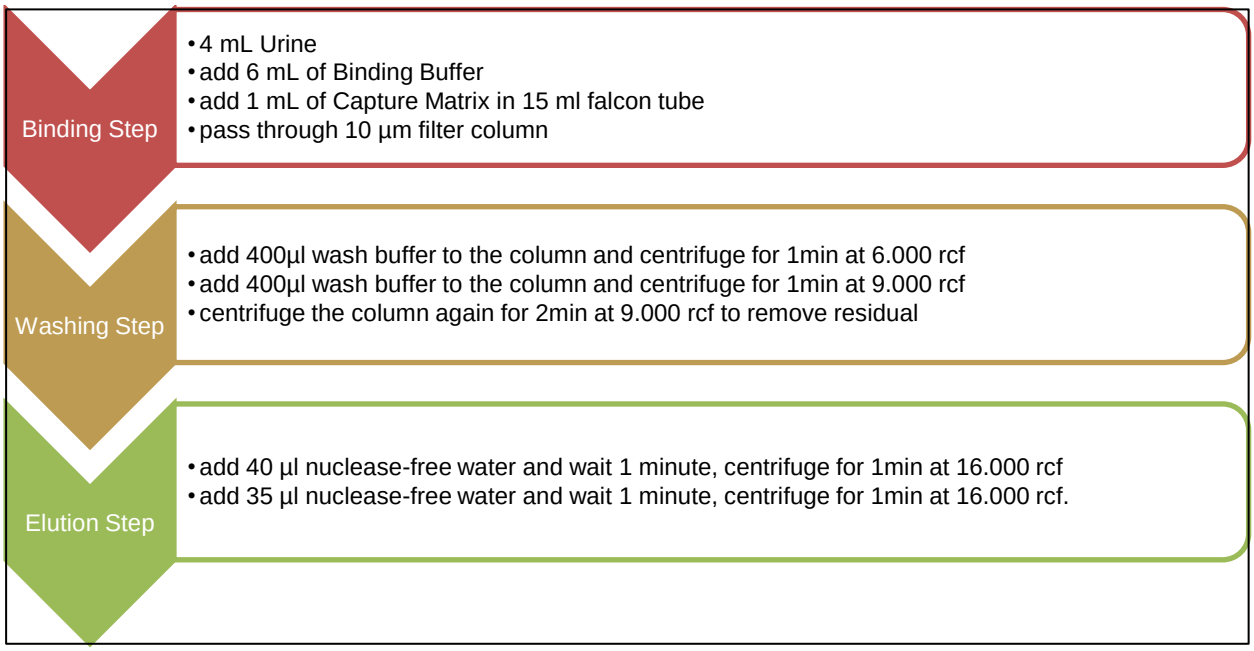


Figure 3.8: Overview of Tr-DNA isolation protocol for the in-house method

3.11 Novel and rapid polymerase chain reaction using an automated analyser

The double-stranded primer-based PCR method works well as a lab-based real-time PCR cycler. To bring the lab-based molecular test to resource-limited settings, we need to implement the assay using the portable and automated POC-capable Alere q analyser. Different assay-related aspects were optimised, e.g., primer: quencher ratio, number of PCR cycles, PCR steps, annealing and elongation temperatures, and duration of each phase. The novel assay using the Alere q must be validated by comparison to the existing SYBR Green-based PCR protocol mentioned earlier [70].

A compact, portable molecular analyser called the “Alere q” analyser is capable for use at point of care (POC). Once the sample has been applied to the single-use cartridge, the analyser automatically performs amplification and detection, including data analysis. The cartridge is a closed system, so that waste remains within the cartridge. The image below (Figure 3.9) shows the Alere q analyser and the cartridge used.

(Source: Alere)

Figure 3.9: Automated molecular analyser the “Alere q” and a single-use assay cartridge

The analyser automatically performs all of the required steps and displays the results on the touch screen. No user input is needed.

For the detection of MTB-specific Tr-DNA, a real-time non-probe-based PCR using a double-stranded primer (DSP) was implemented using the Alere q. This PCR approach

was initially developed for detection of HIV [77]. In the DSP-based PCR, a fluorescent dye is attached to the 5' end of a forward primer, and a reverse complementary oligonucleotide is labelled with a Black Hole Quencher dye (BHQ) at the 3' end. The forward primer binds in solution to its matched reverse oligonucleotide. The quencher prevents the initial fluorescence. However, during amplification in the presence of the target, the bond between the double-stranded primer and the quencher breaks and the fluorescently-labelled primer attaches to the target template [77]. This binding then shows an increase in fluorescence signal.

This technology was applied to the Alere q for the detection of the MTB-specific direct repeat (DR) region using a Cy5-labelled forward primer and a BHQ-labelled complementary reverse primer. The temperature of the annealing phase was adjusted to the primer's melting temperature (Tm) so that the balance was shifted to the primer-template hybrids instead of the primer-quencher hybrids. Figure 3.10, below, is a schematic representation of DSP-based PCR.

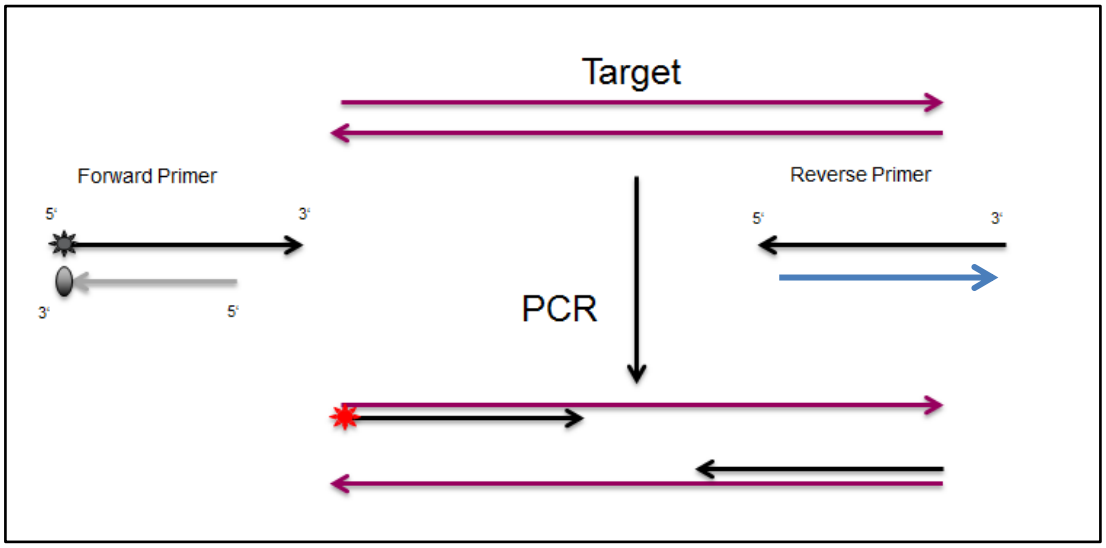


Figure 3.10: Schematic diagram of the double-stranded primer-based PCR principle

The forward and reverse primers are designed to target ultra-short DNA fragments ($\geqslant$ 38 bp). In the absence of a target amplicon in the reaction solution, the fluorescence signal from the forward primer is blocked by the BHQ dye, which is attached to the 3' end of the reverse complementary primer.

3.11.1 Array layout for the Alere q cartridge

The Alere q cartridge contains a microarray chip on which target-specific probes, as well as controls, are spotted in triplicates. The defined index spots for data analysis are spotted five times (Figure 3.11). The microarray is placed in the reaction chamber where the amplification reaction takes place. Internal process controls for the Tr-DNA assay were implemented using the competitive reporter monitored amplification (CMA) method [78].

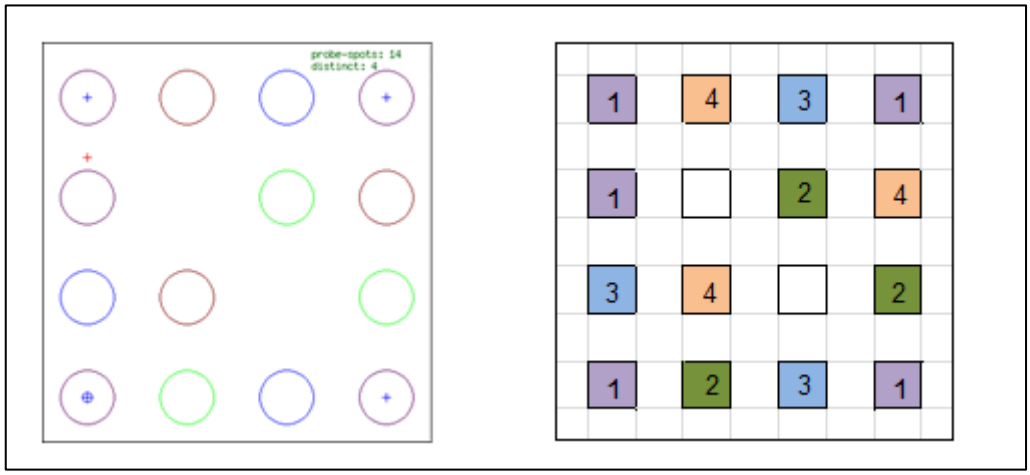


Figure 3.11: Array layout for a single-use Alere q cartridge microarray chip

The spots coloured violet (#1) show the index spots for orientation, green (#2) show the negative hybridization control, blue (#3) show the non-target-specific positive hybridization control, and brown (#4) show the target-specific internal process control.

No fluorescence signal was expected for the negative hybridization control. A fluorescence signal at a defined level has to be detected throughout the assay performance for the positive hybridization control. The internal process control is required to lie within the predefined Ct and lift range to provide a valid test result. Initially, all reverse oligonucleotides present in the reaction solution have Cy5 attached to the 5' and 3' ends. These bind to their complementary immobilised probes on the array, and no signal is given. Figure 3.12 represents the amplification curve generated for the internal process control during the Tr-DNA assay.

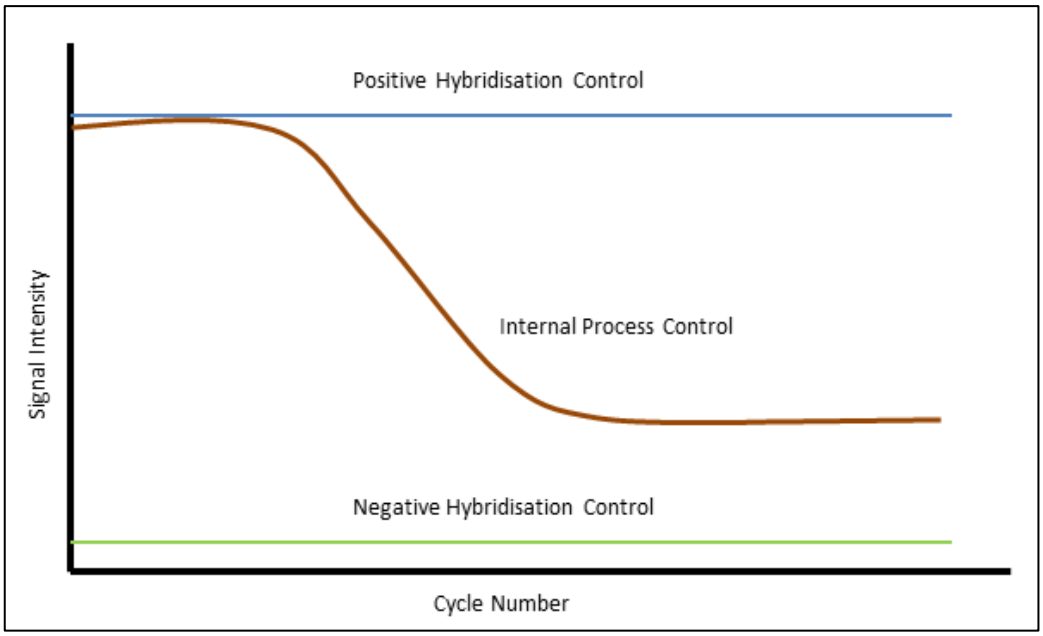
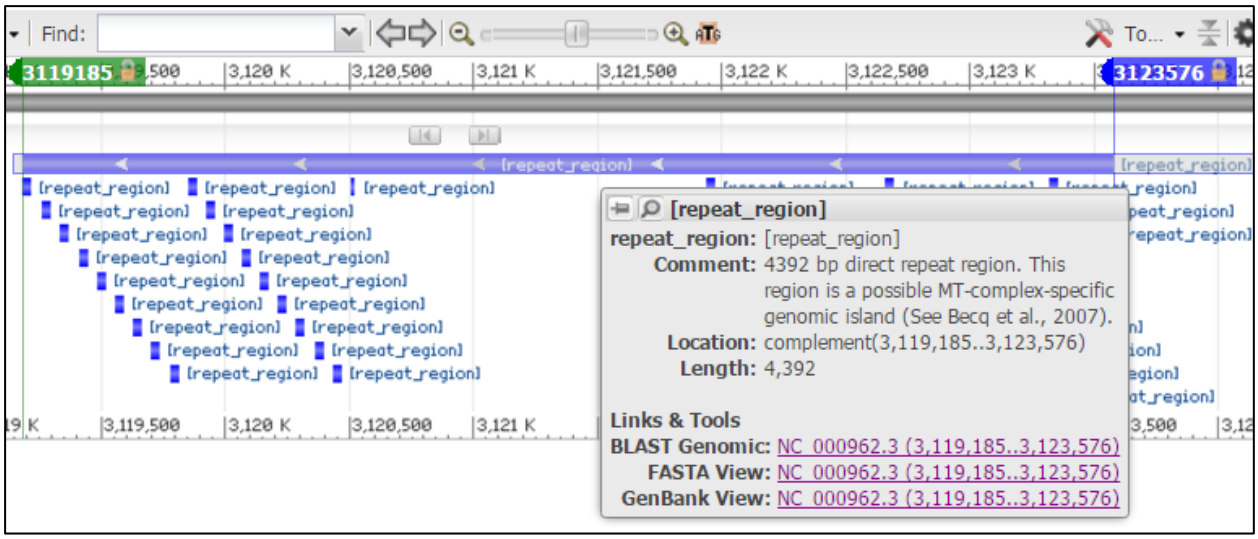


Figure 3.12: Process controls implemented in the Tr-DNA assay

The positive control should remain positive throughout the assay duration, while negative control should not give any unspecific signal. The signal from internal process control (IPC) starts with 100% and as more and more process control amplifies during the assay the signal drops. The IPC should be with in predefined range. These controls make the assay more robust to unwanted amplification and PCR inhibition.

3.11.2 PCR primer design for the DR target region

The forward and reverse primers targeting the *Mycobacterium tuberculosis*-specific DR region were then designed [79]. The primers bound to a 38-bp-long sequence present in the 4392-bp-long DR region [80]. The DR is a highly constant target region repeated 42 times in the MTB genome between 3119185 and 3123576 bp (Figure 3.13), thus increasing the chance of detecting MTB-specific DNA with a high sensitivity. The 5'-Cy5-labelled forward primer had a length of 17 bp. Its reverse complementary primer was 15 bp in length and was BHQ-labelled at its 3' end. The defined reverse primer had a length of 18 bp. The amplified PCR product was 38 bp in total length.



(source: NCBI Ref sequence NC_000962.3)

Figure 3.13: Direct repeat (DR) region located in the *Mycobacterium tuberculosis* genome. This DR region is 4392 bp in length.

3.11.3 PCR reaction mix for detection of MTB-specific Tr-DNA using DR target

The PCR reaction buffer consisted of potassium chloride (KCL) 250 mM, tris-sulfate 375 mM (pH 8.5), EDTA 50 µM, magnesium chloride ($MgCl_2$) 15 mM, Tween-20 1.25%, trehalose 1 M and sodium azide 0.225%. The PCR master mix was prepared by combining molecular grade water, reaction buffer, dNTP mix (containing dATP, dGTP, dCTP, and dTTP), forward primer, reverse primer, quencher, forward primer–IPC, reverse primer–PC, reporter–IPC, reporter–positive hybridization control, and Taq polymerase (Life Technologies, USA). The Table 3.4 shows the final concentration of the master mix reagents. Fresh master mix solution was prepared daily in a master mix room, and an isolated DNA eluate was added in a separate room.

Table 3.4: Reaction mix required for PCR targeting the DR region

Substances	Final concentration	
Molecular grade water	48.30	µl
PCR reaction buffer	20.00	µl
dNTP mix	0.2	mM
Forward primer: Cy5	0.3	µM

Substances	Final concentration	
Reverse primer	0.3	µM
Quencher: BHQ	0.3	µM
Forward primer: IPC	0.3	µM
Reverse primer: IPC	0.3	µM
Reporter: IPC	0.010	µM
Reporter: positive hybridisation control	0.010	µM
Taq	0.250	U/µl
DNA IPC pEX-A-template_320_321_200	10,000	copies/reaction
Final volume	**100**	**µl**

The double-stranded primer-based PCR protocol was implemented using the Alere q analyser, and run using on-board assay software. For the PCR reaction, 10 µl of isolated DNA eluate was added to 90 µl of freshly-prepared master mix solution. Each DNA eluate was measured in triplicates to determine the reproducibility and reliability of the assay. In cases of failure, the test was repeated until three valid results were achieved. The mean Ct-value was taken into consideration for the final TB diagnosis. Different sets of Ct-values for daily analyser quality control (negative and positive control), internal process control (positive and negative) and sample were recorded. To determine the test positivity rate, a capture calibration curve was made spanning a concentration range from 1 to 10,000 copies of H37Rv gDNA per test. Based on the lowest measurable concentration, a cut-off was defined to determine the threshold for defining positives and negatives.

3.12 Quantification of Tr-DNA using IS6110 and DR target amplification

The capture and amplification efficiencies for the MTB-specific IS6110 and DR region were determined using the Tr-DNA-based PCR assay. The urine samples were spiked with serial dilutions of gDNA from the MTB-reference strain H37Rv. Different concentrations of gDNA from the MTB reference strain H37Rv ranging from 1 cp to 10,000 cp. For estimation of DNA capture efficiency, H37Rv was spiked into the urine

and isolated using the in-house method. Furthermore, for estimation of PCR amplification efficiency the H37Rv gDNA was directly spiked in to the master mix solution. The calibration curves for IS6110 were generated on the AB cycler and for the DR region on the Alere q analyser. The calibration curves for the DR region were generated using the 22-min two-step PCR protocol. All experiments were run in triplicates and the mean Ct-values were plotted vs. the concentration of log H37Rv gDNA.

3.13 Controls and precautions to prevent contamination

Contamination of the reagents and lab facility can negatively impact assay quality. Thus, in addition to negative isolation controls, PCR reagents including the target-specific primers must be contamination free. Therefore, differing sets of isolation (extraction), analyser and internal process controls were implemented in the Tr-DNA assay. The internal process controls included an artificial sequence cloned into the plasmid pEX-A vector (Eurofins Scientific, Germany). These checks aided in observing the reagent quality (cross contamination and stability), the performance of the isolation method and Alere q analyser. For DNA isolation, a negative control (normal urine) and positive control (normal urine spiked with H37Rv genome) were isolated in duplicates during the first and last round of isolation. All Alere q analysers were tested running a negative control (molecular grade water) and a positive control (H37Rv genome) in the cartridge daily to monitor analyser performance. The Alere q analysers were inspected for any performance deviation and were sent immediately to technical service if this was detected.

Furthermore, to avoid cross contamination through reagents, all good laboratory practices were taken into consideration, i.e., separating master mix and template handling area. All biosafety cabinets (BSC) were run with the appropriate air circulation and disinfected using DNA-Exitus (VWR Darmstadt, Germany) before and after pipetting. Calibrated pipettes and single-use dual filter pipette tips (PCR clean/sterile) were used. All stock solutions were aliquoted into smaller volumes and stored at the appropriate temperature.

3.14 Statistical analysis

A database was created using Microsoft Excel 2010 (Microsoft Inc. Redmond, USA). Data quality control was performed on a regular basis, including the raw data collected from participants' case reporting forms (CRFs). Results from urine dipstick testing and PCR data were stored in a separate Excel sheet. Bar charts were created using Excel. The study cohort was characterised with descriptive statistics for various demographic data and clinical symptoms. Different sets of statistical tests (chi-square, Kruskal-Wallis one-way analysis of variance, and logistic regression) were performed using Sigma Plot, version 11 (Systat Software GmbH, Erkrath, Germany). The sensitivity (true positive/total positive), specificity (true negative/total negative), negative predictive value (true negative/{true negative + false negative}), positive predictive value (true positive/{true positive+ false positive}), negative likelihood ratio ({100 – sensitivity}/specificity), and positive likelihood ratio (sensitivity/{100 – specificity}) were calculated the for Tr-DNA assay, using the liquid culture as the reference method [81]. The two groups were compared using a Man-Whitney Rank sum test or t-test (two-sided) at an alpha of 0.05, and 95% CIs were calculated using the exact Clopper-Pearson, log method and standard logit model. The Z tests for proportions were performed for sex, HIV status and TB history. Receiver operating characteristic (ROC) curves were generated to show sensitivity and specificity against different diagnostic tests (sputum smear microscopy, Xpert MTB/RIF and liquid culture) [82]. ROC curves were also generated to compare assay performance for IS6110 and DR targets. The Fisher exact Chi-square test was performed to check the statistical difference between male and female regarding HIV prevalence. The multivariate and univariate analyses for potential confounding variables and odds ratios (ORs) was calculated by logistic regression. A Venn four-set diagram using ellipses was generated, showing the relationships between the different diagnostic tests.

4 Results

4.1 Optimisation of the in-house developed Tr-DNA isolation method

4.1.1 Result of synthetic DNA fragment and H37Rv analyses

The in-house developed Tr-DNA isolation method was developed and optimised using synthetic DNA fragments of different lengths (38 bp, 50 bp, 75 bp, 150 bp, and 200 bp) and H37Rv gDNA. The mean Ct-values from three runs for all PCR experiments and the percentage of PCR efficiency are given in Table 4.1. PCR efficiencies were higher for smaller DNA fragments (38bp, 50 bp, and 75 bp), and the H37Rv gDNA showed values close to 100% compared to the 150 bp and 200 bp DNA fragments with values of 81.39% and 90.27%, respectively. The overall excellent performance of DNA fragment amplification indicated process stability and reagent functionality. All PCR negative controls gave, as expected, an undetected result. The limit of detection was determined to be 100 copies for all DNA fragments, and the Ct-values were found to be consistent from a concentration of 100 cp to the highest concentration; this was confirmed by a two-to-three Ct-value decrease for each log step.

Table 4.1: Cycle threshold and percentage of PCR efficiency for DNA fragments and H37Rv gDNA

DNA length	Negative control	Mean Ct-value (DNA concentration)							% PCR efficiency
		10 cp	10^2 cp	10^3 cp	10^4 cp	10^5 cp	10^6 cp	10^7 cp	
38 bp	39.76	37.52	35.72	32.16	28.87	25.49	21.90	18.35	101.79
50 bp	Undetected*	36.50	33.13	29.64	26.24	22.76	19.40	16.45	97.85
75 bp	Undetected*	34.81	31.78	28.68	25.17	21.90	18.64	N/A	102.84
150 bp	39.29	N/A	36.62	33.17	29.77	24.86	21.44	N/A	81.39
200 bp	38.25	N/A	35.94	32.55	N/A	25.24	21.57	18.20	90.27
H37rv gDNA	Undetected*	32.24	28.70	25.37	22.19	18.87	N/A	N/A	99.83

*Undetected: Ct-value $\geqslant$ 40; N/A: Not applicable (not performed)

An amplification curve over the measured concentration range is shown for the 38-bp DNA fragment in Figure 4.1. The calibration curves for all DNA fragments and for the H37Rv genome showed a very high coefficient of determination (R^2) $\geqslant$ 0.995 (Figure 4.2). However, the determined Ct-values were not comparable between the different DNA fragments, and were generally higher than the same concentration of H37Rv gDNA. These results reflect the number of available IS6110 repeats in the H37Rv genome, namely 16 instances in H37Rv compared to one repeat in the synthetic DNA fragments (Appendix 2). Furthermore, H37Rv is more stable than the DNA fragments in solution, which may also have impacted PCR performance.

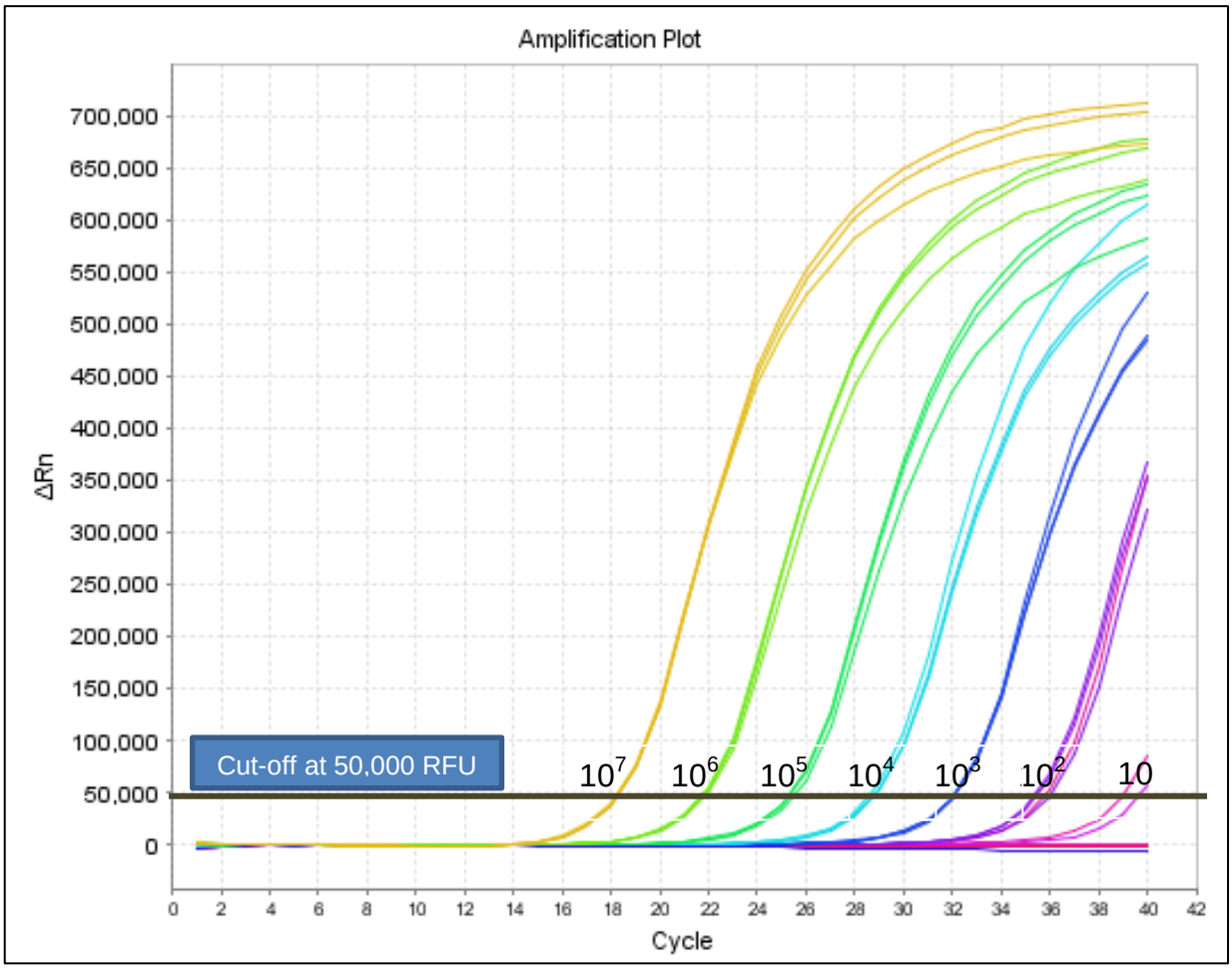


Figure 4.1: PCR amplification shown for the 38-bp DNA fragment for serial dilutions from 10 to 10^7

The thick black line indicates the cut-off value of 50,000 RFU.

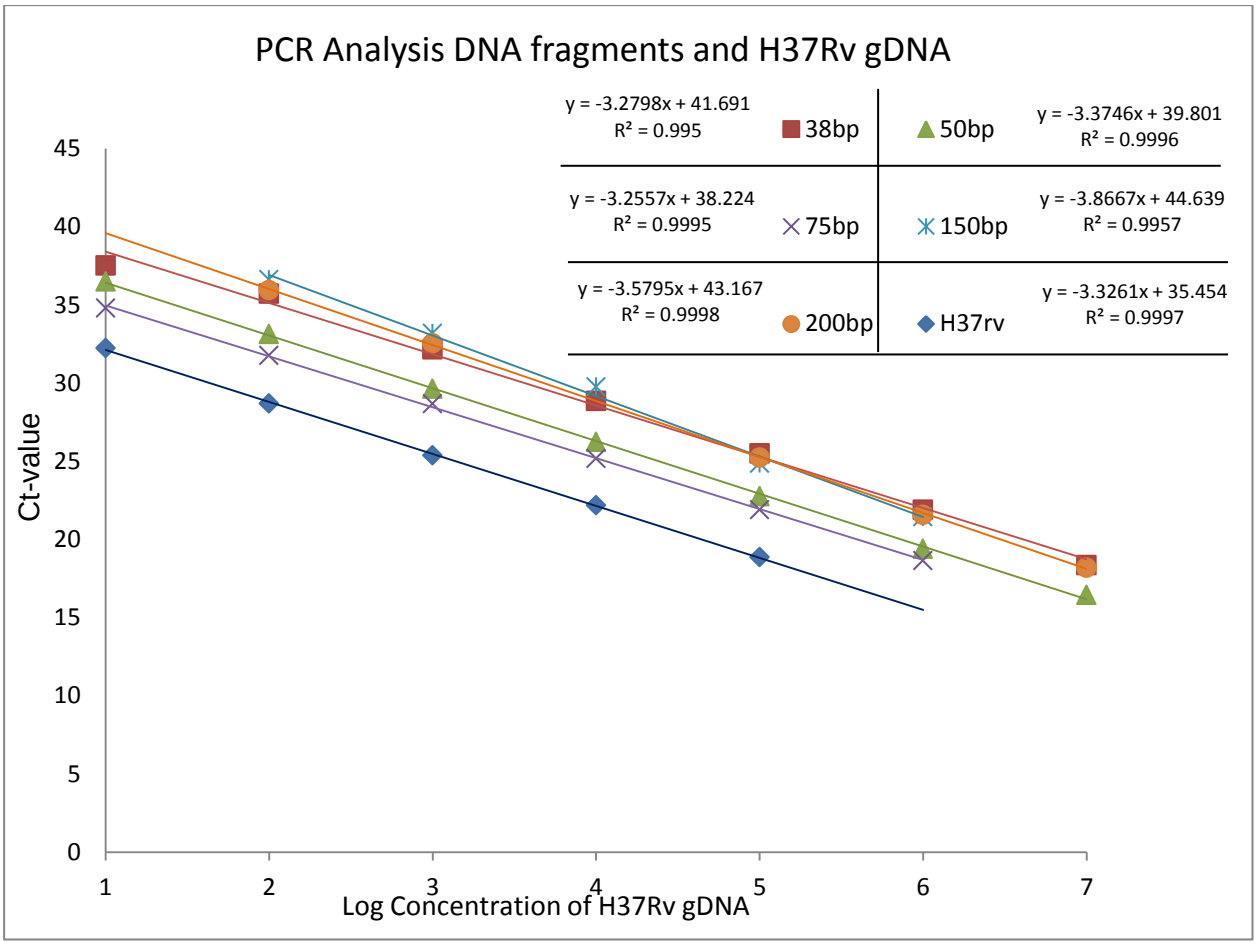


Figure 4.2: Calibration curves for various lengths of DNA fragments and H37Rv gDNA using serial dilution up to 7 log copies

4.1.2 Optimisation of urine and reagent volumes

Different volumes of urine (4.0, 2.0, 1.0 and 0.5 ml) were tested in triplicates. The correct ratio of urine volume to capture the matrix and binding buffer were evaluated using 2500 copies of the 50-bp DNA fragment. Among all tested combinations, no significant differences were found for varying urine volumes ($p = 0.114$; Figure 4.3). Using the largest urine volume (4 ml) also increased the chance of capturing the relatively sparse MTB-specific DNA fragments present in the sample.

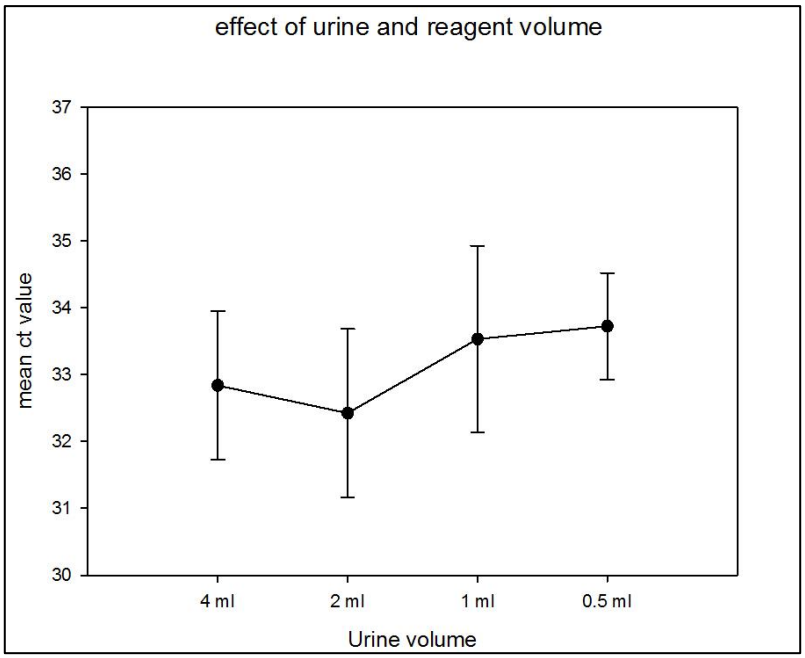


Figure 4.3: Optimisation of urine and reagent volume for the in-house developed method

The vertical lines show the 95% CI and black dot shows the mean cycle threshold (Ct) value for sample measured in triplicates

4.1.3 DNA content measurement for urine samples

Tr-DNA was extracted from five different urine samples using both the in-house and Qiagen methods. The DNA content of eluate was measured using the PicoGreen method and summarised in Table 4.2. The total DNA yield was higher for the in-house method than the Qiagen method ($p = 0.002$). The DNA yield varied from 190 – 3006 ng/ml for the in-house method and from 6.3 – 434 ng/ml for the Qiagen method. These results indicate that the capture matrix used in the in-house method is capable of isolating high amounts of DNA, which may increase the chances of detecting the low concentrations of the target in urine.

Table 4.2: Overview of mean DNA concentration (ng/ml) for the in-house and Qiagen methods, measured using the PicoGreen approach.

DNA fragment length (bp)	In-house method	Qiagen method
	Mean DNA content (ng/ml)	Mean DNA content (ng/ml)
38	933	6.3
50	3006	42
75	292	21
100	384	36
150	282	22
200	190	7.6
H37Rv gDNA	N/A	434

N/A: Not applicable (not performed)

4.1.4 DNA recovery from spiked urine samples

Yield percentages were calculated based on DNA fragment-specific calibration curves generated using pure DNA fragments. Figure 4.4 shows the mean DNA recovery (%) for both the in-house and Qiagen methods. For the Qiagen method, the yield percentage increased with the DNA fragment length, from 22.9% (38 bp) to 68.8% (150 bp), and decreased to 32.3% for the 200 bp DNA fragment. For the in-house method, the yield varied from 19.7% (38 bp) to 27.3% (75 bp), and declined to 16.3% for the 100-bp size and longer. The 200-bp fragment showed the lowest level of DNA recovery (190 ng/ml), and a yield of 3.9%. For the Qiagen method, the 38-bp fragment showed the minimum level of total DNA content (6.3 ng/ml) and had a yield of 22.9%. H37Rv gDNA recovery was 37.8% and 49.3% for the in-house and Qiagen methods, respectively. Overall, the Qiagen method showed a higher yield percentage compared to the in-house method ($p = 0.001$).

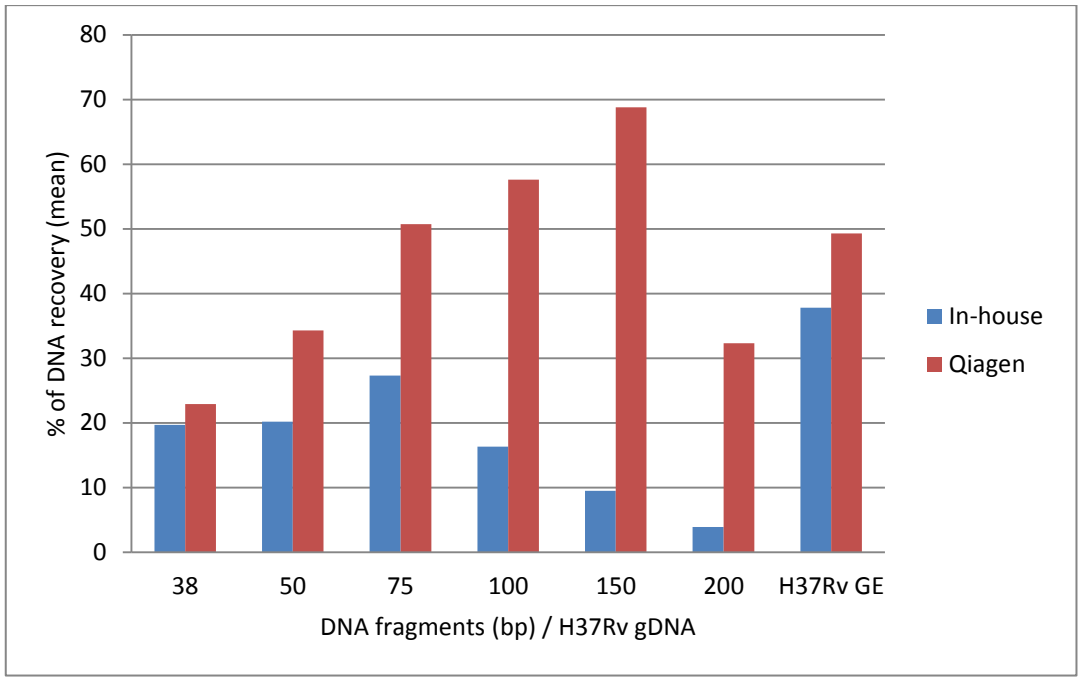


Figure 4.4: Percentage of recovery from spiked urine samples for the in-house and Qiagen methods for DNA fragments and H37Rv gDNA, measured in triplicate

4.1.5 Purity of PCR products

The purity of the PCR products amplified from the DNA fragments (38 bp, 150 bp, and 200 bp) was confirmed using gel electrophoresis. Figure 4.5 shows the specific bands observed for all analysed DNA fragments. Gel electrophoresis showed a very clear separation for different DNA fragments (38 bp, 150 bp, and 200 bp). Furthermore, no unwanted side products were observed, confirming the specificity of target-specific amplification.

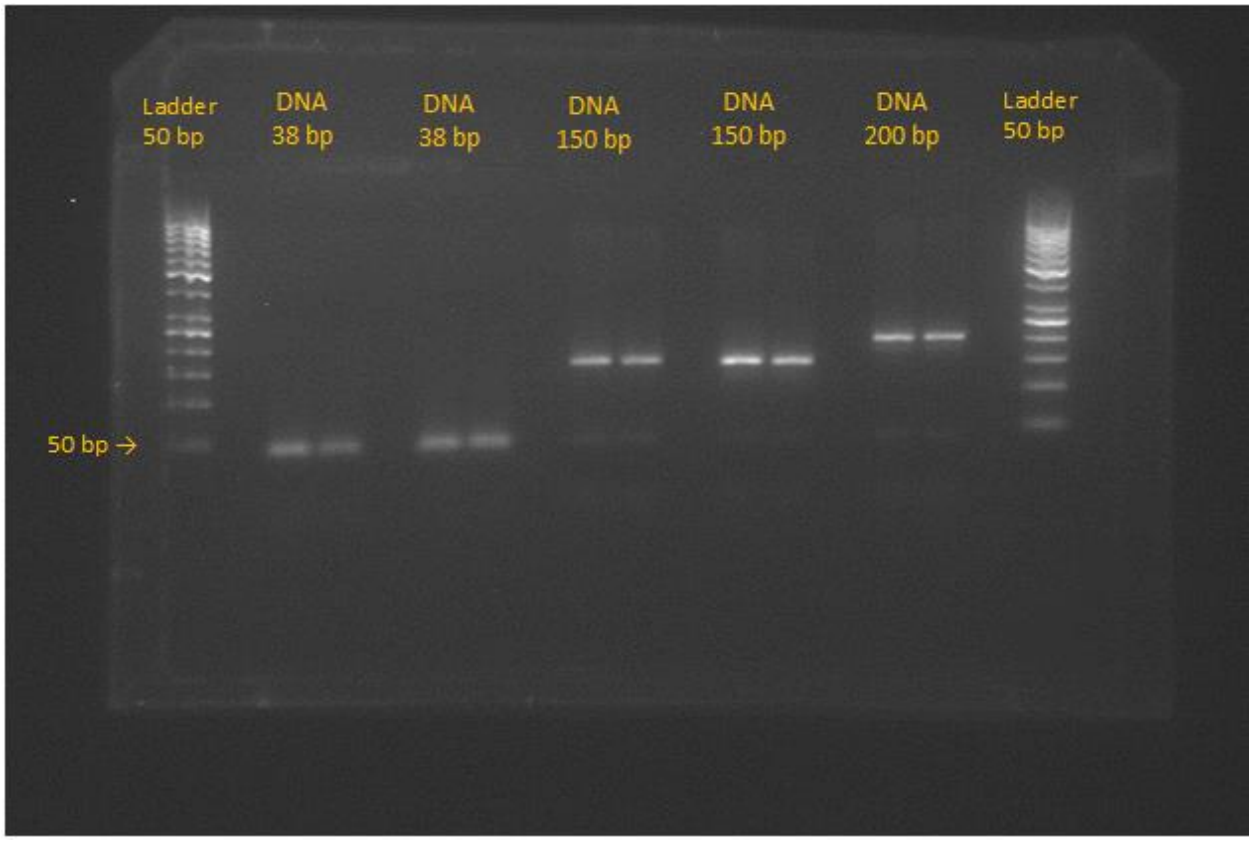


Figure 4.5: Gel electrophoresis bands for the 38-bp, 150-bp, and 200-bp DNA fragments. Size ladders are included in the first and last positions

4.1.6 Evaluation of PCR efficiency

PCR performance data from isolated DNA fragments of different lengths (38 bp, 50 bp, 75 bp, 100 bp, 150 bp, 200 bp, and H37Rv genome) were used as mean delta Ct-values, which were reproducible for both methods (Figure 4.6). Overall, the average delta Ct-values were found to be higher for the in-house method than for the Qiagen method. The standard deviation (SD) values for the in-house method were in the range of 0.09 – 1.36 and for the Qiagen method 0.08 – 1.65. For the in-house method, the lowest value (0.09) was observed for the 150 bp fragment, and the highest value (1.36) for the 200 bp fragment. For the Qiagen method, the minimum value (0.08) was observed for both the 38-bp fragment and H37Rv, and the highest value (1.65) for the 200-bp fragment. The SD value between the two isolation methods showed no difference ($p = 1.00$). The effect of PCR inhibition was the lowest for the amplification of the 50-bp fragment and H37Rv gDNA, and the highest for the 150 bp fragment using in-house method. The Qiagen method showed a low level of inhibition for all processed

DNA fragments. For the 150-bp fragment, a negative delta Ct-value was measured. A negative value indicates that the Ct-value of the eluate was lower than that of the positive control. The minimum impact on PCR efficiency was observed for the H37Rv gDNA, which reached equal mean delta Ct-values.

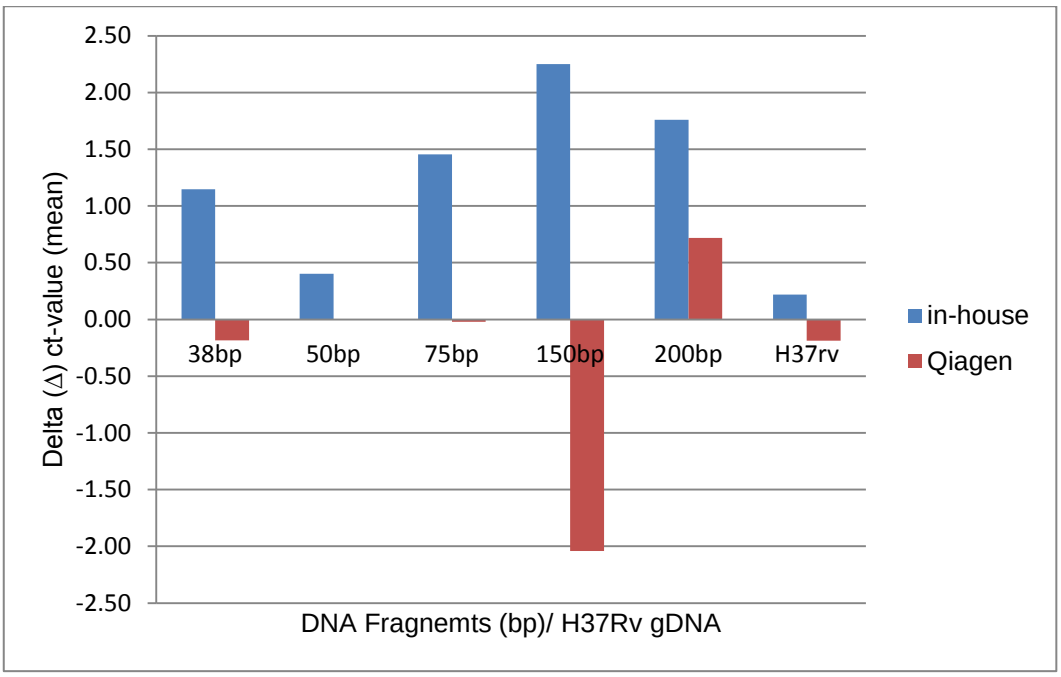


Figure 4.6: Influence of inhibitors on delta cycle threshold (Ct) value for the in-house and Qiagen methods, measured with DNA fragments and H37Rv gDNA

4.1.7 Influence of urine sample on PCR efficiency

The degrees of inhibition among different urine samples were determined using the DNA fragments (38 bp, 50 bp, 75 bp, 100 bp, 150 bp, 200 bp) and H37Rv gDNA. The Ct-values from the different DNA fragment lengths were combined according to the urine number, and the corresponding delta Ct-values were calculated. The mean delta Ct-values (difference between eluate control and inhibition control) for the six different urine samples are shown in Figure 4.7. Clearly, urine sample 9 carried less inhibitors compared to urine sample 7, as indicated by the lowest and highest delta Ct values, respectively. In contrast to in-house method, the Qiagen method showed almost no inhibition. The data show that the urine samples were heterogeneous, carrying different amounts of inhibitory components. The ability of the isolation method to remove inhibitors was therefore affected by the urine samples.

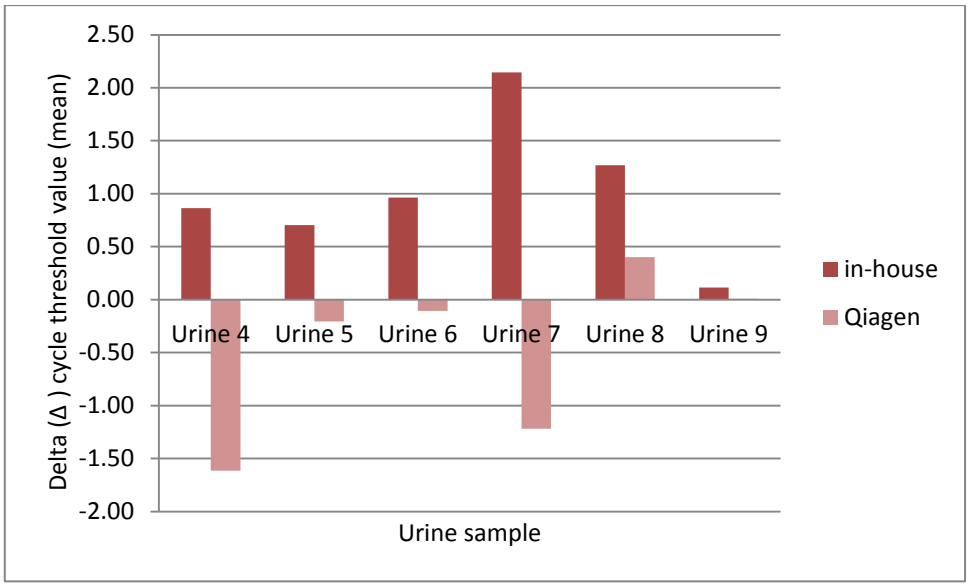


Figure 4.7: Effect of different urine samples on PCR efficiency, shown as delta cycle threshold value for the Qiagen and in-house methods

Difference sets of DNA fragments and H37Rv gDNA was spiked in each urine samples from 4 to 9, and mean delta Ct-value was calculated for urine.

4.1.8 Effect of eluate volume on PCR efficiency

Three different, tested eluate volumes (10 µl, 20 µl, and 40 µl) from three urine samples, together with their mean delta Ct-values, are shown in Figure 4.8. The DNA contents for urine A, B, and C were 399 ng/ml, 280 ng/ml, and 535 ng/ml, respectively. 10 µl of eluate volume showed the lowest delta Ct-values for all three urine samples indicating, less or no PCR inhibition. Increasing the eluate volume to 20 µl further increased the PCR inhibition, which was reflected in the higher delta Ct-values observed: up to 0.83 compared to 0.22 using the 10 µl eluate. Complete PCR inhibition was observed with 40 µl of eluate volume. Overall, the degree of PCR inhibition was directly associated with the eluate amount and urine DNA concentration. Urine B had the lowest level of DNA content and accordingly showed the lowest inhibition.

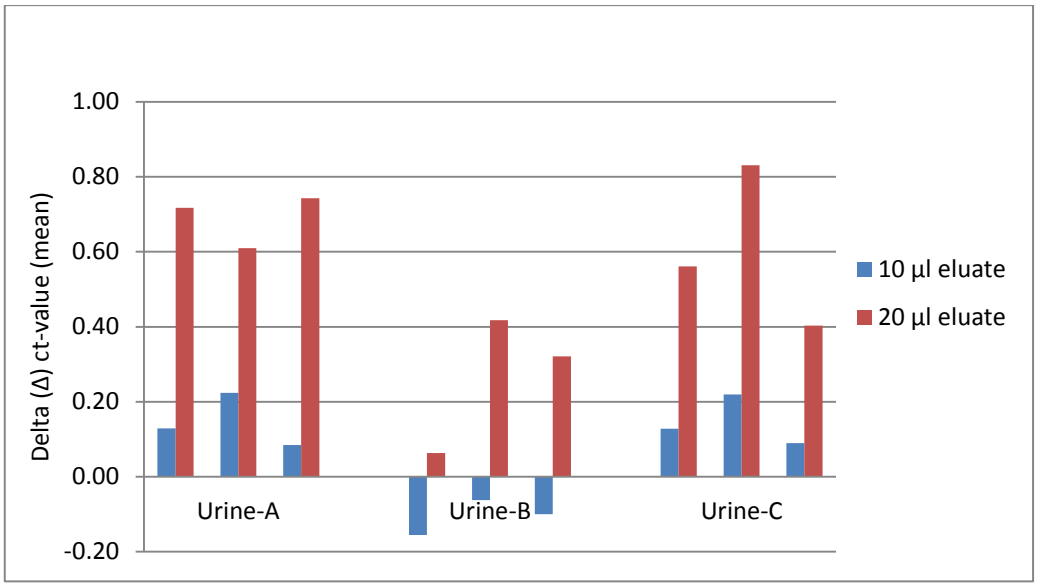


Figure 4.8: Effect of different eluate volumes on PCR efficiency, isolated using the in-house method, shown for three different urine samples

For the 40 µl eluate volume, complete PCR inhibition was observed and a delta Ct-value of 11 was attained.

4.1.9 Effect of eluate dilution on PCR efficiency

The effect of eluate dilution on processed urine samples is shown in Figure 4.9 as mean Ct-values (duplicate measurements). All five urine samples showed reproducible results for all eluate dilution series derived from both isolations. The lowest Ct-value was measured for 1:2 dilutions, which differed statistically from undiluted samples ($p < 0.001$). This result further confirmed the presence of PCR inhibitors, as Ct-value was lower for the 1:2 dilution than that observed for the undiluted sample. From the 1:4 dilutions onwards, an increase in Ct-value was observed, representing the decreasing target concentration. This data suggest that 10 µl may be the optimal eluate volume. All negative controls were undetected (Ct-value $\geqslant$ 40).

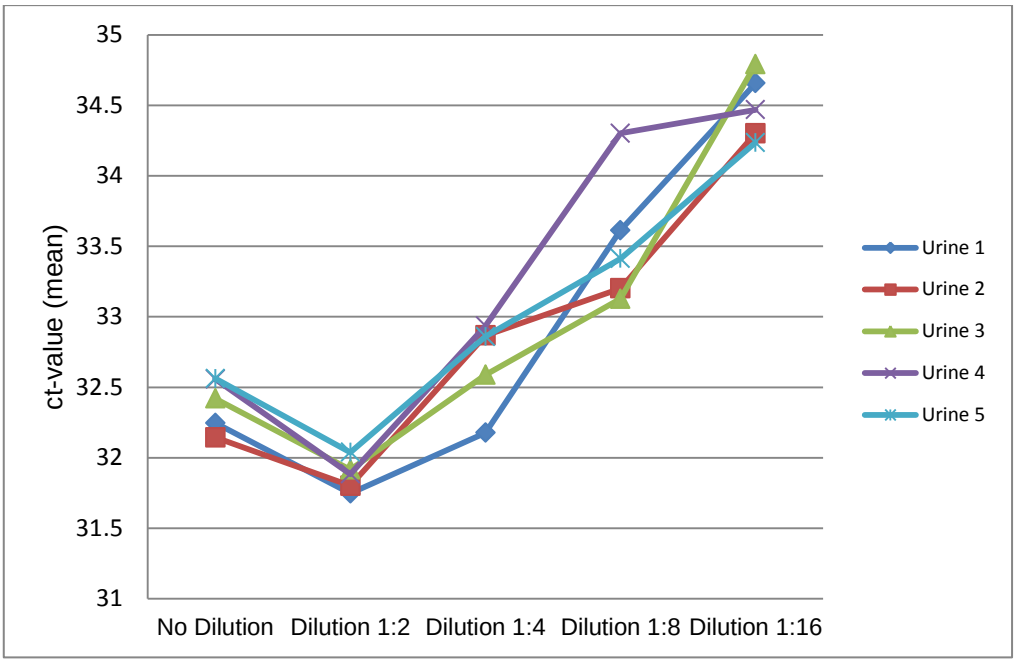


Figure 4.9: Effect of different eluate dilution series on PCR efficiency, isolated using the in-house method and measured for five different urine samples

The eluate was diluted in series, commencing from 1:2 increasing to 1:16 using molecular grade water.

4.1.10 Performance comparison of two Tr-DNA isolation method using clinical samples

The in-house developed method was optimised using a model system based on control urine samples spiked with DNA fragments or H37Rv gDNA. Furthermore, the capabilities of the in-house and Qiagen isolation methods to enrich MTB-specific Tr-DNA from urine were evaluated using clinical samples. Both tested samples were liquid culture positive on MGIT. Tr-DNA was isolated from 4 ml of urine and concentrated in 50 µl eluate; 20 µl of eluate was then added to 80 µl of master mix. Each eluate and the isolation (extraction) controls (negative and positive) were isolated and measured in duplicates. The positive controls for the in-house and Qiagen method were 34.88 and 38.04, respectively (Figure 4.10). The negative control was undetectable (Ct-value ≥ 40) for eluate isolated using both methods. Both methods were able to isolate the MTB-specific Tr-DNA. However, the optimised in-house method showed lower Ct-values for both patient samples as for positive control than those found with the Qiagen method. In summary, preliminary data using clinical samples confirmed the potential of developed in-house method for isolating MTB-specific Tr-DNA from urine.

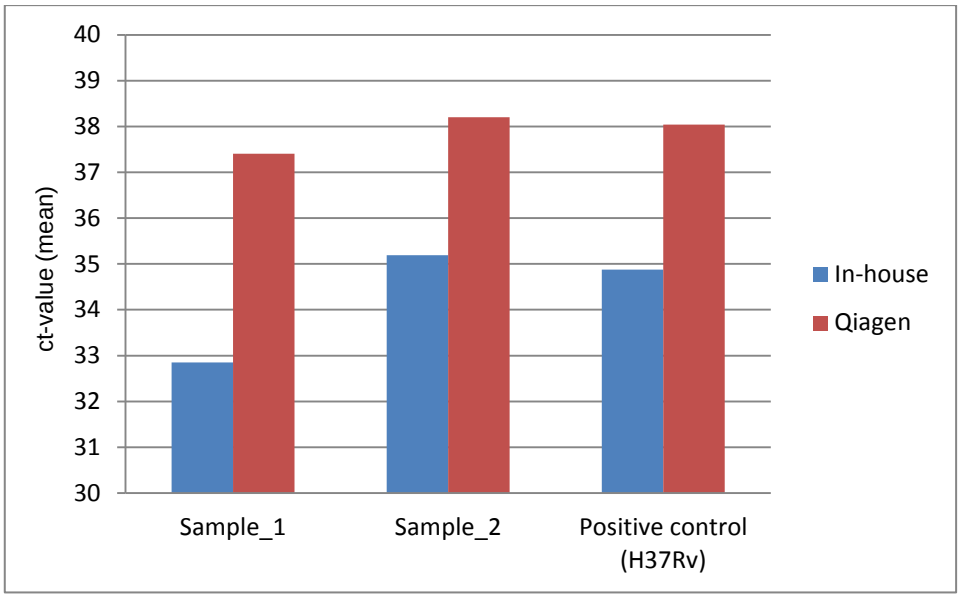


Figure 4.10: Performance comparison between the Qiagen and optimised in-house isolation methods using clinical samples

As a positive control, H37Rv gDNA in a concentration of 100 copies was used. The positive control was spiked in healthy urine and DNA was isolated using both methods.

4.2 Study cohort demographic characteristics

In total, 428 adult pulmonary TB suspects were enrolled between July and November, 2013, at three clinical sites in Langa (n = 162), Gugulethu (n = 209) and Vanguard (n = 57) in Cape Town, South Africa. The baseline demographics and clinical characteristics of the study cohort are presented in Table 4.3. Study participants had a male: female ratio of 1.5:1. The median age of participants was 39 years (range: 19-80 years, inter-quartile range (IQR): 29.96 – 49.16). HIV prevalence was higher among men than women ($p < 0.001$). The CD4 T lymphocyte median cell count was 249 cells/mm^3 (range 8 – 798 cells/mm^3, IQR: 148 – 390 cells/mm^3). Median BMI was 22.13 kg/m^2, with an IQR of 19.43 – 25.30.

Table 4.3: Study participant characteristics (demographic and clinical) at enrolment at three clinical sites in Cape Town, South Africa

All demographic and clinical characteristics were recorded using the case reporting form.

Study cohort characteristics (n = 426)*	Number (percentage)
Sex	
Male	256 (60.09)
Female	170 (39.91)
TB history	
No	262 (61.50)
Yes	164 (38.50)
- **TB therapy completed**	146 (89.02)
- **TB therapy not completed**	18 (10.98)
HIV Status	
Negative	264 (61.97)
Positive	162 (45.07)
ART: Yes	48 (29.63)
ART: No	114 (70.37)
CD4+ T cell count, cells/mm^3	
- **⩽ 200**	64 (39.50)
- **201 – 350**	49 (30.25)
- **> 350**	49 (30.25)
Diabetes mellitus	
No	413 (96.95)
Yes	13 (3.05)
Cough: Current	
No	8 (1.88)
Yes	418 (98.12)
Cough: Longer than 2 weeks	
No	48 (11.27)

Study cohort characteristics (n = 426)*	Number (percentage)
Yes	378 (88.73)
Phlegm production	
No	14 (3.29)
Yes	412 (96.71)
Blood in sputum	
No	363 (85.21)
Yes	63 (14.79)
Weight loss	
No	75 (17.61)
Yes	351 (82.39)
Anorexia	
No	120 (28.17)
Yes	306 (71.83)
Night sweats	
No	83 (19.48)
Yes	343 (80.52)
Fatigue	
No	71 (16.67)
Yes	355 (83.33)
Shortness of breath	
No	203 (47.65)
Yes	223 (52.35)
Fever	
No	308 (72.30)
Yes	118 (27.70)
Chest pain	
No	82 (19.25)
Yes	344 (80.75)

Study cohort characteristics (n = 426)*	Number (percentage)
BMI (kg/m^2)	
< 18.5 (underweight)	64 (15.02)
18.5 – 24.9 (normal range)	250 (58.69)
25.0 – 29.9 (overweight)	66 (15.49)
> 30.00 (obese)	46 (10.80)

*From 428 enrolled patients, two were excluded due to urine sample unavailability. ART: Antiretroviral therapy. BMI classified according to WHO criteria.

4.3 Laboratory testing for TB diagnosis

From 428 enrolled TB suspects, two patients were excluded from the study due to lack of an available urine sample. The liquid MGIT culture, Xpert MTB/RIF, and smear microscopy tests performed on 413 (96.95%), 425 (99.77%), and 424 (99.53%) sputum samples, respectively. Patients were classified as TB positive based on liquid culture and Xpert MTB/Rif test results. However, liquid culture was not performed on 15 samples; here, TB diagnosis was made based on Xpert MTB/RIF test results. One sputum sample was found to be contaminated, and on repeated testing of the reserved sample, the test returned a negative result. Positive TB status was related to HIV infection ($p < 0.001$) and was not related to patient sex ($p < 0.857$).

Figure 4.11, below, shows the flow diagram for the routine TB diagnoses performed in Cape Town. Median time to culture positivity was 16 days (IQR: 13 – 21 days). One patient tested positive for rifampicin mono-resistance (0.57%), and 3 patients (1.71%) tested positive for multi-drug resistance to rifampicin and isoniazid. Among the Xpert MTB/RIF positive results, six (3.08%) patients were rifampicin resistance. The median days to culture positivity among the negative smear group was 21 days, and 15 days for the smear positive group ($p < 0.001$).

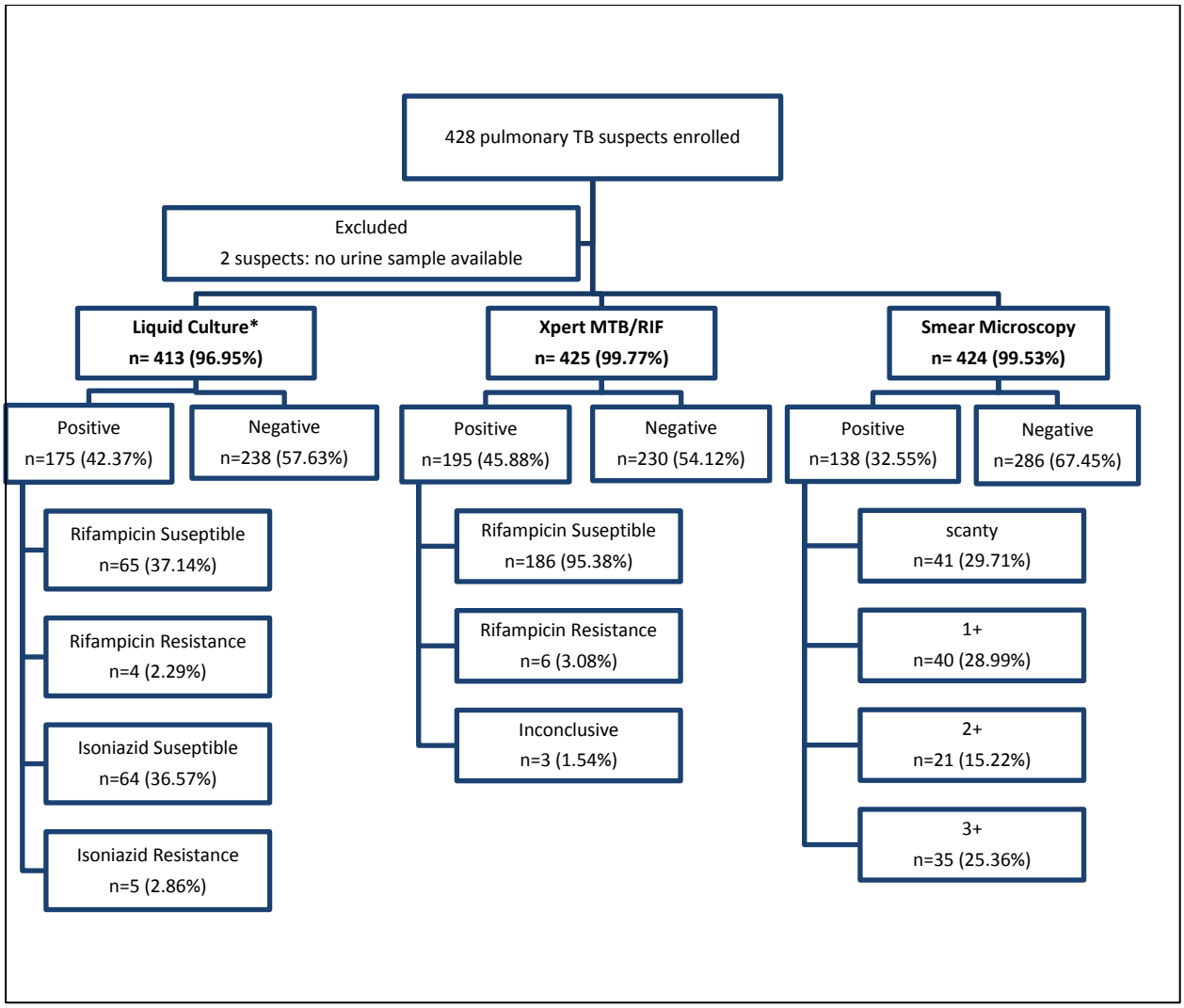


Figure 4.11: Flow diagram showing TB diagnosis using sputum-based tests, liquid MGIT culture, Xpert MTB/RIF molecular test, and smear microscopy

*One liquid culture sample was found to be contaminated, and retesting was performed using reserve sputum. For liquid culture, sputum was incubated at 37°C for up to 42 days (6 weeks), and positive samples underwent further drug susceptibility testing using the MTBDR plus line probe assay (Hain Lifescience, Nehren, Germany). Smear microscopy results was graded from scanty to 3+. All sputum-based tests were performed by the NHLS lab in Cape Town, South Africa.

4.4 Characterisation of urine sample

All urine samples underwent Combur testing and visual control for colour. Most samples were pale straw yellow or transparent yellow. The results of Combur test parameters such as specific gravity, pH, the presence of leukocytes and erythrocytes, and protein score are presented for TB positive and negative samples (Figure 4.12). Specific gravity,

pH, and the presence of leukocytes and erythrocytes were not dependent on patient TB status ($p > 0.05$). However, the protein score was found to be associated with TB status ($p = 0.015$).

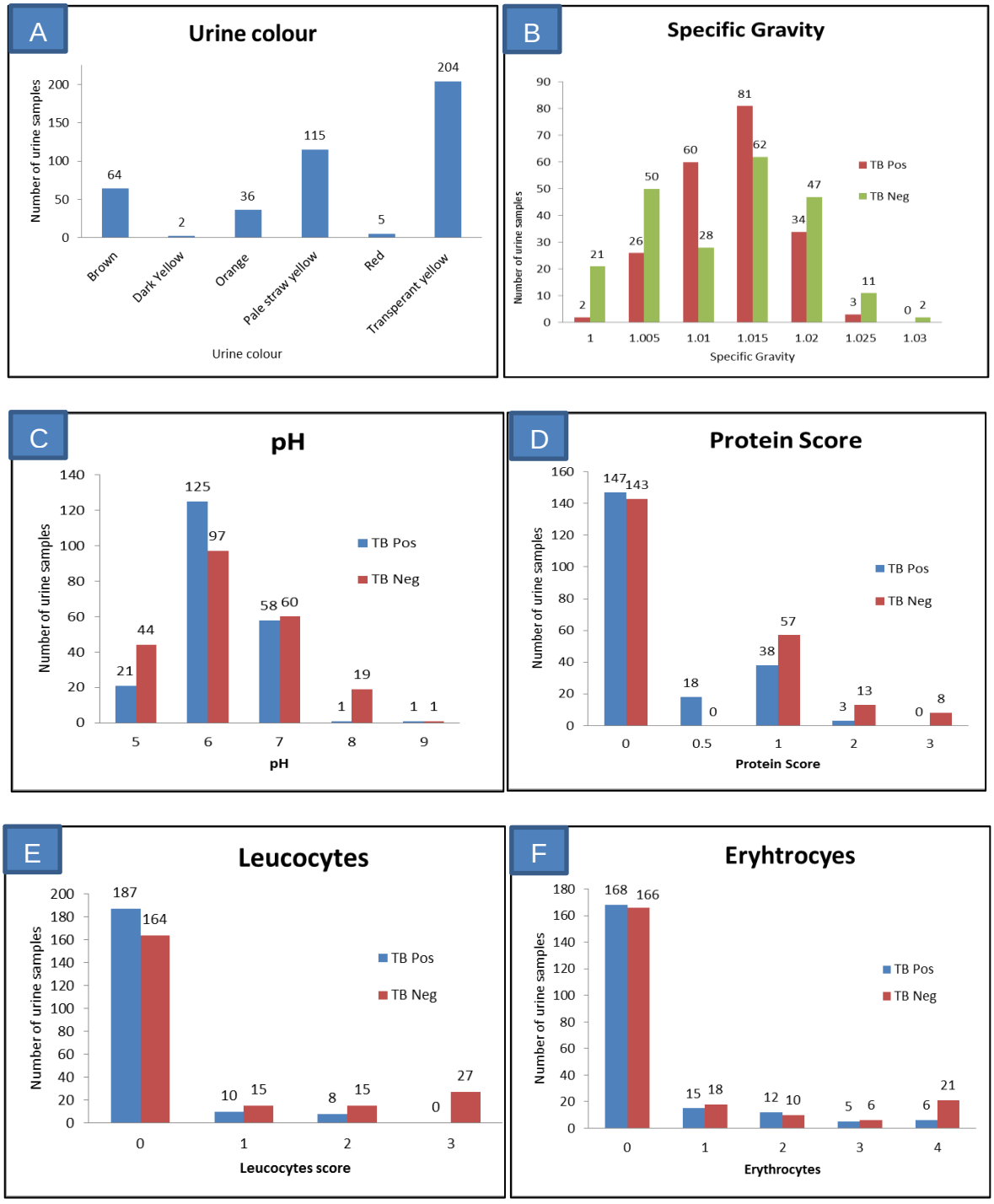


Figure 4.12: Urine sample characterization through visual control and Combur testing.

Sub-figures A-F represents different parameter measures, such as colour (A), specific gravity (B), pH (C), protein score (D), leukocytes (E) erythrocyte (F).

4.5 Implementation and optimisation of PCR method using Alere q

A series of experiments were performed to implement and optimise the DSP-based PCR method using the Alere q. Initially, six different PCR protocols, including variants of a short two and a conservative three-step PCR protocol were analysed for performance (Table 4.4 and Figure 4.13).

Table 4.4: An overview of double-stranded primer-based PCR optimisation using the automated molecular analyser Alere q.

#	Initial denaturation	Annealing	Elongation	Denaturation	Cycle #	Ct value (mean)	Ct value (SD)	Assay time (min)
1	95°C 60 sec	60°C 10 sec	72°C 5 sec	95°C 5 sec	45	33.94	0.11	25
2	95°C 60 sec	60°C 20 sec	70°C 5 sec	95°C 5 sec	40	31.56	0.26	33
3	95°C 60 sec	60°C 20 sec	72°C 5 sec	95°C 5 sec	45	31.71	0.13	30
4	95°C 60 sec	60°C 20 sec	N/A	95°C 5 sec	40	31.75	0.23	22
5	95°C 60 sec	60°C 25 sec	N/A	95°C 5 sec	40	31.36	0.64	27
6	95°C 60 sec	60°C 35 sec	N/A	95°C 5 sec	40	32.06	0.01	32

NB: Six different variations were tested for PCR optimisation. N/S Not applicable (not performed), Sec= seconds

In the two-step PCR method, annealing and elongation were performed simultaneously at a temperature of 60°C. The two tested annealing phase (20 sec vs. 10 sec) shows no difference in performance ($p = 0.07$). Overall, the three-step PCR showed a broader range of values, varying from 31.71-33.94, compared to the two-step PCR (31.36 –

32.06). Furthermore, attempts to optimise the performance by increasing the elongation temperature of the three-step protocol to 72°C and increasing the total number of PCR cycles (45 vs 40) were unsuccessful. Overall, the total time required for the PCR protocols was between 22 and 33 min for all six variants. The lowest PCR analysis time (22 min) was obtained with the two-step PCR protocol (annealing and elongation at 60°C for 20 sec). No significant difference between the six variants was observed ($p > 0.05$). The SDs of the mean Ct values were in the range of 0.01 – 0.64. To reduce the time to results, the shorter two-step PCR protocol was chosen and integrated into the Alere q assay software.

In addition to the PCR protocol, the primer: quencher (P: Q) ratio is also a critical parameter regarding the performance of the DSP-based PCR. We evaluated different P: Q ratios using H37Rv gDNA in triplicates. Table 4.5 shows the PCR data for the P: Q optimisation. A P: Q ratio of 1:1 showed the lowest Ct (33.89) and SD (0.27) values compared to the other ratios tested. The negative controls were undetected for all three tested ratios.

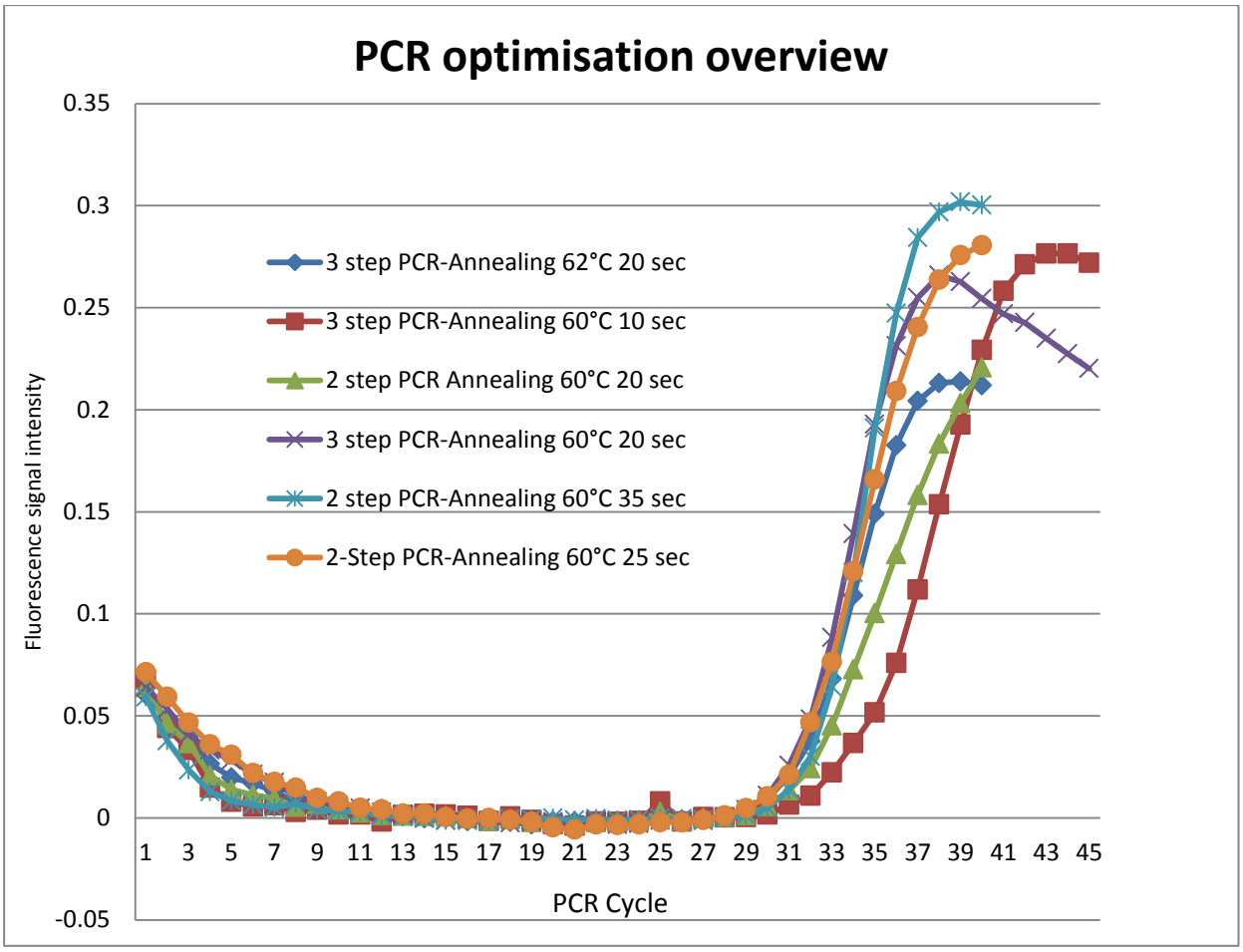


Figure 4.13: Double-stranded primer-based PCR optimisation overview using Alere q. Six different variations were tested for various PCR settings.

The cut-off was defined at a fluorescence signal intensity of 0.1.

Table 4.5: Primer: Quencher (P: Q) ratio optimisation for double-stranded primer-based PCR using H37Rv gDNA in Alere q

Sample	Primer:Quencher ratio	Ct value (mean)[#]	SD
Negative control	1.0:1.0	Undetected*	N/A
	1.0:1.5	Undetected*	N/A
	1.0:2.0	Undetected*	N/A
H37Rv gDNA 100 copies	1.0:1.0	33.89	0.27
	1.0:1.5	33.95	1.42
	1.0:2.0	34.91	1.35

[#]Mean Ct value of three measurements. *Undetected: ⩾ 40 Ct value. SD: standard deviation. N/A: not applicable.

4.6 Detection of Internal Process Control

The specificity of the IPC product was examined using melt curve analysis. This was performed using the standard PCR protocol specific to IS6110 on an AB cycler. Figure 4.14 shows melt curves for both MTB-specific amplicon and IPC, which yielded clearly separated melting temperatures (Tm values). The Tm for the sample and the IPC were 74.5°C and 81.0°C, respectively.

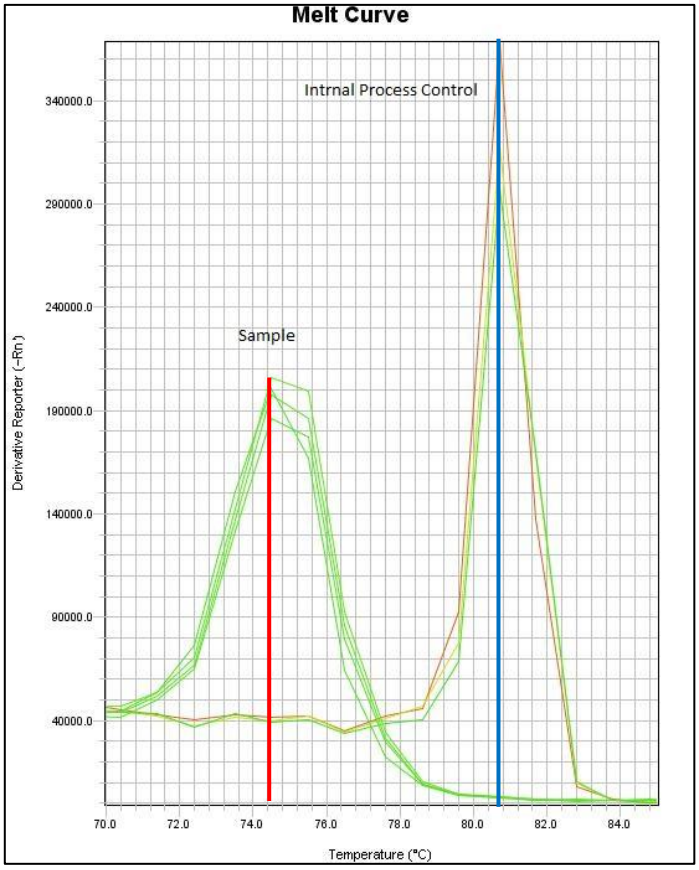


Figure 4.14: Melt curve analysis for the internal process control and MTB-specific target (IS6110),

Experiments were performed on an AB cycler in triplicates, shows the different melting temperatures for both PCR products. The red vertical line shows a Tm of 74.5°C for the target sample; the blue vertical line shows a Tm of 81.0°C for the IPC.

4.7 Calibration curve for target amplification using IS6110 and DR

For the calbration curve, all experiments were run in triplicates and the mean Ct-values were plotted vs the concentration of log H37Rv gDNA (Figure 4.15). The results showed that calibration curves for pure spiked DNA (DNA calibration curve) and DNA isolated

using the in-house method (capture calibration curve) were linear over the complete measured range, from log 0 to log 4 H37Rv gDNA for both target regions (IS6110 and DR). For the IS6110 region, PCR and capture calibration curve showed PCR efficiencies of 94.5% and 104.0% respectively. For the DR region, the PCR and capture calibration curve showed PCR efficiencies of 108% and 104% respectively. A R^2 value of > 0.990 was achieved for all calibration curves. The limit of detection was determined to be five H37Rv gDNA using PCR and a capture calibration curve.

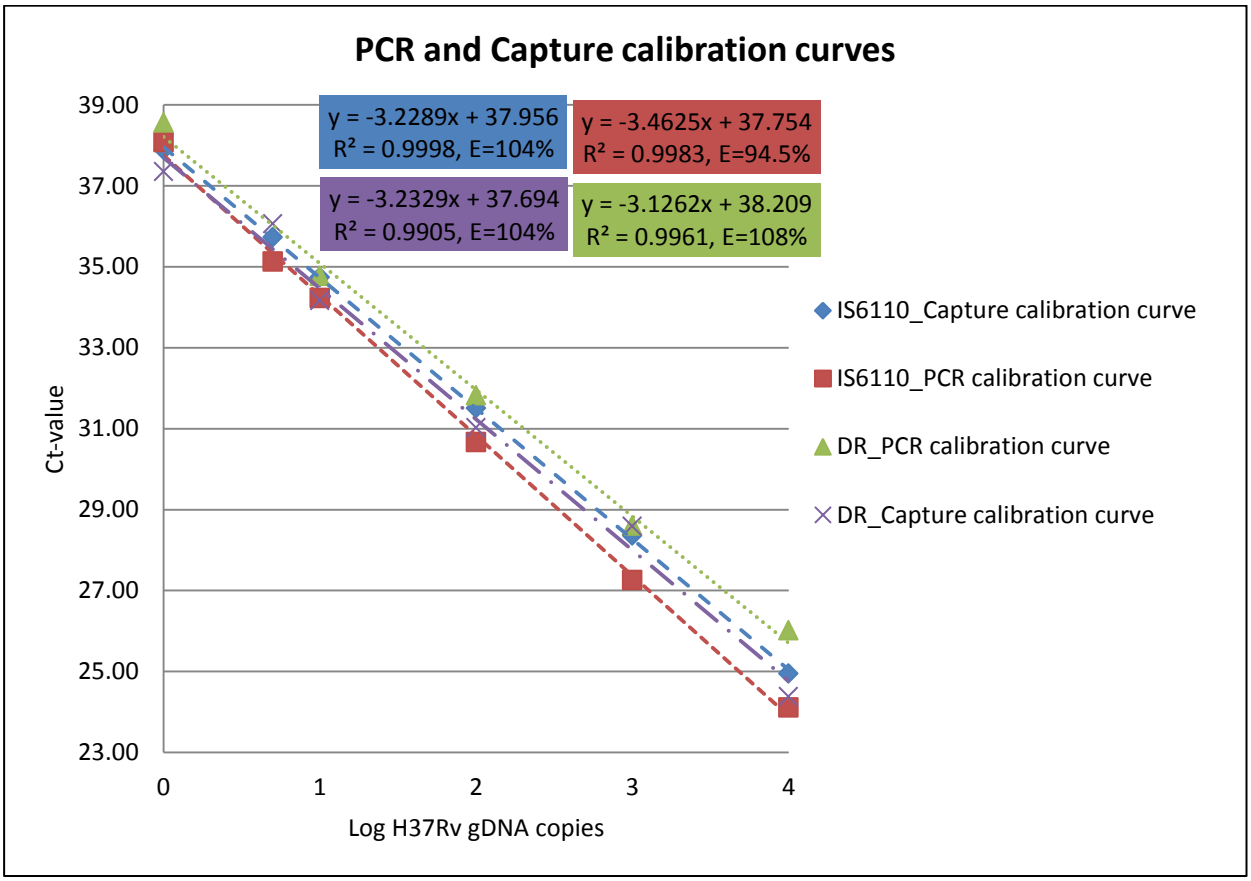


Figure 4.15: Performance characteristics of capture and amplification efficiency for the MTB-specific targets IS6110 and DR.

Serial diluted H37Rv gDNA were tested in triplicates, and the mean Ct-values were plotted against the log copy.

4.8 DNA isolation and quantification from clinical samples

The first 100 samples (50 positive liquid cultures and 50 negative liquid cultures) were isolated in duplicates, and total DNA content was measured using the PicoGreen method. The controls (negative and positive) were included in isolation process to monitor the reagent quality and presence of cross contamination. Each eluate was measured in duplicates, and mean concentration values were shown using box plots (Figure 4.16).

The measured mean DNA content (ng/ml) of female participants (n = 35) was higher than that of male participants (n= 65; p < 0.001). TB-positive participants had a higher DNA content than TB-negative participants (p = 0.020).

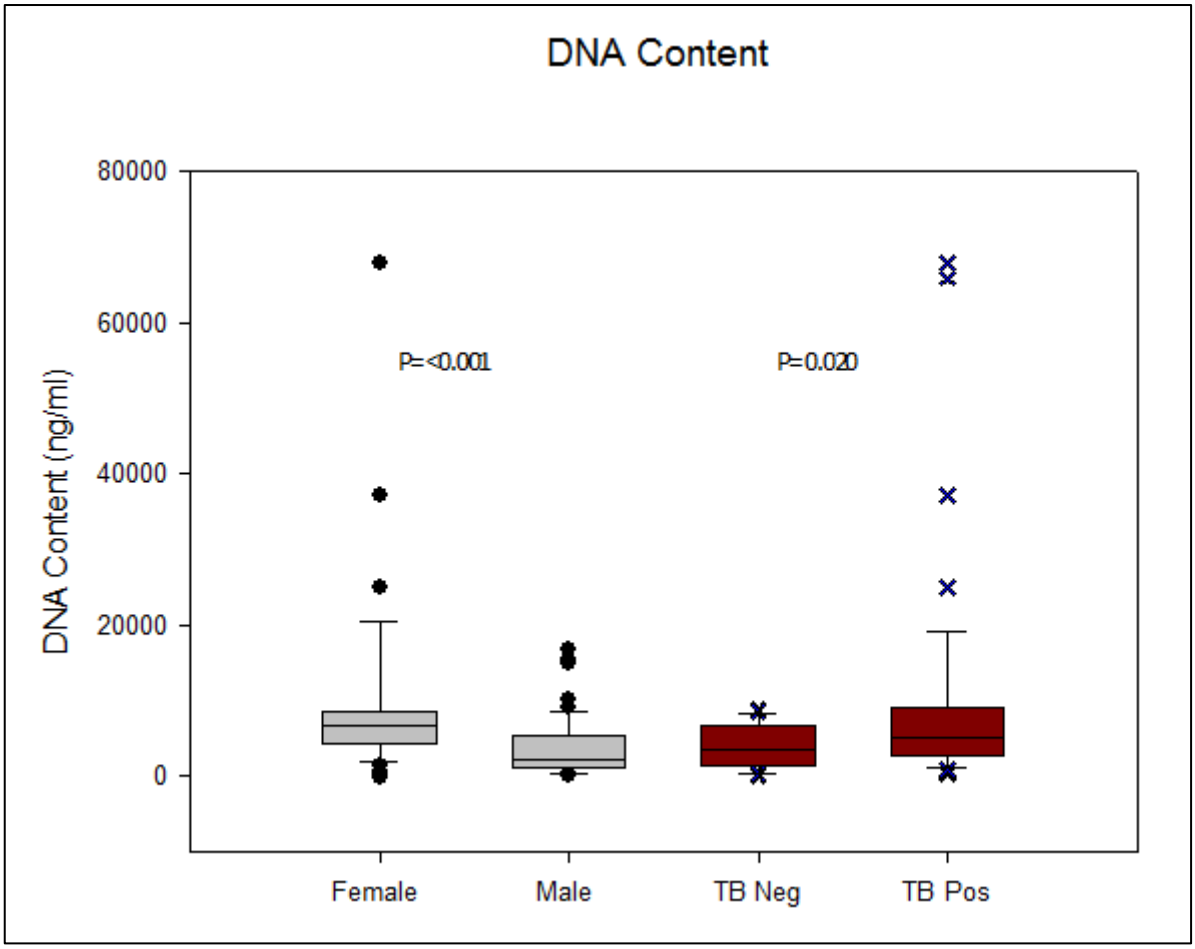


Figure 4.16: DNA content (ng/ml) measured using the PicoGreen method, divided into sex and TB status for the 50 negative and 50 positive liquid culture urine samples.

4.9 Performance comparison of IS6110 and DR target using clinical samples

The sensitivity and specificity of the MTB-specific targets, IS6110 and DR were compared using data generated on an AB cycler and Alere q analyser. The IS6110-based PCR method was performed on an AB cycler using SYBR Green [70]. The DR-based PCR method was conducted in the Alere q analyser using the DSP-based protocol. Fifty

positive and 50 negative liquid culture urine samples were isolated in duplicates, yielding a final eluate volume of 75 µl using the Tr-DNA extraction method. Each eluate was tested in duplicates using both PCR protocols. In total, four measurements were recorded. For the IS6110 a target sensitivity of 50% (95% CI: 35.53 – 64.47) and specificity of 96.23% (95% CI: 87.02 – 99.57), and for the DR region a target sensitivity of 54% (95% CI: 39.32 – 68.19) and specificity of 98.11% (95% CI: 89.93 – 99.95) were determined. The receiver operating characteristic (ROC) curves for both targets are shown in Figure 4.17. Employing the DR target, a higher sensitivity and specificity were reached than when using the IS6110 target.

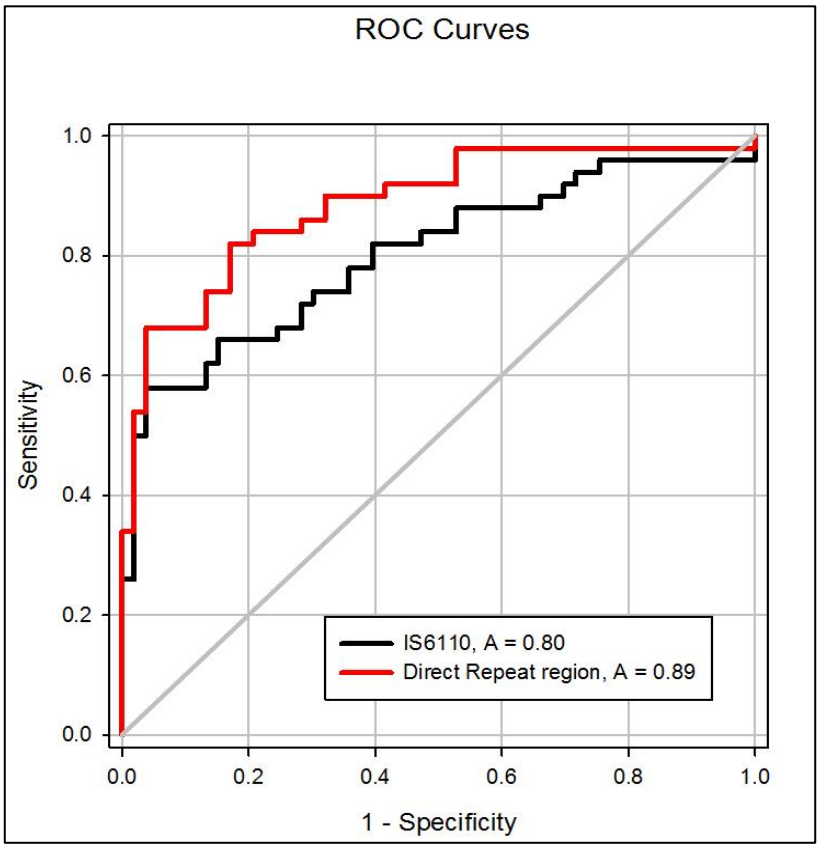


Figure 4.17: Receiver operating characteristic (ROC) curve comparison for the MTB-specific targets IS6110 (black line) and direct repeat (DR) region (red line).

The area under the curve for the IS6110 and DR region were 0.80 and 0.89, respectively.

4.10 Evaluation of cross-contamination

Reagent purity was confirmed by gel electrophoresis for the IS6110 and DR regions. In total, 10 different reagent combinations were tested for contamination and specificity (Figure 4.18). No unspecific side product bands were detected on the gel.

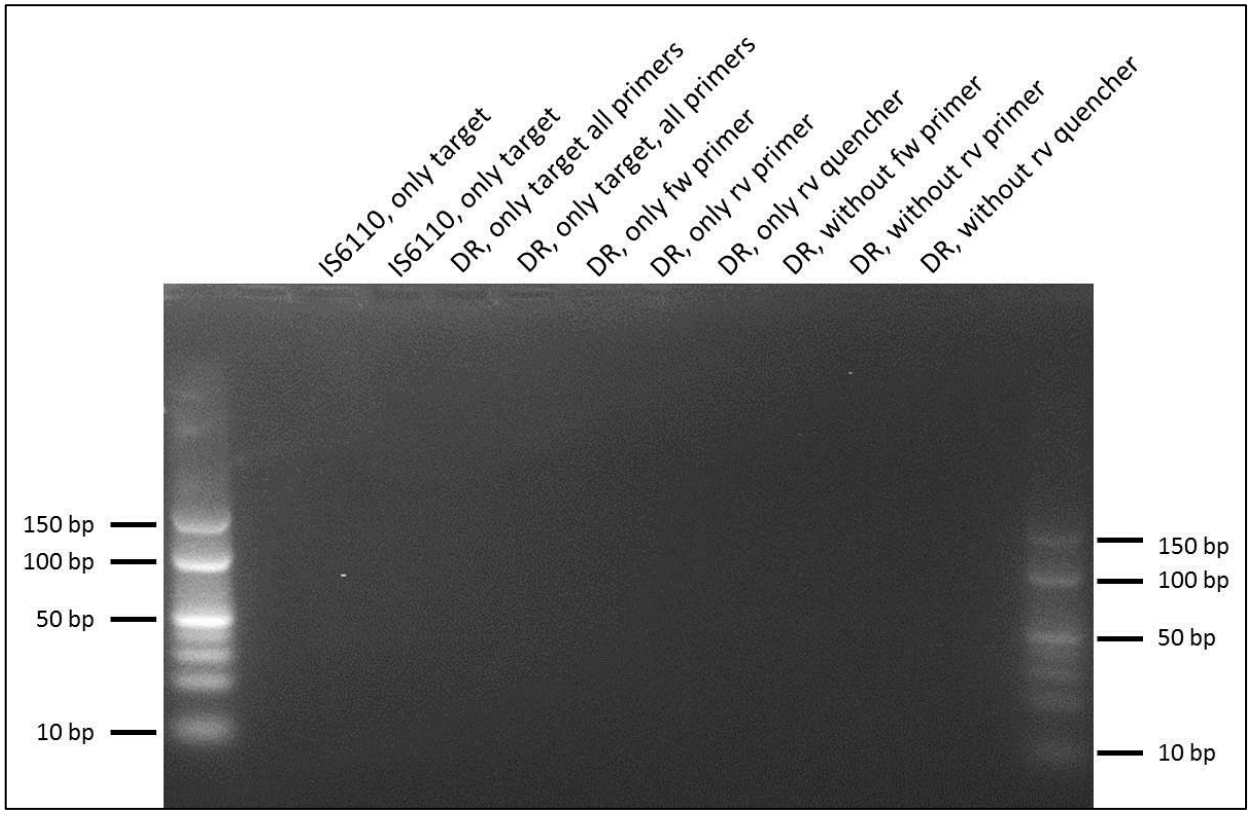


Figure 4.18: Evaluation of reagent purity for cross contamination using gel electrophoresis.

Furthermore, contamination control was performed, namely a 1.5-ml tube containing PBS buffer, was left open to monitor for lab contamination. Normal control urine (containing no spiked DNA fragments) was used as a negative control, and spiked urine containing 3500 H37Rv gDNA was designated the positive control. Table 4.6 shows the mean Ct-values obtained for the experiments performed. Contamination control was found to be negative in all experiments.

Table 4.6: An overview of the experiments performed for reagent purity and cross contamination for IS6110 and DR region targets

	Real-time RT-PCR analyser		Alere q*
Sample	**Ct-value IS6110**	**Ct-value DR**	**Ct-value DR**
Negative control	40.00	40.00	40.00
Positive control	33.06	32.04	31.81
Contamination control	40.00	40.00	40.00

* Only the DR region was measured using the Alere q.

4.11 Evaluation of Alere q analyser performance

In total twelve analysers were used for the Tr-DNA eluate analysis. Daily QC, using 100 copies of H37Rv gDNA, was performed for each Alere q analyser used for the study. A run chart in the form of Levey-Jennings graph with ±3 SD is shown below (Figure 4.19). No significant differences among the 12 Alere q analysers (p = 0.728) were found.

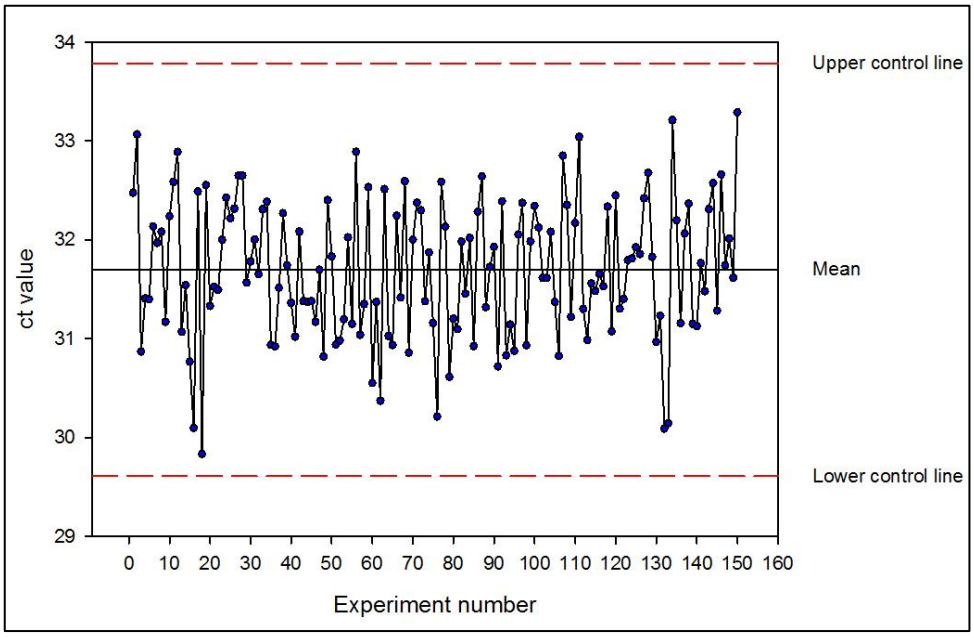


Figure 4.19: Levey-Jennings chart showing the daily performance of the Alere q analysers, measured using 100 H37Rv gDNA.

The dashed red line shows the upper and lower control limits for ±3 SD; the black line shows the mean Ct-values measured,

4.12 Risk factors for Tr-DNA positive test results in clinical samples

Risk factors for Tr-DNA positive test results were evaluated using the univariate and multivariate analysis logistic regression (Table 4.7). The association between patient characteristics (demographics and TB symptoms) and risk (odds ratio) of a positive Tr-DNA test was determined as crude and adjusted odds ratios. Among all tested risk factors, only weight loss was a significant independent predictor of a Tr-DNA positive test, whereas all other parameters were not significant.

Table 4.7: Univariate and multivariate logistic regression analysis showing the association between patient characteristics (demographic and clinical) and risk of a positive Tr-DNA test

	Crude odds ratio	95% CI	*P* value	Adjusted odds ratio	95% CI	*P* value
Sex						
Female	1.0	-	-	1.0	-	-
Male	0.867	0.551-1.365	0.538	0.992	0.605-1.624	0.973
HIV status						
HIV negative	1.0	-	-	1.0	-	-
HIV positive	1.485	0.944-2.336	0.087	1.452	0.896-2.351	0.13
TB history						
No	1.0	-	-	1.0	-	-
Yes	0.839	0.528-1.336	0.460	0.803	0.497-1.3	0.373
Current cough						
No	1.0	-	-	1.0	-	-
Yes	1.899	0.226-15.96	0.555	1.891	0.191-18.676	0.586
Cough > two weeks						
No	1.0	-	-	1.0	-	-
Yes	1.03	0.504-2.106	0.936	1.023	0.465-2.253	0.954
Weight loss						
No	1.0	-	-	1.0	-	-
Yes	2.214	1.09-4.496	0.028	2.258	1.084-4.702	0.03
Night sweats						
No	1.0	-	-	1.0	-	-
Yes	0.947	0.539-1.662	0.848	0.835	0.451-1.547	0.567
Fatigue						

	Crude odds ratio	95% CI	*P* value	Adjusted odds ratio	95% CI	*P* value
No	1.0	-	-	1.0	-	-
Yes	1.629	0.837-3.173	0.151	1.487	0.737-3.002	0.268
Shortness of breath						
No	1.0	-	-	1.0	-	-
Yes	1.233	0.786-1.933	0.362	1.201	0.75-1.923	0.445
Fever						
No	1.0	-	-	1.0	-	-
Yes	0.989	0.6-1.629	0.965	0.959	0.57-1.614	0.876
Chest pain						
No	1.0	-	-	1.0	-	-
Yes	0.927	0.528-1.63	0.794	0.892	0.488-1.63	0.709

4.13 Sensitivity and specificity of Tr-DNA assay using DR target

Initial experiments showed an increased sensitivity for the DR target compared to the IS6110 region. Therefore, the entire study cohort was analysed for the DR target region using an Alere q analyser. All samples were isolated using the in-house developed Tr-DNA method. A flow diagram illustrating the Tr-DNA test cohort is shown in Figure 4.20.

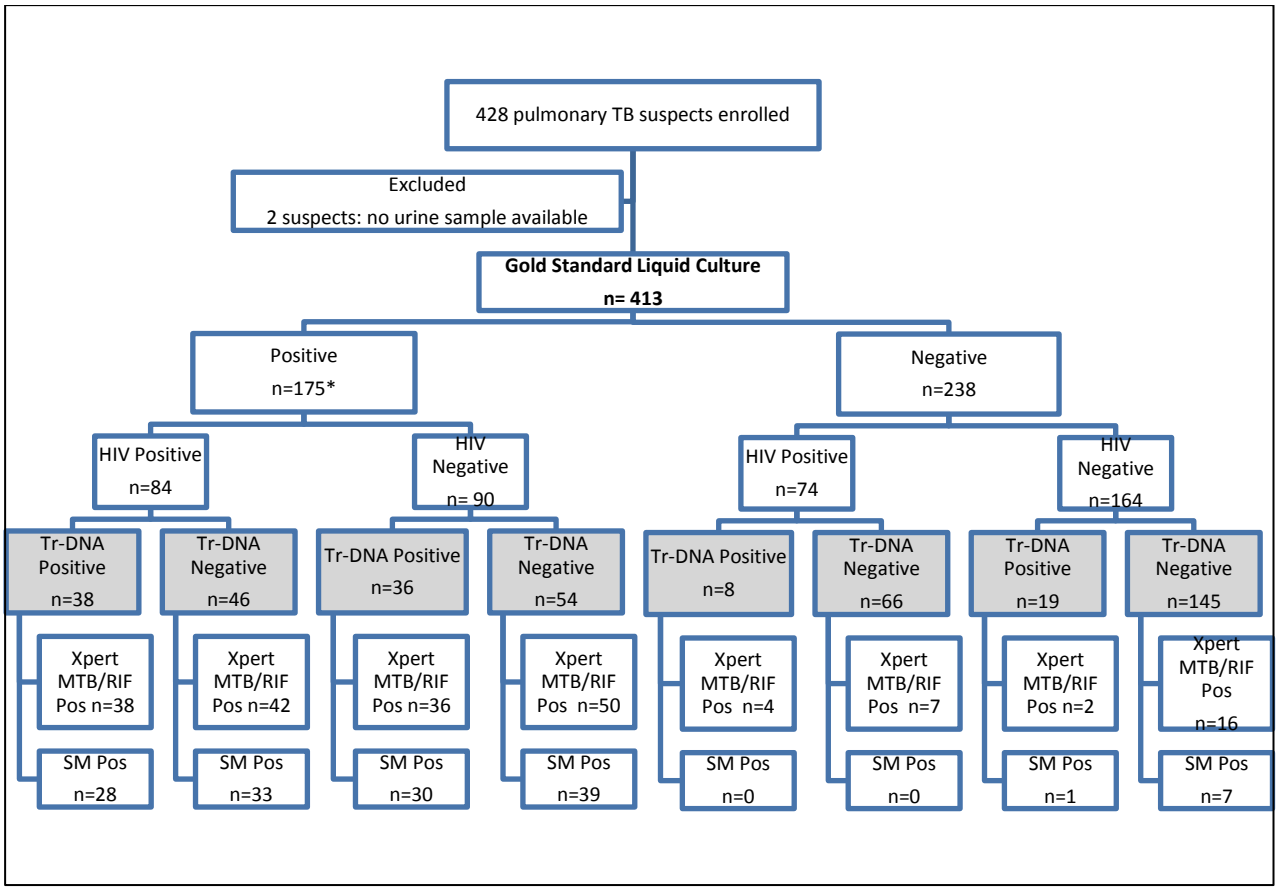


Figure 4.20: Flow diagram showing TB diagnosis made using a urine-based rapid Tr-DNA test carried out on an Alere q analyser.

The performance characteristic of the Tr-DNA assay was compared to the liquid culture among HIV-infected and non-infected patients. *HIV status not available: n = 1.

Tr-DNA assay showed an overall sensitivity and specificity of 42.86% (95%CI: 35.42 – 50.54) and 88.61% (95%CI: 83.86 – 92.36), respectively, when compared with liquid culture. Sensitivity and specificity calculated for sex, HIV status, CD4 count, and clinical site are shown in Table 4.8. For HIV-positive participants, a sensitivity of 45.24% and a specificity of 89.04% were achieved. A low CD4 count (< 200 cells/mm^3) did not show any influence on the sensitivity and the specificity of the Tr-DNA assay. Regarding the three clinical sites, the highest sensitivity for Tr-DNA was measured within the Gugulethu cohort (50.68%) and the lowest Tr-DNA sensitivity (20.83) was found in the Vanguard cohort. The specificity of the Tr-DNA test result between all of the sites ranged from 84.38 – 92.00%. However, Tr-DNA assay positivity was not dependent on clinical site (p = 0.494), CD4 count (p = 0.051) or BMI (p = 0.069). In addition, no difference in

sensitivity was found regarding sex (p = 0.447), HIV status (p = 0.078) or TB history (p = 0.459).

Table 4.8: An overview of the sensitivity, specificity, PLR, NLR, disease prevalence, PPV and NPV for the Tr-DNA assay, calculated for sex, HIV status, CD4 count, and all three clinical sites.

	Sensitivity (95% CI)*	**Specificity (95% CI)***	**PLR (95% CI)+**	**NLR (95% CI)+**	**PPV (95% CI)#**	**NPV (95% CI)#**
Overall Tr-DNA	42.86% (35.42-50.54%)	88.61% (83.86-92.36%)	3.76 (2.54-5.58)	0.64 (0.56-0.74)	73.53% (65.19-80.47%)	67.74% (64.70-70.64%)
Sex						
Male	41.12% (31.70-51.05%)	89.93% (83.68-94.38%)	4.08 (2.36-7.05)	0.65 (0.55-0.77)	75.86% (64.54-84.44%)	66.49% (62.65-70.12%)
Female	45.59% (33.45-58.12%)	86.73% (78.38-92.74%)	3.44 (1.95-6.07)	0.63 (0.50-0.79)	70.45% (57.45-80.81%)	69.67% (64.58-74.32%)
HIV status						
Negative	40.00% (29.81-50.87%)	88.41% (82.50-92.88%)	3.45 (2.11-5.65)	0.68 (0.57-0.81)	65.45% (53.65-75.62%)	72.86% (69.22-76.23%)
Positive	45.24% (34.34-56.48%)	89.04% (79.54-95.15%)	4.13 (2.06-8.27)	0.62 (0.50-0.76)	82.61% (70.33-90.49%)	58.56% (53.38-63.56%)
CD4 count (cells/mm^3)						
⩽200	50.00% (34.90-65.10%)	87.50% (61.65-98.45%)	4.00 (1.06-15.10)	0.57 (0.41-0.81)	92.00% (75.29-97.75%)	37.84% (30.16-46.18%)
201 –	36.84%	89.66%	3.56	0.70	70.00%	68.42%

	Sensitivity (95% CI)*	**Specificity (95% CI)***	**PLR (95% CI)+**	**NLR (95% CI)+**	**PPV (95% CI)#**	**NPV (95% CI)#**
350	(16.29-61.64%)	(72.65-97.81%)	(1.05-12.09)	(0.49-1.01)	(40.73-88.79%)	(60.07-75.73%)
>350	42.11% (20.25-66.50%)	89.29% (71.77-97.73%)	3.93 (1.19-12.95)	0.65 (0.43-0.97)	72.73% (44.74-89.78%)	69.44% (60.27-77.30%)
Clinical sites+						
Gug-ulethu	50.68% (38.72-62.60%)	87.69% (80.78-92.80%)	4.12 (2.47-6.87)	0.56 (0.44-0.72)	69.81% (58.10-79.41%)	76.00% (71.33-80.12%)
Langa	42.31% (31.19-54.02%)	92% (83.40-97.01%)	5.29 (2.35-11.89)	0.63 (0.51-0.77)	84.62% (70.99-92.52%)	60.53% (55.63-65.22%)
Vanguard	20.83% (7.13-42.15%)	84.38% (67.21-94.72%)	1.33 (0.43-4.09)	0.94 (0.73-1.21)	50.00% (24.58-75.42%)	58.70% (52.44-64.68%)

PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; PPV: Positive predictive value; NPV: Negative predictive value
*Confidence intervals for sensitivity and specificity are "exact" Clopper-Pearson confidence intervals
+Confidence intervals for likelihood ratios were calculated using the "Log method"
#Confidence intervals for the predictive values are the standard logit confidence intervals
+Clinical sites are township clinics in Cape Town, South Africa

The combination of the Tr-DNA test and the smear microscopy results showed an increase in TB sensitivity of 83.82% compared to smear microscopy test results alone (sensitivity of 75.14%). Among HIV-positive patients, combining smear microscopy and Tr-DNA tests results showed an increased sensitivity, from 73.49% to 84.34%. The receiver operating characteristics (ROC) curve for the Tr-DNA assay against liquid culture, smear microscopy and Xpert MTB/RIF is depicted in Figure 4.21. A rather poor area under the curve (AUC) of 0.67 was observed for the Tr-DNA assay when compared to the liquid culture, smear microscopy and Xpert MTB/RIF AUCs.

In total, 27 samples tested with the Tr-DNA assay showed discordant results when compared to the liquid culture results after 42 days of incubation. Six of the 21 Tr-DNA

positive/liquid culture negative samples were Xpert MTB/RIF positive, which increased the sensitivity to 46.28% and the specificity to 91.14%. The remaining patient urine samples (n = 21) that had discrepant results between the liquid culture and Tr-DNA assays were further investigated using the Xpert MTB/RIF procedure, as described previously [83]. Overall, only one of the 21 urine samples returned a positive result using Xpert MTB/RIF. A detailed analysis of the false positive results is listed in Table 4.9. Figure 4.22 shows a Venn diagram of all four assays (liquid culture, Xpert MTB/RIF, sputum smear microscopy, and Tr-DNA).

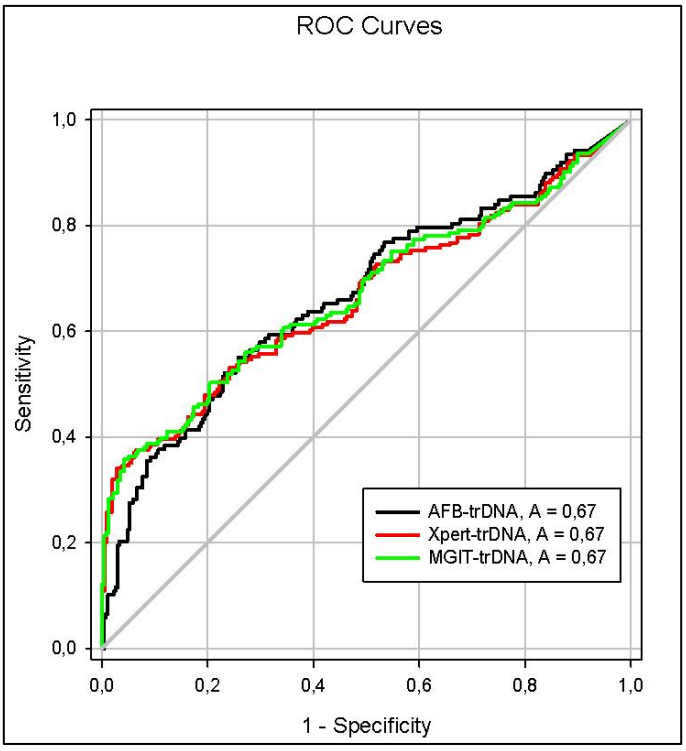


Figure 4.21: Receiver operating characteristic (ROC) curve for Tr-DNA compared to smear microscopy (black line), Xpert MTB/RIF (red line) and liquid MGIT culture (green line).

The area under the curve for all three ROC curves is 0.67.

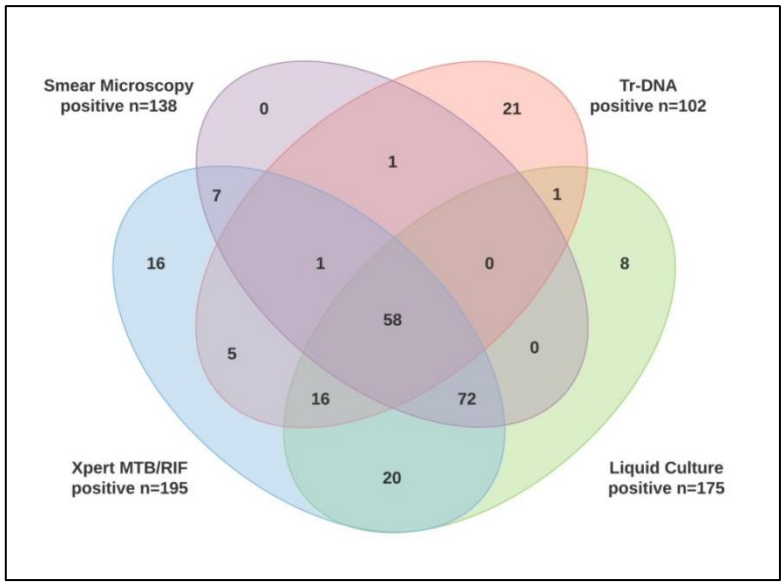


Figure 4.22: Venn diagram showing the number of positive samples diagnosed by sputum smear microscopy (n = 138), Xpert MTB/RIF (n = 195), liquid MGIT culture (n = 175) and Tr-DNA test (n = 102)

In total, 58 patients were diagnosed as positive by all four tests; 21 patients were diagnosed as positive by the Tr-DNA assay alone.

Table 4.9: Xpert MTB/RIF test performed for discordant urine samples that were Tr-DNA positive but sputum liquid culture and Xpert MTB/RIF negative.

Patient no	HIV status	CD4 count (cells/mm^3)	Sex	BMI (kg/m^2)	TB history	Urine: Xpert MTB/RIF
G007	Negative	N/A	Male	30.42	No	Negative
G009	Negative	N/A	Male	19.38	No	Negative
G086	Negative	N/A	Male	21.05	Yes	Negative
G120	Negative	N/A	Male	20.96	No	Negative
G129	Negative	N/A	Male	23.51	No	Negative
G136	Negative	N/A	Male	21.98	Yes	Negative
G138	Negative	N/A	Female	45.33	No	Negative
G140	Negative	N/A	Male	19.87	Yes	Negative
G142	Negative	N/A	Male	24.06	Yes	Negative
G179	Negative	N/A	Male	27.12	No	Negative

Patient no	HIV status	CD4 count (cells/mm^3)	Sex	BMI (kg/m^2)	TB history	Urine: Xpert MTB/RIF
G047	Positive	206	Female	27.68	No	Negative
L082	Negative	N/A	Female	30.43	No	Negative
L115	Negative	N/A	Male	20.17	No	Negative
L134	Negative	N/A	Male	19.25	No	Negative
L152	Negative	N/A	Female	27.10	No	Negative
G031	Positive	483	Female	22.36	No	Negative
L030	Positive	505	Female	20.73	Yes	Negative
G052	Positive	513	Male	34.98	No	**Positive**
V006	Negative	N/A	Male	18.50	Yes	Negative
V020	Negative	N/A	Female	26.22	No	Negative
V022	Negative	N/A	Female	34.72	No	Negative

5 Discussion

This cross-sectional concept study demonstrates that the Tr-DNA assay detects pulmonary TB independent of HIV status and level of immune suppression. Unlike the urine-based TB LAM test, the performance of the Tr-DNA assay was independent of CD4 values [84]. Furthermore, to our knowledge, this is the first study in which *Mycobacterium tuberculosis* (MTB) is diagnosed using a target-specific direct repeat (DR) region. The DR region is present up to 42 times in the MTB genome; this can increase test sensitivity [80]. PCR analysis was performed in triplicates, and included appropriate controls to assure robustness and to avoid cross contamination. All controls worked within defined specifications. Iinternal process and negative controls were run with each DNA eluate tested; this allowed use to observed assay performance. Nevertheless, a moderate Tr-DNA assay sensitivity of 42.86% was observed, further indicates that not all patients with pulmonary TB will have free, circulating MTB-specific DNA passing through the kidneys into urine. The meta-analysis performed on eight Tr-DNA-based studies that used IS6110, rpoB and cfp/hf6 as targets for PCR analysis attained a slightly higher sensitivity (55%) and specificity (94%) than the present study [62]. Furthermore, no correlation was found between the time to liquid culture positivity and Tr-DNA assay Ct value (r^2 = 0.005). The rather low assay performance observed may have been due to several reasons, e.g., time of sample collection (spot vs morning) [62], storage duration of samples (up to three years) [70], and presence of PCR inhibitors in the urine and DNA eluate [85].

MTB-specific Tr-DNA fragments are even shorter; as small as 39 bp have been described [86]. Therefore, we developed and optimised a capture matrix-based in-house nucleic acid method [87, 88] for the capturing of short Tr-DNA fragments (as small as 38 bp). Many different sets of optimisation experiments were performed in comparison with Qiagen method. In addition we implemented in-house method on syringe pump to reduce hands-on time for isolation. A PCR target size of <150 bp has demonstrated Tr-DNA sensitivities of 7 – 79% in alignment with the finding here of 42.86% [54, 65, 89]. Urine-based Tr-DNA molecular tests are useful for diagnosing pulmonary and extra-pulmonary TB in underserved groups (children, HIV-positive and advanced immunosuppressed groups) [90]. However, few Tr-DNA-based studies have been performed with larger patient cohorts (n > 100); those that have showed sensitivities ranging from 15.7% [91] to 44% [92] for pulmonary TB. A lack of reliable nucleic acid

isolation and PCR methods may cause differences in reported sensitivity and specificity. Strong reproducibility of a Tr-DNA test is desired in order for it to be implemented in limited resource settings. However, Kafwabulula et al. (2002) repeated the Sechi and Aceti method for PCR analysis, and reported contradicting results [93] using an identical PCR method for DNA analysis; the only changes made were to the sample size and the Tr-DNA isolation method. Furthermore, Amin et al. (2011) performed a study of extra-pulmonary TB suspects and reported a sensitivity of 46.6% [89], and Moussa et al. (2000) reported a sensitivity and specificity of 96% and 98%, respectively, for genitourinary TB [94].

The total turn-around time (TAT) for the Tr-DNA assay was approximately 45 minutes, from Tr-DNA isolation to the fully-automated PCR process, including data analysis. This short TAT may allow a greater number of patients to be tested for TB, which may in turn further improve the disease control [95] The novel and rapid molecular assay targeting DR target was evaluated against the well-established IS6110 target on subset of study cohort. In preliminary comparison DR target showed high sensitivity and specificity compared to IS6110 target. Therefore, the whole study cohort urine was tested using DR region only. Once the complete cohort was tested using DR target, we observed the reduction in assay performance (sensitivity of 54% observed in preliminary study vs 42.86% for complete cohort).

The major findings of the current study are i) the in-house developed method described here can isolate nucleic acids from urine samples (as low as five copies of H37Rv gDNA), ii) the DSP-based amplification of the short DR target region in a single-use cartridge enables the detection and quantification of MTB-specific Tr-DNA in pulmonary TB, iii) assay performance is independent of HIV status and offers potential benefits to the standard of care when combined with sputum smear microscopy (providing a sensitivity of 83.82%). This study includes a detailed characterization of a large TB suspect cohort, including demographic data, clinical symptoms and Tr-DNA characterization in comparison to routine sputum-based diagnostic tests. The heterogeneity of the urine samples and the possible effect of this on assay performance was unavoidable due to the large sample size involved.

The findings indicate that not all patients with a high bacterial load will excrete MTB into urine. Therefore, other, currently unknown, factors must influence DNA fragment excretion. Although EDTA was added immediately after urine collection to assure DNA frag-

ment stability, the limited sensitivity achieved here may have been associated with a minor degradation process, as the samples were not processed fresh [96]. Furthermore, the excess amount of EDTA added may also have inhibited the PCR process, which may in turn have resulted in reducing assay sensitivity [97]. In order to allow interpretation of the discordant results observed for extra-pulmonary and disseminated TB, participant follow up would be required; this is missing from the current study. Furthermore, negative bacteriological tests results among discordant samples raises the question regarding which diagnostic method should be used as gold standard. Thus, the present study reveals a significant discrepancy between established methods of TB diagnosis and the Tr-DNA assay tested here. Therefore, this study serves as a preliminary investigation on which to base the future research and assay development. The effort to develop a Tr-DNA-based molecular assay that runs on an automated analyser should further be pursued to gain an understanding of the potential benefits it could bring to diagnostics. Likewise, the current in-house developed Tr-DNA isolation method requires technical skills to perform and to make it suitable to be used in limited resource settings; therefore, further simplification is required. This novel Tr-DNA diagnostic test should be evaluated for its impact on public health and its clinical utility to be used for diagnostics [98, 99]. A future Tr-DNA study should include the analysis of fresh urine samples, and participant inclusion criteria should not be limited to pulmonary TB only.

6 Conclusion

TB remains a major problem in developing the country and among HIV-infected patients. The TB diagnosis method is particularly valuable in a limited resource setting for timely initiation of therapy. Newer urine-based molecular methods could be an alternative to the current smear microscopy tests performed using sputum. This first proof-of-concept, cross-sectional study demonstrates the possibility of diagnosing pulmonary TB using a novel Tr-DNA molecular assay implemented using a single-use cartridge, which is processed using an automated molecular analyser. Considering that MTB specific cell-free circulating DNA fragments present in the blood that pass through the kidney in urine. Furthermore, in-house developed method capable enough to extract the DNA fragments from larger urine volume. The overall sensitivity was moderate; however, a higher specificity was obtained. This in-house developed Tr-DNA isolation method also has the potential to be used for other body fluids, such as plasma. Although unsuitable

to be implemented as a stand-alone test, in combination with sputum smear microscopy, this Tr-DNA assay may have the potential to aid TB diagnosis in HIV-endemic regions, where sputum scarce and extra-pulmonary TB are common [100, 101].

7 References

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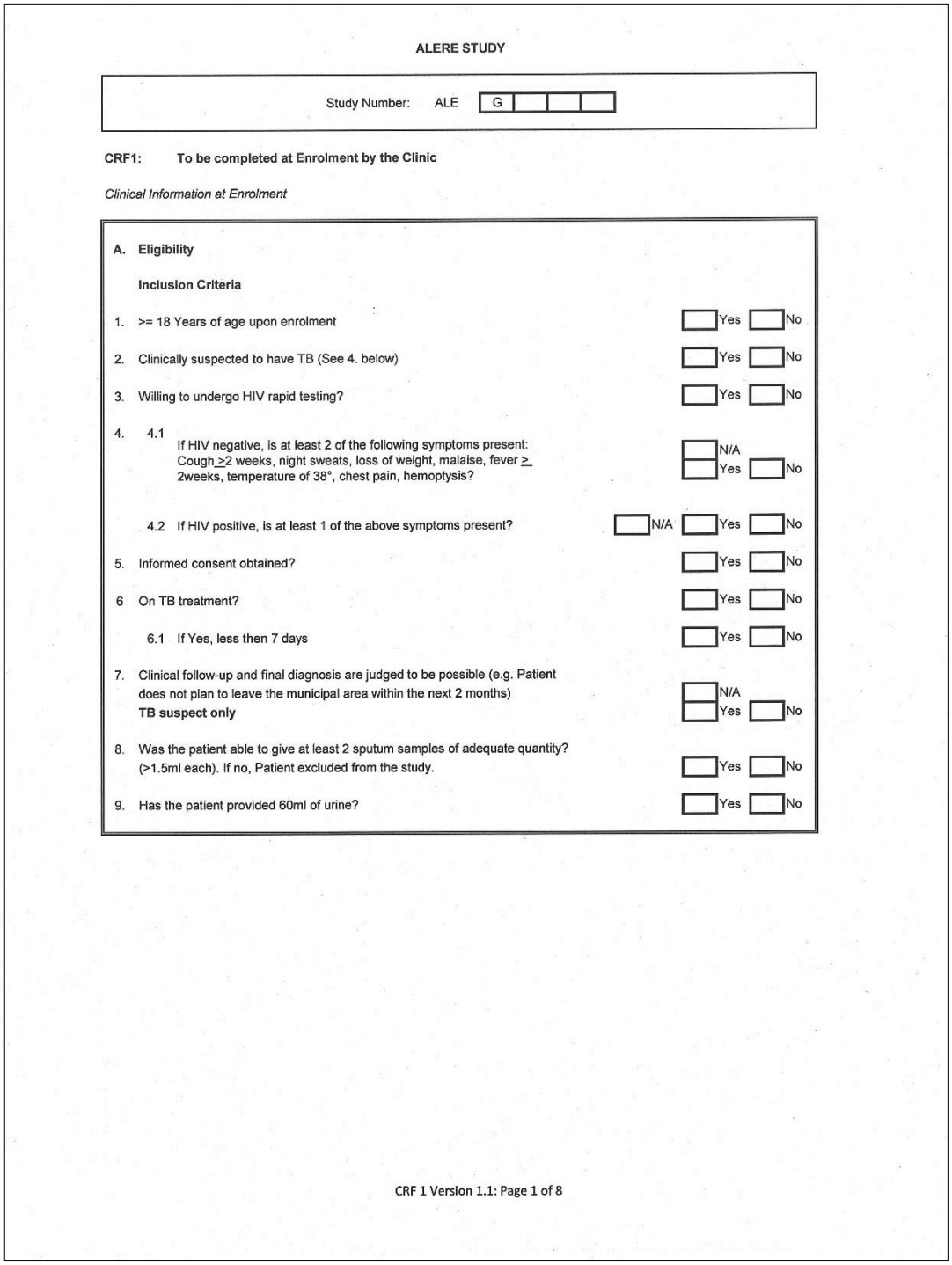
8 Annex

Appendix-1 Case Reporting Form



ALERE STUDY

Study Number: ALE G

CRF1: To be completed at Enrolment by the Clinic

Clinical Information at Enrolment

A. Eligibility

Inclusion Criteria

1. >= 18 Years of age upon enrolment — Yes / No
2. Clinically suspected to have TB (See 4. below) — Yes / No
3. Willing to undergo HIV rapid testing? — Yes / No
4. 4.1 If HIV negative, is at least 2 of the following symptoms present: Cough >2 weeks, night sweats, loss of weight, malaise, fever > 2weeks, temperature of 38°, chest pain, hemoptysis? — N/A / Yes / No

   4.2 If HIV positive, is at least 1 of the above symptoms present? — N/A / Yes / No
5. Informed consent obtained? — Yes / No
6. On TB treatment? — Yes / No

   6.1 If Yes, less then 7 days — Yes / No
7. Clinical follow-up and final diagnosis are judged to be possible (e.g. Patient does not plan to leave the municipal area within the next 2 months) **TB suspect only** — N/A / Yes / No
8. Was the patient able to give at least 2 sputum samples of adequate quantity? (>1.5ml each). If no, Patient excluded from the study. — Yes / No
9. Has the patient provided 60ml of urine? — Yes / No

CRF 1 Version 1.1: Page 1 of 8

ALERE STUDY

Study Number: ALE [G] [ ] [ ] [ ]

B. Patient Information

1. Enrolment Date: DD ____ MM ____ YYYY ____
2. Patient Name: Name: ..... Surname: .....
3. Patient Folder Number: .....
4. Address: Line 1 .....
   Line 2 .....
5. Telephone: Cell: .....
   Alt.Cell: .....
6. Race: [ ]
7. Gender: Male [ ] Female [ ]
8. Date of Birth: DD ____ MM ____ YYYY ____
9. Body weight (kg) .....
10. Height (cm) .....

C. Clinical History

1. History of TB [ ] Not Known [ ] No [ ] Yes →

   1.1 Most Recent TB Treatment (Tx):

   [ ] Stopped without completion -> 1.1.2 Specify approx year ..... [ ] Unknown

   [ ] Completed -> 1.1.3 Specify approx year: ..... [ ] Unknown

   1.2 Specify TB Tx: [ ] 1st Line Tx [ ] 2nd Line Tx (DR-TB) [ ] Unknown

   1.3 Patient considered cured by this TB Tx?: [ ] Yes [ ] No [ ] Unknown

ALERE STUDY

Study Number: ALE G

D. Concomitant Diseases

1. Allergy ☐ Yes ☐ No

   If Yes, specify: .....

2. Diabetes ☐ Yes ☐ No
3. Epilepsy ☐ Yes ☐ No
4. Chronic heart disease ☐ Yes ☐ No
5. Liver disease ☐ Yes ☐ No
6. Renal disease ☐ Yes ☐ No
7. Lung disease ☐ Yes ☐ No
8. Other ☐ Yes ☐ No

   If Yes, specify .....

9. **Medications:** .....
   .....
   .....
   .....

ALERE STUDY

Study Number: ALE G

E. TB Symptoms

1. Current cough ☐ Yes ☐ No
2. Cough ≥ 2 weeks ☐ Yes ☐ No

   (Duration in days .....)
3. Coughing phlegm ☐ Yes ☐ No

3.1 If yes → colour: ☐ Yellow ☐ Green ☐ White ☐ Clear ☐ Blood stained

3.2 If yes → consistency: ☐ Very viscous ☐ Viscous ☐ Non viscous (Watery)

4. Coughing up blood ☐ Yes ☐ No
5. Weight loss (self reported) ☐ Yes ☐ No

   If yes, how much? ☐☐.☐ Kg ☐ Unknown
6. Loss of appetite ☐ Yes ☐ No
7. Drenching night sweats ☐ Yes ☐ No
8. Fatigue ☐ Yes ☐ No
9. Shortness of breath ☐ Yes ☐ No
10. Fever (Current) ☐ Yes ☐ No ☐ Temp
11. Unable to walk unaided ☐ Yes ☐ No
12. Chest pain ☐ Yes ☐ No
13. Other/Comment: .....

.....

.....

F. Substance Abuse

1. Current cigarette smoker ☐ Yes ☐ No
8. Abuse e.g Alcohol/Dagga/Tik ☐ Yes ☐ No

   If Yes, specify: .....

ALERE STUDY

Study Number: ALE G

G. HIV Test Results

1. Known HIV ☐ Negative ☐ Positive

Result date: DD MM YYYY

2. Rapid test: ☐ Not done ☐ Negative ☐ Positive

3. CD4 cell count (Cells/mm^3) (Within last 6 months) Result ..... ☐ Not done

Result date: DD MM YYYY

4. ARV's ☐ Yes ☐ No

Medication name: _____

H. *Sputum 1 at Enrolment*

1 Sputum Information

1.1 Date of collection: DD MM YYYY ☐ Not received

1.2 Time of collection: ☐ ☐ Spot

1.3 Viscosity: ☐ Very viscous ☐ Viscous ☐ Not viscous/watery

1.4 Blood: ☐ Very blood stained ☐ Blood stained ☐ Not blood stained

2 FM/ZN Microscopy

2.1 Direct ZN/FM: ☐ Not done ☐ Neg ☐ Pos ->

Date specimen obtained GP DD MM YYYY ☐ N/A

Grading: ☐ +1 ☐ +2 ☐ +3 ☐ +4

2.2 GeneXpert: ☐ Not done ☐ Neg ☐ Pos ☐ Rif res ☐ Rif susc

Date specimen obtained GP DD MM YYYY ☐ N/A

ALERE STUDY

Study Number: ALE G

2.3 CT Value

Probe	CT Value	Endpoint
D		
C		
E		
B		
SPC		
A		

2.4 MGIT Culture: ☐ Not done ☐ Neg ☐ Pos ☐ Contaminated

Date of MGIT: DD ___ MM ___ YYYY ___ # Days: ☐

2.4.1 Contaminated culture repeated? ☐ N/A ☐ No ☐ Yes

Date of re-inoculation DD ___ MM ___ YYYY ___

Result: ☐ Pos ☐ Neg ☐ Contaminated ☐ Lost

Date of MGIT: DD ___ MM ___ YYYY ___ # Days: ☐

2.5 Speciation Result: ☐ Not done ☐ MTB complex ☐ Non MTB complex ☐ Species

2.6 First line DST result: (HAIN MDRTB plus) ☐ Rif res ☐ Rif susc ☐ INH res ☐ INH susc

I. Initial TB Treatment Decision

1. Patient started on TB treatment: ☐ No ☐ Yes ->

1.1 Date started: DD ___ MM ___ YYYY ___

1.2 TB Treatment decision based on: ☐ Clinical signs/CXR ☐ Smear ☐ Xpert ☐ Culture

ALERE STUDY

Study Number: ALE G

J. Management Plan according to Diagnostic Algorithm

K. *Sputum 2 at Enrolment*

1 Sputum Information

1.1 Date of collection: DD / MM / YYYY ☐ Not received

1.2 Time of collection: ☐ Collect 30 min after Sputum 1

1.3 Viscosity: ☐ Very viscous ☐ Viscous ☐ Not viscous/watery

1.4 Blood: ☐ Very blood stained ☐ Blood stained ☐ Not blood stained

1.5 Sent to lab? ☐ Yes (NAAT)

ALERE STUDY

Study Number: ALE G ☐☐☐

L. *Sputum 3 at Enrolment*

1 Sputum Information

1.1 Date of collection: DD ☐☐ MM ☐☐ YYYY ☐☐☐☐ ☐ Not received

1.2 Time of collection: ☐ Collect on day of results

1.3 Viscosity: ☐ Very viscous ☐ Viscous ☐ Not viscous/watery

1.4 Blood: ☐ Very blood stained ☐ Blood stained ☐ Not blood stained

1.5 Sent to lab? ☐ Yes (Biobank) ☐ No

M. Urine at Enrolment

1.1 Date of collection: DD ☐☐ MM ☐☐ YYYY ☐☐☐☐ Received ☐ Yes ☐ No

N. Blood Samples

1.1 Received? ☐ Yes ☐ No

1.2 Date of collection: DD ☐☐ MM ☐☐ YYYY ☐☐☐☐

O. Compensation paid ☐ Yes

Patient signature: .....

Completed by: ..... Date: DD ☐☐ MM ☐☐ YYYY ☐☐☐☐

Re-checked: ☐ Yes ☐ No Date: ☐☐ ☐☐ ☐☐☐☐

Checked by a 3rd party: ..... Date: ☐☐ ☐☐ ☐☐☐☐

1st Data entry by: ..... Date: ☐☐ ☐☐ ☐☐☐☐

2nd Data entry by: ..... Date: ☐☐ ☐☐ ☐☐☐☐

Appendix-2 Sequence information for the IS6110 target region present in the H37Rv Genome for possible primer binding and detection

Sequence (36bp) 5’-GACGCGATCGAGCAAGCCATCTGGACCCGCCAACAAGA-3’		
Sense/Antisense	From	To
Sense	3891708	3891745
Antisense	3795446	3795483
Sense	3711311	3711348
Sense	3553642	3553679
Sense	3552159	3552196
Antisense	3120912	3120949
Sense	2973038	2973075
Antisense	2785003	2785040
Sense	2636506	2636543
Sense	2550943	2550980
Antisense	2430505	2430542
Sense	2366343	2366380
Sense	1997030	1997067
Antisense	1988091	1988128
Antisense	1542340	1542377
Sense	889950	889987

List of Publication(s)

Following Paper is submitted to the Journal of molecular diagnostics on 27.04.2017.

Patel K, Geldmacher C, Wesolowski M, Rivera-Milla E, Nagel M, Dheda K, Hoelscher M, Labugger I et al. Evaluation of a Urine-Based Rapid Molecular Diagnostic Test with Potential to be Used at Point of Care for Pulmonary Tuberculosis: Cape Town Cohort.

Statement on Pre-release and Contribution

The PhD thesis

“Development and Evaluation of Urine based Rapid Molecular Diagnostic Test for Pulmonary Tuberculosis with Potential for Point of Care: Cape Town Cohort.”

has not been submitted for publication or previously published.

I, Krutarth Patel have made substantial contributions to the study, starting from design of the work, experiments, data collection, analysis and its interpretation.

I have drafted the work and revised it critically for important intellectual properties, and I agree to be accountable for all aspects of the work.

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Affidavit

Krutarth Patel

Name

Street

Zip code, town

Country

I hereby declare, that the submitted thesis entitled

Development and Evaluation of Urine based Rapid Molecular Diagnostic Test for Pulmonary Tuberculosis with Potential for Point of Care: Cape Town Cohort

is the result of my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

The submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

I further declare that the electronic version of the submitted thesis is congruent with the printed version both in content and format.

Place, Date

Signature of PhD Candidate