The evaluation of whole blood cytokine assay for diagnosis of *M.tuberculosis* infection in South African children with household tuberculosis contact

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ABSTRACT

Background: There are critical unmet needs for improved strategies in the detection and diagnosis of *M.tuberculosis* infection in children, and for prevention of tuberculosis disease in children. Bacillus Calmette-Guérin (BCG) vaccination has limited the utility of tuberculin skin testing (TST) in areas with high vaccine coverage.

Objectives: The aim of this study was to estimate the prevalence of *M.tuberculosis* infection in children with household tuberculosis contacts, using QFT-GIT testing in comparison with TST.

Methods: This study was a cross-sectional design to assess the performance of a new T-cell based blood test, namely QuantiFERON-TB Gold In Tube (QFT-GIT), for diagnosis of tuberculosis infection in the children (n=182) of adults (n=124) with pulmonary tuberculosis, additionally to determine the prevalence of *M.tuberculosis* infection in children with household tuberculosis contacts, using QFT-GIT testing in comparison with TST. The study was carried out at Chris Hani Hospital. For children involved in the study, tuberculosis exposure information was obtained, together with TST, QFT-GIT, and HIV testing.

Data obtained from both experiments was statistically analysed using SPSS version 24 to determine whether there was a significant agreement between QFT-GIT and TST on the detection of *M.tuberculosis* prevalence in children with house hold contacts with confirmed *M.tuberculosis* infection.

Results: This study examined the sensitivity and specificity of the QFT-GIT tests compared with the standard TST for diagnosing latent tuberculosis disease in paediatric contacts. Because of the lack of a latent tuberculosis “gold standard”, the specificity and sensitivity of QFT-GIT was calculated with a two-by-two table method. The specificity of the QFT-GIT was 84% and the sensitivity was 85%. There was a good correlation between QFT-GIT and TST (Cohen’s kappa of 0.705). Seventeen percent (17%) of the 182 children tested by QFT-GIT yielded indeterminate results. Age was associated with indeterminate QFT-GIT results in paediatric tuberculosis

contacts. Point prevalence for QFT-GIT was recorded as 31% at baseline and 39.5% after six months indicating variability between QFT-GIT results at baseline and after six months.

Conclusion: It was concluded that the prevalence of tuberculosis infection was common among South African children who live with an adult with active tuberculosis. The agreement between QFT-GIT assay and TST for the diagnosis of latent tuberculosis in children was high. Although TST and QFT-GIT assays appeared comparable, QFT-GIT showed higher positivity rate amongst those contacts with reported household tuberculosis exposure compared to TST. The QFT-GIT assay was a better indicator of the risk of *M.tuberculosis* infection than TST in a BCG-vaccinated population.

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GLOSSARY OF TERMS

Definitions

Chemotactic	movement of cell or organisms either toward or away from chemical stimulus in chemical gradient concentration
Cyclopropanation	cypropropane is introduced into the reaction by the process
Determinate	determined or conclusive
Extra pulmonary tuberculosis	tuberculosis infection in other parts of the body which includes central nervous system in meningitis and bones and joints
Indeterminate	Not defined, established or undetermined
Immunomodulatory	any agent that diminishes immune responses
Latent tuberculosis	*M.tuberculosis* bacilli are contained in oxygen-deprived small granulomas, the bacilli lies dormant with no symptoms
Mycobacterium tuberculosis	Is a small aerobic non-motile bacillus found in nature only growing within the cells of a host. *M.tuberculosis* has a high lipid content which accounts for many of its unique characteristics and divides every 6 to 20 hours. It is determined by

	microscope using auramine-rhodamine stain and fluorescent microscope after being stained with acid solution
Pulmonary tuberculosis	Tuberculosis in the lungs, symptoms includes coughing blood and chest pain. Symptoms are chronic cough with blood-tinged sputum, fever, night sweats, and weight loss
Tuberculosis	Is a deadly infectious disease cause by various strains of mycobacteria including *M.tuberculosis* in humans. It usually affects the lungs but can affect other parts of the body. It is spread through the air when an individual with active disease cough, sneezes or spit. Main symptoms are chronic cough with blood-tinged sputum, fever, night sweats, and weight loss
Autophagy	the process that results after the components of the cytoplasm, these includes organelles and intracellular pathogens in a phagosome are sequestered and delivered to lysosome for degradation
Granuloma	Inflammatory mononuclear cell infiltrate capable of providing a survival niche from which the bacteria may disseminate, but also capable of limiting growth of *M.tuberculosis*

Abbreviations

AFB	Acid Fast Bacilli
Ara*f*	Furanoid arabinose
AG	Arabinogalactan
ARV	Antiretroviral
BCG	Bacillus Calmette-Guerin
CDC	Centre for Disease Control and prevention
CR	Complementary receptors
CD+4	L3T4
CD8+	Ly2
CFP-10	Culture Filtrate protein 10
CH	Chris Hani
CmaA2	Coronamic acid synthetase
CR3	Complement receptor 3
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DC-SIGN	Dendritic-cell specific intercellular adhesion molecule 3-grabbing non-intergrin receptor
DOTS	Directly Observed Treatment Short Course
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ELISPOT	Enzyme-linked immunospot
ELISA	Enzyme linked immunoassay
EPTB	Extra pulmonary tuberculosis
ESAT-6	Early Secretory antigenic Target 6
Esx-1	ESAT-6 systems
Gal*f*	Galactose
GTPase	Rab
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HPCSA	Health professional council of South Africa
IFN-$_{\gamma}$	Gamma Interferon

IGRA	Interferon Gamma Release Assay
LJ	Lowenstein Jensen
INH	Isoniazid
IL	Interleukin
IRC	Independent ethics committee
KZN	Kwazulu Natal
Lam	Lipoarabinomannan
LAMP	Loop-mediated isothermal amplification
LED	Light-emitting diode
NHLS	National Health Laboratory Service
NK	Natural killer
NOD	Nucleotide oligomerization domain
NOS2	Nitric oxide synthase
OD	Optical density
MDP	Muramyl dipeptide
MDR-TB	Multi Drug Resistant Tuberculosis
MODS	Microscopic observation of drug susceptibility
PAMPs	Pathogen associated molecular patters
PAS	Para-aminosalicylic
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PG	Peptidoglycan
PcaA	Cyclopropane synthase
PHA	Phytohemagglutinin
PIM	Phosphatidyl linositolmannoside
PI3P	Phosphatidylinositol 3-phosphate
PPD	Purified Protein Derivative
PRRs	Pattern recognition receptors
QFT-GIT	QuantiFERON-TB Gold In Tube
RIF	Rifampin
RNA	Ribonucleic acid
RMPRU	Respiratory and Meningeal Pathogens Research Unit

RD	Regions of difference
STR	Streptomycin
TB	Tuberculosis
TCR	T cell receptors
TDM	Trehalose 6, 6-dimycolate
TST	Tuberculin Skin Tests
Th1	T helper 1
Th2	T helper 2
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
VUT	Vaal University of Technology
WHO	World Health Organization
XDR TB	Extensively Drug Resistant Tuberculosis
ZN	Ziehl-Neelsen

Symbols

°C	Degrees Celsius
µl	Microliter
%	Percent
<	Less than
>	Greater than

Alphabetical characters

L	litre
m	meter
mL	millilitre
Nm	nanometre

CHAPTER 1
PROBLEM AND ITS SETTING

1.1 INTRODUCTION

Tuberculosis infection continues to be a major cause of death throughout the world. The disease in man is caused by infection with *M.tuberculosis* and remains a serious problem for developing countries globally (Connell et al., 2011; World Health Organization (WHO), 2018). It is projected that 75% of the cases occur in the productive age group between 15 and 54, decreasing productivity and employability because of illness. In addition to the devastating economic costs, tuberculosis imposes indirect negative consequences; children leave school because of their parent's illness (World Health Organization (WHO), 2012; Bukachi et al., 2017). The main reasons observed for the increasing burden of tuberculosis globally are poverty, inadequate case detection, diagnosis, cure, collapse of the health infrastructure in countries experiencing severe economic crisis or civil unrest, and the impact of Human Immunodeficiency Virus (HIV) pandemic (WHO, 2012; Raviglione et al., 2016).

1.1 .1 Global status of tuberculosis

Tuberculosis still remains the leading cause of death from infectious disease (Onubogu et al., 2010; Mohajan, 2015). It is estimated that there were 8.7 million tuberculosis incident cases and 1.4 million deaths in 2011 globally (WHO, 2012; WHO, 2016). Tuberculosis has been a major problem for most health departments in developing countries (Wilkinson et al., 2012; Churchyard et al., 2014). The survey done globally in 2010 showed tuberculosis transmission cases in industrialised countries differ as compared to the cases in the developing world (Raviglione et al., 2012; Sulis et al., 2014). It has been hypothesized that the difference in wealth between industrialized countries and developing countries results in people migrating from poor countries to the industrialized countries and thereby transmitting the bacteria (Sia & Wieland, 2011; Pescarini et al., 2017).

Tuberculosis and HIV co-infection has led to new challenges in controlling the disease especially in developing countries such as South Africa, India and China (Hawn et al., 2014). It is estimated that out of 9.3 million new cases of tuberculosis that occurred in 2007 globally, 1.3 million cases were in HIV positive people (Khan et al., 2010; Onyango, 2011; WHO, 2017).

WHO has introduced tuberculosis control programs such as Directly Observed Treatment Short Course (DOTS) to combat tuberculosis; the program is in response to a number of challenges that have not been addressed, such as the emergence of multi drug resistant TB, the development of co-infection by TB and HIV, poor health systems, insufficient engagement by the government with the communities (Lonnroth et al., 2009; Abubakar, 2013; Wynne et al., 2014). The DOTS programs are prioritising the cure of people with active tuberculosis to prevent transmission and to trace individuals who have been exposed to active tuberculosis and treating those individuals for latent tuberculosis (Lonnroth et al., 2009; Lancella, 2015). The program combines five elements namely:

- Microscopy services
- Political commitment
- Drug supply
- Monitoring systems
- Direct observation of treatment (WHO, 2012; Lei et al., 2015).

However, despite the introduction of DOTS by WHO, the lack of resources in some developing countries forces the governments to continue with tuberculosis control policies which focus mainly on identification and treatment of active tuberculosis at the expense of latent tuberculosis which is neither diagnosed nor treated (Lawn & Zumla, 2011; Raviglione et al., 2016).

Indicated in Figure 1 is the global tuberculosis control report in 2010 by WHO, for surveillance, planning and financing. Most of the reported cases occurred in Asia with 59% followed by Africa with 26%; with minor cases recorded in the Eastern Mediterranean Region with 7%, Europe with 5 % and America with 3% (WHO, 2011; WHO, 2016).

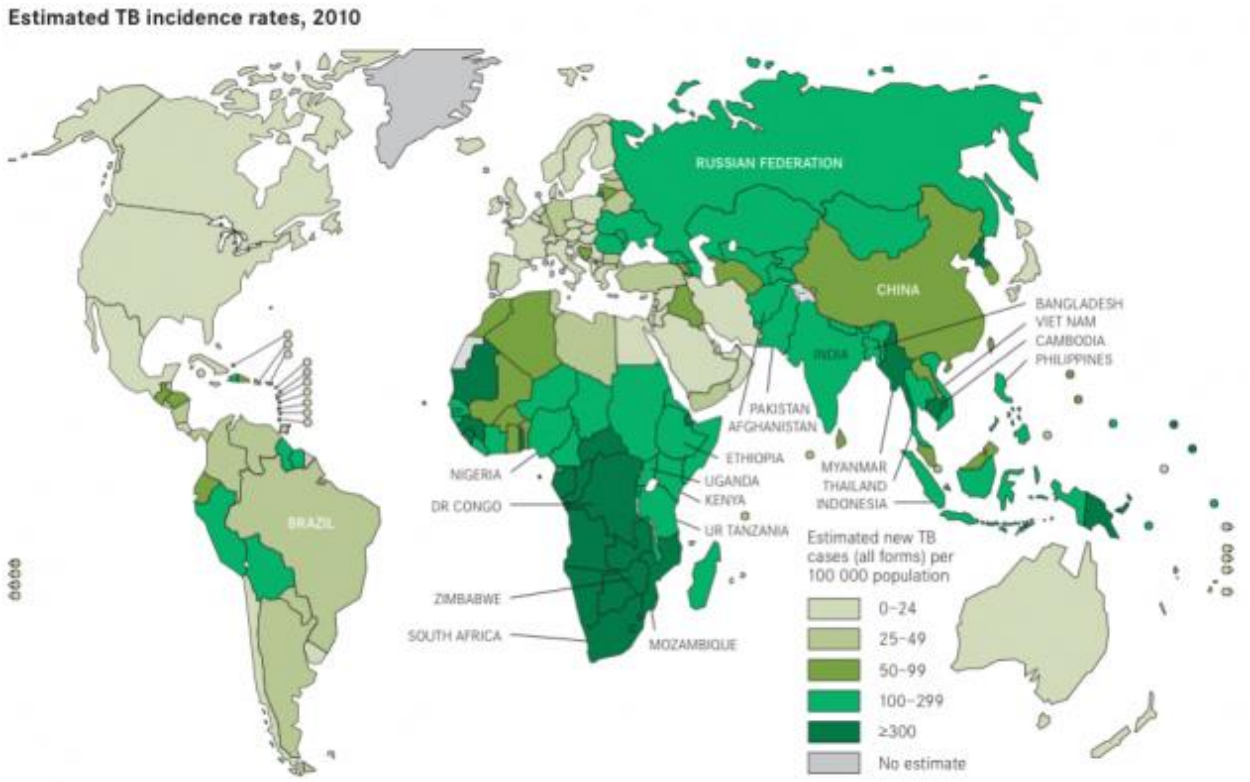


Figure 1: WHO 2010 tuberculosis surveillance (WHO, 2011; WHO, 2016)

Globally the major setback experienced in the control of tuberculosis is the lack of an effective vaccine; the current vaccine is from *Mycobacterium bovis* Bacillus Calmette-Guerin, and does not prevent pulmonary tuberculosis, however it offers some protection against severe forms of tuberculosis such as miliary tuberculosis in children (Parsons et al., 2011; Rhodes et al., 2016; Usman et al., 2017).

Parsons et al., (2011; Sani et al., 2015) have indicated the following factors to be linked to the tuberculosis epidemic in resource poor countries:

- Poverty which is closely linked to malnutrition
- Lack of access to free affordable health care services
- Crowded living conditions
- Dependency of individuals on traditional healers

In addition, the failure to complete the prescribed course of TB treatment in some developing countries has led to strains resistant to anti-tuberculosis drugs (Keshavjee & Farmer, 2012; Sotgiu et al., 2015). In recent years multidrug resistant

tuberculosis (MDR TB) and extensively drug resistant tuberculosis (XDR TB) have emerged as a concern in many developing countries around the world, with poor management of tuberculosis in the treatment being the primary reason (Johnson et al.,2009; Millet, 2013; Seung et al., 2015). The antibiotics used in tuberculosis treatment consist of a combination of isonaizid and rifampin to lessen the chance of the organism surviving and to prevent drug resistant organisms developing (Keshavjee & Farmer, 2012; Kerantzas & Jacobs, 2017). Drug resistant organisms represent a problem in treatment of tuberculosis. However, despite the appearance of MDR TB and XDR TB resistant organisms, the levels of the recorded cases of multi-drug-resistant tuberculosis are low globally, including in developing countries. A common explanation put forward is because of the introduction of programs such as DOTS (Keshavjee & Farmer, 2012; WHO, 2014).

1.1.2 Tuberculosis in South Africa

South Africa is reported to have the fifth highest prevalence of TB cases per capita in the world, with high rates in both children and adults (Wood et al., 2010a; WHO, 2015). In 2007 South Africa represented only 0.7% of the whole world population, however it accounted for 17% of HIV infection reported globally in that year (Karim et al., 2009; Kharsany & Karim, 2015). This high prevalenceof HIV infection means the risk is significantly higher of developing active tuberculosis diseases in those individuals living with HIV than those without HIV (Bassett et al., 2010; Petruccioli., et al 2016). The scourge of HIV infection furthermore has resulted in an increase in the progression of latent tuberculosis into active tuberculosis disease, therefore also an increase in transmission in unprecedented ways (Middelkoop et al., 2009; Blumberg & Ernst, 2016). The increased active tuberculosis in adults because of HIV infection has resulted in a high risk of tuberculosis transmission from adults to children given that transmission from child to child is uncommon (Middelkoop et al., 2009; Siberry, 2014).

South Africa is a country that has a history of violent suppression of native people, poor health standards for black people, and laws which were unjust to black people (Coovadia et al., 2009; Benatar, 2013; Lephakga, 2017). The laws of that era

supported the reservation of land for white people and racial segregation of urban areas, in turn this created a system which failed to provide adequate housing for migrant workers (Coovadia et al., 2009; Benatar, 2013; Capuzzo, 2015). Single sex overcrowded hostels which were poorly ventilated were built. Black South African people were forced to live in these areas which resulted in optimal conditions for tuberculosis transmission and HIV (Coovadia et al., 2009; Benatar, 2013; Li, 2014). In 2011 it was estimated that 8.7 million new cases of tuberculosis were recorded, and 350 000 deaths occurred in people co-infected with tuberculosis/HIV infection in South Africa (Beukes et al., 2010; Marais et al., 2013; Mabunda et al., 2014). Cape Town is in one of the South African provinces amongst the worst affected by tuberculosis; the city had 3.2 million people and 27 000 cases of tuberculosis were recorded in 2006 (Wood et al., 2010a; Blaser et al., 2016). These high tuberculosis cases were mostly because of the effect of the coexistence of HIV infection and tuberculosis (Wood et al., 2010a; Blaser et al, 2016). The distribution of tuberculosis cases is unequal in Cape Town, high incidence cases of tuberculosis are reported in crowded and socially deprived African townships which consist largely of informal shack dwellers (Wood et al., 2010a; Blaser et al., 2016). Kwazulu Natal is another province in South Africa plagued by tuberculosis because of the high prevalence of HIV (Cohen et al., 2011; Assaram et al., 2018). The high prevalence tuberculosis in this province is estimated to be 1094 cases per 10 000 persons per year (Cohen et al., 2011; Naidoo et al., 2014).

Programs like DOTS have been implemented in South Africa since 1996 to curb the transmission of tuberculosis (Mukinda et al., 2012; Arshad et al., 2014). The programme has shown some success however it has been slow to reach the goals set by the WHO of 70% detection rate, as of 2009 the detection rate was less than 60, while 85% was set as a success target for treatment, the success target for treatment stood at 76% in 2008 (Mukinda et al., 2012; Glaziou et al., 2015).

Tuberculosis diagnosis in South African health departments is still based on conventional methods which can take up to four weeks for definitive results of tuberculosis to be available (Beukes et al., 2010; Alli et al., 2011; Cudahy & Shenoi, 2016). This creates challenges for clinicians because of the delay in starting

treatment leads to fatality in some cases (Beukes et al., 2010; Alli et al., 2011; Naidoo et al., 2017).

1.1.3 Tuberculosis in Soweto, Gauteng

Soweto is classified as a lower middle-class urban area with a population of 3.5 million reported in 2010 and is part of the city of Johannesburg in Gauteng, South Africa. Soweto is bordering the city's mining areas and accommodates mainly black people migrating from rural areas (Dramowski et al., 2011). Between 2003 and 2008 the prevalence of HIV infection in South Africa was estimated to be approximately 10-12% (Statistics South Africa, 2017). Soweto has an approximately 3% of under-18 year's olds who are infected by HIV as reviewed by Nunes et al., (2011; (Statistics South Africa, 2017). The prevalence of tuberculosis cases reported in adults from Soweto was 262 per 100 000 in 2004 (Karstaedt & Bolhaar, 2014). The prevalence of tuberculosis in children living in Soweto is unknown.

1.1.4 Tuberculosis in children

Out of 8.6 million new cases worldwide of tuberculosis infection reported in 2012 worldwide, an estimated 530 000 occurred in children. Of these, there were 74 000 deaths in HIV negative children (WHO, 2013; WHO, 2014). Childhood tuberculosis remains one of the major diseases afflicting children throughout the world; however, it is still a neglected aspect of the disease in most developing countries (Nicol et al., 2009; Esposito et al., 2013; Marais & Schaaf, 2014). The negligence arises from the observation that children do not often transmit the disease and attention to them contributes little to fighting the spread of tuberculosis (Nicol et al., 2009; Seddon & Shingadia et al., 2014). Most programmes focus on diagnosis and cure of adult smear positive patients, because that's where most cases of tuberculosis transmission originate (Nicol & Zar, 2011; Marais, 2017). However, the greater population of children when exposed to active tuberculosis disease develop neither signs nor symptoms despite establishing latent infection nor harbouring the bacterium. This presents a future risk in adulthood (Swaminathan & Rekha, 2010; Lange et al., 2014). Most cases of adult active tuberculosis results from reactivation

of latent tuberculosis acquired during childhood (Swaminathan & Rekha, 2010; Lange et al., 2014).

The exact number of annual cases of childhood tuberculosis is unknown. It was reported that ofthe 8.6 million tuberculosis cases that occurred in 2012, 1 million cases were estimated to have occurred in children less than 15 years of age (Scriba et al., 2011; Seddon & Shingadia, 2014).

The risks of children developing tuberculosis disease are greatest after exposure to sputum-positive adult (Achkar & Ziegenbalg, 2012; Luzzati et al., 2017). The children are at high risk because the immune system in young children is less developed (Achkar & Ziegenbalg, 2012; Simon et al., 2014). TB disease in children normally takes about 12 months to progress from asymptomatic carriage to active primary disease (Paul et al., 2013; Lancella et al., 2015).

Exposure to adults with smear-positive pulmonary tuberculosis has increased the risk of latent tuberculosis infection inchildren living in 22 countries recorded as high burden countries which includes South Africa (Achkar & Ziegenbalg, 2012; Churchyard et al., 2014). The risks are estimated to be around 30-40% in children less than 12 years. In adolescents the risks are 15% with 24% in children between 1-5 years (Piccini et al., 2014). Infants less than a year are more at risk at 43%, however the risk is assessed using TST test which suffers from several limitations including cross reactivity with Bacillus Calmette-Guerin (BCG) vaccine and false positives from previous and cleared natural exposure (Paulet al., 2013; Pai et al., 2014; Andrews et al., 2015). Latent tuberculosis has a high risk in infants of progression to active tuberculosis. The protective T-cell-mediated immune response is less effective during the first months of life therefore *M.tuberculosis* growth is not contained (Mack et al., 2009; Sakamoto et al., 2012; Sanduzzi et al., 2016).

1.1.5 HIV epidemic association with Tuberculosis

The emergence of Human immunodeficiency virus (HIV) has worsened the tuberculosis problem globally (Wilkinson et al., 2012; Swaminathan, 2016).

Tuberculosis is now identified as the leading cause of mortality in people living with HIV infection within developing countries (Pawlowski et al., 2012; Bruchfeld et al., 2015).

In 2007 it was reported that 1.37 million (15%) of 9.3 million new cases of tuberculosis were in tuberculosis/HIV co-infected people with sub-Saharan Africa accounting for 74% of the burden of tuberculosis/HIV co infections, followed by South Asia with 11% (Naidoo et al., 2011).

In individuals who are co-infected with *M.tuberculosis* and HIV, the pathogens accelerate the rate of immunological function's deterioration. Therefore, if not treated, it results in increased and premature death in the individuals harbouring the pathogens (Pawlowski et al., 2012; Shankar et al., 2014). HIV infection increases the risks of latent tuberculosis progression to active tuberculosis and the creation of new tuberculosis disease or re-infection (Pawlowski et al., 2012; Kiazyk & Ball, 2017). Pulmonary tuberculosis accounts for about 75% cases in all patients infected with HIV, as compared to other opportunistic pathogens (Swaminathan & Narendran, 2008; Rabie & Goussard, 2016).

Developing countries with a high prevalenceof HIV have seen an increase in pulmonary tuberculosis because of the weakened immune systems in advanced stages of HIV disease, delayed diagnosis and treatment of tuberculosis (Kingkaew et al., 2009; Padmapriyadarsini et al., 2011; Seid & Metaferia, 2018).
Tuberculosis is usually restricted to the lungs in individuals with intact cellular immune systems however, in persons with immune suppression such as in HIV infection, *M.tuberculosis* bacilli frequently disseminate beyond the lungs and cause disease in other organs as reviewed by O'gara et al., (2013; Lapausa et al., 2015). The delay in diagnosing tuberculosis in HIV patients has been linked with 45% to 85% deaths that occur in this population when appropriate treatment is initiated not later than 3 weeks after the presentation of active tuberculosis (Rao et al., 2009; Nogueira et al., 2018). The delay in treatment is caused by various illnesses imitating tuberculosis (Ren et al., 2009; Alavi et al., 2015).

In resource poor countries with a high prevalence of tuberculosis, children with HIV carry an increasing proportion of the overall burden of TB (Ren et al., 2009; Martinson et al., 2011; Seddon & Shingadia, 2014). The burden arises for the reason that tuberculosis and HIV share many clinical features. It is often difficult to diagnose tuberculosis in HIV-infected children (Venturini et al., 2014). Many of HIV infected children could be saved if improvements in diagnosis and treatment of tuberculosis were available (Venturini et al., 2014).

The HIV epidemic and associated co-infection with tuberculosis has caused a heavy burden on the already fragile public health system in South Africa (Karim et al., 2009; Hartel et al., 2018). Tuberculosis was an epidemic in South Africa; most people were harbouring the bacterium in latent state. The emergence and high rate of HIV infection has resulted in people harbouring latent tuberculosis progressing to active tuberculosis leading South Africa to have the most serious tuberculosis epidemics in the world (Karim et al., 2009; Gopalan et al., 2016).

In South Africa, the prevalenceof HIV infection is estimated at 40% of the global burden of tuberculosis (Dellar et al., 2015). This has been associated with increases in the rate of smear negative tuberculosis disease among HIV-infected individuals and the prevalence of undiagnosed tuberculosis among this population in 2008 (Lawn et al., 2009; Dellar et al., 2015). HIV infection changes the presentation of smear-negative pulmonary tuberculosis from a slowly progressive disease with low bacterial load and reasonable prognosis, to one with reduced pulmonary cavity formation and sputum bacillary load (Marais et al., 2011; Campos et al., 2016). Positive sputum dominates when the CD4 cell counts are high in HIV infection when immune function is relatively intact; the opposite is observed in advanced HIV disease stages which are dominated with smear-negative sputum (Munawar & Singh, 2012; Campos et al., 2016).

The incidence of tuberculosis disease in infants is 1596 per 100 000 among HIV-infected infants, a rate 24.2 times higher than infants not infected with HIV (Hesseling et al., 2009a; Seddon & Shangadia, 2014).

The implementation of highly active antiretroviral therapy (HAART) has shown a dramatic reduction in the prevalence of adult tuberculosis compared to those not receiving it (Marais et al., 2011; Pathmanathan et al., 2017). However, the prevalence of active tuberculosis continues to be higher in HIV infected adults receiving HAART than in HIV negative patients (Marais et al., 2011; Gunda et al., 2018). Drugs that inhibit the replication of HIV such as Antiretroviral (ARV) have been used to prevent the immune system form deterioration. The introduction of ARV has resulted in improved quality of life forpeople infected with HIV and decreased the risks of active tuberculosis development (Marais et al., 2011; Meintjes et al., 2017).

1.1.6 Challenges with Tuberculosis diagnosis

Tuberculosis diagnosis is still delayed by some short comings with acid fast bacilli (AFB) smears requiring extensive labour. Culturing mycobacteria is very time consuming (Parida & Kaufmann, 2010; Ryu, 2015). To have a conclusive culture result it takes an average of 3 to 6 weeks for the results to be available, which limits the detection and diagnosis of active disease effectively (Kocagoz et al., 2012; Ryu, 2015). TST has been the sole test used to detect latent tuberculosis; the test uses a mixture of antigens obtained as protein precipitated from *M.tuberculosis* supernatants in liquid form (Mark et al., 2009; Mehta et al., 2015).

The recently developed QuantiFERON-TB in Tube relies on the detection of a host cell mediated immune response to *M.tuberculosis*-specific antigens. The target antigens: early secreted antigenic target 6-kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10) are absent from all BCG vaccine strains and most non-tuberculosis mycobacteriaand are thus specific for circulating MTB (Dosanjh et al., 2011; Arlehamn et al., 2014). The test is based on measuring released gamma interferon (IFN-γ) which are inflammatory cytokines produced by the cell's immune system in response to foreign antigens (Jahdali et al., 2013; Bhat et al., 2017). Released gamma interferon from T cells previously sensitised with mycobacterial antigens can be measured by Enzyme Linked Immunoassay (ELISA) or enzyme linked immunospot (Jahdali et al., 2013; Lombardi et al., 2017).

1.2 BACKGROUND OF THE STUDY

The study was conducted in South Africa, at the Chris Hani Hospital in Soweto by the Respiratory and Meningeal Pathogens Research Unit (RMPRU). The location was selected because of the high prevalence of tuberculosis in the catchment area surrounding the hospital.

Adults with pulmonary tuberculosis were recruited to join the study from the Chris Hani Hospital admission ward for tuberculosis patients. Once consent had been obtained for the adults' participation in the study, these adults were asked to provide information about their children (aged 6 months to 16 years) and potential tuberculosis exposure of their children at home. Knowledge and attitudes about diagnosis of tuberculosis infection and chemoprevention was obtained from the adult participants through use of a structured interview. The information was compared to the inclusion and exclusion criteria set for the study and if the criteria were met the children were invited to the RMPRU clinic for blood withdrawal and the children were then tested for *M.tuberculosis* infection using the QFT-GIT and the conventional tuberculin skin test.

Tuberculin skin testing (for children with a prior negative tuberculin skin test) and QFT-GIT testing of children were performed at study entry and approximately 6 months later. The study was designed to assess the performance of a new T-cell based blood test, namely QuantiFERON-TB In Gold Tube, for diagnosis of tuberculosis infection in children of adults with pulmonary tuberculosis, and to determine the prevalence of *M.tuberculosis* infection among these children.

1.3 SIGNIFICANCE OF THE STUDY

In South Africa, there are critical unmet needs for the improvement of strategies in the diagnosis of latent *M.tuberculosis* infection in children, and for prevention of tuberculosis disease in children (Wood et al., 2011c; Kumar et al., 2015). Bacillus Calmette-Guérin (BCG) vaccination has limited the utility of tuberculin skin testing (TST) (Parida & Kaufmann, 2010; Seddon et al., 2016). However, the QFT-GIT test

uses antigens which are absent from the BCG vaccination, indicating that the tests should not be affected by BCG, and therefore may be a more accurate diagnosis of latent *M.tuberculosis* infection (Parida & Kaufmann, 2010: Ndzi et al., 2016). Results from this study contribute towards the improvement and the understanding of the prevalence of tuberculosis infection among BCG-vaccinated children who are household contacts of adults with pulmonary tuberculosis.

This study will evaluate the usefulness and performance of QFT-GIT in South African children. The ability to accurately diagnose latent tuberculosis, without being confounded by BCG and non-tuberculosis mycobacteria, would allow for detection of those children who are truly infected and therefore may benefit from further evaluation and treatment, and prevent those who are not infected from having to undergo treatment for tuberculosis disease.

1.4 MOTIVATION

The difficulties in diagnosing *M.tuberculosis* in children using TST are hampered by the test's limitation which includes human error when interpreting the results and cross reaction with environmental mycobacteria (Starke, 2012; Pai et al., 2014). A recently developed whole blood assay is not affected bythis limitation and was used to quantitatively measure IFN-γ production by lymphocytes specific to the *M.tuberculosis* antigens ESAT-6, and CFP-10; its use and assessment had been limited in children and the dissertation aims were to document the diagnosis (Abdalhamid et al., 2010; Pai et al., 2014).

Infants are unable to produce sputum samples consciously which makes the bacteriological diagnosis of tuberculosis difficult. As a result, clinicians have had to resort to using clinical symptoms, TST, radiological and history of the contact as an indication for diagnosis of active and latent tuberculosis as reviewed by Mandalakas et al., (2011; Pai et al., 2014). A better procedure for detection of active and latent tuberculosis in infants is urgently required.

1.5 AIM

The main aim of this study was to estimate the prevalence of *M.tuberculosis* infection in children with household tuberculosis contact, by using QFT-GIT testing in comparison with TST.

1.6 OBJECTIVES

The objectives of the study were to:

- To determine the sensitivity of the QFT-GIT tests compared with the standard TST for diagnosing latent tuberculosis infection in paediatric contacts (Reported in 4.4).
- Compare the TST specificity results with the QFT-GIT results (Reported in 4.5 and 4.6).
- Determine within-subject variability of the QFT-GIT and TST tests at baseline and approximately 6-month time period (Reported in 4.7).
- Determine factors associated with indeterminate QFT-GIT results in paediatric tuberculosis contacts (Reported in 4.8).
- Assess the effect of HIV status of adult'scases on probability of tuberculosis transmission to paediatric contacts (Reported in 4.9).

1.7 STUDY RELEVANCE

Various studies have shown that IFN gamma tests are highly sensitive and specific for detecting *M.tuberculosis* infection in adults, however in children there is limited data reporting on a comparison between QFT-GIT and TST in developing countries where there is a high burden of tuberculosis (Basu et al., 2012; Sharma et al., 2017). Results from this study will improve the understanding of the prevalence of tuberculosis infection among BCG-vaccinated children who are household contacts of adults with pulmonary tuberculosis. This study will aid in understanding the usefulness and performance of QFT-GIT in South African children. The ability to accurately diagnose latent tuberculosis, without confounding by BCG and non-tuberculosis mycobacteria, would allow for detection of those children who are truly

infected and therefore may benefit from further evaluation and treatment, and prevent those who are not infected from having to undergo treatment for tuberculosis disease.

1.8 STRUCTURE OF THE DISSERTATION

This dissertation was divided into five chapters. Chapter one provides information on the background and framework of the study, indicating the problem and its setting. An extensive literature review is reported in chapter two. Chapter three reports on the methods used. In chapter four results are reported and discussed. The conclusions, discussions and recommendations relating to the study are included in chapter five. A reference list compiled using the Harvard style of referencing, as prescribed by the guidelines of the Vaal University of Technology (VUT) and the annexures complete the document.

CHAPTER 2
LITERATURE STUDY

2.1 INTRODUCTION

Tuberculosis still remains a leading cause of mortality globally from a single infectious disease followed by human immunodeficiency virus (HIV). Tuberculosis is caused by a highly aerobic bacillus called *M.tuberculosis* (World Health Organization, 2013; Moon et al., 2017). The disease is transmitted through the air by released aerosols when people who have active tuberculosis cough, sneeze, or spit (Knechel, 2009; Dontwi et al., 2014).

Standard diagnostic tests for active tuberculosis disease detection are based on microscopic examination, microbiological culture, radiological tests, and recently molecular tests (Monkongdee et al., 2009; Dunn et al., 2016). For diagnosis of latent tuberculosis, TST is still used as a gold standard after its discovery almost 110 years ago by Robert Koch (Cattamanchi et al., 2011; Ritacco & Kantor, 2015).

Great strides in diagnosing latent tuberculosis were made in recent years with development of blood based tests such as enzyme linked immunospot (ELISPOT) and QuantiFERON assays which promise more reliable detection of latent tuberculosis infection (Kakkar et al., 2010; Lalvani et al., 2011; Bae et al., 2016).The new blood based tests show promising data to supersede the century old TST (Lalvani & Pareek, 2010; Lalvani et al., 2011; Bae et al., 2016).

2.2 HISTORICAL PERSPECTIVE OF TUBERCULOSIS

An *M.tuberculosis* bacillus was discovered by Robert Koch in 1882. The tubercle bacillus was discovered preserved in mummies 4000 years old, demonstrating the bacilli were present in ancient Egyptian times (Donoghue, 2011; Young, 2017). *M.tuberculosis* belongs to the mycobacterium genus, Actinomycetales order and Actinomycytes class (Sakamoto, 2012; Barka et al., 2015). Most Mycobacteria are found in water or soil, but few are facultative intracellular pathogens usually infecting

mononuclear phagocytes (macrophages) in humans (Sakamoto, 2012; Weiss & Schaible, 2015).

Evolutionary origin of *M.tuberculosis* is uncertain however it is speculated to have originated from soil where it is believed to have initially infected cattle and evolved roughly from 15 000 to 20 000 years ago from *M.bovis* (Smith et al., 2009; Hauer et al., 2015). Domestication of cattle which occurred between 10 000 to 25 000 years ago is believed to have allowed transmission of mycobacterial pathogens to humans (Smith et al., 2009; Brites & Gagneux, 2015).

The above mentioned speculation is now doubtful since recent data by deoxyribonucleic acid (DNA) sequencing methods reveals that there is a difference in characteristics between *M.tuberculosis* and *Mycobacterium bovis* (Russell, 2013; Zimpel et al., 2017). Another hypothesis is that *M.tuberculosis* complexes are derived from a common ancestor since the bacteria are characterized by 99.9% similarity at the nucleotide level and have identical 16S ribonucleic acid (RNA), but what is intriguing about the hypothesis is that the bacteria differ in their host tropisms, phenotypes and pathogenicity (Sakamoto, 2012; Brites & Gagneux, 2015).

For the past 100 years tuberculosis is estimated to have killed 100 million people globally. A decline in tuberculosis mortality was observed in the twentieth century in industrialised countries (Lonnroth et al., 2009; Jackson et al., 2016). The decline was attributed to improvement in sanitation and housing conditions, introduction of BCG vaccination and the use of streptomycin, isoniazid and rifampicin as antimicrobial agents for anti-tuberculosis treatment (Lonnroth et al., 2009; Jackson et al., 2016).

2.3 PHYSICAL CHARACTERISTICS OF MYCOBACTERIUM TUBERCULOSIS

2.3.1 Cell wall of *Mycobacterium tuberculosis*

The cell wall of *M.tuberculosis* is the main distinctive feature of the bacterium contributing to its many characteristics as reviewed by Fakuda et al., (2013; Alderwick et al., 2015). *M.tuberculosis* is among the few groups of bacteria that are

able to survive within phagocytic cells of the host; this shows the bacterium cell wall has properties that protect the bacterium against host microbicidal processes (Meena & Meena, 2011; Querol & Rosales, 2017). The cell wall barrier of the bacterium is responsible for the intracellular survival of the bacterium by directly modulating the immunological reaction between mycobacteria and the host (Malen et al., 2011; Peddireddy et al., 2017).The cell wall contributes to the main characteristic that distinguishes the mycobacteria such as, resistance to harsh environment, acid fast staining properties, low permeability of the cell wall and the intrinsic resistance to many hydrophobic antibiotics as reviewed by Gengenbacher & Kaufmann (2012; Gygli et al., 2017).

The pathogen has a thick waxy cell wall made up of peptidoglycans, polysaccharides, glycolipids, and long fatty acids known as mycolic acid which make the bacterium unique among prokaryotes as reviewed by Gengenbacher & Kaufmann (2012; Marrakchi et al., 2014). The peptidoglycans are the main components of the cell wall of *M.tuberculosis* and responsible for the properties of shape forming and chemical structure that resembles that found in other bacteria (Gupta et al., 2010; Velayati & Farnia, 2012; Grzegorzewicz et al., 2016). The cell wall of *M.tuberculosis* has esterified arabinogalactan distal ends with high-molecular weight fatty acids (Mishra et al., 2011; North et al., 2014). The cell wall structure is called mycolyl- arabinogalactan-peptidoglycan complex (mAGP) (Mishra et al., 2011; Abrahams & Besra, 2016). The bacterium cell wall has one distinguishing characteristic: the absence of bacterial typical structures such as lipoteichoic acids found in gram positive bacteria and lipopolysaccharides found in gram negative bacteria (Gupta et al., 2010; Malanovic & Lohner, 2016). *M.tuberculosis* is therefore classified as Gram positive even though when the gram stain is performed the cell wall does not retain the dye. This is because of the high lipid and mycolic acid content of its cell wall which is impermeable to Gram staining (Knechel, 2009; Sakamoto, 2012; Jackson, 2014).

2.3.1.1 Peptidoglycan

The foundational structure of *M.tuberculosis* and other mycobacterial species is peptidoglycan. It is the backbone of mAGP and provides shape, strength and the rigidity of the cell wall in *M.tuberculosis* (Mishra et al., 2011; Kieser et al., 2015). The type of peptides cross-linking are identified as peptidoglycan (PG) classes; A1γ type is the common one in *M.tuberculosis* PG structure (Both et al., 2013; Catalão & Pimentel, 2018). The PG is insoluble with alternating *N*-acetlyglucosamine (GLcNAc) and modified muramic acid residues [*N*-acetyl-β-D-glucosaminyl-(1→4)-*N*-acetylmuramic acid] (Both et al., 2013; Rush et al., 2017). In the muramic acid of the peptidoglycan, the *N*-acetyl group is further oxidized to *N*-glycoly groups in *M.tuberculosis* [*N*-glycolymuramic acid] (Favrot & Ronning, 2012; Alderwick et al., 2015). The residues of muramic acid are also modified by tetrapeptide [L-alanyl-D-isoglutaminyl-meso-diaminopimely-D-alanine] side chain (Favrot & Ronning, 2012; Alderwick et al., 2015).

2.3.1.2 Arabinogalactan

In *M.tuberculosis* a heteropolysaccharide chain of furanoid arabinose (Ara*f*) and galactose (Gal*f*) is called arabinogalactan (AG). The residues of furanosyl are arranged into three differential regions: the arabinan, the non-reducing terminal segments of arabinan and the galactan core (Asmarani et al., 2016). The galactan core of AG has 5- and 6-linked β-D- Gal*f* residues. A chain of arabinan chain linkage consists of linear 5-linked α-Ara*f* residues with 5.5- Ara*f* introducing branching residues. Arabinan chains linkage occurs at C-5 of the 6-linked β-D- Gal*f* residues (Pan et al., 2011; Wu et al., 2017). Four clusters of mycolic acid are then attached to arabinofuranosyl terminal motifs of non-reducing ends of arabinan chain via ester linkage. The galactan core of AG is finally attached to the C-6 position of muramic acids of the PG via a phosphodiester linkage of α-L-rhamnopyranose (Rhap)-(1→3)-D-N-acetylglucosamine (GlcNAc)-(1→phosphate) (Pan et al., 2011; Angala et al., 2014).

2.3.1.3 Mycolic acids

Mycolic acids are complex, hydroxylated, branched-chain fatty acids released by all mycobacteria and contain α-alkyl and β-hydroxy fatty acids which are responsible for the hydrophobic characteristics of the mycobacterial cell wall (Barkan et al., 2009; Marrakchi et al., 2014). Mycolic acids have functional groups such as keto, methoxy, cyclopropane rings and epoxy ester groups (Barkan et al., 2009; Teramoto et al., 2015). *M.tuberculosis* such as a meromycolate branch and α-branch mycolic acids, where the meromycolate branch defines the heterogeneity (Barkan et al., 2009; Portevin et al., 2014). The meromycolate branch has polar moieties such as methoxy groups and ketones; furthermore, functional groups such as methyl branches, cyclopropanes and unsaturations (Barkan et al., 2012; Marrakchi et al., 2014). *M.tuberculosis* in particular contains unsaturated mycolates containing cyclopropanes known as α-mycolic acids. The most abundant mycolic acid found in the cell wall of *M.tuberculosis* is α-mycolic acid followed by methoxy and keto-mycolates (Leger et al., 2009; Barkan et al., 2012; Groenewald et al., 2014). The inner leaflet of the *M.tuberculosis* cell has the majority of mycolic acids covalently bound via carboxylate ester to form the non-extractable tetramycolyl-pentarabinosyl unit. Mycolic acids play an important role in maintaining the rigid cell shape of *M.tuberculosis.* Furthermore, they play a part in the resistance to chemical injury and protect *M.tuberculosis* against hydrophobic antibiotics (Meena & Meena, 2011; Carel et al., 2014).

In figure 2 below, *M.tuberculosis* stained with Acid-fast dye solution is illustrated; the bacterium appears red and as tight, rope-like rods (Timothy, et al., 2006; Saxena et al., 2014).

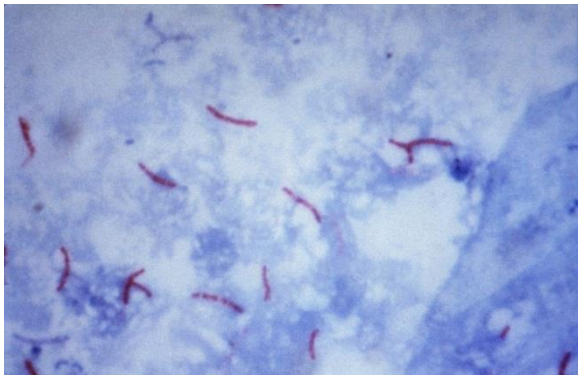


Figure 2: *M.tuberculosis* stained with Ziehl-Neelsen acid fast stain technique under microscope (Timothy et al., 2006; Saxena et al., 2014)

2.4 *M.TUBERCULOSIS* TRANSMISSION

Practically all transmission of *M.tuberculosis* is from person to person; usually by mucous droplets called droplet nuclei that become released into the air when an individual with pulmonary tuberculosis coughs or sneezes (Knechel, 2009; Ahmad, 2011; Fennelly & López, 2015). The released droplet remains in the air for minutes to hours after expectoration (Knechel, 2009; Ahmad, 2011; Fennelly & López, 2015).

Most of the released bacilli are stopped by the upper parts of the airways where the mucus secreting goblets exist. The body has mucociliary escalators containing mucus-producing goblet cells that trap the foreign substances as indicated in figure 3 and ciliated epithelium which are continuously beating and pushing the trapped foreign substances in mucus up and out into the throat (Racaniello, 2009; Whitsett & Alenghat, 2014).

Some bacilli however pass this first defence mechanism and are carried in droplets small enough (5 to 10 microns) to reach the alveolar spaces. In the early weeks of infection after the droplets are inhaled some dormant bacteria in infected macrophages migrate to regional lymph nodes (Kingkaew et al., 2009; Miranda et al., 2012; McClean & Tobin, 2016).

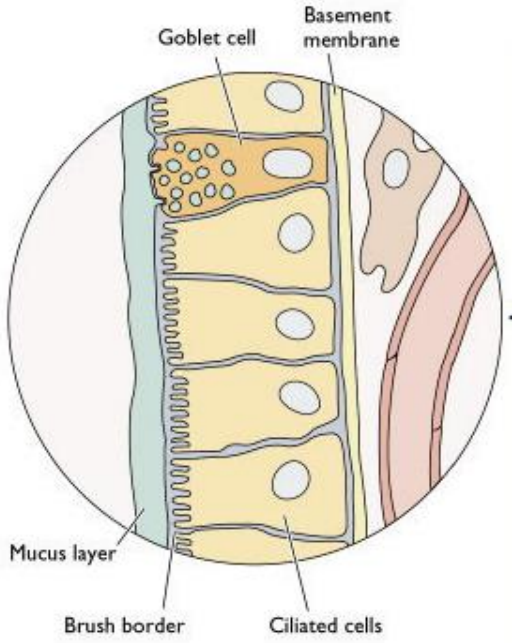


Figure 3: Mucociliary mechanism (Racaniello, 2009)

In the lymph nodes they access the blood stream and spread throughout the body (Kingkaew et al., 2009; Miranda et al., 2012; McClean & Tobin, 2016). *M.tuberculosis* not only infects the lungs but can spread to various parts of the body such as joints, bones, central nervous system and urinary tract (Kingkaew et al., 2009; Miranda et al., 2012; McClean & Tobin, 2016).

It is estimated that 2 billion people are latently infected with tuberculosis. Out of these 2 billion cases, reinfection and reactivation cases emerge as reviewed by Gengenbacher & Kaufmann (2012; Chapman & Lauzardo, 2014) indicated in figure 4. When *M.tuberculosis* droplets are inhaled, after incubation which can be between 4-12 weeks, infected alveolar macrophages can follow three routes: Firstly alveolar macrophages either destroy the invading pathogens resulting in a less understood mechanism called abortive infection; secondly the macrophages engulf the invading pathogen resulting in formation of solid granuloma structures and development of latent tuberculosis. *M.tuberculosis* is able to avoid the host cellular mechanism such as phagolysosome fusion used by the body to kill invading intracellular pathogens in these states as indicated in figure 4 (Simeone et al., 2012; Zarelli et al., 2018). The bacterium is able to avoid lysosomal fusion by manipulating the host signal transduction pathways resulting in phagosomes that are unable to fuse to lysosomes

(Simeone et al., 2012; Zarelli et al., 2018). After the body has initiated phagocytosis by dendritic cells and macrophages, *M.tuberculosis* remains dormant in macrophage's compartments that maintain characteristics of an early endosome (Vandal et al., 2009; Korb et al., 2016).

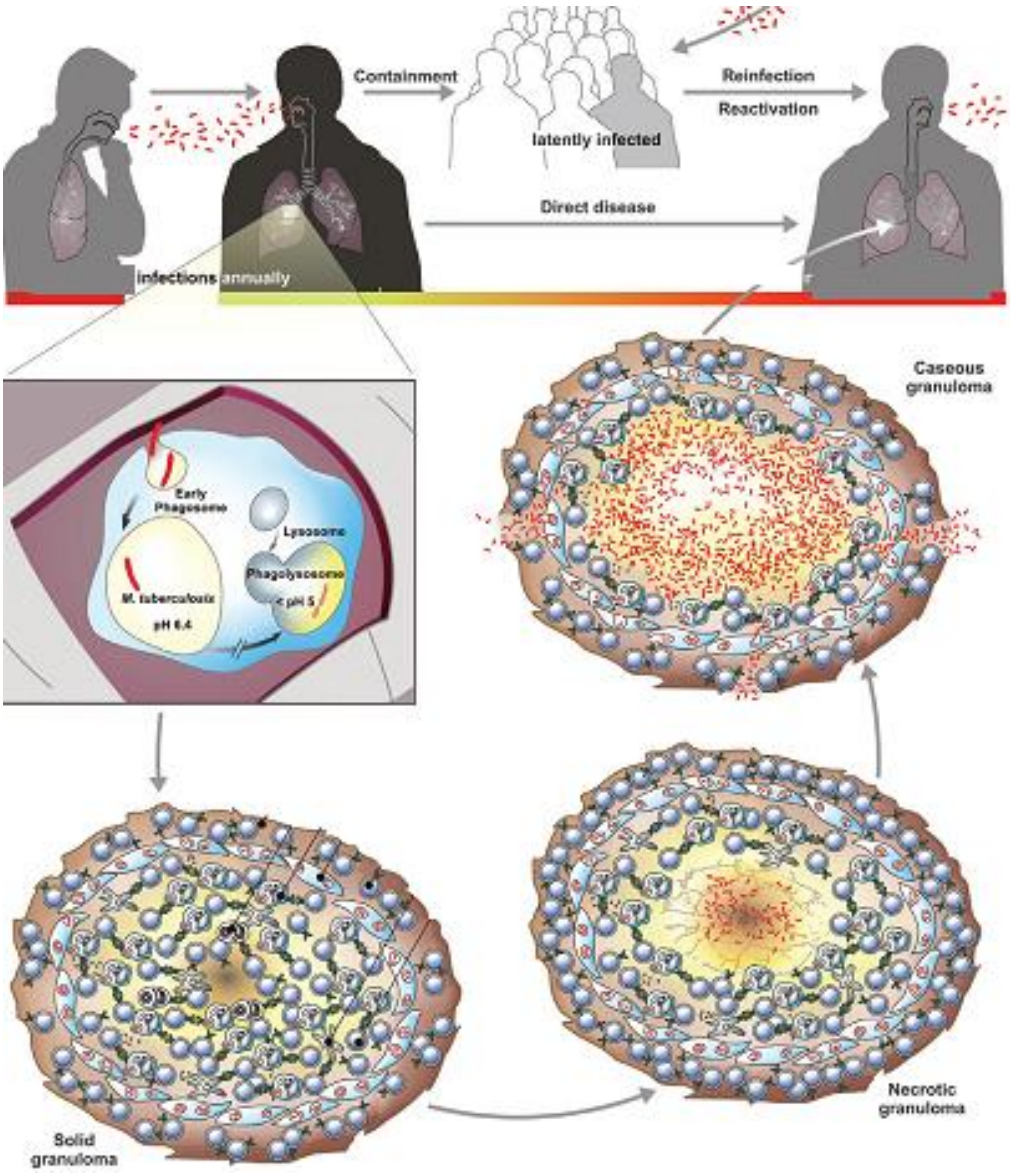


Figure 4: Transmission of droplet nuclei released into the air and route of infection of *M.tuberculosis* in humans (Gengenbacher & Kaufmann, 2012)

The bacterium persists in the macrophage compartments which has a pH range from 6.5 to 4.5 depending on the macrophage's activation state as indicated in figure 4 (Vandal et al., 2009; Baker et al., 2014). Once the host's immune system is compromised, necrotic granuloma formation begins (dying of tissue), allowing

M.tuberculosis to grow within the granuloma, leading to the macrophages failing to contain the bacterium in granulomas resulting in caseous granulomas (cell death) and formation of active tuberculosis as reviewed by Gengenbacher & Kaufmann (2012; Diedrich et al., 2016) indicated in figure 4).

It is estimated on average that individuals with smear positive sputum transmit tuberculosis to at least ten contacts annually (Abebe et al., 2007; Asefa & Teshome, 2014). Knechel, (2009; Forrellad et al., 2013; Migliori et al., 2018) indicated that the factors determining the virulence of tuberculosis transmission are:

- The virulence of the bacilli
- The number of bacilli in the droplets
- The degree of ventilation
- Vaccination
- Immune status of the host

At the primary site of tuberculosis infection, granulomas get formed by macrophages engulfing the invading pathogens (Knechel, 2009; Forrellad et al., 2013; Saleh et al., 2016). The granulomas remain intact in individuals with strong cellular immune systems; however, if the host's cellular immune status changes and the local immune system fail to control the invading bacillus, the granulomas leak the bacilli and the infection spreads to other areas of the lung and is finally transmitted to other organs of the body (Philips & Ernst, 2009; Ottenhoff et al., 2012; Bozzano et al., 2014).

2.5 VIRULENCE OF *MYCOBACTERIUM TUBERCULOSIS*

There is different virulence factors involved in *M.tuberculosis* pathogenesis. The major factors that have been put forward include: the ability of the bacterium to remain in a dormant state; its ability to divide rapidly in the infected hosts; its intracellular niche and its complex cell wall which contains lipids that allow the bacterium to be impermeable to attack by the host immune system or with antimicrobials (Forrellad et al., 2013; Prozorov et al., 2014).

Three major mycolic acids, alpha mycolate, methoxymycolate and ketomycolate play important roles in pathogenesis of *M.tuberculosis* (Barkan et al 2009; Pandrangi, 2015). Two enzymes in *M.tuberculosis* known as cyclopropane synthase (PcaA) and coronamic acid synthetase (cmaA2) in alpha mycolate are required for cyclopropanation. It is believed that cyclopropanation plays a crucial role as an immunomodulatory lipid modifier (Barkan et al., 2009; Barkan et al., 2012; Poger & Mark, 2015). Deficiency in cyclopropanation or loss of PcaA causes early growth defects in the lungs and failure to activate macrophage innate immune responses while on the other side, loss of cmaA2 results in hyper-virulence and hyper inflammatory innate to immune activation in macrophages (Barkan et al., 2009; Barkan et al., 2012; Orme & Basaraba, 2014).

The ability of *M.tuberculosis* to survive in a host with an intact immune system shows that the organisms can evade the innate and adaptive immunity. The bacterium does so by preventing phagolysosome formation and by subversion of the host's cell death pathway (Divangahi et al., 2010; Mitchell et al., 2016). Living *M.tuberculosis* bacilli in granulomas inhibit phago-lysosomal fusion, which destabilizes this significant phase of the macrophage defence mechanism (Welin et al., 2011; Pires et al., 2016).

M.tuberculosis such as H37Rv are virulent strains that induce the tumour necrosis factor of human macrophages and actively inhibit the induction of macrophage apoptosis; however it has been reported that regardless of how the apoptosis is induced, either by pathogen itself or by cytotoxic lymphocyte, the viability of *M.tuberculosis* is lowered (Divangahi et al., 2010; Abebe et al., 2011; Olsen et al., 2016). The cell wall components of *M.tuberculosis* such as cord factor trehalose 6, 6-dimycolate (TDM) which is found in virulent *M.tuberculosis* strains and not present in avirulent strains also assists in persistence of *M.tuberculosis* (Forrellad et al., 2013; Nobre et al., 2014).

M.tuberculosis virulence depends on multiple proteins, for this literature review the two molecules CFP-10 and ESAT-6 that are key to the methods used are discussed. Virulence of *M.tuberculosis* depends on the secretion of ESAT-6 and its protein partner CFP-10 by the ESX-2/ VII secretion system encoded in ESX 1 genomic locus

near the origin of replication (Simeone et al., 2009; Solans et al., 2014). The ESX-1 system is absent from *Mycobacterium bovis* and *Mycobacterium microti*; in *M.tuberculosis* it is responsible for the export of ESAT-6 and CFP-10 (Simeone et al., 2009; Solans et al., 2014). Translocation of *M.tuberculosis* from the phagosome into the cell cytoplasm of the hosts at later stages of infection is facilitated by CFP-10 and ESAT-6 (Mahmoudi et al., 2013; Welin et al., 2015).

The antigens CFP-10 and ESAT-6 depend on each other for their secretion and stability, and form a tight dimer together (Sreejit et al., 2014). ESAT-6 and CFP-10 antigens are capable of modulating adaptive and innate immune responses. ESAT-6 and CFP-10 weaken the innate immune response by IL-12 production inhibition from macrophages (Guo et al., 2012b; Refai et al., 2015).
Deactivation of ESAT-6 results in a decrease in the virulence of *M.tuberculosis* (Sreejit et al., 2014). This was demonstrated by the deletion of the Regions of difference (RD) 1 for *M.tuberculosis* and gene knock-in of RD1 in *M.bovis* in mice (Wang et al., 2009a; Saikolappan et al., 2012; Ru et al., 2017). *In vitro,* mutation in RD1 reduces cytolysis of type 2 pneumocytes, macrophages, pulmonary necrosis, cell-to-cell spread, and bacterial dissemination in the lungs (Kinhikar et al., 2010; Espitia et al., 2012; Parasa et al., 2014).

ESAT-6 antigen is able to bind to the basolateral surface of pneumocytes of the host's proteins laminin and results in lung epithelial cell lysis; therefore the antigen also causes the dissemination of *M.tuberculosis* in the lung (Boggaram et al., 2013; Scordo et al., 2016). Furthermore, through epithelial cell matrix MMP-9 (metallo-proteinase-9) induction, ESAT-6 contributes to the formation of granuloma, an important step in pathology of tuberculosis (Boggaram et al., 2013; Azikin et al., 2017).

2.6 MODE OF INFECTIONOF *M.TUBERCULOSIS*

Tuberculosis infection requires inhalation of particles less than 5µm in diameter to go into to the upper respiratory tract and isdeposited deep in the lungs, usually in the airspaces of the lower lung (Yadavet al., 2010; Yang et al., 2014). Once

Mycobacterium tuberculosis, has gained entry into the cell by surviving phagocytosis, it challenges the innate immune responses of the host macrophages (Yadav et al., 2010; Lerner et al., 2015). Alveolar macrophages are the primary source of tuberculosis infection because they allow the invading *M.tuberculosis* to replicate, and are able to distributeviable tubercle bacilli within the lung and to other parts of the body (Pawlowski et al., 2012; Woo et al., 2018).

Specialized epithelial cells known as M cells which transfer particles across epithelial barriers to an intraepithelial lymphoid pocket created by the modification of the basolateral surface, is the route of entry for most enteric pathogens across the mucosa into the body (Kerrigan & Brown, 2009; Shakya et al., 2016). *M.tuberculosis* mode of entry into macrophages is through complementary receptor (CR) - mediated phagocytic pathways as CR ligation does not trigger phagocyte microbicidal responses as indicated in figure 5 (Kerrigan & Brown, 2009; Restrepo et al., 2014). Throughout this phagosomal arrest *M.tuberculosis* persists at a stage where no harm can be done to the bacterium while delivery of nutrients continues (Taneja et al., 2010; Puri et al., 2013; Ryndak et al., 2016). The acquisition of *M.tuberculosis* to the phagosome is through dendritic-cell specific intercellular adhesion molecule 3-grabbing non-intergrin receptor (DC-SIGN) and complement receptor 3 (CR3), complexglycolipids-lipoarabinomannan (Lam)-phosphatidyl linositolmannoside (PIM)-through the mannose receptors as indicated in figure 5 (Libero & Mori, 2005; Rajaram et al., 2017).

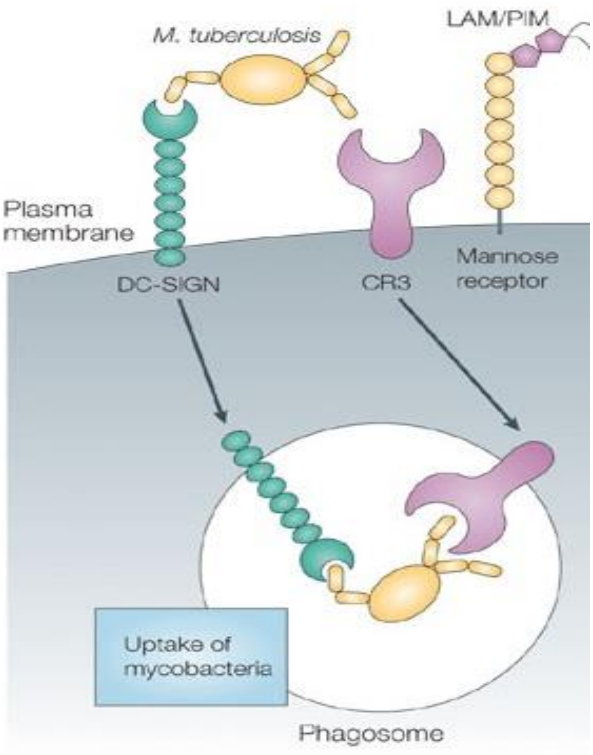


Figure 5: *M.tuberculosis* acquisition (adapted from Libero & Mori, 2005)

2.7 IMMUNE RESPONSE AGAINST *M.TUBERCULOSIS* INFECTION

2.7.1 Innate immune response

For the body to initiate a response against the invading pathogens, the immune system must be able to recognize the invader (Kleinnijenhuis et al., 2011; Yatim & Lakkis, 2015). Activation of the innate immune system starts with pattern recognition of microbial structures called pathogen associated molecular patters (PAMPs) (Kleinnijenhuis et al., 2011; Pahar et al., 2017).

The cell wall components of *M.tuberculosis* play a major role in recognition and interaction with the host's cells because of lipids such as mycolic acids, trehalose containing lipids and several lipoglyco conjugates (Tahlan et al., 2012; Wright et al.,

2017). The surface of *M.tuberculosis* is dominated by biosynthetically related mannosylated lipoglyco conjugates which mediate recognition by pattern recognition receptors (PRRs) and entry of the bacillus into the host's cells through C-type lectin (Sakamoto, 2012; Mishra et al., 2017). *M.tuberculosis* cell wall components are responsible for macrophage activation and are recognized by at least two different recognition receptors: the toll-like receptors (TLR) and the nucleotide oligomerization domain (NOD)-like receptors which are also responsible for mediating engulfed pathogens as indicated in figure 6 (Torrado & Cooper, 2013; Leissingern et al., 2014). TLR2 and TLR1 heterodimer play the most important role in recognition of atriacylated lipoprotein derived from *M.tuberculosis*, while NOD2 recognizes muramyl dipeptide (MDP) which results in activation of NF-kB, leading to the production of inflammatory cytokines and direct antimicrobial activity by both receptors (Torrado & Cooper, 2013; Blanc et al., 2017).

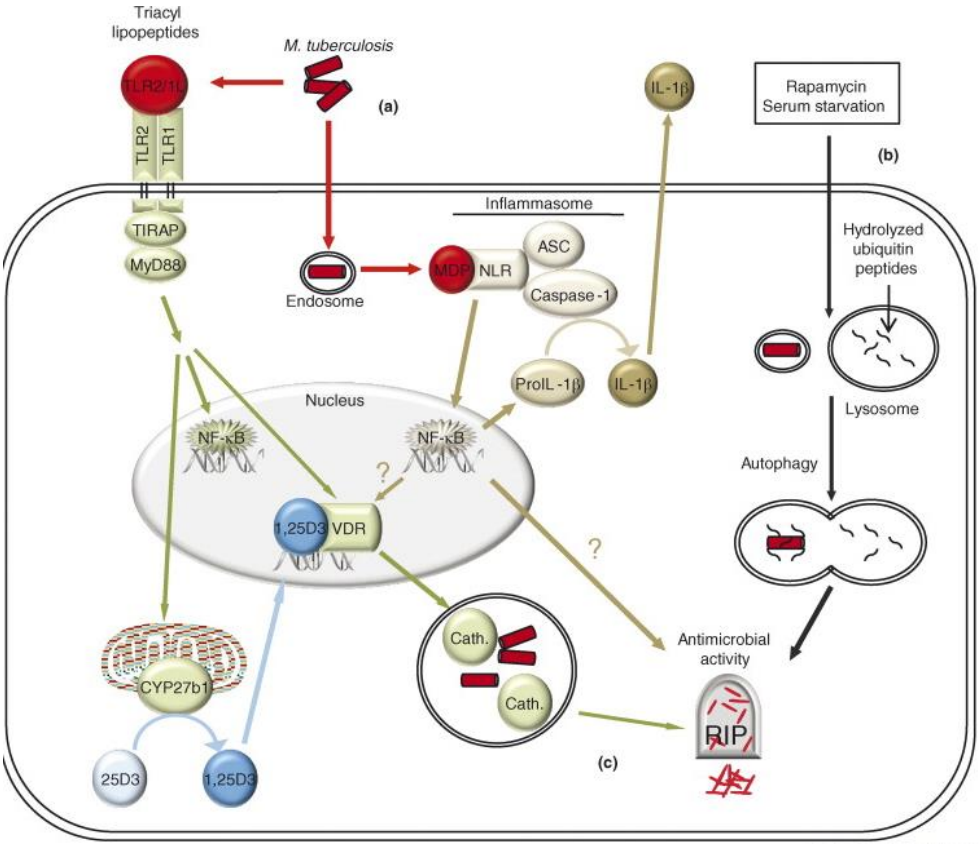


Figure 6: Host recognition mechanisms of human macrophages against invading intracellular *M.tuberculosis* (Liu & Modlin, 2008)

Figure 6 (a) indicates two families of pattern recognition receptors, the NOD-like receptors and Toll-like receptors which mediate recognition of *M.tuberculosis* (b) antimicrobials such as rapamicin results in delivery of hydrolyzed ubiqutin peptides to the lysosome and induction of autophagy leading to antimicrobial activity (c) Murine macrophages utilize iNOS; macrophage in humans have evolved to release antimicrobial peptides upon TLR stimulation (Liu & Modlin, 2008; Mortaz et al., 2015).

2.7.2 Humoral immune response

Human immunity is capable of mounting a defence against the invading *M.tuberculosis* by releasing phagocytic macrophages that are able to ingest and destroy *M.tuberculosis* even though they have not previously encountered the organism. However, the defence against the invading pathogen depends on the host's cellular immune system (Yu & Xie, 2012; Cadena et al., 2016). Macrophages from the cellular immune system use several mechanisms to kill the invading bacterium, such as production of nitric oxide (NO), vitamin D-induced antimicrobial activity and autophagy, which is defined as the process that results after the components of the cytoplasm, these include organelles and intracellular pathogens in phagosome, are sequestered and delivered to lysosome for degradation (Yu & Xie, 2012; Jamaati et al., 2017).

The body, once it has recognized the invading bacterium mounts a defence against *M.tuberculosis* infections by releasing T cells that produce specific patterns of cytokines, which can be broadly divided into T helper 1 (Th1) and T helper 2 (Th2) (Ottenhoff & Kaufmann, 2012; Choi et al., 2018). Immunity against *M.tuberculosis* invasion depends on interaction between activated T cells, leucocytes, macrophages, and the following pro-inflammatory cytokines:

- IFN-γ
- Interleukin (IL)-12
- IL-1 Beta (β)
- IL-6
- TNF-α

- IL-10
- IL-4

(Hasan et al., 2009; Choi et al., 2018).

Macrophages unaided cannot control intracellular mycobacterial growth; infected macrophages secrete tumor necrosis factor-α **(**TNF-α) and chemokines which play a most important role in coordinating the recruitment of T lymphocytes to the site of infection in formed granulomas (Csoka et al., 2012; Gonzalez et al., 2016). Activated T cells interact with macrophages and secrete IFN-γ cytokine that promotes macrophages to kill intracellular mycobacteria (Csoka et al., 2012; Gonzalez et al., 2016).

Another important cell that plays a major role in recognition and destruction of *M.tuberculosis* is human peripheral CD8 T cells which show specificity towards *M.tuberculosis* antigens, such as ESAT-6, on stimulation with this antigen (Sakamoto, 2012; Arlehamn et al., 2014). Natural immunity can stop and kill some bacillary growth by priming and activating antigen-specific Ly2 (CD8+) and L3T4 (CD+4) T cells. These T cells produce cytokines such as IFN gamma whose role is to activate macrophages and recruitment of cells to the site of infection which normally occurs in the lung in tuberculosis disease (Sakamoto, 2012; Nicholson, 2016). The lysis of *M.tuberculosis* infected macrophages is caused by CD8+ T cells through a Fas-independent granule exocytosis pathway and the Fas-FasL interaction which leads to apoptotic death of infected target cells (Sakamoto, 2012; Lin & Flynn, 2015).

M.tuberculosis infection in human hosts follows three potential outcomes once the invading pathogen enters the hosts: (1) bacterial death (2) possible bacterial survival or growth (3) bacterial death or restriction of growth (Philips & Ernst, 2009; Ban˜uls et al., 2015). As indicated in figure 7 (a) the maturation of phagosome can be prevented by *M.tuberculosis*resulting in the bacterium continuing to grow by inhibiting phosphatidylinositol 3-phosphate (PI3P) generation. GTP-bound GTPase (Rab7) active recruitment is impared by the bacterium while GTPase (Rab5) is retained in an early endosome-like compartment; (b) Phagosomal membrane is permeabilized in the ESAT-6 systems (Esx-1), which results in direct cytosolic

access; (c) in some instances this step results in the bacterium being able to escape into the cytosol; (d) in cases where the infection is caused by *Mycobacterium marinum*, the ubiquitin system of the host recognizes the cytosolic bacteria and it is resequestered in a membrane-bound compartment; (e) the replication of the invading bacterium is shorterned; (f) Vitamin D together with interferon gamma is able to overcome the step of early endosome-like arrest; in so doing it encourages bacterial delivery to autolysosomes resulting in bacterial growth being halted or killed (Philips & Ernst, 2009; Cambier et al., 2014).

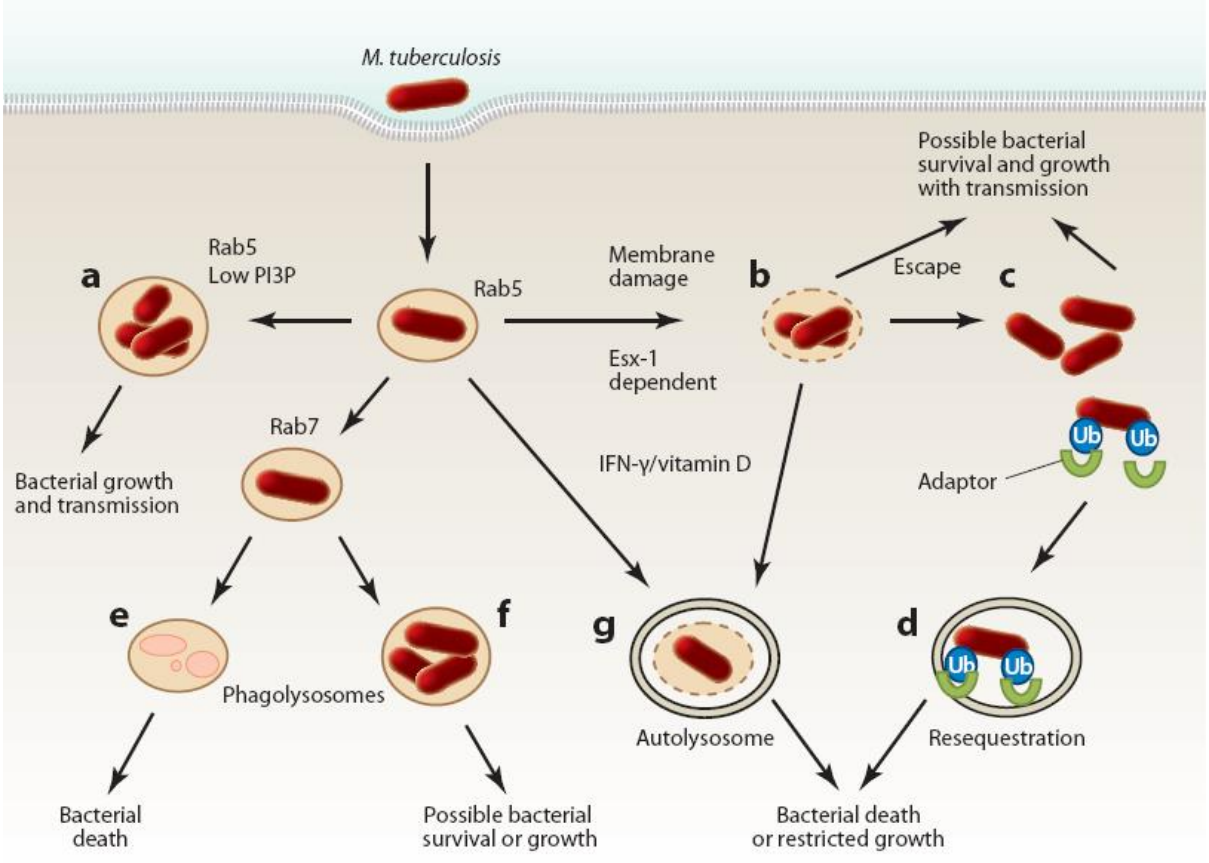


Figure 7: Host Immune system protection against tuberculosis infection (Philips & Ernst, 2009; Cambier et al., 2014)

The persistence of tuberculosis and signals by cytokines and chemokines result in formation of immunological structures called granulomas as mentioned before. They are the key components of host defence against invading intracellular pathogens (Huynh et al., 2011; Marino et al., 2015). Immediately after *M.tuberculosis* is engulfed inside the granulomas, it is exposed to harsh conditions which include

limited oxygen and increased nitric oxide resulting from inhibition of reactive nitrogen intermediates' produced by nitric oxide synthase (NOS2) inhibitor (Huynh et al., 2011; Jamaati et al., 2017).

Cytokines are protein molecules which mediate intercellular communication in the immune system and are produced by different cell types. (Duque & Descoteaux, 2014). Cytokines are released by cytokine-secreting immunological cells. The roles played by cytokines in immunity are classified based on the two types of the T helper cells: Th1 and Th2 cells (Wang et al., 2009b; Pennock et al., 2013; Annunziato et al., 2015). The fate of tuberculosis infection depends on these T helper cells in determining whether the disease progresses, resolves or becomes latent (Wang et al., 2009b; Cayabyab et al., 2012; Lyadova & Panteleev, 2015).

In humoral immunity response, which is the first defensive line against infection, phagocytes, T cell receptors (TCR), T cells, natural killer cells, mast cells, neutrophils, eosinophils, including pro-inflammatory cytokines such as IL-1,IL-6, IL-12, IL-18, IFNs and TNF-α play major roles in the immune response (Elios et al., 2011; Zimmerman et al., 2014). In response against tuberculosis infection, Th1 is responsible for the production of IFN-γ, IL-2 and lymphotoxin while IL-4, IL-5, IL-6, IL-9 and IL-13 are produced by Th2 (Wang et al., 2009b; Lyadova & Panteleev, 2015).

2.7.2.1 Interferon-gamma

In the 1950s, it was recognized that virus infected cells under certain conditions develop resistance to a second viral infection. This led to a conclusion that a virus infected cell has a mechanism that interferes with another virus infection (Schneider et al., 2014). In 1957 when Isaacs and Lindenmann described a small protein and named it "interferon"; which seemed likely to be secreted and produced by cells after the detection of pathogen-associated molecular patterns, now known as PAMPs, by pattern recognition receptors (Schneider et al., 2014). In 1960 IFN-γ was discovered and was found to have multiple pathological and biological functions. IFN-γ has been shown to play a major role in signalling in diseases such as arthritis, diabetics,

multiple sclerosis and *M.tuberculosis* disease (Chen & Liu, 2009; Khalilullah et al., 2014).

(a) Production of IFN-γ

The interferons are produced by natural killer cells as part of the innate immune response, and by cytotoxic T lymphocyte (CTL) effectors cells once antigen-specific immunity develops (Sutherland et al., 2010; Muller & Stoiber, 2017). Interferons are a group of cytokines secreted by Th1 mediated cells and are divided into type I, II, III classes according to their amino acid sequence (Chen & Liu, 2009; Turner et al., 2014). IFN-γ falls into type II of the group and it is secreted by mitogenically activated T cells and natural killer cells (Chen & Liu, 2009; Turner et al., 2014).
The production of IFN-γ usual occurs two to four weeks after the initial infection; by this period the production of CD4+ and CD8+ T cell responses is clear. These cells produce IFN-γ to enhance the innate immunity of the effector mechanism which is critical for differentiation of specific T cells and clonal expansion (Rueda et al., 2010; Sakai et al., 2016). CD4+ T cells are the main source of IFN-γ, which is the cytokine that plays a major role in anti-TB response in both mice and humans (Rueda et al., 2010; Chen & Kolls, 2013; Kumar, 2017). The deficiency of CD4+ has been demonstrated to result in high susceptibility to *M.tuberculosis* infection in mice (Rueda et al., 2010; Matthews et al., 2012; Allie et al., 2013; Teixeira et al., 2017).

As indicated in Figure 8, the diagram shows different types of interferon that cells produce: interferon alpha and interferon beta are common types and released by cells of the immune system. IFN-γ is released primarily by T cells and sends signals to stop the growing of the invading bacterium and it is also involved in host defence mechanisms (Godsell, 2010; Pennock et al., 2013; Lerner & Gutierrez, 2015).

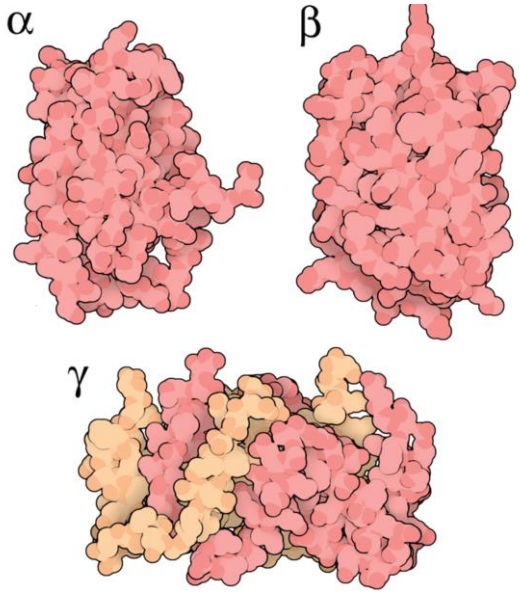


Figure 8: Diagram of IFN-α, IFN-β and INF-γ as depicted by Protein Data Bank (Godsell, 2010)

The interferon alpha and IFN-γ differ in their binding site: IFN-γ is a dimeric protein, its two copies of receptor bind on either side, while interferon alpha is monomeric, and therefore two different receptor chains bind to different portions of the protein (Godsell, 2010). Interferon alpha and interferon beta are proteins with four helix bundles belonging to type 1 interferons while IFN-γ belongs to type 2 and has no structure similarities to type 1 interferons (Randal & Kossiakoff, 2001; Piehler et al., 2012; Ng et al., 2015).

(b) Functional characteristics of IFN-γ

Type II or IFN-γ is a cytokine that contributes to protective immunity against viral and intracellular bacterial infections by activating macrophages to more effectively eliminate the invading organisms by inhibiting replication of invading pathogens (Billiau & Matthys, 2009; Lwasaki & Pillai, 2014). IFN-γ is involved in activating alveolar macrophages in order to produce a variety of molecules involved in linking mycobacterium and inhibiting the growth of the bacterium (Chowhudhury et al., 2014).

The production of IL-12 cytokine is induced by dendritic cells, macrophages and neutrophils; in turn IL-12 stimulates T cells and natural killer cells to produce IFN-γ

(Billiau & Matthys, 2009; Duque & Discoteaux, 2014). A combination of IL-12 and IFN-γ assists in coordination of pathogen recognition by innate immune cells, furthermore with the induction of adaptive immunity by creating a positive feedback loop, which amplifies Th1 response in tuberculosis disease (Billiau & Matthys, 2009; Thompson et al., 2011; Gasteiger et al., 2017).

Interferon gamma plays an important role in restriction of bacterial growth in humans and mice against *M.tuberculosis* by restricting the progression of the disease (Desvignes & Ernst, 2009; Lin & Flynn, 2015). Patients with mutations in the IFN-γ receptor genes are highly susceptible to mycobacterios is even when infected with species of tuberculosis that are less virulent (Billiau & Matthys, 2009; Alejo et al., 2014). The same is observed in mice with a defect in the IFN-γ receptor. When infected with *M.tuberculosis*, the mice die rapidly from bacterial load in the lungs. Treatment of cultured macrophages by IFN-γ in mice has been shown to result in autophagy and acidification of phagosomes in mycobacterium, leading to intracellular killing of *M.tuberculosis* (Desvignes & Ernst, 2009; Pacheco et al., 2013; Kimmey & Stallings, 2016).

2.7.2.2 Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α) has been identified as one of most important pro inflammatory cytokines found in the immune response to tuberculosis infection (Sakamoto, 2012; Joshi et al., 2015).TNF-α is involved in many biological functions in tuberculosis immunity such as (a) the activation of immune and endothelial cells, (b) thymocyte proliferation and (c) induction of apoptosis (Sakamoto, 2012; Akdis et al., 2016).

(a) Production of TNF-α

TNF-alpha is a proinflammatory cytokine produced by alveolar macrophages after they are stimulated by inhaled *M.tuberculosis* (Sakamoto, 2012; Gonzalez et al., 2018). The main instigators of TNF-alpha production are bacterial cell wall

components, parasites, fungi, tumor cell membrane components and viruses (Jozefowski et al., 2011; Fu et al., 2017).

(b) Functional characteristics of TNF-α

TNF-α plays a major role in stimulating monocytes, macrophages, and dendritic cells; it also plays a key role in maintenance of mycobacterial antigen-induced granulomas (Sakamoto, 2012; Duque & Descoteaux, 2014). Depletion of TNF-α *in vitro* in mice using TNF-α neutralizing antibody prevented granuloma formation in response to mycobacterium infection (Philips & Ernst, 2009; Redford et al., 2011; Silva et al., 2018). This indicates that the delayed recruitment of chemokines and recruitment of CD11b+ cells consequently result in inability of the hosts to contain infection; the mice were rapidly killed upon infection with *M.tuberculosis* (Philips & Ernst, 2009; Keeton et al., 2014). Mutations in the TNF-α gene in humans with tuberculosis have not been found. Furthermore, no positive associations between gene polymorphisms of TNF-α and disease susceptibility have been established (Philips & Ernst, 2009; Qidwai et al., 2012; Murdaca et al., 2014).

2.7.2.3 Interleukin-12

Interleukin-12 (IL-12) is produced mainly by phagocytic cells after phagocytosis of tuberculosis and plays the key role in host defence against *M.tuberculosis* (Sakamoto, 2012; Coronel & Arreola, 2016). IL-12 production results in production of IFN-γ cytokines which play crucial roles in immunity against *M.tuberculosis* (Sakamoto, 2012; Groen et al., 2015). Individuals with active tuberculosis have been found to have ineffective protective immunity to mycobacterial antigens and Th1 cytokine; IFN-γ is suppressed as compared to individuals who are healthy tuberculin reactors (Sutherland et al., 2010; Adrian et al., 2015).

(a) Production of IL-12

The primary producers of IL-12 are antigen-presenting cells, phagocytic cells, monocytes, macrophages, dendritic cells, and neutrophils in response to stimulation by *M.tuberculosis* antigens (Sakamoto, 2012; Ma et al., 2015).

(b) Functional characteristics of IL-12

IL-12 induces proliferation of T and NK cells are indicated in figure 9. It also stimulates IFN-γ production and cytotoxic activity. IL-12 plays a crucial role in polarisation of CD4+ T cells to the TH1 phenotype that mediates immunity against intracellular pathogens (Dinarello, 2009; Muraille et al., 2014). The development of Th1 CD4+ T cells is stimulated by IL-12 because it is a potent inducer of IFN-γ production by natural killer (NK) cells and T cells, resulting in enhancement of cell mediated cytotoxicity (Dinarello, 2009; Luckheeram et al., 2012; Vacaflores et al., 2016). IL-12 and IFN-γ conversely antagonise Th2 differentiation and production of IL-4, IL-5 and IL-13 (Abdi & Singh, 2010; Santarlasci et al., 2013; Bao & Reinhardt, 2015).

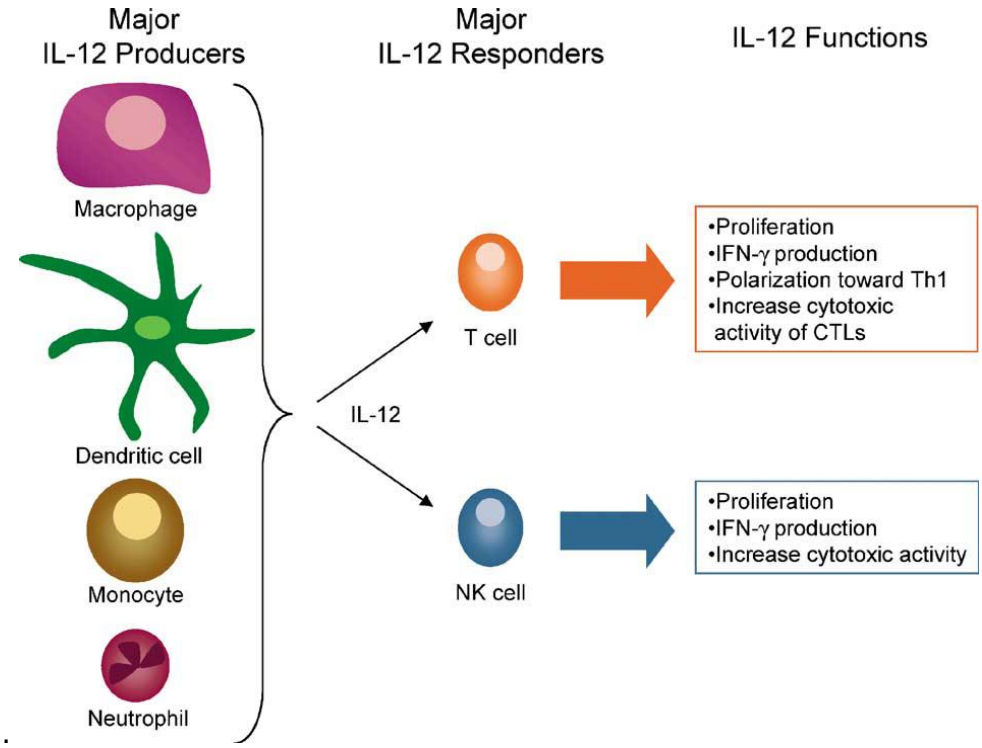


Figure 9: Responders of IL-12 and cellular sources (Wartford et al., 2003)

Bacteria or bacterial products are strong instigators of Th1 CD4+ T cell response because of their ability to stimulate IL-12 production by dendritic cells (Abdi & Singh, 2010; Weyand et al., 2011; Larussa et al., 2015). However, bacterial products can also have opposite effects, for example lipoprotein stimulates the production of IL-12 by macrophages while mannosylated lipoarabinomannans inhibit the production of IL-12 by human dendritic cells (Abdi & Singh, 2010; Kleinnijenhuis et al., 2011; Søndergaard et al., 2014). The major role played by IL-12 in regulation of adaptive immune response and T cell proliferation by inducing naïve CD4+ T cells to induce their differentiation and expansion is indicated in figure 9. Macrophages, dendritic cells, monocytes and neutrophils are the major producers of IL-12. The responders are T cell and NK cell (Wartford et al., 2003; Ma et al., 2015).

2.7.2.4 Interleukin-4

Interleukin-4 (IL-4) was identified in 1982 by Paul, Vitetta, Howard, and colleagues (Martinez et al., 2009; Paul, 2015). IL-4 is a member of short-chain four-helix bundle cytokines that attach to shared receptor subunits, but at amino acid level they exhibit only 25 percent identity; this account significantly in structural differences that influence receptor binding (Dyken & Locksley, 2013; Kuan & Ziegler, 2014). The initial type 2 immunity response consists of different complex orchestration of cytokines and cells, usually taking place at mucosal barriers (Dyken& Locksley, 2013; Bamias et al., 2014). Many molecules and cell types are involved in different aspects of recruitment and activation of adaptive and innate effector cells, however most of these activities are coordinated by IL-4 and IL-13 cytokines which are closely related and play both overlapping and distinct roles in type 2 immunity (Dyken & Locksley, 2013; Coquet et al., 2015).

(a) Production of IL-4

T-helper-2 lymphocytes, basophils, natural killer cells, and mast cells are the producers of a key regulatory cytokine IL-4 (Martinez et al., 2009; O'Brien et al., 2016). Cytokine production profiles during tuberculosis infection showed that IFN-γ was released in individuals with mild disease while individuals at advanced stages of the disease had increased levels of IL-4 and decreased IFN-γ production (Martinez et al., 2009; Ashenafi et al., 2014). Polymorphism disruption in cytokines genes may shift this balance between Th1 and Th2 cytokines genes which will determine the progression and outcome of tuberculosis (Martinez et al., 2009; Longhi et al., 2013; Wang et al., 2015).

(b) Functional characteristics of IL-4

IL-4 together with IL-13 is responsible for sequestration and delivery to lysosomes for degradation of intracellular *M.tuberculosis* (Cooper, 2009; Ma et al., 2013; Duque & Descoteaux, 2014).

IL-4 possesses *in vitro* and *in vivo* anti-inflammatory properties; as such IL-4 prevents IFN-γ production and macrophage activation (George et al., 2000; Wager & Wormley, 2014). It therefore leads to enhancement of differentiation of Th2 cells and minimizes procoagulant activity expression by activated endothelial cells (George et al., 2000; Wager & Wormley, 2014).

2.7.2.5 Interleukin-1β

(a) Production of IL-1β

Interleukin-1β (IL-1β) is released by monocytes, macrophages and dendritic cells and is found in excess at the primary site of tuberculosis infection (Verway et al., 2013; Paolo & Shayakhmetov, 2016).

(b) Functional characteristics of IL- 1β

IL-1 plays an important role in regulating transcription and signal transduction in particular those of Th1 responses. This is evident as differences that occur when autoinflammatory and immunopathologies diseases occur in the suppression of IL-1 beta (Cavalcanti et al., 2013; Sivangala & Sumanlatha, 2015).

2.7.2.6 Interleukin-6 (IL-6)

(a) Production of IL-6

Interleukin-6 is found atthe primary site of tuberculosis infection and has both pro and anti-inflammatory properties (Guo et al., 2012a; Monastero & Pentyala, 2017). When mycobacterial infections occur, IL-6 is produced early to mount an immune response against the invading pathogen, however it can be harmful to the hosts because of its pro-and anti-inflammatory properties which can lead to inhibition of TNF-α and IL-1β production in mycobacterium infections (John, 2012; Landskron et al., 2014).

2.7.2.7 Interleukin-10 (IL-10)

(a) Production of IL-10

Macrophages and T lymphocytes produce IL-10 when infection by *M.tuberculosis* occurs. Interleukin-10 is considered an inhibitory cytokine unlike IFN-γ and TNF-α, playing a role in regulating a balance between immunopathological and inflammatory responses (Cavalcanti et al., 2013; Adrian et al., 2015). In people with severe tuberculosis disease, macrophages have been found to be supressed *in vivo*, however when IL-10 is inhibited this suppression is partially reverted (Cavalcanti et al., 2013; Rajaram et al., 2014).

(b) Functional characteristics of IL-10

Interleukin-10 subdues the Dendritic cells (DC) and macrophage functions, including IL-12 and TNF production required for Th1 responses and killing of intracellular pathogens; therefore in *M.tuberculosis* infection IL-10 may affect the course of the disease (Redford et al., 2010; Cilfone et al., 2015).

2.8 TUBERCULOSIS SPECIFIC ANTIGENS

The subtractive DNA hybridization of pathogenic *M.bovis* bacillus Calmette Guerin strain and DNA micro array analysis of *M.tuberculosis* H37Rv has led to discovery of regions of difference (RDs) (Samten et al., 2009; Prozorov et al., 2013; Solans et al., 2014). *M.tuberculosis* RD1 genome encodes both immunogenic proteins CFP-10 and ESAT-6 which are found to be specific for detection of infection with *M.tuberculosis* (Samten et al., 2009; Bottai et al., 2011; Chegou et al., 2014). The RDs are present in *M.tuberculosis* but absent in all BCG strains and most environmental mycobacteria with the exceptions of the opportunistic pathogen, *Mycobacterium marinum* (Simeone et al., 2009; Ber, 2015). The discovery of these potent T cell sensitizer antigens has altered latent tuberculosis detection and led to a new generation of *M.tuberculosis* specific diagnostic tests (Aichelburg et al., 2009; Hareli et al., 2013; Latorre & Domínguez, 2015). These new specific diagnostic tests

use ELISA to measure the production of IFN-γ concentration in response to ESAT-6 and CPF-10 in supernatant (Aichelburg et al., 2009; Shakak et al., 2013; Yi et al., 2016).

T-lymphocytes strongly recognise ESAT-6, and it is a target for cell-mediated immunity and results in IFN gamma production in people infected with tuberculosis (Kumar et al., 2010; Arlehamn et al., 2014). *M.tuberculosis* secretes these small proteins ESAT-6 and CFP-10 (Yu & Xie, 2012; Nicholson et al., 2015). ESAT-6 is highly conserved among mycobacterium; the identity of *M.leprae, M.smegmatis, M.marinum, M.kansasii,* and *M.bovis* is 38%, 72%, 92, 98% and 100 % respectively (Yu & Xie, 2012; Kumar et al., 2016).

Cryoelectron microscopy has shown that the lysis of red blood cells and macrophages by pore formation in their membranes is caused by ESAT-6. The antigen contributes to the translocation of *M.tuberculosis* from the phagolysosomes to the cytoplasm in myeloid cells and induces apoptosis of macrophages (Kinhikar et al., 2010; Simeone et al., 2015).

2.9 TUBERCULOSIS

Tuberculosis is reported to be responsible for millions of deaths around the world (O'Gara et al., 2013; Khan, 2017). In sub-Saharan Africa the tuberculosis incidence rate was estimated to be twice that of the South-East Asia region with estimation of 350 cases per 100 000 population (WHO, 2010b; Sandhu, 2011; Kyu et al., 2018).

This deadly infectious disease in humans is caused by threetuberculosis complexes:

- *M.tuberculosis*
- *M.bovis*
- *M.africanum*

M.tuberculosis is the main cause of infection in humans with *M.bovis* being the causative agent in cattle (O'Gara et al., 2013; Hlokwe et al., 2017). *M.tuberculosis* is classified as being a highly aerobic bacterium; therefore, it grows well in ventilated places such as in the upper lobes of the lungs (Sakamoto, 2012; Koch & Mizrah, 2018). When conditions are suited such as at 37 degrees Celsius and under optimal

oxygen supply and supply of nutrients, a single bacterium forms white to light-yellow colonies on agar after an incubation period of 3 to 4 as reviewed by Gengenbacher & Kaufmann (2012; Carr, 2017). As compared to other bacteria, the bacterium does not form spores; however, it has the capacity to remain dormant, and a state during which the bacterium still has phenotypic drug resistance as reviewed by Gengenbacher & Kaufmann (2012; Dunn et al., 2016).

Tuberculosis disease in high incidence countries is not easy to control because of several factors which include; the case findings depend on individuals presenting themselves to health care services and serial sputum is required. This results in individuals requiring several visits to the health care services which creates extra costs and prohibits the individual from returning, leading to a significant problem of patient dropout (Parsons et al., 2011; Mhimbira et al., 2017).

Tuberculosis symptoms at the beginning of infection are often nonspecific and include: weight loss, low grade fever accompanied by chills and night sweats, blood tinged sputum, and bad cough that can last three weeks (Knechel, 2009; Fogel, 2015). However, different symptoms will be observed for an individual with bone tuberculosis, thus tuberculosis symptoms vary depending on where the bacilli are growing (Knechel, 2009; Delogu et al., 2013; Turner et al., 2017).

Tuberculosis usually occurs in the lungs; however, the bacterium can spread to other parts of the body. In individuals with compromised or impaired cellular immune systems, the inhaled droplet nuclei reach the alveoli of the lung, multiply and spread through the lymphatic system to the lymph nodes and through the blood stream to other organs (Schutz et al., 2010; Fogel, 2015).

Schutz et al., (2010; Valour et al., 2014) indicated the organs to which tuberculosis can spread, to be:

- Digestive and urogenital tracts
- Skin
- Bones
- Joints
- Nervous system
- Lymph nodes

- Kidneys

This kind of tuberculosis is called extra-pulmonary tuberculosis, and it is far less common as compared to pulmonary tuberculosis (Schutz et al., 2010; Valour et al., 2014).

Tuberculosis in humans follows four basic stages:

- In the first stage after 3 to 8 weeks of *M.tuberculosis* infection, the bacilli becomes implanted in alveoli from where the bacterium is spread by the lymphatic circulation to regional lympnodes in the lung resulting in formation of Ghon complex.
- The next stage which lasts for about 3 months results in the bacterium being carried to other organs of the body, including lungs, by hematogenous circulation which results in formation of other forms of tuberculosis such as meningitis tuberculosis or miliary tuberculosis which leads to fatality.
- During the third stage lasting for about 3 to 7 months, inflammation of the pleural surfaces can occur resulting in severe chest pains.
- In the last stage which is known as latent tuberculosis, the disease does not progress and remains in a latent state and may take up to 3 years for progression to active tuberculosis to occur (Mack et al., 2009; Fogel, 2015).

To treat tuberculosis successfully various factors are considered which include costs, duration of the therapy and drug side effects (Ahmad & Mokaddas, 2010; Shin & Kwon, 2015). The treatment is said to be successful if an individual with tuberculosis presents a negative sputum after 2 months of therapy, while treatment failure is indicated by a positive sputum after 4 months of treatment owing to factors such as incomplete therapy, costs of drugs, multiple drug combinations used in the treatment, adverse reactions relating to drugs and HIV co-infection that leads to persistence of the disease because of acquired resistance (Ahmad & Mokaddas, 2010; Cihlar & Fordyce, 2016).

A new form of tuberculosis which is resistant to conventional drugs has been detected known as Multidrug-resistant TB (MDR-TB) which is estimated to have 500 000 cases each year globally (Daley, 2010; Falzon et al., 2017). At least 1 case of

extensively drug-resistant TB (XDR-TB) has been reported in approximately 57 countries with high rate of tuberculosis (Daley, 2010; Ahmed et al., 2013; Prasad et al., 2017).

2.9.1 Multi and Extensive drug resistant Tuberculosis

Globally shortly after anti-tuberculosis drugs, streptomycin (STR), isoniazid (INH) and para-aminosalicylic (PAS) were introduced, development of resistance by *M.tuberculosis* clinical isolates to the drugs was observed (Johnson et al., 2009; Eldholm et al., 2014). To combat the drug resistant strain of tuberculosis, rifampicin was introduced to be used in combination therapy (Johnson et al., 2009; Field, 2015). The introduction of the drug resulted in a decline in drug resistant and drug susceptible tuberculosis (Johnson et al., 2009; Ahmad & Mokkaddas, 2014).

Ironically the decline in drug resistant tuberculosis led to a decline in funding and tuberculosis interests, therefore no monitoring of drug resistant tuberculosis was done for the past 20 years until the appearance of the HIV/AIDS pandemic (Johnson et al.,2009; Hwang & Keshavjee, 2014). The disease caused the increase in transmission of tuberculosis coupled with outbreaks of multi-drug resistant tuberculosis resistant to INH and rifampicin (Johnson et al., 2009; Cohen et al., 2016). Because of the scourge of HIV infection, simultaneous treatment of tuberculosis and HIV resulted in malabsorption and suboptimal therapeutic blood levels of rifampin (RIF) and INH (Ahmad & Mokaddas, 2010, Ahmad & Mokkaddas, 2014). This combination of drugs increases the chances of formation of multi drug resistant tuberculosis regardless of whether the treatment regime is adhered to or not (Ahmad & Mokaddas, 2010; Nguta et al., 2015). Surveillance of drug resistant tuberculosis was resumed in 1990 in industrialised countries; however, the incidence of drug resistant tuberculosis in the developing world still remains unclear (Johnson et al., 2009; Matteelli et al., 2014).

In developing countries non-adherence to drug regimens has led to the development of multi-drug resistant tuberculosis which is estimated to have had 489 000 new cases occurring in 2006 (Thomas et al., 2010; Kempker et al., 2015). The appearance of drug resistant tuberculosis compromises the success achieved in

treatment of susceptible tuberculosis in sub-Saharan Africa (Thomas et al., 2010; Sloan & Lewis, 2016).

A strain of tuberculosis resistant to the first line of defence of drugs like INH and rifampin and second line injectable agents like amikacin, capreomycin or kanamycin and any fluoroquinolone, is said to be XDR-TB (Thomas et al., 2010; Jnawali & Ryoo, 2013; Varghese & Hajo, 2017).The XDR-TB strain is distributed widely with cases detected in 57 countries worldwide as mentioned (Thomas et al., 2010; Prasad et al., 2017). Transmission of the newly evolved tuberculosis resistant strain into a new host results in acquiring tuberculosis already resistant to first line drugs (Ahmad & Mokaddas, 2010; Muller et al., 2015).

In South Africa the first case of XDR-tuberculosis was reported in 2006 (Abubakar et al., 2013; Chatterjee & Pramanik, 2015). When the spoligotyping was done, it was discovered that since 1994 the majority of cases found were caused by *M.tuberculosis* belonging to the strain found mostly in one province of South Africa, Kwazulu Natal (KZN) ST60 (Stavrum et al., 2009; Phasa et al., 2017).

Located south west of KZN province is the Eastern Cape, another South African province which had tuberculosis cases estimated to have accounted for 705 of 100 000 cases per population in South Africa in 2006 (Kvasnovsky et al.,2011; Nanoo et al., 2015). In 2008 reported tuberculosis cases had changed, Eastern Cape had the highest MDR-tuberculosis caseload in the whole of South Africa (Kvasnovsky et al., 2011; Pietersen et al., 2015).

2.9.2 Latent Tuberculosis

Latent tuberculosis is a state whereby the mycobacterium remains dormant in the host. The bacterium occur low number in this manifestation of tuberculosis; the latent state can continue until there is a weakening of the host's immune system, therefore resulting in the multiplication of the bacterium and manifestation of clinical disease (Mack et al., 2009; Peddireddy et al., 2017). It is estimated that one third of the world population is latently infected with *M.tuberculosis* (Bartalesi et al., 2009; Houben &

Dodd, 2016). A lifetime risk of developing active tuberculosis among this population is 5 to 10% either within 1 to 2 years after primary tuberculosis infection (Bartalesi et al., 2009; Lancella et al., 2015). The available programs worldwide focus on diagnosis and treatment of active tuberculosis to prevent the transmission of tuberculosis; however, most new cases of active tuberculosis have been linked to progression from latent tuberculosis (Lalvani & Pareek, 2009; Andrews et al, 2012; Rakotosamimanana et al., 2015). The linkage was recognized because most of the recorded tuberculosis cases appear once the cellular immune system is compromised. Therefore, the estimated billion people living with latent tuberculosis represent a major barrier to thecomplete elimination of tuberculosis (Rangaka et al., 2015).

Latent tuberculosis is difficult to treat because of several characteristics of the bacillus; the bacterium is within the macrophages therefore not actively dividing. Most bacteria are easily killed when they are actively multiplying by antimicrobial drugs (Hauck et al., 2009; Baky, 2016). In the latent state tuberculosis cannot be effectively treated because the bacterium is not dividing, therefore difficult to detect and quantify (Hauck et al., 2009; Baky, 2016). Despite being in the latent state, these dormant bacilli still trigger a strong cellular response which serves as an amplified marker for the presence of latent tuberculosis infection by releasing small number of active organisms out of granulomas (Ahmad & Mokaddas, 2010; Matty et al., 2015).

Latent tuberculosis risk of progression to become active in children differs from latent tuberculosis in adults; furthermore, the active tuberculosis disease symptoms in children are not specific therefore other childhood illnesses can be mistaken for tuberculosis (Kakkar et al., 2010; Kim et al., 2012; Lancella et al., 2015; Mulenga et al., 2015).

Latent tuberculosis has a high risk of progression to active disease in children with 8.6-10% in children between 0-14 years old and 24 % in children between 1-5 years old, and as high as 40-50% in infants (Alavinia et al., 2012; Carvalho et al., 2018). The progression usually occurs within 12 months of tuberculosis infection. Childhood tuberculosis is usually recent; therefore, the need for immediate diagnosis and

treatment of asymptomatic infection is essential (Getahun et al., 2012; Hamzaoui et al., 2014).

Cells such as Th1-type T cells have been reported to play an essential role in long-term immunity and clinical latency against *M.tuberculosis* (Kim et al., 2013; Lyadova & Panteleev, 2015). Individuals, who lack Th-1 type T cells because of impaired cellular immunity caused by depletion of cells in HIV infection, are vulnerable to tuberculosis infection (Chen & Kolls, 2013; Day et al., 2017). The production of IFN-γ and IL-2 associated with cell mediated immunity and macrophage activation characterises the responses of T-helper cells to HIV/tuberculosis infection (Hanza et al., 2010; Slight et al, 2013; Dobrakowski et al., 2016).

In the host infected with HIV, T helper cell's such as Th1 and Th2 dominance play major roles in effective containment of latent tuberculosis within the granulomas (Veerdink et al., 2009; Chen & Kolls, 2013; Amelio et al., 2017). HIV infection results in T-cell depletion and furthermore leads to switching of Th1 to Th2 dominance; the occurrences result in latent tuberculosis progression to active tuberculosis as illustrated in figure 10 (Matrajt et al., 2014).
Once HIV is taken up through DC-SIGN, the trans-infection of CD4+ T-cells is allowed. This leads to both defects and loss of CD4+ cells which play major roles in effective immune response against *M.tuberculosis.* The role cytokines play in the host infected with HIV in driving the interaction between CD4+ T-cells in infected macrophages, is illustrated in figure 10 (Siawaya et al., 2007; Baxter et al, 2014).

Dendritic cells (DC) which are essential in production of an effective adaptive immune response have the ability to carry antigens to lymphoid tissues and to use a DC-SIGN-dependent for uptake of HIV which facilitates T-cell trans-infection (Tsegaye & Pohimann, 2010; Sandgren et al., 2013; Ahmed et al., 2015). Mycobacteria shed molecules, interacting with DC and macrophages through DC-SIGN, result in secretion of the anti-inflammatory cytokine IL 10, thereby suppressing DC function and maturation and in so doing also minimizing the ability of host's response to invading pathogens as illustrated in figure 10 (Husain & Norazmi, 2013; Victor, 2017).

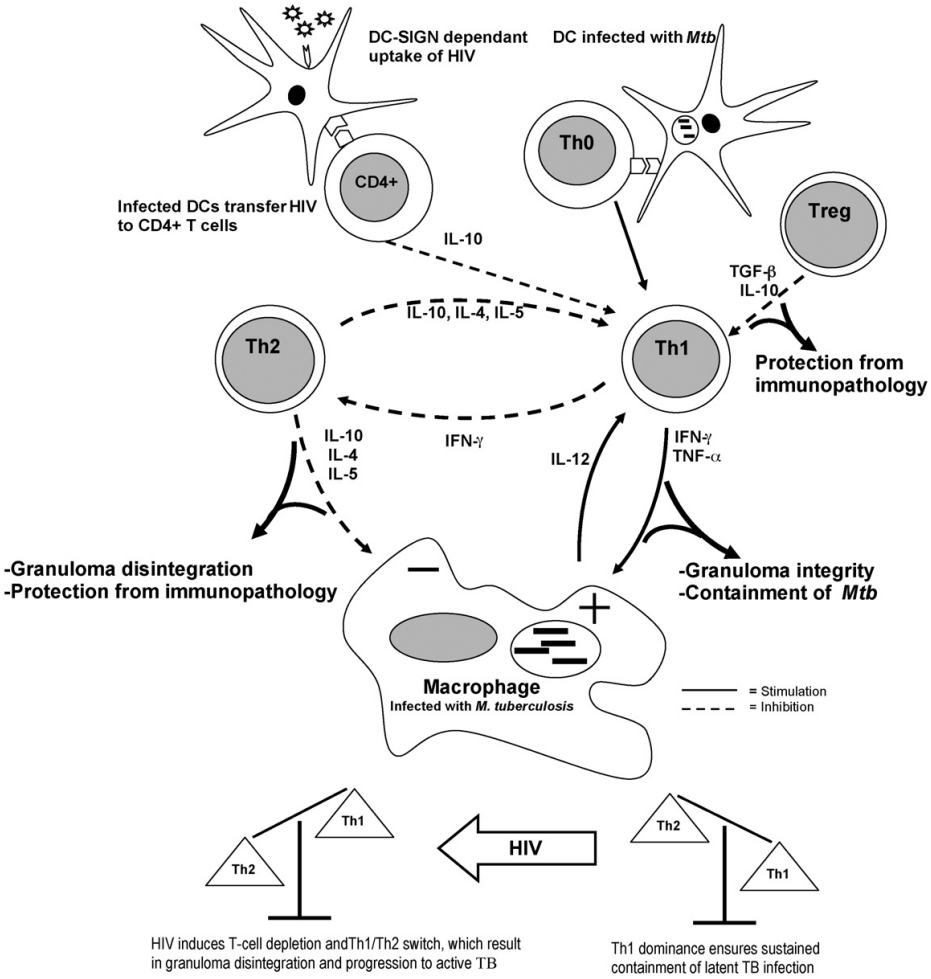


Figure 10: Explanation of immunopathogenisis of latent tuberculosis in TB/HIV co-infected host (Siawaya et al., 2007)

2.10 BCG VACCINATION

Mycobacterium bovis derived BCG vaccine has been used for nearly a century and is still the oldest most widely used vaccine as the preventive method for transmission of pulmonary tuberculosis in adults (Yamamura et al., 2010; Luca & Mihaescu, 2013; Singh et al., 2015). However, the effectiveness of the BCG vaccination in young children against pulmonary tuberculosis is variable (Tameris et al., 2013; Dockrell & Smith, 2017). The vaccine was developed in 1920 by Albert Calmette and Camille Guerin from *M.bovis* (Yamamura et al., 2010; Luca & Mihaescu, 2013; Bottai & Brosch, 2016).

Calmette and Guerin were working with virulent strains of the tubercle bacillus by sub culturing the bacterium and testing the organisms in different culture media. While working they noted that one of the glycerine-bile potato culture mixtures had grown a bacillus that seemed less virulent; with this discovery they changed the course of their research to focus on sub culturing the attenuated strain to consider its use in vaccine development. They continued their research throughout world war one in 1919 until they were satisfied with the results, they found in animal research, showing that the bacteria were unable to cause tuberculosis; the newly developed vaccine was used in humans in 1921 (Chapman et al., 2010; Jivani et al., 2016).

The vaccine is effective in protecting children against tuberculosis but fails to prevent transmission of adult pulmonary tuberculosis disease (Yamamura et al., 2010; Roy et al., 2014). BCG vaccination is estimated to be administered to over 100 million children worldwide per annum (Nuttall & Eley, 2011; Harris et al., 2016). Several trials have been done to assess the efficacy of BCG; these trials concluded that the BCG has 60-80% protective efficacy in children against tuberculosis, in particular in severe forms like meningitis; against pulmonary tuberculosis the vaccine protection varies depending on the geographical area (Roy et al., 2014). The BCG vaccine has been found not to be effective when given to people who are already infected with the environmental mycobacterium, which explains the geographical variation. Furthermore the efficacy of the vaccine is limited by the tests used to assess its efficacy such as TST which suffers from various limitations, including not being able to distinguish the positive results caused by the vaccine from that caused by contact with *M.tuberculosis* (Roy et al., 2014).

The BCG vaccine has a good safety profile with no serious side effects; the complications recorded in vaccinated people are ulceration and lymphadenitis at the site where the vaccine was injected in <1% to 10% of cases (Poudel & Chitlangia, 2014). The vaccine has caused rare deaths in 1 to 20 cases per million doses where the vaccinated individuals developed a BCG disseminated infection called BCGosis. The cases occurred in children who were suffering from severe immunodeficiency disease, who was HIV infected and had chronic granulomatous disease (Poudel & Chitlangia, 2014).

Malnutrition, measles, and HIV infection can cause false positive TST reactions in children who are vaccinated with BCG; therefore, the vaccine is not always the cause of false positive reactions (Ling et al., 2011; Prendergast, 2015). It is estimated that 75% of the 130 million children born in 2002 were vaccinated with BCG worldwide (Hesseling et al., 2009b; Dunem et al., 2015).

South Africa has carried out BCG vaccination in new-borns since 1973 (Hesseling et al., 2009b; Dlamini & Maja, 2016). All HIV infected children born in South Africa receive BCG vaccine which increases the risk for developing the vaccine's side effects such as BCG adenitis, as observed in one study done in the Western Cape, where 6% children enrolled in HIV public programme developing the disease (Hesseling et al., 2009b; Ravie et al., 2011; Gasper et al., 2017).

BCG has been shown to prevent disseminated tuberculosis in HIV- unexposed children, however, BCG has been associated with serious adverse events in HIV Infected infants, Including disseminated BCG disease and BCG immune reconstitution inflammatory syndrome (IRIS) (Blakney et al., 2015). Because of these adverse events WHO considers known HIV infection as a contraindication to BCG Vaccination (Blakney et al., 2015).

2.11 PERFORMANCE OFQUANTIFERON-TB GOLD IN TUBE IN CHILDREN

The QGT-GIT performance in children has not been extensively evaluated but the available data in studies done in children suggest that it performs better than TST (Bua et al., 2013; Pai et al., 2014). Table 1 represents different agreements reported between QFT-GIT and TST in different populations.

Table 1: Agreement between QFT and TST in healthy population with varying risks for LTBI (Pattnaik et al., 2012)

Study/Year	Country	Risk Group	Total participants	BCG Vaccinated (In %)	Actual Agreement (In %)
Brock et al., 2004	Denmark	Contacts of Persons with TB	45	0	93
Pai et al., 2005	India	Health Care Workers	719	71	81
Kang et al., 2005	Korea	Close & casual contacts of persons with TB	120	73	53
Porsa et al., 2006	USA	Prisoners	409	-	89
Ferrara et al.,2006	Italy	Hospitalized adults	286	18	73
Harada et al.,2006	Japan	Health care workers	304	91	13
Dogra et al.,2006	India	Hospitalized children	97	82	94
Mahomed 2006	South Africa	Healthy adults	358	81	68
Tsiouris et al.,2006	South Africa	Paediatric contacts	184	73	78
Lee et al., 2006	Korea	Healthy adults	131	100	74
Nakaoka et al.,2006	Australia	Paediatric contacts	75	49	81
Connel et al.,2006	Australia	Paediatric contacts	75	49	64

The evaluation of the sensitivity and specificity of TST and QFT-GIT is complicated by the absence of reference standards for latent tuberculosis. Furthermore, the limitations of TST are well known in terms of sensitivity and specificity. These limitations may place children at risk of over or under treatment for latent tuberculosis infection as reviewed by Mandalakas et al., (2011; Doan et al., 2017). In the study conducted at Dr. Sani Konuk Training Hospital in Baerirkoy, Turkey, in children aged 3 months to 14 years, the sensitivity and specificity of QFT-GIT was estimated to be 65.85% and 82.14% respectively (Onur et al., 2012). The estimated specificity of 97.8% was reported by Asil and colleagues in BCG vaccinated children aged 1-15 old from 11-day care, 24 primary and junior high schools in Iran (Asl et al., 2015).

Rose and colleagues recruited children less than 15 year of age with culture or chest x-rays confirming tuberculosis and children with no symptoms to assess the specificity and sensitivity of QFT-GIT and TST at Muheza district hospital in Tanzania (Rose, et al., 2012). The sensitivity of QFT-GIT in children confirmed to have TB infection was 19% and 6% respectively. Children with no symptoms QFT-GIT and TST had sensitivity of 10% and 3% respectively (Rose et al., 2012). WHO has reported that in nineteen studies done simultaneously, assessing sensitivity and specificity of QFT-GIT among low- and middle-income settings in 2067children, the pooled sensitivity and specificity of QFT-GIT was 83% and 58% respectively (WHO, 2010a: WHO, 2011; Starke, 2014).

There are few studies that report the data on the performance of QFT-GIT on an age-related performance assessment (Bua et al., 2013; Du et al., 2018). Jenum and colleagues reported that children less than two years of age are prone to developing TB disease, not as a result of reactivation but because of primary infection (Jenum et al., 2014). The indeterminate results of QFT-GIT in children have been seen to be age-related; this creates a concern that age may affect the sensitivity of QFT-GIT in children as reviewed by Herrera et al., (2011; Tebruegge et al., 2014). Topic and colleagues reported that more indeterminate results were found in children less than 3 years old than in older children in a study conducted by Conell et al., (Topic et al., 2011; Mandalakas et al., 2015).

2.12 DIAGNOSIS OF TUBERCULOSIS

With the increase in tuberculosis disease observed in resource poor countries because of synergy of tuberculosis and HIV, the WHO has introduced two programs to improve the sensitivity and turnaround time for diagnosis of tuberculosis. The two introduced programs focus on replacement of the normal microscope with a light-emitting diode (LED) microscope for sputum smear microscopy, and the use of molecular line probe assays to improve the sensitivity and turnaround time for diagnosis of tuberculosis (Parsons et al., 2011; James et al., 2015).

The diagnosis of *M.tuberculosis* is complicated by various characteristics of the bacterium such as: (a) its cell wall has a high lipid concentration which makes it impermeable to stains and dyes, (b) the bacterium grows slowly in comparison with other pathogenic bacteria, (c) and it requires special techniques for microscopic observation (Emovon, 2009; Sia & Wieland, 2011; Liu et al., 2018).
Before tuberculosis can be confirmed, various tests have to be performed to have conclusive information (Narredy & Muthukuru, 2014). The data includes epidemiological materials, radiography information, microbiological data including acid-fast smears, mycobacterial cultures, and more recently results from nucleic acid amplification testing of *M.tuberculosis* (Narredy & Muthukuru, 2014). However conventional methods for laboratory diagnosis of active tuberculosis have limitations: acid-fast staining has limited sensitivity, and cultureis time consuming (Narredy & Muthukuru, 2014). Furthermore, acid-fast staining of bacilli in sputum smears has a low sensitivity in children because more than 10^4 of bacilli per ml of sputum are required (Narredy & Muthukuru, 2014).

Diagnosis of tuberculosis disease in children has several interfering factors. These factors are firstly, HIV epidemic because most lung diseases associated with HIV disease produce the same symptoms as tuberculosis (Tsai et al., 2013; Agyeman & Asenso, 2017). Secondly, methods like radiological testing were not developed for HIV-infected children (Tsai et al., 2013; Hamzaoui et al., 2014). Thirdly, the diagnosis of latent tuberculosis in HIV infected children is difficult because of false negatives in the tuberculin skin test (Druszcynska et al., 2012; Salgame et al., 2015). Fourthly,

difficulties obtaining usable sputum sample in children because infants cannot voluntarily produce sputum, and lastly childhood tuberculosis is normally extrapulmonary or involves exclusively intrathoracic lymph nodes. Invasive procedures are required for acquiring usable samples in children (Graham, 2010; Grant et al., 2012; Elhassan et al., 2016). The tuberculosis detection in children is still based on positive acid-fast stains for confirmation of active tuberculosis (Graham, 2010; Caulfield et al., 2016).

Emovon (2009; Tsai et al., 2016) proposes the following methods expectorating sputum:

- Saline nebulizer sputum transtracheal aspirate which is a test that requires the patients to inhale a nebulised hypertonic saline solution therefore promoting coughing and allowing expectoration of the lungs.
- Bronchoalveolar lavage is a test which uses the insertion of a bronchoscope into the lower part of the respiratory tract and introducing saline into the airways and aspirating the fluid through a suction channel.

These tests are used because most cases of tuberculosis involve pulmonary tuberculosis disease; however, these tests are uncomfortable for children (Emovon, 2009; Tsai et al., 2016). For extra pulmonary tuberculosis which involves various organs of the body, Emovon (2009; Scott et al., 2014) indicated that the following specimens can be used for tuberculosis diagnosis:

- Urine
- Pleural fluid
- Pericardial fluid
- Peritoneal fluid
- Joint aspirate
- Gastric aspirate
- Pentral spinal fluid (CSF)
- Pus
- Blood bone marrow biopsy
- Lymph node,

- Skin and bones depending on tuberculosis clinical presentation

The conventional acid-fast staining method accounts for only 50% case detection in HIV-tuberculosis patients (Rao et al., 2009; Afred et al., 2014). Decreased tuberculin reactivity, atypical radiographic features, and confusion with other HIV-related infections hinder diagnostic methods of tuberculosis in HIV-infected people (Rao et al., 2009; Padmapriyadarsini et al., 2011; Cudahy, 2016).

The diagnosis of latent tuberculosis has changed a little during the past century; reliable diagnosis of latent tuberculosis is complicated by absence of a practical gold standard (McNerney et al., 2012; Aggerbeck et al., 2018). However new diagnostic methods have been proposed that use nucleic acid amplification and IFN gamma to detect *M.tuberculosis* complex. These diagnostic tests may provide quick and specific results for identifying the bacterium (McNerney et al., 2012; Lee et al., 2016).

2.12.1 Tuberculin Skin Testing

The tuberculin skin test has been the method of choice used to diagnose latent *M.tuberculosis* infection for almost a century (McNerney et al., 2012; O'Shea et al., 2014). The test is relatively easy to use, and it is inexpensive; however, this test has several important limitations: firstly, BCG vaccinated people and people infected with non-tuberculosis mycobacteria can cause falsely positive TST results, because the antigens used for the TST are also present in the BCG vaccine and non-tuberculosis mycobacteria (Guillen, 2011; Seddon et al., 2016). Secondly, the TST has a relatively poor sensitivity, especially in people on chemotherapy, steroids, and individuals who are cellular immune system compromised such as in HIV infection, at the extremes of age, and with advanced tuberculosis disease (Bakir et al., 2009; Sharma et al., 2012; Lin et al., 2016). Lastly, the TST requires two patient-provider interactions, one to administer the test and another to inspect the forearm within a certain timeframe in order to determine the test result (Abdalhamid et al., 2010; Mancuso et al., 2011; Baker et al., 2017).

The TST is an intradermal injection mixture of Purified Protein Derivative (PPD), a crude mixture of *M.tuberculosis* proteins, and it measures a delayed-type

hypersensitivity (DTH) reaction two to four weeks after tuberculosis infection (Yew & Leung, 2007; Guillen, 2011; Mon et al., 2014). Diagrammatic representation of TST for diagnosing *M.tuberculosis* is indicated in figure 11. The PPD is intradermally injected into the volar surface of the forearm and induration of any delayed-type hypersensitivity response is measured 72h later (Lalvani, 2007; Hoft et al., 2018).

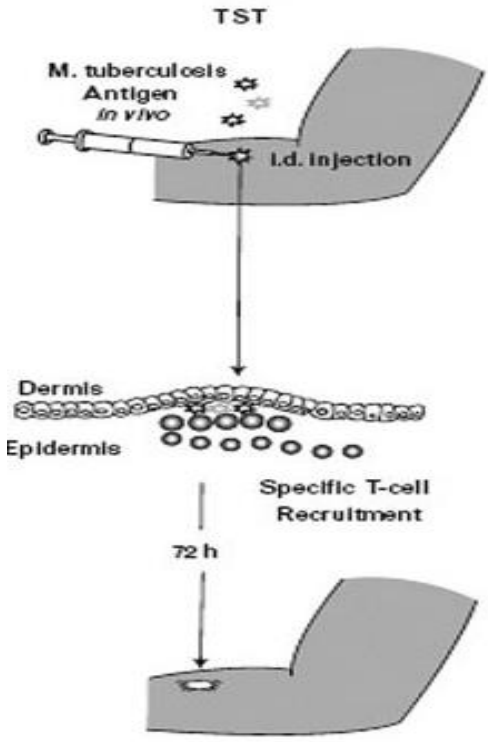


Figure 11: TST tests for diagnosis of latent tuberculosis (Lalvani, 2007)

In a study conducted in India, children under the age of four years showing signs of malnutrition, when TST was performed for diagnosis of latent tuberculosis, results were non-reactive even when apparent signs of progression to active tuberculosis were observed indicating that the tests were ineffective in some groups (Dheda et al., 2005; Jenum et al., 2014). Other than its limitation because of cross reactivity with BCG, other external factors which influence the TST tests are: the interpretation of the results, as it depends on human discretion on reading the diameter, and the need for a return visit to have the tests read (Tsara et al., 2009; Lobue & Castro, 2012; Zenner et al., 2017).

Despite having several shortcomings like inability to distinguish between latent and active tuberculosis, the tests are clinically useful and lead to the prevention means to

be taken against tuberculosis infection in individuals with positive tests (Hauck et al., 2009; Roy et al., 2014). The cut-off point between 5 to 10 mm has regularly been employed as a positive result in diagnosing latent tuberculosis (Orainey, 2009; Moon & Hur, 2013; Janagond et al., 2017). The actual sensitivity of the tests is not known because of the absence of a gold standard in diagnosing latent tuberculosis infection (Orainey, 2009; Moon & Hur, 2013; Aggerbeck et al., 2018).

2.12.2 Direct Microscopy Examination

Tuberculosis is traditionally detected by smear microscopy from an individual who has suspected active tuberculosis infection with Ziehl-Neelsen (ZN) acid fast stain (Wilson, 2011; Ratsavong et al., 2017). Diagnosis of tuberculosis infection by microscope is still used as a diagnostic tool in most countries with limited resources. It is used as the first line test, and it can be performed on simple microscopes (Wilson, 2011; Singhal et al., 2015). The test has several advantages when compared to new methods such as: it is less expensive as compared to new methods, it requires few resources to introduce the method and lastly the test has been in use in many laboratories, therefore the infrastructure is already in place (Wilson, 2011; Alfred et al., 2014).

However, several disadvantages have been recorded: the procedure is labour intensive, it is less sensitive about 30-70% than culturing, the detection limit is about 5000-10 000 acid fast bacilli per ml of sputum, 3 sputa must be collected, dark room is needed, and an expensive light source microscope (Emovon, 2009; Madico et al., 2016). Mercury vapour light used in this microscopy can be hazardous if the bulb is broken (Emovon, 2009; Nance et al., 2012; Baird et al., 2014). Furthermore, the sputum smear sensitivity varies because it is dependent on the diligence taken when collecting the specimen, how the smears were prepared, and examination of the smears (Parsons et al., 2011; Anyim et al., 2014). Tuberculosis smear microscopy is highly insensitive in children because of low bacillary loads in the sputum (Parsons et al., 2011; Lu et al., 2017).

2.12.3 Fluorescent light emitting diode (LED) Microscopy

Because of the limitations of conventional light microscopy using ZN stain, such in that fluorescent microscope are expensive, the bulbs blow and are expensive to replace, and they use a fair amount of electricity. WHO has recommended the phasing in of LED microscope which has an improved sensitivity and takes less time to perform, to replace the conventional fluorescence microscopy (Wilson, 2011; Imaz et al., 2017). A sputum smear containing *M.tuberculosis* as seen under LED microscope fluorescence is indicated in figure 12, glowing light green.

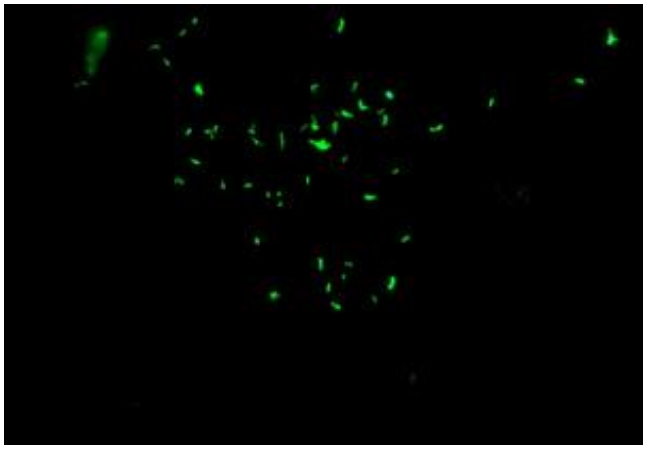


Figure 12: *M.tuberculosis* fluorescence positive (Wilson, 2011)

WHO has opted for the use of the LED microscope because it is easier to improve on the existing technology than developing newer technology, for example smear microscopy is widely used for diagnosis of tuberculosis therefore infrastructure is already available for performing the LED test (Bosse et al., 2015). Secondly, introducing LED tests will require fewer resources than introducing a new technology. Thirdly the LED microscope would likely be less expensive than new technologies therefore will be used even in poor resource countries (Bosse et al., 2015. Lastly, since the light microscope has been in use in many laboratories, health care providers are familiar with a microscope and know the limitation and strengths of the tests and how to interpret the results (Wilson, 2011; Coulibaly et al., 2016).

2.12.4 Culture of *Mycobacterium tuberculosis*

Diagnosis of tuberculosis using culture media such as Ogawa and Lowenstein Jensen (LJ) is used for active pulmonary tuberculosis (Soto et al., 2009; Rueda et al., 2015). Globally most cases of tuberculosis are diagnosed by a direct examination of sputum, however culture diagnosis is used to support the results as the tests are more sensitive and are able to detect 10-1000 viable organisms per ml of specimen (Khechine & Drancourt, 2011; Madico et al., 2016).

Culturing *M.tuberculosis* is based on re-examination of positive acid-fast-stained sputum by microscopy and growth on agar-based media such as Lowenstein Jensen which is used as a standard media for culturing *M.tuberculosis* (Khechine & Drancourt, 2011; Asmar et al., 2015). The test has the advantage of low cost; however, the test has its limitations, and most notable being the time it takes to get definitive results (Khechine & Drancourt, 2011; Caulfield & Wengenack, 2016). Detectable growth can take up to four to eight weeks of culturing (Garberi et al., 2011; Caulfield & Wengenack, 2016).

The mycobacterial growth indicator tube (MGIT) system is part of the new-generation of rapid tests introduced for the detection of mycobacteria. The test is based on using fluorescence detection of mycobacterial growth in a tube containing a fluorescence quenching-base oxygen sensor together with a modified Middlebook 7H9 medium (Hasan et al., 2013; Wescott et al., 2017). The MGIT automated system has the advantage of maximum recovery of mycobacteria, furthermore the MGIT 960 system has more than 900 samples in one instrument, it is easy to interpret the results and it is rapid (it takes 4-13 days) to have results (Hasan et al., 2013; Mahomed et al., 2017). The disadvantages of the Bactec MGIT 960 system are the required labour-intensive biochemical methods for species identification in positive cultures (Lu et al., 2011; Mahomed et al., 2017).

2.12.5 Microscopic observation drug susceptibility assay

Microscopic observation drug susceptibility (MODS) assay is a recently developed method which uses broth microtiter plates designed to detect *M.tuberculosis* complex from sputum specimens (Ha et al., 2009; Kirwan et al., 2016). The method is based on liquid culture which detects growing, living mycobacteria based on distinctive characteristics and morphology of *M.tuberculosis* in liquid medium (Ha et al., 2009; Alva et al., 2013; Pinhata et al., 2017).*M.tuberculosis* in liquid culture appears as cording, strings or tangles and grows faster in liquid medium than in solid medium (Ha et al., 2009; Brum et al., 2016). *M.tuberculosis* grows faster in liquid medium therefore the inhibition of growth in wells containing drugs, indicates the resistance of the drugs, however the liquid medium has an increased risk of contamination (Wilson, 2011; Jung et al., 2018).

The test is used to detect the resistance of *M.tuberculosis* to first line of defence drugs, rifampin and isoniazid. The assay uses microtitre plates containing Middlebrook 7H9 broth medium, antimicrobial agents and growth supplements (Wilson, 2011; Sieniawska et al., 2018). Anti-tuberculosis drugs are added in wells at different concentrations and inoculated with the clinical sample, sealed and examined periodically for growth (Wilson, 2011; Fowler et al., 2017). *M.tuberculosis* appears as cordlike tangles in wells not inoculated with antibiotics under a microscope. Samples positive for drug susceptibility for *M.tuberculosis* complex are indicated by formation of cording as indicated in figure 13 (Caviedes & Moore, 2007; Sanogo et al., 2017).

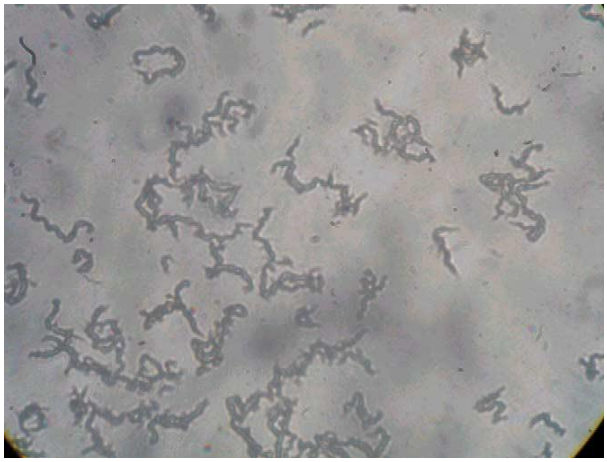


Figure 13: *M.tuberculosis* in MODS at 40X magnification (Caviedes & Moore, 2007)

2.12.6 MDR-XDR tuberculosis Colour Test

The test in principle is based on visually observing colour changes in 4 quadrants of an agar plate, caused by growing tubercle bacilli on Thin-Layer Agar (TLA) medium (Wilson, 2011; Fox et al., 2017). The performance characteristics of the MDR-XDR tuberculosis tests is limited, however available data suggest the tests have high sensitivity and specificity for drug resistance detection and the contamination rate appears to be lower than that of liquid and solid media (Wilson, 2011; Zhang et al., 2017). *M.tuberculosis,* when grown in media containing antibiotics only grew in the clear quadrant which servers as positive control (red dots) but was inhibited in coloured antibiotic containing quadrants as indicated in figure 14.

Figure 14: MDR-XDR tuberculosis colour tests for drug susceptible tuberculosis (Finddiagnostics, 2012)

2.12.7 Molecular tests

2.12.7.1 Conventional polymerase chain reaction

The detection of mutated genes is very important in diagnostic area in congeal and metabolic disease (Hakeem et al., 2013; Tysarowska et al., 2015). Currently three tests have been proposed to improve the sensitivity and diagnosis of pulmonary tuberculosis by directly amplifying *M.tuberculosis* DNA from sputum samples. They are: Amplified *M.tuberculosis* Direct tests (Gen-Probe), the BD ProbeTec ET Direct tuberculosis assay (Beckton Dickinson Diagnostics) and the Amplicor *M.tuberculosis* polymerase chain reaction (PCR) assays (Roche Molecular Systems). These tests are available for direct detection of *M.tuberculosis* in clinical specimens (Davis et al., 2009; Linasmita et al., 2012; Tarhan, 2018).

Even with the introduction of these three technologically advanced tests, time still remains a limitation with the tests requiring a minimum of 2 to 5 days to reliably give a definitive diagnosis of pulmonary tuberculosis (Alcaide & Coll, 2011; Oommen & Banaji, 2017). The most promising assay is the polymerase chain reaction (PCR) test. The test allows amplification of target DNA or RNA molecules exponentially (Alcaide & Coll, 2011; Pathak & Das, 2015). Clinically the tests promise a great success in diagnosis of tuberculosis by detecting and identifying fastidious or slow growing organisms such as mycobacteria directly from the clinical specimen and amplifying the DNA (Alcaide & Coll, 2011; Shrivastava et al., 2017).
One of the conventional testsavailable is the commercial kit known as the amplicor. The test amplifies a specific segment of the 16s rRNA gene, followed by hybridisation and colorometric detection (Alcaide & Coll, 2011; Pickering et al., 2018).

2.12.7.2 Real-time polymerase chain reaction

Real-time PCR is based on simultaneous amplification and fluoremetric detection by labelled probes of different DNA targets (Alcaide & Coll, 2011; Gupte & Kaur, 2016). Real time PCR has several advantages including the fact that DNA extractions occur

in single tubes, thus it minimizes the chances of cross-contamination (Alcaide & Coll, 2011; Kalle et al., 2014). PCR has been shown to be the more reliable method as a diagnostic test (Leeuwen et al., 2007; Gholoobi et al., 2014). The highly sensitivity of PCR can be its major disadvantage because any form of contamination of the sample by even trace amounts of DNA can produce misleading results (Garibyan & Avashia, 2014).

2.12.7.3 The GeneXpert Automated System

The Gene Xpert test is a semi-quantitative nested real-time PCR in-vitro diagnostic tests used for the detection of rifampin resistance associated mutations of the *rpoB*gene in samples collected from patients to rifampicin. The test is also used for the detection of *M.tuberculosis* complex DNA in sputum samples or sputa that is either AFB smear negative or positive (Hakeem et al., 2013; Atashi et al., 2017).
The Gene Xpert has the following advantages: it is more sensitive than sputum smear microscopy in detecting TB; furthermore, it has similar accuracy as culture. Xpert is very important because it can detect RIF-resistant TB in less than 2 hours. Disadvantage of Gene Xpert is that its cost is very high; it costs more than smear microscopy. The cost is roughly US$17,000 and it uses cartridges that cost US$9.98 for each test. This does not include the running costs and human resource as reviewed by Piatek et al., 2013; Kendall et al., 2017).

2.12.8 Loop-mediated isothermal amplification

Molecular techniques show promising results in rapid detection of tuberculosis; however, these methods are technically difficult and require considerable expertise, require expensive equipment and a huge amount of space is needed (Nagdev et al., 2011; Purohit & Mustafa, 2015). To deal with these limitations of current molecular methods, a new molecular test called Loop-mediated isothermal amplification (LAMP) was developed. The method has been shown to be highly specific and sensitive, owing to the six primer pairs used in the tests which recognize eight distinct regions in the DNA of concern (Nagdev et al., 2011; Sahoo et al., 2016).

The DNA of interest is amplified by LAMP with high efficiency under isothermal conditions using six or eight primers. The process results in the creation of large amounts of DNA and the high specificity of the reaction makes it possible to detect amplification by visually inspecting fluorescence or turbidity without the need for gel electrophoresis as indicated in figure 15 (Boehme et al.,2007; Karthik et al., 2014). The test has advantages because it uses a closed-tube system; therefore, it minimizes the risk of workspace contamination with amplicon (Boehme et al., 2007; Karthik et al., 2014).

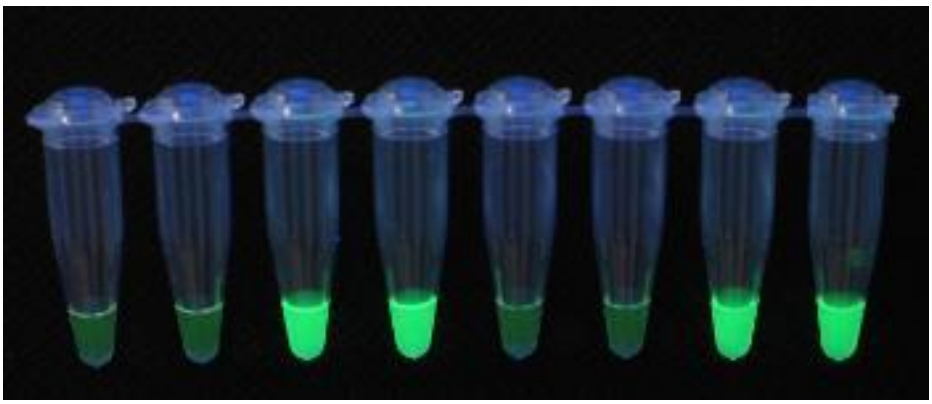


Figure 15: LAMP assay (Boehme et al., 2007)

2.12.9 Enzyme-linked immunospot tests

The greatest advances in detection of latent tuberculosis infection were made in the recent years with discovery of antigens encoded in the RD1 region, ESAT-6 and CFP-10 of the genomic segment of *M.tuberculosis.* The discovery has led to improvement in diagnosis of latent tuberculosis (Druszcynska et al., 2012; Petruccioli et al., 2016).

The discovery of ESAT-6 and CFP-10 antigens has resulted in the creation of diagnostic tests based on the stimulation of sensitized T-lymphocytes followed by measurements of the released gamma interferon either by ELISPOT or ELISA (Nafae et al., 2013; Lange et al., 2017). These ESAT-6 and CFP-10 antigens elicit strong, specific T-cell responses because these antigens are targets of Th1 T-cells in *M.tuberculosis* disease. The antigens are not present in non-tuberculosis

mycobacteria and all strains of BCG with exemption of *M.szulgai, M.marinum, M.kansai, M.flavescens* and *M.gastrii* (Lalvani & Pareek, 2009; Ru et al., 2017).
The ELISPOT assay was developed in the 1990s as a diagnostic tool for detecting latent tuberculosis as reviewed by Nafae et al., (2013; Lima et al., 2017). The test is based on counting individual antigen specific T cells. When the T cells from a person with *M.tuberculosis* encounter two antigens ESAT-6 and CFP-10 *in vivo*, they become sensitized; when the T cells re-encounter these antigens *ex vivo* in the overnight incubation, they release the cytokine IFN-γ (Lalvani, 2007; Vidal et al., 2014). After the 24 hours incubation the T cells produce IFN-γwhich is then detected with an antibody-enzyme conjugate in conjunction with a chromogenic substrate to produce a dark spot, which is a trail of an individual *M.tuberculosis*-specific T cell (Segall & Covic, 2010; Jacobs et al., 2014).

ELISPOT assay has advantagesinthat it directly measures the response of the Th1 cell mediated immune response, however the disadvantage is that the blood has to be used within 12 hours after collection and special expertise is required in isolation of the white blood cells from the blood (Lalvani, 2007; Horvat, 2015).

Diagrammatic representation of ELISPOT and ELISA for diagnosing *M.tuberculosis*: ELISPOT is illustrated in figure 16 (top to bottom): separation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation from blood takes place, cells are washed, counted and incubated with *M.tuberculosis* ESAT-6 and CFP-10 antigens in a 96-well ELISPOT microtitier plates for 16 to 20 hours. T cells are sensitised by antigens to secrete IFN. The visible spots counted each represents the footprint of T cell that responded (Lalvanni, 2007).

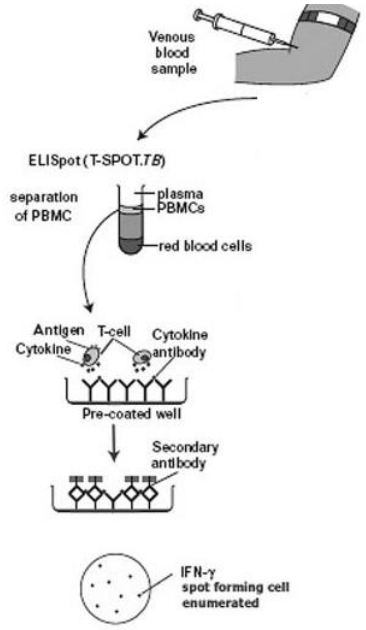


Figure 16: ELISPOT and ELISA for diagnosing *M.tuberculosis* (Lalvanni, 2007)

2.12.10 QuantiFERON-TB Gold In Tube

The QuantiFERON-TB Gold In Tube (Cellestis limited, Carnegie, Australia) assay and ELISPOT (Oxford immunotec, Oxford, UK) are recent commercially available tests that detect IFN-γ producing T-cells (Achkar et al., 2010; Riaze et al., 2012; Smit et al., 2016). The QuantiFERON-TB Gold In Tube and ELISPOT are designed to replace TST. The tests operate on T-cell based approaches since tuberculosis has been shown to evoke a strong T-helper 1 type cell-mediated immune response. During infection IFN-γ is released from *M.tuberculosis* specific T-cells and used as a marker of infection (Achkar et al., 2010; Mzinza et al., 2015).

A QuantiFERON® TB-Gold test uses whole blood incubated with CFP-10 and ESAT-6 antigens in a 24-well plate for 24 hours. If the individual being tested has tuberculosis, the T cells will recognize the recombinantantigens and secrete IFN gamma (Lalvanni, 2007; Wlodarczyk et al., 2014). Centrifugation of the plasma is performed to harvest the plasma. Antibodies specific for IFN-γ are coated in the bottom of each well. Detection of cytokine-bound antibodies is detected by the use of an antibody conjugated to enzymes that catalyses a colorimetric reaction. In each well, released IFN-γ concentration is measured using optical density and standard curve plotted as illustrated in figure 17 (Lalvanni 2007).

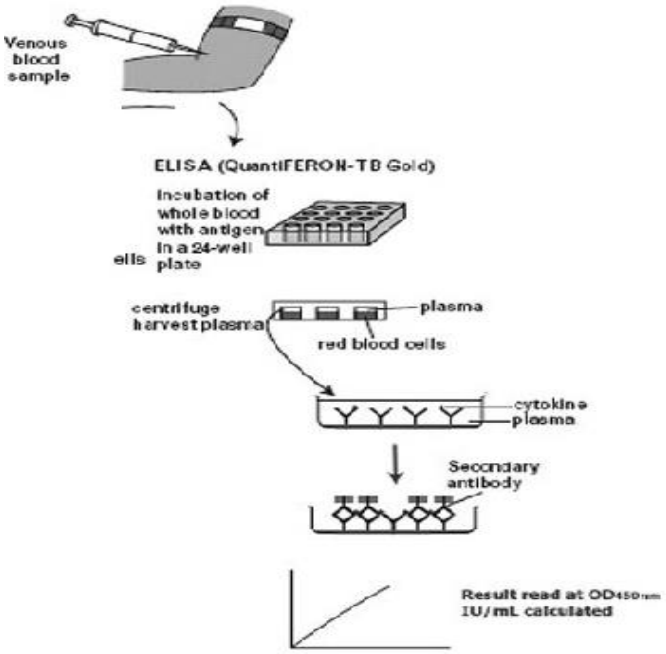


Figure 17: QuantiFERON®TB-Gold tests (Lalvanni, 2007)

The QuantiFERON® TB-Gold test in principle is based on that, fixed blood volume of whole blood is incubated overnight with *M.tuberculosis* specific antigens ESAT-6 and CFP-10. The antigens will cause stimulation of sensitized T lymphocytes resulting in IFN-gamma production, followed by measurements of released IFN-γ using ELISA (Helmy et al., 2012; Zhao et al., 2017). The test has several advantages as compared to TST such as the need for fewer patient visits, avoidance of subjective readings, and the ability to perform serial testing (Helmy et al., 2012; Zhao et al., 2017). A major limitation is the higher material costs (especially in developing countries) of the tests kits and the venous blood needed for their use in young children (Helmy et al., 2012; Zhou et al., 2014).

2.12.11 Radiography

The chest X-ray examination remains a key tool for clinical detection of tuberculosis, however its low specificity and poor film quality in poor resource setting can lead to over-diagnosis (Waitt et al., 2013; MacPherson et al., 2018). As a result, to confirm pulmonary tuberculosis, respiratory samples (expectorated sputum) are submitted to

a bacteriological laboratory for microscopic examination and for mycobacterial culture to support the X-ray results (Hoog et al., 2011; Ryu, 2015).

The tests use radiographs to show enlargement of hilar, mediastinal, or sub carinal lymph nodes and lung parenchyma changes (Khan & Starke, 1995; Bhalla et al., 2015). The test shows abnormalities in airways caused by obstruction and resulting from enlargement of intra thoracic nodes. Young children rarely have cavitation; consequently, the test has limitations in children for detection of tuberculosis (Khan & Starke, 1995; Graham et al., 2015).

2.13 CONCLUSION

Tuberculosis diagnosis in children still remains a challenge which requires urgent attention. The development of IFN gamma assays for diagnosis in children shows a promise however the performance of these tests in paediatrics still remains a subject that requires further study. The information that is available from the limited published studies shows the tests may have an increased sensitivity, specificity and are feasible in the clinical environment in evaluating latent tuberculosis in children. The study aims to add more information on the use of IFN gamma assays in children and the performance of the assay will aid in allowing detection of those children who are truly infected and therefore may benefit from further evaluation and treatment.

CHAPTER 3
METHODOLOGY

3.1 INTRODUCTION

The limitations of the TST to diagnose latent tuberculosis place a burden on developing world healthcare systems (Al-Orainey, 2009; Kasprowicz et al., 2011; Park et al., 2018). The introduction of blood-based tests such as QGT-GIT has provided a new opportunity for diagnosis of latent tuberculosis (Parkash et al., 2009; Banaei et al., 2016).

This study will aim to improve the understanding of prevalence of tuberculosis infection among BCG-vaccinated children who are household contacts of adults with pulmonary tuberculosis using the QuantiFERON-TB In Tube test.

3.2 ETHICAL CONSIDERATION

The study protocol and informed consent documents for both the principal and sub-study were approved by the IRBs/Ethics Committees of University of Witwatersrand (Johannesburg, South Africa, approved on the 03 September 2007, protocol M070121), and Johns Hopkins University, (Baltimore, Maryland, USA).
All procedures conducted during this study adhered to the declaration of Helsinki (2008) and protection of human subjects (21 CFR 50) as well as the Health Profession Council of South Africa (HPCSA) guidelines. Codes of conduct were extended by adhering to the Medical Research Council (MRC) of South Africa guidelines. Any amendments to the protocol or consent materials were reviewed and approved by the IRC before they were implemented (Annexure 1).

3.3 STUDY DESIGN

This was a cross sectional observational study to evaluate the performance of a newly introduced T-cell based test known as QuantiFERON-TB In Tube, for diagnosis of tuberculosis infection in children of adults with pulmonary tuberculosis,

and to determine the transmission rate and prevalence of *M. tuberculosis* among these children. The study was carried out in Chris Hani hospital in Soweto.

Adults with pulmonary tuberculosis were recruited by referral from the nurses once a parent had a confirmed tuberculosis result from Chris Hani Hospital in-patient wards, as well as from a local Mofolo community clinic. Adults were asked to provide information about their children and potential TB exposure of their children. The children were tested for *M. tuberculosis* infection with the QFT-GIT and the conventional tuberculin skin test.

3.4 ENROLLMENT AND SELECTION OF STUDY PARTICIPANTS

3.4.1 Adult Cases

Inclusion criteria were as follows:

- Age > 18 years
- Diagnosis of pulmonary TB within the preceding 3 months. Diagnosis can be based on microbiologic tests, or histopathology, or clinician diagnosis, or any combination of these methods. Pleural TB alone does not meet the inclusion criterion.
- Availability of chest radiograph (film or written report) performed within one month prior to or 2 weeks following TB diagnosis
- Presence, in the home environment, of at least one child (age ≥ 6 months and ≤ 16 years, for whom the adult is the biologic parent or legal guardian)
- Willingness to have TB contact investigation performed for child/children (age ≥ 6 months and ≤ 16 years, for whom the adult is the biologic parent or legal guardian) in the home environment
- Willingness for child/children (age ≥ 6 months and ≤ 16 years, for whom the adult is the biologic parent or legal guardian) in the home environment to be tested for HIV infection (unless written results are available for a negative test performed within the preceding 12 months or less, or a positive test performed anytime)
- Informed consent

Exclusion Criteria:

- Incarcerated or institutionalized
- Inability to provide informed consent
- Children whom diagnosis of active TB disease, treatment for active TB disease, diagnosis of latent *M. tuberculosis* infection, or treatment for latent *M. tuberculosis* infection at any time prior to TB diagnosis in the corresponding adult case

3.4.2 Paediatric Contacts

Inclusion Criteria:

- age ≥ 6 months to ≤ 16 years
- for children aged 7 years or older, consent was needed from adult to participate in the study

3.5 SAMPLING STRATEGY

It was estimated based on previous routine recordings of patient attendance at Chris Hani Hospital and Mofolo clinic that the prevalence of *M.tuberculosis* in children was 0.40. A sample size of 164 children was required to estimate a 95% confidence interval for the prevalence of 0.40 ± 0.075. To account for potential clustering, the number was increased by 50%, to 246. The sample size was increased 10%, to 273 children was done to allow for a 10% dropout (TST not read, blood not able to be drawn).

3.5.1 Internal validity

To avoid discrepancies and variation in TST readings, one trained staff phlebotomist was appointed to administer the tuberculin to the children and to measure TST reading. Standard procedures supplied by the manufacturer were followed exactly for clinical and laboratory procedures.

3.5.2 External validity

The tubes were transported within the required time frames which were within 16 hours after collectionand the blood tubes were stored at room temperature which was the proposed temperature according to the manufacturer package insert.
Only tubes supplied with the QuantiFERON®-TB Gold kit were used for blood collection and incubation.

3.6 TUBERCULIN SKIN TESTING

3.6.1 Principle

As discussed in 2.12.1 the tuberculin skin test is based on intradermal injecting intermediate strength purified derivative protein and measuring the delayed-type hypersensitivity responses 48 to 72 hours (Yew & Leung, 2007; Nayak & Achariya, 2012; Lewinsohn et al., 2017).

i. Materials provided

1. Purified Protein Derivative ampule

ii. Materials required but not provided

1. Sterile syringes
2. Sterile needles

3.6.2 Procedure

The tuberculin test was performed by a trained and registered phlebotomist by intradermal injecting 0.1ml (2 TU) of intermediate-strength purified protein derivative (PPD; Biovac SA, Manchester RD, Wadeville) using a sterile, new needle and syringe, into the dorsal side of the forearm. Transverse indurations at the TST site of injection were measured after 48-72 hours by the trained phlebotomist in evaluation

of Mantoux. Indurations greater than 5mm were regarded as a positive result and less than 5mm as negative as per the manufacturer's recommendation.

3.7 BLOOD COLLECTION

The children were brought by parents to the respiratory and meningial pathogen research unit (RMPRU) clinic and the TST administered 72 hours earlier was read. In pre-analytical procedure the following was adhered to make sure quality assurance was followed when collecting blood:

- Only tubes supplied by the manufacturer were used and consisted of TB Antigen (red cap), Mitogen (purple cap) and Nil control (grey cap). These tubes contained dried antigens coated on the inner wall therefore after blood collection, the tubes needed to be mixed thoroughly.
- To ensure sufficient volume required was collected, the tubes were filled to the black mark on the outside of the tube.
- After the blood was collected in the tubes, the samples were transported at room temperature (no refrigeration).
- Blood tubes were incubated upright at 37 degrees Celsius for 16 to 24 hours within 16 hours after collection.
- After incubation, the tubes were centrifuged to separate cells from plasma with the help of the gel plug in the tubes.
- Care was taken not to disturb the gel, only plasma was harvested.

The sample tubes were labelled with participant's study number and date of collection. Despite the manufactures' recommendation to collect 1ml in each reduced vacuum tube directly from the patient, to prevent significant discomfort in the child participants associated with this procedure, a single 5ml syringe was used to collect 3ml of blood which was then immediately distributed equally between the QFT-GIT tubes. Each tube was inverted ten times to mix the blood with antigens and transported at room temperature within 16 hours to RMPRU laboratory in protective Styrofoam boxes to prevent breakage or contamination. The QFT-GIT tubes were incubated upright at 37 degrees Celsius for 16-20 hours. After the incubation, the tubes were centrifuged at 2000 rcf for 15 minutes and the plasma aliquoted into

labelled cryotubes vials and stored at - 20 degrees Celsius until the assay was performed.

3.8 QUANTIFERON-TB TEST

3.8.1 Principle

QuantiFERON-TB gold in-tube (QFT-GIT) Test detects the in vitro cell-mediated immune response to *M. tuberculosis* with the method of enzyme-linkedimmunosorbent assay (ELISA) by using early secretory antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB 7.7 as reviewed by Karima et al., (2009; Asl et al., 2015).

i. Materials provided

1. Microplate strips
2. Nil control, TB antigen and mitogen control tubes
3. Diluent, Lyophilized conjugate (100X concentration)
4. Lyophilised Human IFN-γ standard
5. Wash buffer (20X concentration)
6. Enzyme substrate and enzyme stopping solution

II. Materials required but not provided

1. 37 degrees Celsius incubator, calibrated pipettes
2. Microplate shaker, washer and reader

3.8.2 QuantiFERON®-TB Gold Validation

Specificity QuantiFERON®-TB Gold kit

A study involving 866 participants was carried out in United State of America (USA) to assess the specificity of QuantiFERON®-TB Gold kit. Blood was drawn for

QuantiFERON®-TB Gold and TST was performed. Risk factors for TB and demographic information were collected and determined using a standard survey at the time of sample collection and testing.

Out of 866 participants, 432 had no known risks factors of *M.tuberculosis* infection, and determinate results were available for 391 participants for both QuantiFERON®-TB Gold and TST. None were BCG vaccinated. Another study was done in Japan to support the specificity of the QuantiFERON®-TB Gold in participants with low risks of *M.tuberculosis* infection, approximately 90% of study participants had received BCG vaccination. The results obtained from both specificity studies are indicated in table 2 (CELLESTIS LIMITED QuantiFERON®-TB Gold insert package).

Table 2. Validation of QuantiFERON®-TB Gold specificity in participants with no reported risk of *M.tuberculosis* infection

Study	BCG Status % Vaccinated	Total tested	No.QFT-G Indeterminate	No.QFT-G Positive/No Valid Tests	QFT-G Specificity (95%CI)	No.TST Positive/ No. Tested	TST Specificity (95% CI)
USA (unpublished)	0%	391	1	3/390	99.2% (97.6-99.8)	6/391	98.5% (96.5-99.4)
Japan (unpublished)	˜90%	190	4	3/186	98.4% (95-99.6)	-	-
TOTAL		581	5/58(0.9%)	6/576	99.0%	-	-

Adapted from Package insert

Sensitivity QuantiFERON®-TB Gold kit

Participants from Japan and Australia who were confirmed to be culture positive for tuberculosis were used to test the sensitivity of QuantiFERON®-TB Gold test as indicated in table 3.

Table 3: QuantiFERON®-TB Gold: Participants with confirmed *M.tuberculosis* infection by culture method

STUDY		Disease Confirmed by	No.QFT-Gold positive/No.Valid Tests	QFT-Gold Sensitivity (95% CI)
Japan TB Patients Validation study		Culture	24/27	89% (72-96%)
Austarlian TB Patients Validation Study	Pulmonary	Culture	7/10	70% (40-89%)
	Extra-pulmonary		17/17	100% (82-100%)
TOTAL			48/54	89% (78-95%)

Adapted from Package insert

At present there's no standard test for latent tuberculosis infection therefore a microbiological culture result is regarded as a true reflection of *M.tuberculosis* disease. The blood collection for sensitivity of the QuantiFERON®-TB Gold kit was carried out after less than 8 days of study participant initial treatment for tuberculosis. The findings from the two groups of *M.tuberculosis* positive culture are summarized in table 3.

3.8.3 Human IFN-γ ELISA procedure

All plasma samples and reagents, except for conjugate 100X concentrate, were brought to room temperature (22 °C ± 5 °C) before use. At least 60 minutes were allowed for equilibration. The kit standard was reconstituted by adding distilled water following the instructions as indicated on the label of the vial, mixed gently to avoid frothing and to ensure complete solubilisation. The reconstituted kit standards were used to produce a 1 in 4 dilution series of IFN-γ in Green Diluents (GD) following manufacturers package insert (CELLESTIS LIMITED QuantiFERON®-TB Gold package insert). Freeze dried conjugate was reconstituted to a concentrate 100X

with 0.3 ml of distilled water. The conjugate was mixed gently to minimize frothing and to ensure complete solubilisation. Prior to assay, the frozen plasmas were vortexed to ensure that IFN-γ is evenly distributed throughout the sample. Freshly prepared 50 µl of working strength conjugate diluted with distilled water was added to the required ELISA wells with a multichannel pipette. A Pipette with 100 µl range was used to add 50 µl of test plasma samples into appropriate wells. Finally, 50 µl conjugate was added to each of the Standards 1 to 4. The conjugate and plasma samples/standards were mixed thoroughly using a micro plate shaker for 1 minute. Each plate was covered with a lid and incubated at room temperature (22 °C ± 5 °C) for 120 ± 5 minutes. During the incubation, one-part Wash Buffer 20X Concentrate was diluted with 19 parts of distilled water and mixed thoroughly. Wells were washed with 400 µl of working strength wash buffer for at least 6 cycles. To remove residual wash buffer, plates were tapped face down on an absorbent towel. A volume of 100 µl of Enzyme Substrate Solution was added to each well and mixed thoroughly using a micro plate shaker. The plate was covered with a lid and incubated at room temperature (22 °C ± 5 °C) for 30 minutes. Following the 30-minute incubation, 50 µl of Enzyme Stopping Solution was added to each well and mixed. Enzyme Stopping Solution was added to wells in the same order and at approximately the same speed as the substrate. The Optical Density (OD) of each well was read within 5 minutes of stopping the reaction with a micro plate reader fitted with a 450 nm filter and with a 620 nm to 650 nm reference filters. OD values were used to calculate the results.

3.9 CALCULATION OF RESULTS

QuantiFERON®-TB Gold IT software was used to analyse the raw data and calculate results. Software training was provided to the investigator by aCellestis agent. The software generated a standard curve and provided a test result for each subject. The software made use of a log (e) - Log (e) standard curve by plotting the Log (e) of the mean OD (y-axis) against the Log (e) of the IFN-gamma concentration of the standards in IU/ml (x-axis), omitting the zero standard from these calculations. The software calculated the best fit for the standard curve by regression analysis.

3.10 TEST INTERPRETATION

The following the set of criteria were used by the software to determine the results indicated in Table 4 (adapted from QFT-GIT package insert):

Table 4: Interpretation values of QFT-GIT results

Nil [IU/mL]	TB Antigens minus Nil [IU/mL]	Mitogen minus Nil [IU/mL]	Quantiferon-TB [IU/mL]	Report/Interpretation
≤ 8.0	< 0.35	≥ 0.5	Negative	*M.tuberculosis* infection NOT likely
	≥ 0.35 and <25 % of Nil value	≥ 0.5		
	≥ 0.35 and ≥25% of Nil value	Any	Positive	*M.tuberculosis* infection likely
	< 0.35	< 0.5	Indeterminate	Results are indeterminate for TB-Antigen responsiveness
	≥ 0.35 and <25 % of Nil value	< 0.5		
> 8.0[3]	Any	Any		

3.11 QUALITY ASSURANCE

The investigator was trained with dummy samples collected from the trainer and research team for a period of 3 days. On the first day, 6 ml of blood was drawn into two sets of Mitogen, Nil and Antigen tubes and incubated at 37 degrees Celsius. The second day the incubated tubes were spun down, and plasma isolated into duplicate vials. One vial was analysed on the same day with the investigator observing the entire procedure. The other vial was stored at minus 20 degrees Celsius. The next day, the stored plasma vial was defrosted by the trainer and analysed, and the results compared. On day three the samples were run in duplicate by the investigator and the trainer to intentionally subject the samples to wrong volumes of reconstituted

kit standard to demonstrate the unsuccessful standard curve run of the experiment and the successful run. The demonstrations showed the robustness of test results was dependent on the generation of an accurate standard curve. Therefore, results derived from the standards were examined before test sample results could be interpreted. For the ELISA to be valid, the following criteria had to be met:

- The mean OD value for Standard 1 had to be ≥ 0.600
- The %CV for Standard 1 and Standard 2 replicate OD values had to be ≤ 15%
- Replicate OD values for Standard 3 and Standard 4 had to not vary by more than 0.040 optical density units from their mean
- The correlation coefficient (r) calculated from the mean absorbance values of the standards had to be ≥ 0.98

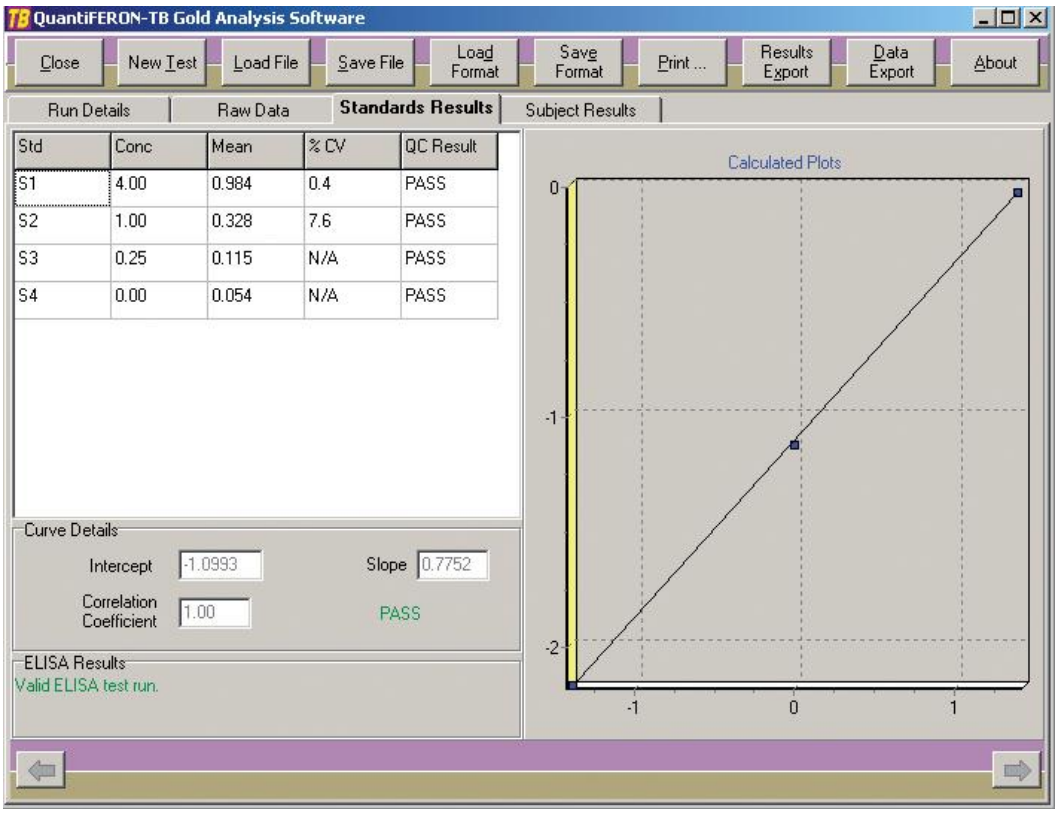
In this study manufacturer's package insert guidelines for pre-analytical (incubation, transportation and processing) and analytical (testing) quality assurance (QuantiFERON-TB Gold package insert) were followed.

The accuracy of the tests results depended on the generation of an accurate standard curve. For quality assurance of the data, QuantiFERON-TB Gold software supplied with the kits from Cellestis Ltd., Carnige, and Austalia for raw data analysis and interpretation of the results was used. The software screen indicated different values of the prepared standards and different concentrations of the standards. For each of the prepared standard a QC result was done by the software indicated with PASS if the standard met the set criteria. The quality control screen had an option for the user to print the standard curve, save file, load format, load files, save format, export data and results. Furthermore slope, intercept and Coefficience of variance of the standard curve were shown in the screen. The software automatically performed quality control (QC) checks, Coefficience of variance (CV), mean, generated the standard curve and provided a test result for each of the participants.

For the ELISA test run to be valid (PASS); it depended on the standard curve generated by the QFT-GIT software. The intercept and correlation coefficient values were indicated on the slope refer to figure 18. Figure 18 is an example of what the standard curve presentation looks like if it is a valid run. This figure was adapted from the kit insert (QuantiFERON-TB Gold package insert). If the standard curve

didn't meet the set criteria, the software would report it as invalid (FAIL) and the run needs to be repeated. Once the software had a valid standard curve, the analysis of raw data began and interpretation of the results. Under subject results screen the following were available, Nil, TB Ag, Mitogen TB Ag-Nil, Mitogen-Nil and subject results. For Nil, TB Ag, Mitogen TB Ag-Nil, Mitogen-Nil values were recorded for each participant.

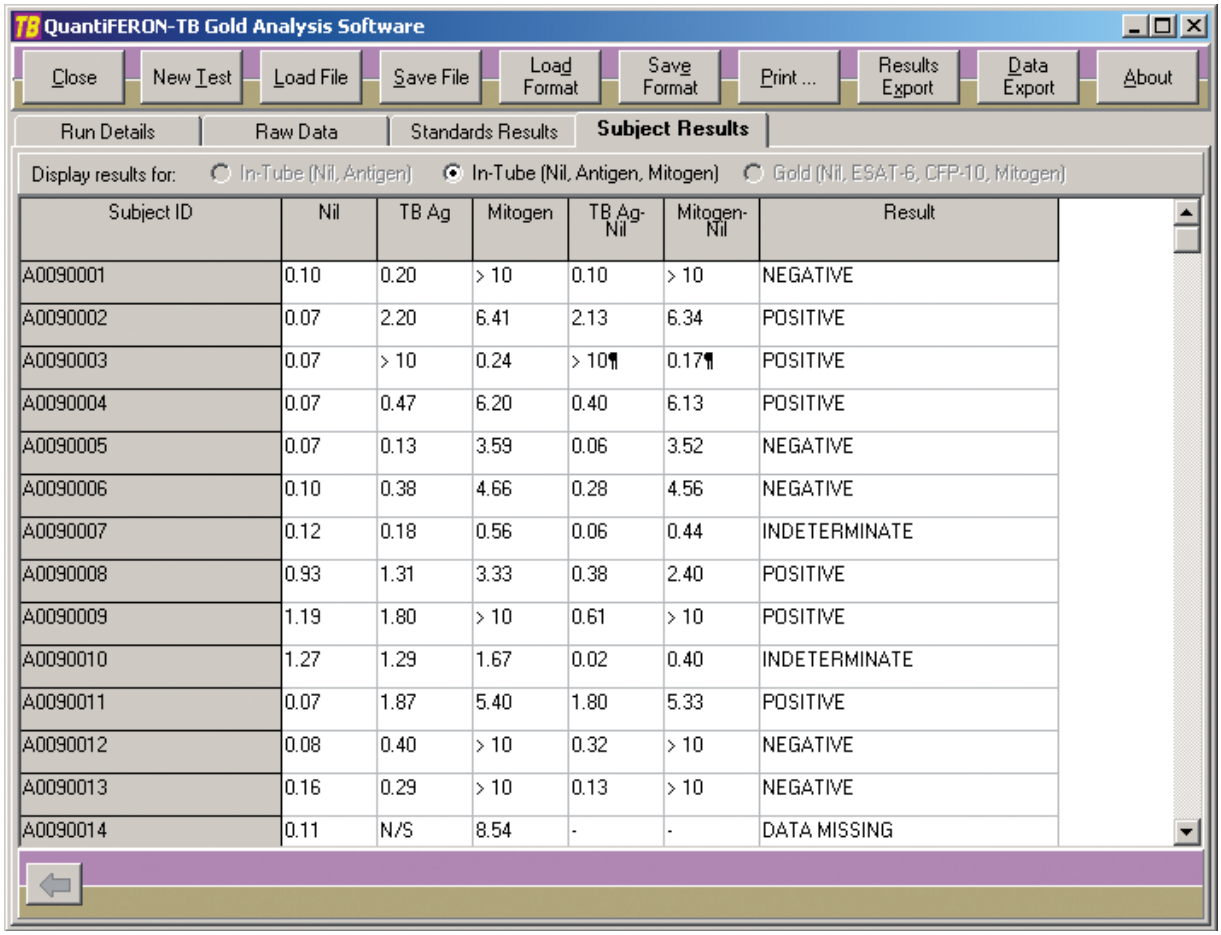
For the tests to be positive, a cut off limit of 0.35-1.0 IU/ml and for negative 0.20-0.34 IU/ml was used which was calculated by the QFT-GIT software. In the event of the software encountered the subject results which had positive results, however the mitogen response minus nil was less than 0.35 IU/ml, the software will put a ¶ symbol. This helped to indicate warning of false positive results that may be because of a mix-up of TB antigen and Mitogen droplets during the addition of samples. The quality assurance was within the accepted ranges for all the samples included in this study based on the PASS reading on the Quality control screen calculated by QFT-GIT software.



Adapted from QuantiFERON-TB Gold analysis software package insert (v2.61)

Figure 18: The standard curve of QuantiFERON-TB Gold analysis software

The results were available for each of the subject ID; furthermore, an option to view in Tube Nil, Antigen or In Tube Nil, Antigen and Mitogen and Gold Nil, ESAT-6, CFP-10 Mitogen was available. The subjects screen had an option for the user to print the standard curve, save file, load format, load files, save format, export data and results. Furthermore slope, intercept and Coefficience of variance of the standard curve were shown in the screen as indicated in figure 19. Figure 19 is an example of what the positive results presentation looks like. This figure was adapted from the kit insert (QuantiFERON-TB Gold package insert).



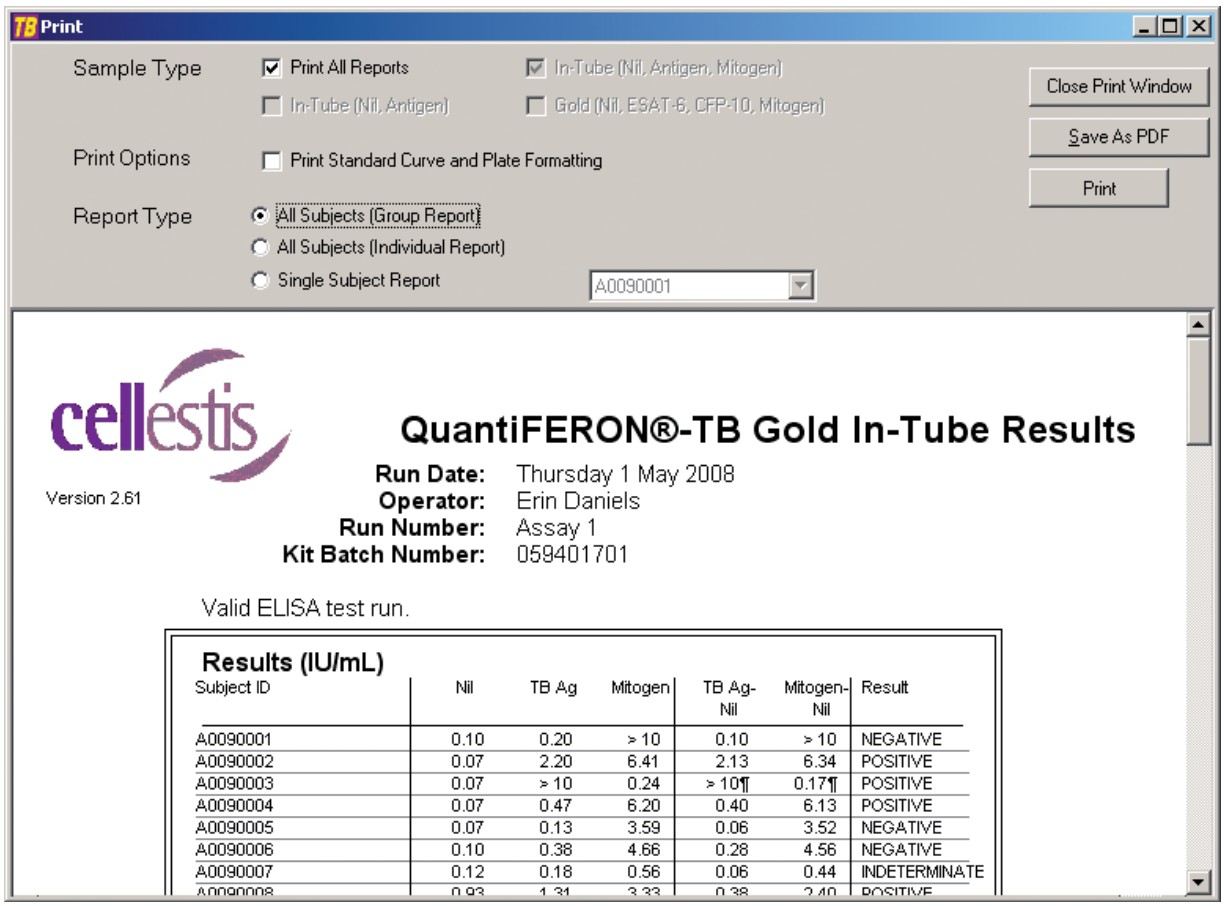
Subject ID	Nil	TB Ag	Mitogen	TB Ag-Nil	Mitogen-Nil	Result
A0090001	0.10	0.20	> 10	0.10	> 10	NEGATIVE
A0090002	0.07	2.20	6.41	2.13	6.34	POSITIVE
A0090003	0.07	> 10	0.24	> 10¶	0.17¶	POSITIVE
A0090004	0.07	0.47	6.20	0.40	6.13	POSITIVE
A0090005	0.07	0.13	3.59	0.06	3.52	NEGATIVE
A0090006	0.10	0.38	4.66	0.28	4.56	NEGATIVE
A0090007	0.12	0.18	0.56	0.06	0.44	INDETERMINATE
A0090008	0.93	1.31	3.33	0.38	2.40	POSITIVE
A0090009	1.19	1.80	> 10	0.61	> 10	POSITIVE
A0090010	1.27	1.29	1.67	0.02	0.40	INDETERMINATE
A0090011	0.07	1.87	5.40	1.80	5.33	POSITIVE
A0090012	0.08	0.40	> 10	0.32	> 10	NEGATIVE
A0090013	0.16	0.29	> 10	0.13	> 10	NEGATIVE
A0090014	0.11	N/S	8.54	-	-	DATA MISSING

Adapted from QuantiFERON-TB Gold analysis software package insert (v2.61)

Figure 19: The results screen of QuantiFERON-TB Gold

To keep a quality assurance file of the results, print-outs of the results were done. The software had an option to save the results as PDF; furthermore, print options for the standard curve were available. The option to print the final results for an individual or for all subjects was indicated in the screen. The software had an entry

for the run date, the operator, the run number and the kit batch for quality assurance and easy tracking of subjects as indicated in figure 20. Figure 20 is diagram representing an illustration of different options available to report the results. This figure was adapted from the kit insert



Adapted from QuantiFERON-TB Gold analysis software package insert (v2.61)

Figure 20: Diagram showing options available for printing the results

3.12 DATA ANALYSIS

The study data were captured on an excel sheet. A two-by-two table was used to calculate the sensitivity and specificity of QFT-GIT in detecting latent tuberculosis in children living with an adult with tuberculosis as indicated in table 8.To determine the sensitivity of QFT-GIT (a new diagnostic test) in detecting the presence of the infection, the results of TST (the routine tests used as a standard indicator of latent

tuberculosis in South Africa) were used and compared to that of the QFT-GIT. The following were set as criteria for validity of tests :

- If both the TST and QFT-GIT had a positive result, the test was said to reflect a true positive (TP).
- The detection of a positive result by QFT-GIT and a negative detection by TST, the results were said to reflect a false positive (FP)
- The test results were regadered as true negative (TN) when both the TST and QFT-GIT had negative results.
- With detection of positive results by TST and negative detection by QFT-GIT, the results were said to be false negative (FN) (Parikh et al, 2008; Trevethan, 2017).

To evaluate the sensitivity and specificity of QFT-GIT in this study, the TST was selected as a true positive indicator in this study as it is the sole test in South African context detecting latent tuberculosis in children living with an adult infected with tuberculosis, the calculations were done following the formula set by two-by-two standard criteria and the following formula:

A) Sensitivity = a / (a+c) x 100 or True Postive /(True Positive + False Negative) x 100 (Parikh et al., 2008; Trevethan, 2017).

B) Specificity = d / (b+d) x 100 or True Negative/(True Negative + False Positive) x 100 (Parikh et al., 2008; Trevethan, 2017).

The incidence rate of children living with adults with tuberculosis was determined by the following formula:
A) Incidence rate of the disease = (Number of cases) / (Person – Time at Risks)

The number of new cases was a straightforward count of new cases
To calculate (person-time at risks) the following formula was used:

B) [(Number of people at risk at the **beginning** of the time interval + Number of people at risk at the **end** of the time interval) / 2] x (Number of time units in the time interval)

(Vandenbroucke & Pearce, 2012; Cheung et al., 2017).

To evaluate the point prevalence in children living with adults who have tuberculosis disease, the following formula was used:

A). Point prevalence = Number of cases / Population size (Smik et al., 2012; Alexander et al., 2015).

The incidence rate relationship between IFN gamma assay and TST of children living with adults who have been diagnosed with tuberculosis disease was calculated using Microsoftexcel data analysis tool pack. All categorical variables were allocated codes, with negative allocated 1 and positive 2 and entered into excel data analysis. Statically data analysis was run through regression to obtain a p value.

The overall results of this study were expressed as percentages for categorical variables. Correlations between categorical variables were assessed by using chi-square tests and fisher's exact test. The correlations between QFT-GIT and TST for diagnosing latent tuberculosis infection were done using SPSS version 24. The data was entered into SPSS version 24 software and Cohen's kappa was used for the analysis. The data was in string variables and was converted into numerical variables for statistical analysis. All categorical variables were allocated codes, with negative allocated 1 and positive 2 Cohen's kappa statistics were used for concordance between TST and QFT-GIT with p-value of ≤ 0.05 considered significant. The association of indeterminate results with gender between QFT-GIT and TST was done using SPSS version 24. The data was entered into SPSS version 24 software and Chi-square kappa was calculated.

3.13 CONCLUSION

Two tests, namely IFN gamma assay and TST were used to detect latent tuberculosis in children. IFN gamma assay was used to detect latent tuberculosis in whole blood whereas PPD was injected into the arm and the diameter read after 48-72 hours for TST. Procedure for IFN gamma assay was followed according to the manufacturer's insert package to ensure reliable results were reported.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

The ability of diagnostic tests to correctly detect the presence of the disease is defined as sensitivity (Parikh et al., 2008; Baratloo et al., 2015). Furthermore, the specificity is defined as the ability of a diagnostics test to correctly exclude the presence of the disease when it is not present (Zhou et al., 2011; Baratloo et al., 2015). To evaluate the performance of IFN-gamma assay and TST is challenged by the lack of a gold standard test for latent tuberculosis. Most studies use culture confirmed results in evaluating the sensitivity of IFN-gamma assay; however latent tuberculosis and active tuberculosis are two different conditions (Young et al., 2009; Metcalfe et al., 2011; Dutta & Karakousis, 2014). There is a substantial difference in immunological and clinical presentation between latent and active tuberculosis (Young et al., 2009; Rozot et al., 2013; Dutta & Karakousis, 2014).

4.2 STUDY POPULATION

The study recruited 273 childrenexposed to adults with tuberculosis disease.

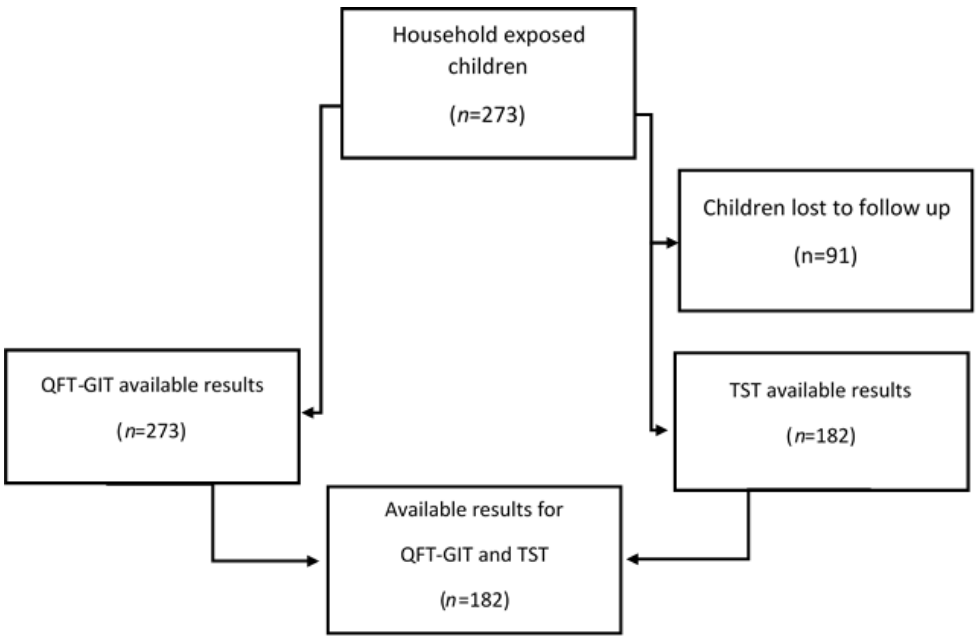


Figure 21: Flow diagram of study participants

Final collection of 273 (100%) samples of children who are exposed to adults with tuberculosis for QFT-GIT, were collected and 182 (66.6%) for TST. The study had 91(33.3%) participants who dropped out (43 adults withdrew, 40 relocated and 8 violated the study protocol by registering in other studies) as indicated in figure 21.

The study recruited 167 adults with tuberculosis disease and 273 children exposed to the adults with tuberculosis disease as indicated in table 5. The number of parents recruited was 117 female and 50 male. The children comprised of 144 female and 129 male. The majority of adult participants with tuberculosis were African (n=160). A total of 41% (n=68) of adults with tuberculosis cases results were confirmed by AFB positive sputum, 48% (n=80) were confirmed by culture positive sputum (sputum smear was negative for AFB) and 11% (n=19) diagnosed by clinical and radiology findings.

Table 5: Demographics of study participants at enrolment

		Adults Participants N=167	Paediatric Contacts N=273
Gender	Female	117 (70%)	144 (52%)
	Male	50 (30%)	129 (48%)
Ethnicity	African/Black	160 (96%)	
	Coloured/Mixed race	5 (3%)	Not obtained
	Unspecified or Other	2 (1%)	
TB diagnosis category	Smear Positive TB (Positive sputum AFB smear)	68 (41%)	
	Smear Negative TB (Positive sputum culture, negative smear)	80 (48%)	n/a
	Clinical TB (culture & smear positive)	19 (11%)	

The majority of the children involved in the study spent an average of 7 to 10 hours with the adult with tuberculosis disease at night. Most children (67%) exposed to adults with tuberculosis disease were sleeping in a different bed but in the same room as the tuberculosis positive adults. Thirty three percent (33%) of children exposed to adults with tuberculosis disease were sleeping in the same bed with the adult. A total number of 153 (92**%**) adult participants were HIV-infected.

Five percent of children exposed to adults with tuberculosis desease (n=14) tested positive for HIV infection. Of the total recruited pediatric contacts 52% were females and the median age of the children was 6 years with 42% younger than 5 years old. A total number of 94% (n=257) recruited children exposed to adults with tuberculosis disease were vaccinated, 1.8% (n=5) unvaccinated and the rest unknown (Table 6).

Table 6: Characteristics of study participants at enrolment

		Adults Cases N=167	**Paediatric Contacts N=273**
Age	Mean	34 years (95% confidence interval)	6 years (95% confidence interval)
	< 2 y	n/a	51
	2-5 y	n/a	76
	6-10 y	n/a	91
	> 10 y	n/a	55
HIV	Infected	153 (92**%**)	14 (5**%**)
	Uninfected	4 (2**%**)	251 (93**%**)
	Unknown	10 (6**%**)	8 (2**%**)
Vaccination (BCG)	Vaccinated	n/a	257 (94%)
	Unvaccinated	n/a	5 (1.8%)

4.3 STUDY RESULTS

The study had a final sample collection of 66.6% (n=182) for TST and 100% (n=273) samples of children who are exposed to adults with tuberculosis for QFT-GIT (Figure 22). Six percent (n=17) children had indeterminate QFT-GIT resultsfor QFT-GIT. In the remaining 94% (n=256) determinate QFT-GIT results, 31% (n=80) were positive and 69% (n=176) were negative. For TST out of 67% (n=182) determinate, 69% (n=126) were negative, 31% (n=56) positive and a high number 33% (n=91) of participants dropped out. The TST results for the dropped-out participants were not included in all study data analysis.

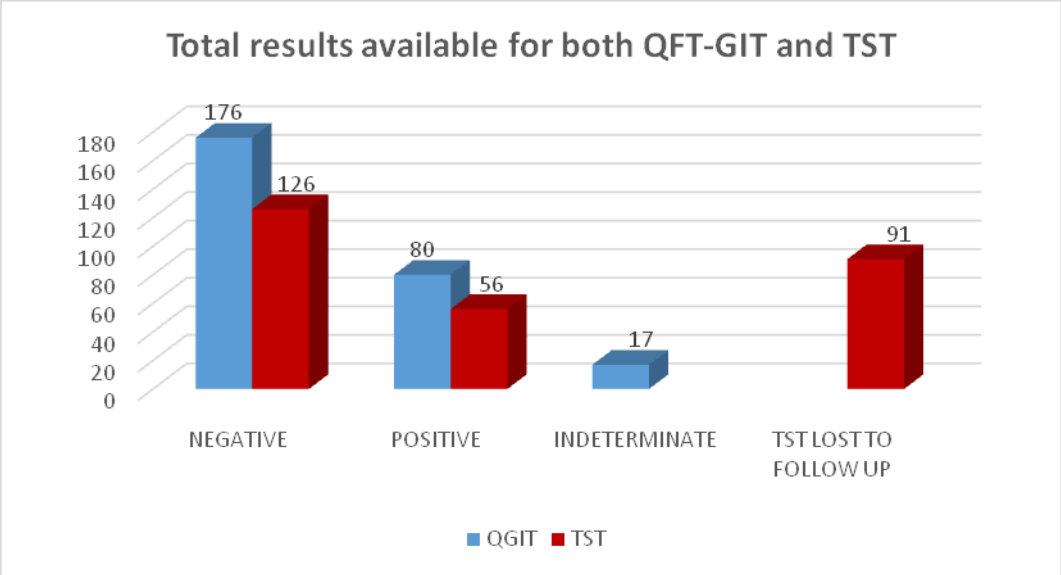


Figure 22: Graph showing total results available for both QFT-GIT and TST

A total number of 168 study participants had determinate results for both whole blood IFN gamma tests and TST tests. The IFN gamma had 61% (n=111) negative and 31% (n=57) positive. TST having 31% (n=56) positive and 69% (n=126) negative. Eight percent (n=14) of the QFT-GIT assay tests where inderterminate because of low mitogen response (Figure 23).

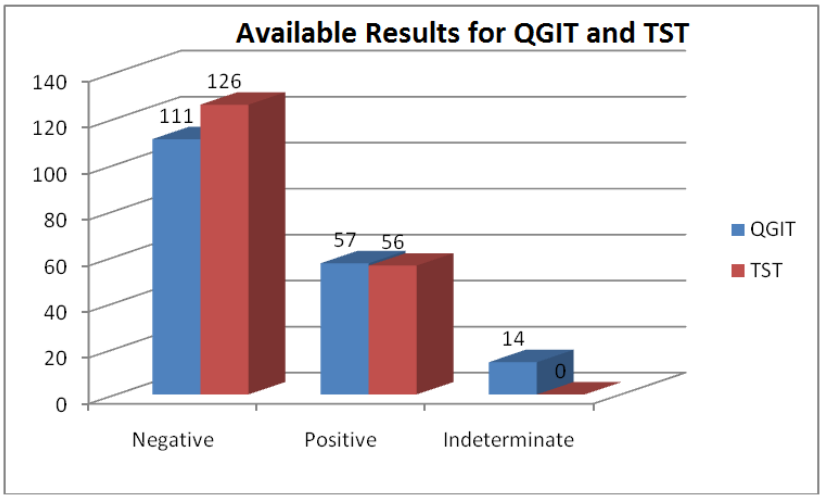


Figure 23: Graph showing results available for both QFT-GIT and TST

A total of 273 children living with adults infected by tuberculosis were enrolled in the study. 100% (273) sample analysis for QFT-GIT was done, for TST only 66.6% (n=182) was done. Out of the 182 determinate TST tests, 30.7% (n=56) were positive and 69.78% (n=126) negative.

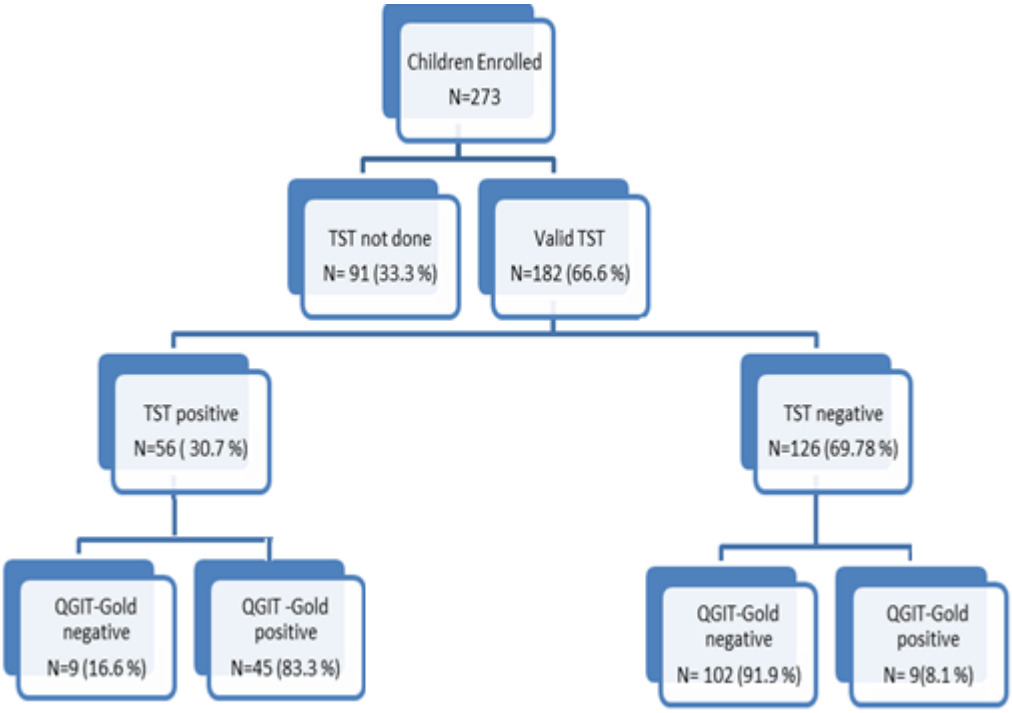


Figure 24: Summary of the distribution of results at base line for QFT-GIT

Of the 30.7% (n=56) children that tested positive TST,QFT-GIT results were: 83.3% (n=45) positive, 16.6% (n=9) negative. Out of the reported 126 negative TST results, QFT-GITresults were 91.9% (n=102) negative, 8.1% (n=9) positive (Figure 24).

4.4 SENSITIVITY AND SPECIFICITY BETWEEN QFT-GIT AND TST RESULTS

The positive (True positive) rate of 24.7% (n=45) was recorded between TST and QFT-GIT. The relationship between TST and QFT-GIT in negative (True Negative) results was 56% (n=102). QFT-GIT negative/TST positive (False Negative) was 4.9% (n=9) and was QFT-GIT Positive/TST negative (False Positive) was 6.6% (n=12). 1.1% (n=2) was QFT-GIT indeterminate/TST positive while QFT-GIT indeterminate/TST negative was 6.6% (n=12) as indicated in Figure 25.

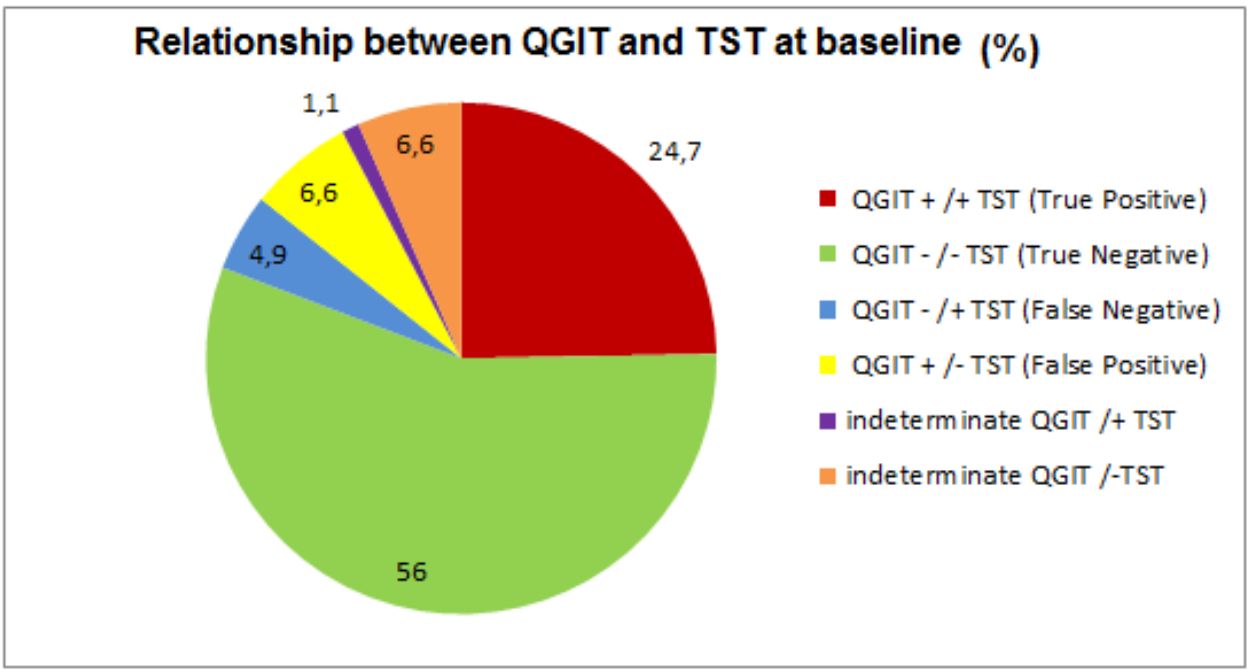


Figure 25: Graph showing agreement between QFT-GIT and TST at baseline sample collection

In the present study there was an overall agreement of 87.5% at baseline between TST and QFT-GIT (Table 7).

Calculations of relationship between TST and QFT in children at baseline

TST (+) QFT – GIT (+) + TST (-) QFT- GIT (-) / 168

45 + 102 / 168

= 87.5%

Table 7: Baseline relationship between TST and QFT-GIT assay results in children

TST cut-off ≥ 5mm	QFT-GIT positive	QFT-GIT negative	Total	Relationship (%)
TST positive	45	9	54	87.5
TST negative	12	102	114	
Total	57	111	168	

After 6 months followup as indicated in Figure 26 the positive (True positive) rate of 30.7% (n=56) was recorded between TST and FT-GIT. The relationship between TST and QFT-GIT in negative (True Negative) results was 49.4% (n=90). QFT-GIT negative/TST positive (False Negative) was 4.9% (n=9) and QFT-GIT Positive/TST negative (False Positive) was 6% (n=11). 0.5% (n=1) was QFT-GIT indeterminate/TST positive while QFT-GIT indeterminate/TST negative was 4.3% (n=8). These were the same children (n=182) from TST and QGIT baseline group.

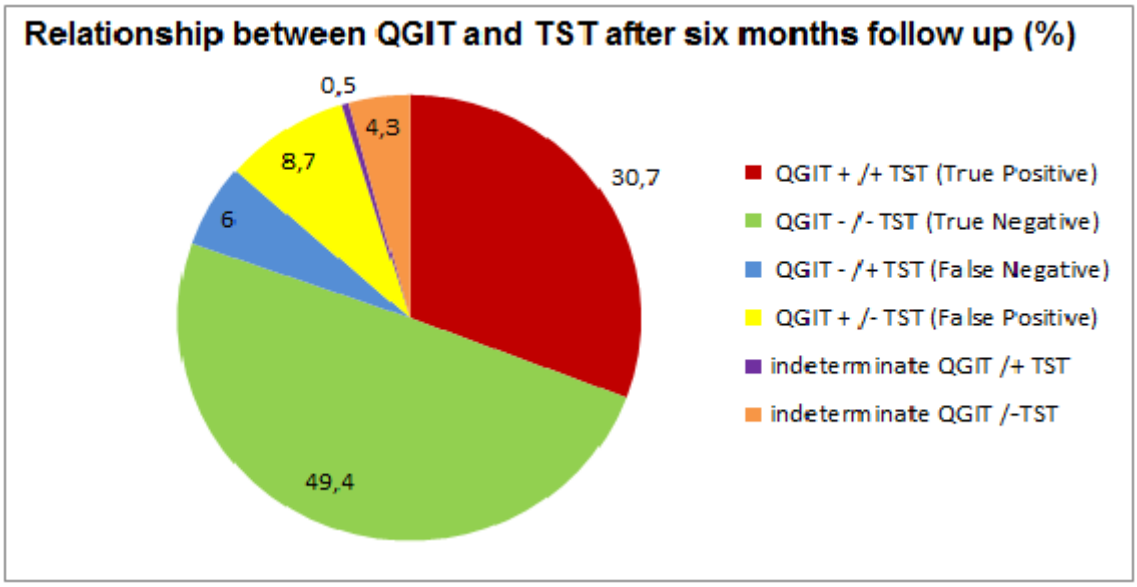


Figure 26: Graph showing agreement between QFT-GIT and TST after six months follow up sample collection

For collection after six months follow up, a total number of 173 of the same baseline children exposed to adults with tuberculosis disease had both QFT-GIT and TST determinate tests results avalable, 56 tested positive by QFT-GIT and TST, 16 by negative TST/positive QFT-GIT. Negative TST/QFT-GIT negative was 96, with negative QFT-GIT/TST positive at 11. In the present study there was an overall agreement of 84.4% after six months follow up between QFT-GIT and TST. The indeterminate QFT-GITsamples were not included as indicated in Table 8.

Calculations of relationship between TST and QFT in children after 6 months

TST (+) QFT – GIT (+) + TST (-) QFT- GIT (-) / 173

56 + 90 / 173

= 84.4%

Table 8: Relationship between TST and QFT-GIT results in children 6 months after TB treatment began in the parent contacts

TST cut-off ≥ 5mm	QFT-GIT positive	QFT-GIT negative	Total	Relationship (%)
TST positive	56	11	67	84.4
TST negative	16	90	106	
Total	72	101	173	

Table 9 represents a standard two-by-two table used in calculating the sensitivity and specificity of QFT-GIT. Study populations are represented by N, and represent children tested for a latent tuberculosis with QFT-GIT. The symbols a,b,c and d represent the number of participants. The indeterminate QFT-GIT results were not included.

Table 9 : Two-by-two table after six months follow up

Test result	Disease		Totals
	Present	Absent	
Positive	A True Positives (TP) 56	B False Positives (FP) 12	a+b 56 + 12
Negative	C False negatives (FN) 11	D True negatives (TN) 96	c+d 11 + 96
Totals	a + c 56 + 11	b + d 12 + 96	N 175

a,b,c,d=number of participants

QFT-GIT sensitivity after six months sample collection

Sensitivity = a / (a+c) x 100 or True Postive /(True Positive + False Negative) x 100

= 56 / (56+11) x 100

= 84%

QFT-GIT specificity after six months

Specificity = d / (b+d) x 100 or True Negative/(True Negative +False Positive) x 100

= 90 / (90+16) x 100

= 85%

The sensitivity of the QFT-GIT was 84% and the specificity was 85% calculated using TST results as the routine test.

4.5 CORRELATIONS BETWEEN QFT-GIT AND TST RESULTS

The baseline correlation between QFT-GIT and TST was good. A significant correlation was statistically observed; the Cohen's kappa results was 0.718 Kappa showed that there was a statistically significant correlation between QFT-GIT and TST (p< 0.001). The correlation between QFT-GIT and TST had a Cohen's kappa statistic of 0.675 which was moderate after six months follow up.Kappa showed that there was a statistical significant correlation between QFT-GIT and TST (p< 0.001) after six month follow up.The evaluated results indicated there is a statistical significant correlation between QFT-GIT and TST at baseline and after six months follow up.

4.6 VARIABILITY BETWEEN BASELINE AND AFTER SIX MONTHS

4.6.1 Variability between TST results at baseline and after six months

In total, 182 children living with adults who have tuberculosis disease were screened for latent tuberculosis infection with TST. In this study 30.7% (n=56) positives (Point Prevalence) detected by TST at baseline sample collection were compared to the

37% (n=68) positives (Point Prevalence) after six months follow-up as indicated in figure 27.

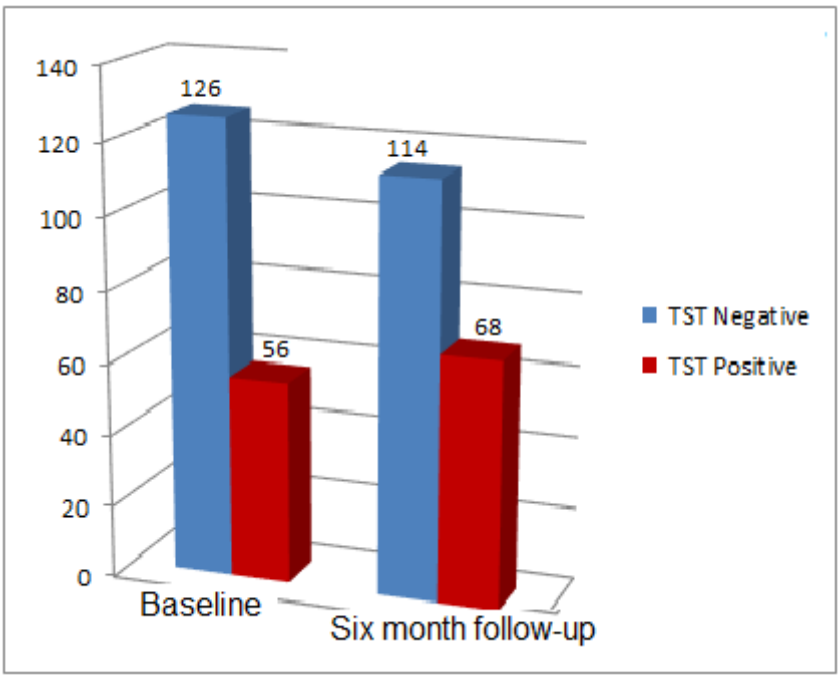


Figure 27: Graph showing variability between TST results at baseline and after six months

The incidence rate of children according to the TST contracting latent tuberculosis was calculated as follows:

Incidence rate of the disease = (Number of cases) / (Person – Time at Risks)

(126 children at risk at the beginning of March + 114 children at risk at the end of six months.) / 2] x 6 months = [(240 / 2) x 6] months = 696 person/months of risk

Note: Since 12 children got latent tuberculosis from baseline to six-month collection, the incidence rate was then calculated as follows:

(12 new cases) / (696 person-months of risk)

Incidence rate of children living with adults with tuberculosis disease was

=1.7 %.

4.6.2 Variability between QFT-GIT results at baseline and after six months

The study had a total of 182 children living with adults infected with tuberculosis tested with QFT-GIT for latent tuberculosis infection. In this study 31% (n=57) positives (Point Prevalence) were detected by QFT-GIT at baseline sample collection, 60.9% (n=111) negatives and 7.6% (n=14) indeterminate results. After six months 39.5% (n=72) positives (Point Prevalence) were detected, 55.4% (n=101) negatives and 5.4% (n=10) indeterminate as indicated in figure 28.

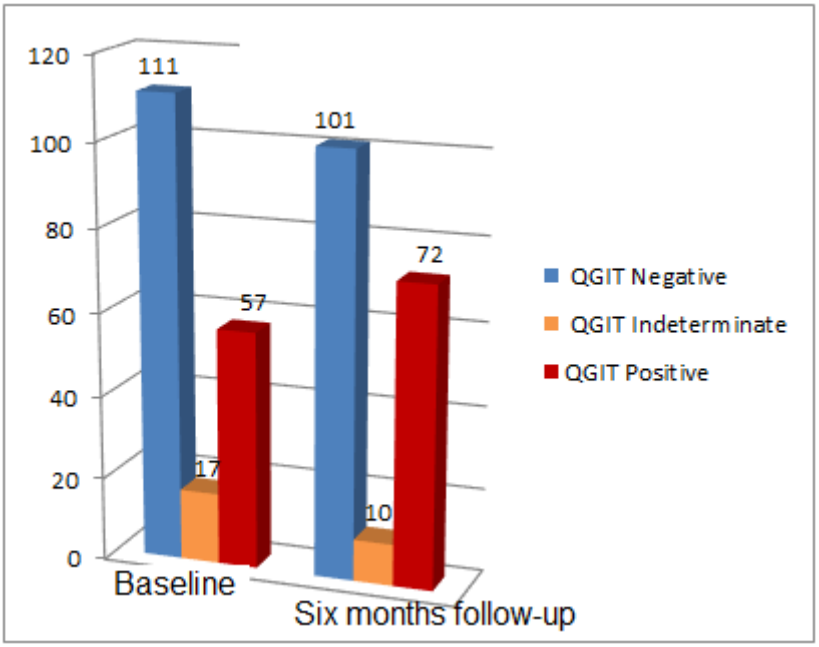


Figure 28: Graph showing variability at baseline and follow-up results (6 months) determined by QFT-GIT

The study had a total of 182 children living with adults infected with tuberculosis tested with QFT-GIT and TST for latent tuberculosis infection after six months. In this study 6% (n=11) TST negatives reported at baseline were detected as positives after six months follow up. None of the TST positive recorded at baseline converted to negative after six months. The study had a 12% (n=22) QFT-GIT negative at baseline sample collection, that were report as QFT-GIT positives after six months. Interestingly enough the study reported 5% (n=9) QFT-GIT positive detected at

baseline now being detected as QFT-GIT negative after six months as indicated in figure 29.

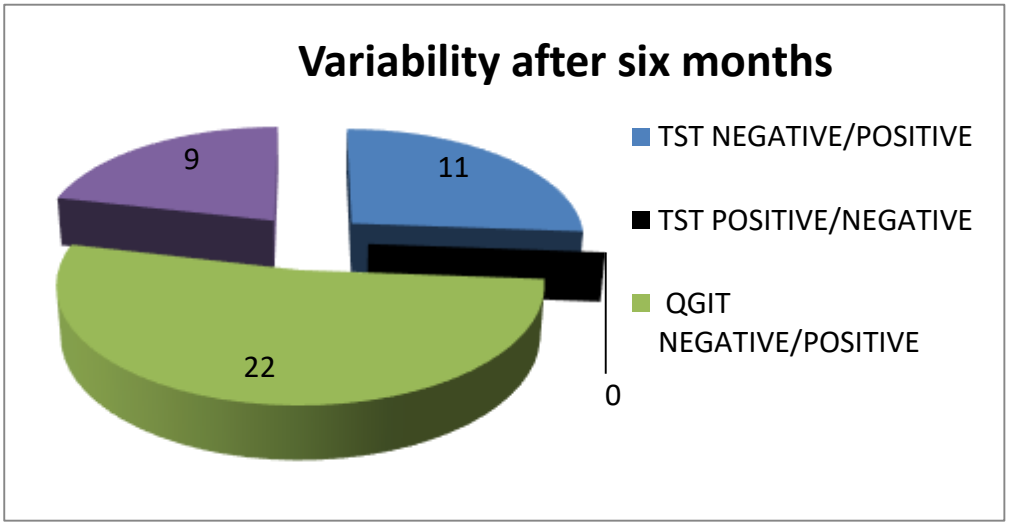


Figure 29: Graph showing variability after six months between TST and QFT-GIT on the same participant tested at baseline and after six months.

The incidence rate of children living with adults with tuberculosis as determined by the QFT-GIT was:

[(111 (negative) children at risk at the baseline + 101 (negative) children at risk at the end of six months follow-up) / 2] x 6 months = [(212 / 2) x 6] months = 636 person-months of risk.

Note: Since 15 children got latent tuberculosis from baseline to six months follow-up, the incidence rate was then calculated as follows:

(15 new cases) / (636 person-months of risk)

Incidence rate of children living with adults infected who havetuberculosis disease was

= 2.3 %.

The incidence rate relationship between QFT-GIT and TST of children living with adults infected by tuberculosis was significant (p<0.001)

In this study the indeterminate results were not included when variability of the IFN-gamma assay and TST after six months was assessed. The indeterminate results fell under the study objectives therefore until the factors that influence the IFN-gamma assay are determined; the indeterminate represented a result which was neither negative nor positive in a clinical setting which was the setting this study was based on.

4.7 THE EFFECT OF HIV STATUS OF ADULTS PROBABILITY OF TUBERCULOSIS TRANSMISSION TO PEDIATRIC CONTACTS

A total of 92**%** (n=153) adult participants were HIV-infected. Out of the total 92% HIV positive adults, only 9.1% (n=14) HIV positive children were recorded. One child had inderterminate results, 12 had negative results and 1 tested positive for latent tuberculosis. For HIV negative adults, 3 children tested positive for latent tuberculosis infection and 11 tested negative.The pecentages of HIV negative adults was relatively low. The association between HIV status of adult participants and the probability of tuberculosis transmission to paediatric contact was considered to be not statistically significant. The Fisher's exact test had the two-tailed P value of 0.5956, as indicated in table 10.

Table 10: The effect of HIV status of adults probability of tuberculosis transmission to paediatric contacts

	QFT-GIT Positive	QFT-GIT Negative	Total
HIV Positive Adult	1	12	**13**
HIV Negative Adult	3	11	**14**
Total	**4**	**23**	**27**

4.8 FACTORS ASSOCIATED WITH INDETERMINATE QFT- GIT TESTS

4.8.1 Indeterminates associated with Age

The number of inderterminates was grouped into different age denominators as indicated in table 11. The number of inderterminates was at 15 % (8/51) in age group less than two years old. In children between 2-5 years the numbers of indeterminates decreased to 6.5% (5/76). At 6-10 years the indeterminates were 3% (3/91) with >10 years at 1.8% (1/55).

Table 11: Frequencies of Indeterminate QFT-GIT results by age

Age	Indeterminate results/ Different age denominators (%)
< 2	8/51 (15)
< 2-5	5/76 (6.5)
6-10	3/91 (3)
>10	1/55 (1.8)

There was a significant positive relationship between the probability of getting a QFT-GIT results and the age ($p<0.01$).

4.8.2 Association of indeterminate results with gender

A total of 273 children comprising of 144 males and 129 females were recruited into the study. The indeterminate results were reported in 6.2% (n=8) and 4.2% (n=6) in male.Gender of the study participants showed no significant relationship in association with indeterminate results.

4.8.3 Association between HIV status and indeterminate results

The study had 14 HIV positive children living with adults infected by tuberculosis which was relatively low. Out of a total of 7.1% (n=14) children who were HIV positive, only 1 indeterminate result was recorded in children living with adults infected by tuberculosis.

4.8.4 BCG vaccination associated with indeterminate QFT-GIT

To assess the effect of BCG association with indeterminate results on QFT-GIT in children living with adults infected with tuberculosis, 8.4% (n=15) children were indeterminate out of 182 vaccinated children. In all vaccinated children, no indeterminate results were recorded. Out of 5 children who were unvaccinated only 2 had indeterminate results; however, these results were not included in this study bacause these children didn't return for TST readings.

4.9 DISCUSSION

The current study evaluated the new diagnostic test called IFN-gamma release assay for diagnosis of *M.tuberculosis* in children living with adults infected by tuberculosis.

To estimate the sensitivity of the IFN-gamma assay the indeterminate results were excluded, a two-by-two statistical calculation was used comparing IFN-gamma assay to TST because the TST is used as a routine test, therefore a "gold standard" for latent tuberculosis diagnosis. The IFN-gamma assay sensitivity was 84% after six months follow up visit. IFN-gamma release assays therefore, had high sensitivity. These results were similar to the 81.8% recorded by Vallada and colleagues comparing TST and IFN-gamma assay in children vaccinated with BCG in Brazil (Vallada et al., 2014). Verhagen and colleagues also reported a similar sensitivity range of 88% in Ware children under 16 years of age in the Orinoco Delta in North-eastern Venezuela who were living with household contacts with culture confirmed tuberculosis (Verhagen et al., 2014). The recent meta-analysis assessing the latent tuberculosis sensitivity and specificity of QFT-GIT and TST in children in low, moderate and high income countries reported a slightly higher sensitivity and specificity of QFT-GIT and TST. The pooled sensitivity of TST and QFT-GIT was 80% and 83-84% respectively. The pooled specificity of TST and QGIT was 85% and 91-94% respectively (Tieu et al., 2014).

The present analysis demonstrates that, in predominantly BCG vaccinated children exposed to adults with tuberculosis disease, there was a good relationship between IFN-gamma assay and TST assay in overall ranges of 87.5% at baseline and 84.4% after six months. The Cohen's kappa statistic was 0.705 which was good at baseline and after six months follow-up sample collection the Cohen'skappawas 0.673 which was moderate. It is comparable with a study conducted in rural India by Dogra and colleagues in children exposed to adults with tuberculosis disease whereby the relationship of IFN-gamma assay and TST was 95.2% (Dograet al., 2007). The correlation kappa statistic was 0.7 (95 % CI 0.53, 0.92) (Dogra et al., 2007).

The obtained results create confidence in the use of TST and IFN-gamma assay in detecting latent tuberculosis in children. Although these tests have high association, it should be noted that the two do not measure the exact same components of the cellular immune response.

Subject variability was found to have occurred when point prevalence at baseline was compared to six months collection point prevalence. The increase of 6.3% for TST was recorded while for IFN-gamma assay an increase of 8.5% point prevalence was recorded. The incidence rate per month between the IFN-gamma assay and TST was 2.3% and 1.7% respectively. It should be noted that TST can subsequently boost the TST and QFT response therefore giving a "false positive" in QFT results after 6 months. Van Zyl-Smit and colleagues in healthy medical students, volunteers and health care workers in Cape Town, South Africa reported observing boosting effect after 7 days post TST as compared to day 0 for QFT (van Zyl-Smit et al., 2009; Lewinsohn et al., 2017). The same observation was noted in this study after six months with participants found to have 5% (n=9) QFT-GIT positive detected at baseline now being detected as QFT-GIT negative after six months as indicated in figure 29. Furthermore, the conversion of QFT-GIT complicates the true rate of tuberculosis transmission among children in cross sectional studies. There was a significant positive relationship between IFN-gamma assay incidence rate per month and TST incidence rate per month (p=0.0007).

In this study age, gender, vaccination and HIV status as possible contributing factors for the indeterminate were investigated. The previous studies have reported that HIV

status in adults result in indeterminate, therefore in this study the aim was to assess if the same results will be reported in children (Tebruegge et al., 2014). Corncening the age and vaccination most studies have reported that when QFT-GIT testing was done on elderly (>65), the number of indeterminate was high because of immunologic status; therefore the aim was to see whether the effects of age and vaccination in children will be a contributing factor of indeterminate results (Cho et al., 2012; Seddon et al., 2016). In the study that was done at Johns Hopkins University, it was reported that females had 75% possibilities of indeterminate results using QFT-GIT, therefore the aim was to assess if gender of the participants was a contributing factor for possible indeterminate results (Fabre et al., 2014).

Six percent (n=17) of the results were indeterminate by IFN-gamma assay test. A total of 47.1% (n=8) of children with indeterminate results were less than two years old as indicated in table 14; age seemed to play a most important role with the majority of indeterminate results found in children less than two years old. The older the child was, the less recorded indeterminate results occurred. The indeterminate results possibly were a result of low mitogen response; these findings were also reported by Lucas and colleagues in Western Australia on 524 African and Burmese immigrant children aged 5 months to 16 years (Lucas et al., 2010). The study had 15% indeterminate results recorded due to failed mitogen response in children less than 3 years old (Lucas et al., 2010). Kampmann et al., (2006) also reported similar findings of children less than 5 years old who produced low IFN-gamma release assay responses to *M.tuberculosis* antigens in Italy.Furthermore IFN-gamma assay indeterminate results were recorded in 15 study participants with negative TST results. This finding would suggest that a large fraction of those negative TST could actually be false-negative results. Since TST does not have an internal positive control, this hypothesis cannot be verified. Gender, HIV status and BCG vaccination did not show to have any significant contribution to indeterminate results.

The study had 92% (n=153) of HIV infected adults diagnosed with tuberculosis disease. The number of HIV infected children participating in the study was relatively low at 9.1% (n=14). In the study done by Huang and colleagues on 2% (n=99) HIV positive and 98% (n=4106) HIV negative adults in Lima Peru, assessing the risk of tuberculosis transmission to children (Huang et al., 2014). The study reported that

the children were less likely to be infected with tuberculosis by adults who were HIV positive than adults who were HIV negative (Huang et al., 2014). In the present study, to fully assess the effect of adult's HIV positive status on probability of transmitting tuberculosis to children was hampered by the low number of negative adults enrolled in the study.

Although TST and IFN-gamma assay appeared comparable, IFN-gamma assay showed a higher positivity rate amongst those contacts with reported household tuberculosis exposure compared to TST, indicating that test might be more sensitive than TST. Furthermore, the scar from mantoux testing is avoided. The data have shown that TST is adversely affected by external factors with 33% (n=91) dropouts recorded. The participant dropouts were also recorded by Arend and colleagues in the study conducted in DiakonessenhuisUtrch/Zeist hospital patients; the study enrolled 15 515 participants and administered TST. The TST readings were available for 92% (n=14 128) with 8% (n=1387) patients lost because of not returning for TST readings (Arend et al., 2007). Ncayiyana and colleagues in the study conducted in Johannesburg, South Africa; reported 97% (n=45) participants in whom a TST was placed, TST was read. Participants dropouts of 3% (n=13) was recorded after 48-72h because the participants could not be traced (Ncayiyana et al., 2016).

4.10 CONCLUSION

The sensitivity of IFN-gamma assay was high when compared to TST in children living with tuberculosis infected adults. Regarding the comparison between IFN-gamma assay and TST, the association between the two tests was high. Both TST and IFN-gamma assay had within-subject variability after the 6-month time period. Even though the study had low indeterminate results, age was reported as the main factor that contributed to indeterminate results. The effects of HIV on probability of transmitting tuberculosis from adults infected to exposed children were not fully assessed because of the low number of HIV negative adults.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 INTRODUCTION

Currently the tests available for detection and diagnosis of *M.tuberculosis* infections focusing on identification of the active bacterium have limitations in diagnosis of latent tuberculosis in children (Kampmann et al., 2009; Yang et al., 2014). This study was assessing the feasibility of using IFN-gamma assay and TST tests in children as possible diagnostic tools. Furthermore, it evaluated the prevalence of *M.tuberculosis* infection among children of adults with tuberculosis cases using IFN-gamma assay and TST methods.

5.2 RESEARCHER'S CONTRIBUTION

The investigator was responsible for making sure the phlebotomist reported the study participants' data on appropriate forms. The phlebotomist was trained by the researcher on which tubes to use to collect blood and how to correctly incubate the blood tubes. The blood analysis, quality control, quality assurance and data capturing were performed by the investigator. The interpretation of the collected data and sending the quality assured data to the principal study coordinator to USA John Hopkins University was done by the researcher. The researcher was also responsible for compiling and finalization this report.

5.3 LIMITATION OF THE STUDY

Limitations that were noted in the study firstly were: the lack of microbiologically confirmed tuberculosis in paediatrics. Therefore, the researcher was unable to determine the sensitivity of IFN-gamma assay as intended. To partly overcome this limitation the researcher used the two by two calculation using TST as a standard test to estimate sensitivity of IFN-gamma assay. Secondly, theresearcher was unable to obtain information on a microbiological diagnosis of tuberculosis for every adult case, and almost half of the cases had been diagnosed with tuberculosis based

on clinical and radiographic findings. This situation is not uncommon in resource-limited settings. Thirdly, because of the high drop out of study participants not returning for TST reading, the researcher was not able to evaluate the prevalence of *M.tuberculosis* by means of the TST as intended. Fourthly, the study had insufficient HIV negative adult recruits to draw a reliable conclusion for assessing the effect of HIV on probability of transmission to paediatric contacts. Lastly only the relationship between vaccination with BCG and indeterminate IFN-gamma assay results was evaluated in this study, instead of the complete effect of BCG vaccination on children living with adults infected by tuberculosis because of cross sectional data, which focus more on one period.

5.4 MAIN FINDINGS

5.4.1 Literature

In South Africa, tuberculosis cases have increased six times over the past 20 years, because of increasing HIV prevalence (Wood et al., 2010c; Mahtab & Coetzee, 2017). Despite programs like DOTS being introduced by the WHO in South Africa which aimed for a 100% case detection from 1996 to 2005, by 2003 the case detection was 65% which was still below the target. However, the introduced annual programs focus on surveying the prevalence of active tuberculosis in antenatal women and household surveys, leading to limited information describing the prevalence of latent tuberculosis in the general population, including children (Wood et al., 2010c; Bunyasi et al., 2017).

Compared to adults, children have been found to have a higher risk of acquiring tuberculosis disease transmission from adults with active tuberculosis (Nicol et al., 2009; Nachiappan et al., 2017). Therefore, there is an urgent need to advance the current tools in diagnosing tuberculosis in this population. The tests currently available are time consuming and with the exception of TST, all the tests are based on diagnosing and identification of tuberculosis bacterium, which makes them unsuitable in latent tuberculosis infection (Sester et al., 2011; Wang et al., 2018).

The latest developments in microbiology and basic immunology have allowed the development of IFN-gamma release assay. The test uses *M.tuberculosis* antigens that are absent from BCG strains of *M.bovis* to stimulate the IFN-γ production from blood as reviewed by Schluger & Burzynski (2012; Rhodes et al., 2014). The QuantiFERON assays promise more reliable detection of latent tuberculosis infection and prevention of false positives by cross reaction with BCG (Cattamanchi et al., 2011; Babayigit et al., 2014).

5.4.2 Relationship between the IFN-gamma assay and TST

The findings in the study demonstrated that there was an association between IFN-gamma assay and TST diagnosing latent tuberculosis in South African children living with adults infected by tuberculosis. The same association was observed between the two tests when performed after six months.

5.4.3 HIV status of parents increase the probability of tuberculosis transmission to paediatric contacts

This study had a high proportion of HIV infected adult index cases (92%), but relatively low HIV infected children (n=14). The number of HIV negative adults was not sufficient (8%) to perform the statistical analysis assessing the effect of the status of HIV adults on the probability of transmitting tuberculosis to children as intended.

5.4.4 The sensitivity of IFN-gamma assay and TST for diagnosing tuberculosis disease in paediatric contacts

Microbiological results are currently used as a “gold standard” for assessing latent tuberculosis sensitivity. However, microbiological tests are intended for detecting active tuberculosis diagnosis while latent tuberculosis is a different condition. In the present study IFN-gamma assay had a high sensitivity when TST was used as a “gold standard”. Because there is no diagnostic “gold standard” for latent

tuberculosis diagnosis, it should be noted that the sensitivity and specificity of IFN-gamma assay and TST in latent tuberculosis diagnosis still needs further evaluation.

5.4.5 Factors associated with indeterminate IFN-gamma assay results in paediatric tuberculosis contacts

Age seemed to play the most important role in factors associated with indeterminate. The majority of indeterminate results were found in younger children. The younger the children were, the higher the rate of indeterminate recorded. Gender, HIV status and BCG vaccination did not show to have any significant contribution in indeterminate results found.

5.4.6 To determine within-subject variability of the IFN-gamma assay and TST tests over an approximately 6-month time period

The findings available for the six months collection period suggested that there was variability in both IFN-gamma assay and TST. The children who were recorded as negative at baseline collection were now positive after six months. However, the new positives cases that were recorded after six months are not known whether they are from transmission from the adults, whether they are acquired from conversion or whether they developed from the environment. The individual result of QFT-GIT infection in this study generates questions of how reliable the test is. The data suggest that repeat testing over time may be of benefit to TB exposed household contacts.

5.5 SIGNIFICANCE OF THE STUDY

This study showed that IFN-gamma assay was a reliable and sensitive diagnostic tool to detect latent tuberculosis in immunized children older than two years. A big advantage of the IFN-gamma assay compared to TST was the prevention of participant dropouts. No follow up was needed for the IFN-gamma release assay, while the participants needed to return for TST reading. This study contributes to the

limited available data on the prevalence of latent tuberculosis in South Africa amongst children living with tuberculosis positive adults.

5.6 CONCLUSION

Independent of which method was used to assess latent tuberculosis prevalence in South African children of adults with tuberculosis cases in Soweto, the data showed tuberculosis point prevalence was high amongst the children living with adults infected by tuberculosis. The data showed that, despite a high rate of BCG vaccination in the study population, TST still is a good diagnostic tool for detection of children with latent tuberculosis infection. The reported data show correlation with IFN-gamma assay which is reported that the test is not affected by BCG vaccination. Based on the data, the IFN-gamma assay was a better indicator of the risk of *M. tuberculosis* infection than TST in a BCG-vaccinated population, when to compared on the ability to circumvent patient dropout.

5.7 RECOMMENDATIONS

5.7.1 Community

Based on the data collected it is recommended that adults with active tuberculosis sleep in a different room from children because of risk the of tuberculosis transmission. These findings should be communicated to adults with tuberculosis disease. Although treatment of TB or HIV/AIDS was not within the spectrum of this study, positive results were reported, and participants were referred to local medical care.

5.7.2 Policy makers

The IFN-gamma assay needs to be performed independently at different government laboratories and the relationship between the IFN-gamma assays results assessed in children exposed to active tuberculosis adults. Furthermore, the data collected be used to determine the possibility of replacing the TST; this was also suggested by

Samea and colleagues (Samea et al., 2013) and if not, it is recommended that the cost of introducing a new test to the current diagnosis of latent tuberculosis infection, be examined.

5.7.3 Scientific community

Larger studies are required to assess the use of the IFN-gamma in children not vaccinated with BCG to determine the specificity and the sensitivity of the assays in this population. The data showed the IFN-gamma assay performed better the older the child was. Whether these data demonstrate the tests perform better with older children or it has better sensitivity, remains to be studied further. The IFN gamma assay performance needs to be studied in a high tuberculosis burden setting, more importantly in children with active tuberculosis. Both IFN-gamma assay and TST need to be simultaneously performed to maximize the diagnosis of latent tuberculosis in children. In spite of the discontinuation of the QGIT test in 2017, this study still contributes valuable scientific information.

5.8 FURTHER RESEARCH NEEDED

Further studies are needed to assess the potential use of IFN-gamma assay as a screening test for latent tuberculosis in routine clinics, because of the improved specificity. The study had a low number of HIV positive children; more studies are required in these populations to evaluate IFN-gamma assay. Furthermore, in this study all the children who participated were BCG vaccinated, therefore, more studies are needed to assess the effect of BCG on IFN-gamma assay in children who are not vaccinated. The study had a low number of HIV negative adults; therefore, further studies are required in this population with a more balanced HIV uninfected arm, to assess the probability of HIV negative adults transmitting tuberculosis to children. Further studies that look at long term follow up which include more robust endpoints rather than just comparison with TST are needed.

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7. ANNEXURES

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)
Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1294 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

3 September 2007

Mrs Charlene Conradie
Research Operations Coordinator
Perinatal HIV Research Unit
CH Baragwanath Hospital
University

Dear Charlene

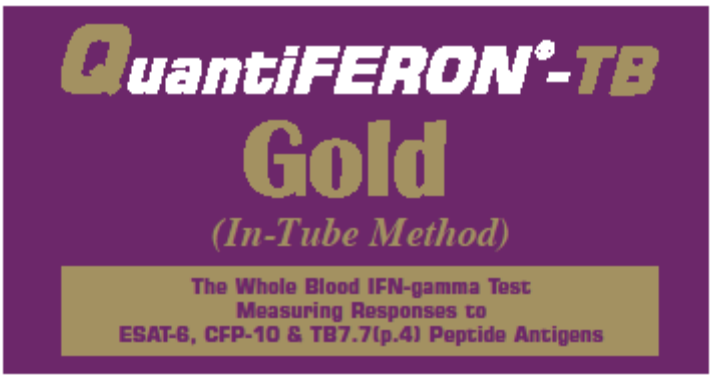
RE: Protocol M070121: Whole Blood cytokine Assay for Diagnosis of Mycobacterium Tuberculosis in South African Children with Household Tuberculosis Contact

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has approved the protocol amendment to the abovementioned study. Copy attached.

Yours sincerely,

Anisa Keshav (Ms)
Secretary
Human Research Ethics Committee (Medical)

Annexure 1: Human research ethics committee (Medical)



PACKAGE INSERT

For *In Vitro* Diagnostic Use

cellestis

Annexure 2: QuantiFERON-TB Gold Package Insert

QuantiFERON®-TB Gold (QFT®) ELISA
Package Insert

Σ 2 x 96 (catalog no. 0594-0201)

Σ 20 x 96 (catalog no. 0594-0501)

The whole blood IFN-γ test measuring responses to ESAT-6, CFP-10, and TB7.7(p4) peptide antigens

IVD For in vitro diagnostic use

CE

REF 0594-0201, 0594-0501

QIAGEN, 19300 Germantown Road
Germantown, MD 20874 USA

EC REP QIAGEN GmbH, QIAGEN Strasse 1
40724 Hilden, GERMANY

1075115 Rev. 07

www.QuantiFERON.com

QIAGEN®

Contents

1. Intended Use

QuantiFERON-TB Gold (QFT®) is an in vitro diagnostic test using a peptide cocktail simulating ESAT-6, CFP-10, and TB7.7(p4) proteins to stimulate cells in heparinized whole blood. Detection of interferon-γ (IFN-γ) by enzyme-linked immunosorbent assay (ELISA) is used to identify in vitro responses to those peptide antigens that are associated with *Mycobacterium tuberculosis* infection.

QFT is an indirect test for *M. tuberculosis* infection (including disease) and is intended for use in conjunction with risk assessment, radiography, and other medical and diagnostic evaluations.

2. Summary and Explanation of the Test

Tuberculosis is a communicable disease caused by infection with *M. tuberculosis* complex organisms (*M. tuberculosis*, *M. bovis*, *M. africanum*), which typically spreads to new hosts via airborne droplet nuclei from patients with respiratory tuberculosis disease. A newly infected individual can become ill from tuberculosis within weeks to months, but most infected individuals remain well. Latent tuberculosis infection (LTBI), a non-communicable asymptomatic condition, persists in some, who might develop tuberculosis disease months or years later. The main purpose of diagnosing LTBI is to consider medical treatment for preventing tuberculosis disease. Until recently the tuberculin skin test (TST) was the only available method for diagnosing LTBI. Cutaneous sensitivity to tuberculin develops from 2 to 10 weeks after infection. However, some infected individuals, including those with a wide range of conditions hindering immune functions, but also others without these conditions, do not respond to tuberculin. Conversely, some individuals who are unlikely to have *M. tuberculosis* infection exhibit sensitivity to tuberculin and have positive TST results after vaccination with bacille Calmette-Guérin (BCG), infection with mycobacteria other than *M. tuberculosis* complex, or undetermined other factors.

LTBI must be distinguished from tuberculosis disease, a reportable condition which usually involves the lungs and lower respiratory tract, although other organ systems may also be affected. Tuberculosis disease is diagnosed from historical, physical, radiological, histological, and mycobacteriological findings.

QFT is a test for cell-mediated immune (CMI) responses to peptide antigens that simulate mycobacterial proteins. These proteins, ESAT-6, CFP-10, and TB7.7(p4), are absent from all BCG strains and from most nontuberculous mycobacteria with the exception of *M. kansasii*, *M. szulgai*, and *M. marinum*.(1) Individuals infected with *M. tuberculosis* complex organisms usually have lymphocytes in their blood that recognize these and other mycobacterial antigens. This recognition process involves the generation and secretion of the cytokine, IFN-γ. The detection and subsequent quantification of IFN-γ forms the basis of this test.

The antigens used in QFT are a peptide cocktail simulating the proteins ESAT-6, CFP-10, and TB7.7(p4). Numerous studies have demonstrated that these peptides antigens stimulate IFN-γ responses in T cells from individuals infected with *M. tuberculosis,* but generally not from uninfected or BCG-vaccinated persons without disease or risk for LTBI.(1–32) However, medical treatments or conditions that impair immune functionality can potentially reduce IFN-γ responses. Patients with certain other mycobacterial infections might also be responsive to ESAT-6, CFP-10, and TB7.7(p4), as the genes encoding these proteins are present in *M. kansasii*, *M. szulgai*, and *M. marinum*.(1, 23) The QFT test is both a test for LTBI and a

helpful aid for diagnosing *M. tuberculosis* complex infection in sick patients. A positive result supports the diagnosis of tuberculosis disease, but infections by other mycobacteria (e.g., *M. kansasii*) could also lead to positive results. Other medical and diagnostic evaluations are necessary to confirm or exclude tuberculosis disease.

Principles of the Assay

The QFT system uses specialized blood collection tubes, which are used to collect whole blood via venepuncture, that contain antigens representing certain *M. tuberculosis* proteins or controls. Incubation of the blood occurs in the tubes for 16 to 24 hours, after which, plasma is harvested and tested for the presence of IFN-γ produced in response to the peptide antigens.

The QFT test is performed in two stages. First, whole blood is collected into each of the QFT blood collection tubes, which include a Nil tube, TB Antigen tube, and a Mitogen tube.

The Mitogen tube can be used with the QFT test as a positive control. This may be especially warranted where there is doubt as to the individual's immune status. The Mitogen tube may also serve as a control for correct blood handling and incubation.

The tubes are shaken to mix antigen with the blood and should be incubated at 37°C ± 1°C as soon as possible, and within 16 hours of collection. Following a 16 to 24 hour incubation period, the tubes are centrifuged, the plasma is removed and the amount of IFN-γ (IU/ml) measured by ELISA. The QFT ELISA uses a recombinant human IFN-γ standard, which has been assayed against a reference IFN-γ preparation (NIH Ref: Gxg01-902-535). Results for test samples are reported in International Units (IU) relative to a standard curve prepared by testing dilutions of the secondary standard supplied with the kit.

Heterophile (e.g., human anti-mouse) antibodies in serum or plasma of certain individuals are known to cause interference with immunoassays. The effect of heterophile antibodies in the QFT ELISA is minimized by the addition of normal mouse serum to the Green Diluent and the use of $F(ab')_2$ monoclonal antibody fragments as the IFN-γ capture antibody coated to the microplate wells.

A test is considered positive for an IFN-γ response to the TB Antigen tube that is significantly above the Nil IFN-γ IU/ml value. If used, the plasma sample from the Mitogen tube serves as an IFN-γ positive control for each specimen tested. A low response to Mitogen (<0.5 IU/ml) indicates an indeterminate result when a blood sample also has a negative response to the TB antigens. This pattern may occur with insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, incorrect filling/mixing of the Mitogen tube, or inability of the patient's lymphocytes to generate IFN-γ. The Nil sample adjusts for background, heterophile antibody effects, or non-specific IFN-γ in blood samples. The IFN-γ level of the Nil tube is subtracted from the IFN-γ level for the TB Antigen tube and Mitogen tube (if used).

Time Required for Performing Assay

The time required to perform the QFT assay is estimated below; the time of testing multiple samples when batched is also indicated:

37°C ± 1°C incubation of blood tubes: 16 to 24 hours

ELISA: Approx. 3 hours for one ELISA plate
(28 to 44 individuals)
<1 hour labor
Add 10 to 15 minutes for each extra plate

3. Components and Storage

Blood Collection Tubes*	300 tubes	200 tubes	100 tubes
Catalog no.	T0590-0301	0590-0201	T0593-0201
Number of preps	100	100	100
QuantiFERON Nil Tube (gray cap, white ring)	100 tubes	100 tubes	
QuantiFERON TB Antigen Tube (red cap, white ring)	100 tubes	100 tubes	
QuantiFERON Mitogen Tube (purple cap, white ring)	100 tubes		100 tubes
QFT Blood Collection Tubes Package Insert	1	1	1
High Altitude (HA) Blood Collection Tubes (for use between 1020 and 1875 meters)*	300 tubes	200 tubes	100 tubes
Catalog no.	T0590-0505	0590-0501	T0593-0501
QuantiFERON HA Nil Tube (gray cap, yellow ring)	100 tubes	100 tubes	
QuantiFERON HA TB Antigen Tube (red cap, yellow ring)	100 tubes	100 tubes	
QuantiFERON HA Mitogen Tube (purple cap, yellow ring)	100 tubes		100 tubes
QFT Blood Collection Tubes Package Insert	1	1	1

* Not all product configurations are available in every country. Please refer to QIAGEN customer care (details on www.qiagen.com) for more information on what configurations are available for ordering.

ELISA Components	2 Plate Kit ELISA	Reference Lab Pack
Catalog no.	0594-0201	0594-0501
Microplate Strips (12 x 8 wells) coated with murine anti-human IFN-γ monoclonal antibody	2 sets of 12 x 8-well Microplate Strips	20 sets of 12 x 8-well Microplate Strips
Human IFN-γ Standard, lyophilized (contains recombinant human IFN-γ, bovine casein, 0.01% w/v Thimerosal)	1 x vial (8 IU/ml when reconstituted)	10 x vials (8 IU/ml when reconstituted)
Green Diluent (contains bovine casein, normal mouse serum, 0.01% w/v Thimerosal)	1 x 30 ml	10 x 30 ml
Conjugate 100X Concentrate, lyophilized (murine anti-human IFN-γ HRP, contains 0.01% w/v Thimerosal)	1 x 0.3 ml (when reconstituted)	10 x 0.3 ml (when reconstituted)
Wash Buffer 20X Concentrate (pH 7.2, contains 0.05% v/v ProClin® 300)	1 x 100 ml	10 x 100 ml
Enzyme Substrate Solution (contains H_2O_2, 3,3′, 5,5′ Tetramethylbenzidine)	1 x 30 ml	10 x 30 ml
Enzyme Stopping Solution (contains 0.5M H_2SO_4)†	1 x 15 ml	10 x 15 ml
QFT ELISA Package Insert	1	1

† Contains sulfuric acid. See page 9 for precautions.

Materials Required But Not Provided

- 37°C ± 1°C incubator. CO_2 not required
- Calibrated variable volume pipets for delivery of 10 µl to 1000 µl with disposable tips
- Calibrated multichannel pipet capable of delivering 50 µl and 100 µl with disposable tips
- Centrifuge capable of centrifuging the blood tubes at least 3000 RCF (g)
- Microplate shaker capable of speeds between 500 and 1000 rpm
- Deionized or distilled water, 2 liters
- Microplate washer (for safety in handling plasma samples, an automated washer is recommended)
- Microplate reader fitted with 450 nm filter and 620 nm to 650 nm reference filter

Storage and Handling

Blood Collection Tubes

- Store blood collection tubes at 4°C to 25°C.

Kit Reagents

- Store kit reagents refrigerated at 2°C to 8°C.
- Always protect Enzyme Substrate Solution from direct sunlight.

Reconstituted and Unused Reagents

For instructions on how to reconstitute the reagents, please see Section 6 (page 14)

- The reconstituted kit standard may be kept for up to 3 months if stored at 2°C to 8°C.

  Note the date on which the kit standard was reconstituted.
- Once reconstituted, unused Conjugate 100X Concentrate must be returned to storage at 2°C to 8°C and must be used within 3 months.

  Note the date on which the conjugate was reconstituted.
- Working strength conjugate must be used within 6 hours of preparation.
- Working strength wash buffer may be stored at room temperature for up to 2 weeks.

4. Warnings and Precautions

For in vitro diagnostic use

Warnings

- A negative QFT result does not preclude the possibility of *M. tuberculosis* infection or tuberculosis disease: false negative results can be due to stage of infection (e.g., specimen obtained prior to the development of cellular immune response), co-morbid conditions which affect immune functions, incorrect handling of the blood collection tubes following venipuncture, incorrect performance of the assay, or other immunological variables.
- A positive QFT result should not be the sole or definitive basis for determining infection with *M. tuberculosis*. Incorrect performance of the assay may cause false-positive responses.
- A positive QFT result should be followed by further medical evaluation and diagnostic evaluation for active tuberculosis disease (e.g., AFB smear and culture, chest X-ray).
- While ESAT-6, CFP-10, and TB7.7(p4) are absent from all BCG strains and from most known nontuberculous mycobacteria, it is possible that a positive QFT result may be due to infection by *M. kansasii*, *M. szulgai*, or *M. marinum*. If such infections are suspected, alternative tests should be investigated.

Precautions

For in vitro diagnostic use only.

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

CAUTION: Handle human blood as if potentially infectious. Observe relevant blood handling guidelines.

The following risk and safety phrases apply to components of the QuantiFERON-TB Gold ELISA Kit.

Hazard Statements

QuantiFERON Enzyme Stopping Solution

Contains: sulfuric acid. Warning! May be corrosive to metals. Causes skin irritation. Causes serious eye irritation. Wear protective gloves/ protective clothing/ eye protection/ face protection.

QuantiFERON Enzyme Substrate Solution

Warning! Causes mild skin irritation. Wear protective gloves/ protective clothing/ eye protection/ face protection.

QuantiFERON Green Diluent

Contains: tartrazine. Warning! May cause an allergic skin reaction. Wear protective gloves/ protective clothing/ eye protection/ face protection.

QuantiFERON Wash Buffer 20x Concentrate new

Harmful to aquatic life with long lasting effects. Avoid release to the environment.

Further information

Safety Data Sheets: www.qiagen.com/safety

- Deviations from the *QuantiFERON-TB Gold (QFT) ELISA Package Insert* may yield erroneous results. Please read the instructions carefully before use.
- Do not use kit if any reagent bottle shows signs of damage or leakage prior to use.
- Blood samples should be transported to the laboratory at ambient temperature (22°C ± 5°C). Do not transport on ice or refrigerate.
- **Important:** Inspect vials prior to use. Do not use Conjugate or IFN-γ Standard vials that show signs of damage or if the rubber seal has been compromised. Do not handle broken vials. Take the appropriate safety precautions to dispose of vials safely. Recommendation: Use a vial de-crimper to open the Conjugate or IFN-γ Standard vials to minimize risk of injury from the metal crimp cap.
- Do not mix or use the Microplate Strips, Human IFN-γ Standard, Green Diluent, or Conjugate 100X Concentrate from different QFT kit batches. Other reagents (Wash Buffer 20X Concentrate, Enzyme Substrate Solution, and Enzyme Stopping Solution) can be interchanged between kits providing the reagents are within their expiration periods and lot details recorded. Discard unused reagents and biological samples in accordance with Local, State, and Federal regulations.
- Do not use the blood collection tubes or ELISA kit after the expiration date.
- Ensure that laboratory equipment such as plate washers and readers have been calibrated/validated for use.

5. Specimen Collection and Handling

QFT uses the following collection tubes:

1. QuantiFERON Nil tubes (gray cap with white ring; use between sea level and 810 m)
2. TB Antigen tubes (red cap with white ring; use between sea level and 810 m)
3. QuantiFERON Mitogen tubes (purple cap with white ring; use between sea level and 810 m)

High Altitude (HA) Tubes

1. QuantiFERON HA Nil tubes (gray cap with yellow ring; use between 1020 m and 1875 m)
2. HA TB Antigen tubes (red cap with yellow ring; use between 1020 m and 1875 m)
3. QuantiFERON HA Mitogen tubes (purple cap with yellow ring; use between 1020 m and 1875 m)

Antigens have been dried onto the inner wall of the blood collection tubes so it is essential that the contents of the tubes be thoroughly mixed with the blood. The tubes must be transferred to a 37°C ± 1°C incubator as soon as possible and within 16 hours of collection

The following procedures should be followed for optimal results:

1. For each subject collect 1 ml of blood by venipuncture directly into each of the QFT blood collection tubes. This procedure should be performed by a trained phlebotomist.
   - Standard QFT blood collection tubes should be used up to an altitude of 810 meters. High Altitude (HA) QFT blood collection tubes should be used at altitudes between 1020 and 1875 meters.
   - If using QFT blood collection tubes outside these altitude ranges, or if low blood draw volume occurs, blood can be collected using a syringe, and 1 ml immediately transferred to each of the three tubes. For safety reasons, this is best performed by removing the syringe needle, ensuring appropriate safety procedures, removing the caps from the 3 QFT tubes and adding 1 ml of blood to each (to the black mark on the side of the tube label). Replace the caps securely and mix as described below.
   - As 1 ml tubes draw blood relatively slowly, keep the tube on the needle for 2–3 seconds once the tube appears to have completed filling, to ensure that the correct volume is drawn.

     The black mark on the side of the tubes indicates the 1 ml fill volume. QFT blood collection tubes have been validated for volumes ranging from 0.8 to 1.2 ml. If the level of blood in any tube is not close to the indicator line, it is recommended to obtain another blood sample.
   - If a "butterfly needle" is being used to collect blood, a "purge" tube should be used to ensure that the tubing is filled with blood prior to the QFT tubes being used.
   - Alternatively, blood may be collected in a single generic blood collection tube containing lithium heparin as the anticoagulant and then transferred to QFT tubes. Only use lithium heparin as a blood anticoagulant since other anticoagulants interfere with the assay. Fill a blood collection tube (minimum volume 5 ml) and

gently mix by inverting the tube several times to dissolve the heparin. Blood should be maintained at room temperature (22°C ± 5°C) before transfer to QFT tubes for incubation, which **must** be initiated within 16 hours of blood collection.

2. Immediately after filling the tubes, shake them ten (10) times just firmly enough to ensure that the entire inner surface of the tube is coated with blood, to dissolve antigens on tube walls.
   - Tubes should be between 17°C – 25°C at the time of blood filling.
   - Over-energetic shaking may cause gel disruption and could lead to aberrant results.
   - If blood has been collected in a lithium heparin tube, samples must be evenly mixed before dispensing into QFT tubes. Ensure that the blood is thoroughly mixed by gentle inversion immediately prior to dispensing. Dispense 1.0 ml aliquots (one per QFT tube) into an appropriate Nil, TB Antigen, and Mitogen tube. This is best performed aseptically, ensuring appropriate safety procedures, removing the caps from the three QFT tubes and adding 1 ml of blood to each (to the black mark on the side of the tube label). Replace the tube caps securely and mix as described above.
3. Label tubes appropriately.
   - Ensure each tube (Nil, TB Antigen, Mitogen) is identifiable by its label or other means once the cap is removed.
4. Following filling, shaking, and labeling, the tubes must be transferred to a 37°C ± 1°C incubator as soon as possible, and within 16 hours of collection. Prior to incubation, maintain the tubes at room temperature (22°C ± 5°C). Do not refrigerate or freeze the blood samples.

6. Directions for Use

Stage 1 — Incubation of Blood and Harvesting of Plasma

Materials provided

- QFT blood collection tubes (Refer to Section 3)

Materials required (but not provided)

- Refer to Section 3

Procedure

1. If the blood is not incubated immediately after collection, re-mixing of the tubes by inverting 10 times must be performed immediately prior to incubation.
2. Incubate the tubes UPRIGHT at 37°C ± 1°C for 16 to 24 hours. The incubator does not require CO_2 or humidification.
3. After incubation at 37°C ± 1°C, blood collection tubes may be held between 4°C and 27°C for up to 3 days prior to centrifugation.
4. **A**fter incubation of the tubes at 37°C ± 1°C, harvesting of plasma is facilitated by centrifuging the tubes for 15 minutes at 2000 to 3000 RCF (g). The gel plug will separate the cells from the plasma. If this does not occur, the tubes should be re-centrifuged at a higher speed.
   - It is possible to harvest the plasma without centrifugation, but additional care is required to remove the plasma without disturbing the cells.
5. Plasma samples should only be harvested using a pipet.
   - After centrifugation, avoid pipetting up and down or mixing plasma by any means prior to harvesting. At all times, take care not to disturb material on the surface of the gel.
   - Plasma samples can be loaded directly from centrifuged blood collection tubes into the QFT ELISA plate, including when automated ELISA workstations are used.
   - Plasma samples can be stored for up to 28 days at 2°C to 8°C or, if harvested, below –20°C (preferably less than -70°C) for extended periods.
   - For adequate test samples, harvest at least 150 µl of plasma.

Stage 2 — Human IFN-γ ELISA

Materials provided

- QFT ELISA kit (Refer to Section 3).

Materials required but not provided

- Refer to Section 3.

Procedure

1. All plasma samples and reagents, except for Conjugate 100X Concentrate, must be brought to room temperature (22°C ± 5°C) before use. Allow at least 60 minutes for equilibration.
2. Remove strips that are not required from the frame, reseal in the foil pouch, and return to the refrigerator for storage until required.

   Allow at least 1 strip for the QFT standards and sufficient strips for the number of subjects being tested (refer to Figures 2A and 2B for 3-tube and 2-tube formats, respectively). After use, retain frame and lid for use with remaining strips.
3. Reconstitute the Human IFN-γ standard with the volume of deionized or distilled water indicated on the label of the standard vial. Mix gently to minimize frothing and ensure complete solubilization. Reconstitution of the standard to the stated volume will produce a solution with a concentration of 8.0 IU/ml.

   Note: The reconstitution volume of the kit standard will differ between batches.

   Use the reconstituted kit standard to produce a 1 in 4 dilution series of IFN-γ in Green Diluent (GD) (see Figure 1). S1 (Standard 1) contains 4 IU/ml, S2 (Standard 2) contains 1 IU/ml, S3 (Standard 3) contains 0.25 IU/ml, and S4 (Standard 4) contains 0 IU/ml (GD alone). The standards should be assayed at least in duplicate.

Recommended procedure for duplicate standards	Recommended procedure for triplicate standards
a. Label 4 tubes "S1", "S2", "S3", "S4".	a. Label 4 tubes "S1", "S2", "S3", "S4".
b. Add 150 µl of GD to S1, S2, S3, S4.	b. Add 150 µl of GD to S1.
c. Add 150 µl of the kit standard to S1 and mix thoroughly.	c. Add 210 µl of GD to S2, S3, S4.
d. Transfer 50 µl from S1 to S2 and mix thoroughly.	d. Add 150 µl of the kit standard to S1 and mix thoroughly.
e. Transfer 50 µl from S2 to S3 and mix thoroughly.	e. Transfer 70 µl from S1 to S2 and mix thoroughly.
f. GD alone serves as the zero standard (S4).	f. Transfer 70 µl from S2 to S3 and mix thoroughly.
	g. GD alone serves as the zero standard (S4).

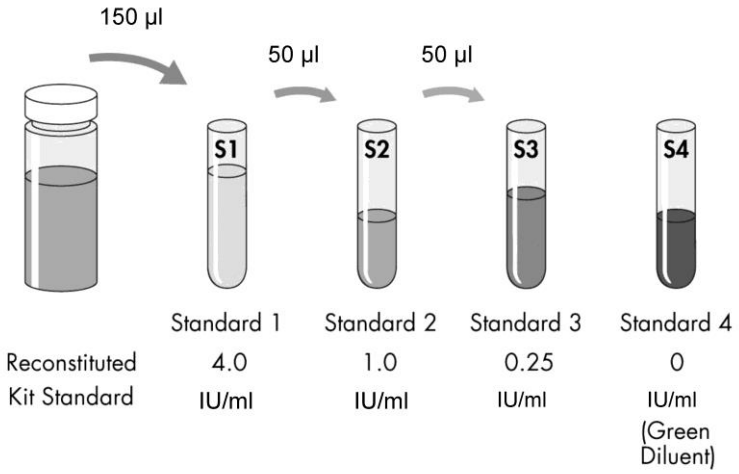


Figure 1. Preparation of standard curve. Prepare fresh dilutions of the kit standard for each ELISA session.

4. **Reconstitute freeze dried Conjugate 100X Concentrate with 0.3 ml of deionized or distilled water. Mix gently to minimize frothing and ensure complete solubilization of the conjugate.**

   Working strength conjugate is prepared by diluting the required amount of reconstituted Conjugate 100X Concentrate in Green Diluent as set out in Table 1 – Conjugate Preparation.

Table 1. Conjugate preparation

Number of strips	Volume of Conjugate 100X Concentrate	Volume of Green Diluent
2	10 µl	1.0 ml
3	15 µl	1.5 ml
4	20 µl	2.0 ml
5	25 µl	2.5 ml
6	30 µl	3.0 ml
7	35 µl	3.5 ml
8	40 µl	4.0 ml
9	45 µl	4.5 ml
10	50 µl	5.0 ml
11	55 µl	5.5 ml
12	60 µl	6.0 ml

- Mix thoroughly but gently to avoid frothing.
- Working strength conjugate should be used within 6 hours of preparation.
- Return any unused Conjugate 100X Concentrate to 2°C to 8°C immediately after use.
- Use only Green Diluent.

5. For plasma samples harvested from blood collection tubes and subsequently frozen or stored for more than 24 hours prior to assay, thoroughly mix before addition to the ELISA well.
   - If plasma samples are to be added directly from the centrifuged QFT tubes, any mixing of the plasma should be avoided. At all times take care not to disturb material on the surface of the gel.
6. Add 50 µl of freshly prepared working strength conjugate to the required ELISA wells using a multichannel pipet.
7. Add 50 µl of test plasma samples to appropriate wells using a multichannel pipet (Refer to recommended plate layout on page 16 and 17, Figures 2A and 2B). Finally, add 50 µl each of the Standards 1 to 4.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	1A	1M	*S1*	*S1*	*S1*	13N	13A	13M	21N	21A	21M
B	2N	2A	2M	*S2*	*S2*	*S2*	14N	14A	14M	22N	22A	22M
C	3N	3A	3M	*S3*	*S3*	*S3*	15N	15A	15M	23N	23A	23M
D	4N	4A	4M	*S4*	*S4*	*S4*	16N	16A	16M	24N	24A	24M
E	5N	5A	5M	9N	9A	9M	17N	17A	17M	25N	25A	25M
F	6N	6A	6M	10N	10A	10M	18N	18A	18M	26N	26A	26M
G	7N	7A	7M	11N	11A	11M	19N	19A	19M	27N	27A	27M
H	8N	8A	8M	12N	12A	12M	20N	20A	20M	28N	28A	28M

Figure 2A. Recommended sample layout for Nil, TB Antigen, and Mitogen tubes (28 tests per plate).

- S1 (Standard 1), S2 (Standard 2), S3 (Standard 3), S4 (Standard 4)
- 1N (Sample 1. Nil plasma), 1A (Sample 1. TB Antigen plasma), 1M (Sample 1. Mitogen plasma)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	5N	9N	13N	17N	*S1*	*S1*	25N	29N	33N	37N	41N
B	1A	5A	9A	13A	17A	*S2*	*S2*	25A	29A	33A	37A	41A
C	2N	6N	10N	14N	18N	*S3*	*S3*	26N	30N	34N	38N	42N
D	2A	6A	10A	14A	18A	*S4*	*S4*	26A	30A	34A	38A	42A
E	3N	7N	11N	15N	19N	21N	23N	27N	31N	35N	39N	43N
F	3A	7A	11A	15A	19A	21A	23A	27A	31A	35A	39A	43A
G	4N	8N	12N	16N	20N	22N	24N	28N	32N	36N	40N	44N
H	4A	8A	12A	16A	20A	22A	24A	28A	32A	36A	40A	44A

Figure 2B. Recommended sample layout for Nil and TB Antigen tubes (44 tests per plate).

- S1 (Standard 1), S2 (Standard 2), S3 (Standard 3), S4 (Standard 4)
- 1N (Sample 1. Nil plasma), 1A (Sample 1. TB Antigen plasma)

8. Mix the conjugate and plasma samples/standards thoroughly using a microplate shaker for 1 minute at 500 to 1000 rpm.
9. Cover each plate with a lid and incubate at room temperature (22°C ± 5°C) for 120 ± 5 minutes.
    - Plates should not be exposed to direct sunlight during incubation.
10. During the incubation, dilute one part Wash Buffer 20X Concentrate with 19 parts deionized or distilled water and mix thoroughly. Sufficient Wash Buffer 20X Concentrate has been provided to prepare 2 liters of working strength wash buffer.

Wash wells with 400 µl of working strength wash buffer for at least 6 cycles. An automated plate washer is recommended.

- Thorough washing is very important to the performance of the assay. Ensure each well is completely filled with wash buffer to the top of the well for each wash cycle. A soak period of at least 5 seconds between each cycle is recommended.
- Standard laboratory disinfectant should be added to the effluent reservoir, and established procedures followed for the decontamination of potentially infectious material.

11. Tap plates face down on absorbent, lint-free towel to remove residual wash buffer. Add 100 µl of Enzyme Substrate Solution to each well and mix thoroughly using a microplate shaker.
12. Cover each plate with a lid and incubate at room temperature (22°C ± 5°C) for 30 minutes.
    - Plates should not be exposed to direct sunlight during incubation.
13. Following the 30-minute incubation, add 50 µl of Enzyme Stopping Solution to each well and mix.
    - Enzyme Stopping Solution should be added to wells in the same order and at approximately the same speed as the substrate in step 11.
14. Measure the Optical Density (OD) of each well within 5 minutes of stopping the reaction using a microplate reader fitted with a 450 nm filter and with a 620 nm to 650 nm reference filter. OD values are used to calculate results.

7. Calculations and Test Interpretation

QFT Analysis Software is used to analyze raw data and calculate results. It is available from www.QuantiFERON.com. Please ensure that the most current version of the software is used.

The software performs a quality control assessment of the assay, generates a standard curve, and provides a test result for each subject, as detailed in the Interpretation of Results section.

As an alternative to using the QFT Analysis Software, results can be determined according to the following method.

Generation of Standard Curve

(if QFT Analysis Software is not used)

Determine the mean OD values of the kit standard replicates on each plate.

Construct a $\log_{(e)}$-$\log_{(e)}$ standard curve by plotting the $\log_{(e)}$ of the mean OD (y-axis) against the $\log_{(e)}$ of the IFN-γ concentration of the standards in IU/ml (x-axis), omitting the zero standard from these calculations. Calculate the line of best fit for the standard curve by regression analysis.

Use the standard curve to determine the IFN-γ concentration (IU/ml) for each of the test plasma samples, using the OD value of each sample.

These calculations can be performed using software packages available with microplate readers, and standard spreadsheet or statistical software (such as Microsoft® Excel®). It is recommended that these packages be used to calculate the regression analysis, the coefficient of variation (%CV) for the standards, and the correlation coefficient (r) of the standard curve.

Quality Control of Test

The accuracy of test results is dependent on the generation of an accurate standard curve. Therefore, results derived from the standards must be examined before test sample results can be interpreted.

For the ELISA to be valid:

- The mean OD value for Standard 1 must be ≥ 0.600.
- The %CV for Standard 1 and Standard 2 replicate OD values must be ≤15%.
- Replicate OD values for Standard 3 and Standard 4 must not vary by more than 0.040 optical density units from their mean.
- The correlation coefficient (r) calculated from the mean absorbance values of the standards must be ≥ 0.98.

The QFT Analysis Software calculates and reports these quality control parameters.

If the above criteria are not met the run is invalid and must be repeated.

The mean OD value for the Zero Standard (Green Diluent) should be ≤ 0.150. If the mean OD value is > 0.150 the plate washing procedure should be investigated.

Interpretation of Results

QFT results are interpreted using the following criteria:

Note: Diagnosing or excluding tuberculosis disease, and assessing the probability of LTBI, requires a combination of epidemiological, historical, medical, and diagnostic findings that should be taken into account when interpreting QFT results (Tables 2 and 3).

Table 2. When Nil, TB Antigen, and Mitogen tubes are used

Nil (IU/ml)	TB Antigen minus Nil (IU/ml)	Mitogen minus Nil (IU/ml)*	QFT result	Report/Interpretation
≤8.0	< 0.35	≥ 0.5	Negative	*M. tuberculosis* infection NOT likely
	≥ 0.35 and < 25% of Nil value	≥ 0.5	Negative	*M. tuberculosis* infection NOT likely
	≥ 0.35 and ≥ 25% of Nil value	Any	Positive†	*M. tuberculosis* infection likely
	< 0.35	< 0.5	Indeterminate‡	Results are indeterminate for TB-Antigen responsiveness
	≥ 0.35 and < 25% of Nil value	< 0.5	Indeterminate‡	Results are indeterminate for TB-Antigen responsiveness
> 8.0§	Any	Any	Indeterminate‡	Results are indeterminate for TB-Antigen responsiveness

* Responses to the Mitogen positive control (and occasionally TB Antigen) can be outside the range of the microplate reader. This has no impact on test results. Values > 10 IU/ml are reported by the QFT software as > 10 IU/ml.

† Where *M. tuberculosis* infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate in the QFT ELISA. If repeat testing of one or both replicates is positive, the individual should be considered test positive.

‡ Refer to the "Troubleshooting Guide" section for possible causes.

§ In clinical studies, less than 0.25% of subjects had IFN-γ levels of > 8.0 IU/ml for the Nil value.

The magnitude of the measured IFN-γ level cannot be correlated to the stage or degree of infection, level of immune responsiveness, or likelihood for progression to active disease. A positive TB response in persons who are negative to Mitogen is rare, but has been seen in patients with TB disease. This indicates the IFN-γ response to TB Antigen is greater than that to Mitogen, which is possible as the level of Mitogen does not maximally stimulate IFN-γ production by lymphocytes (see Figure 3).

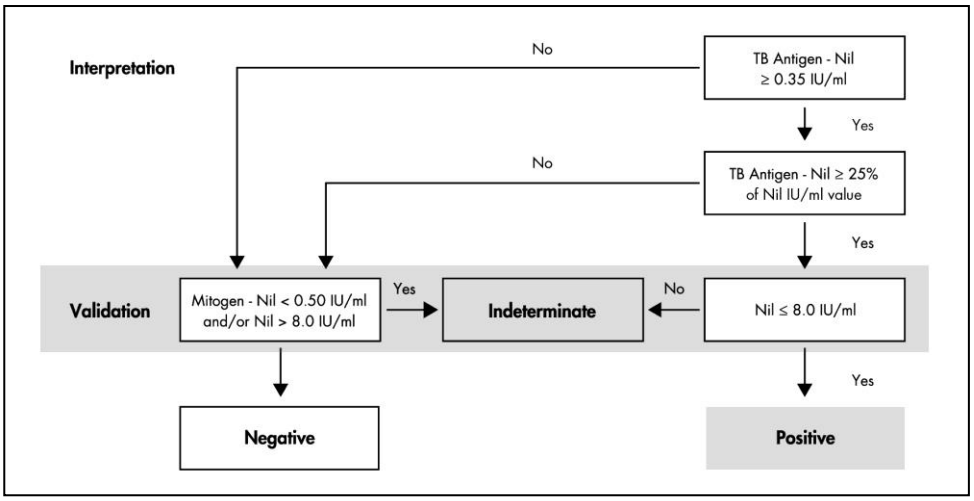


Figure 3. Interpretation flowchart where Nil, TB Antigen, and Mitogen tubes are used.

Table 3. When only QuantiFERON Nil and TB Antigen are tubes used

Nil (IU/ml)	TB Antigen minus Nil (IU/ml)	QFT result	Report/Interpretation
≤8.0	< 0.35	Negative	*M. tuberculosis* infection NOT likely
	≥0.35 and < 25% of Nil value	Negative	*M. tuberculosis* infection NOT likely
	≥ 0.35 and ≥ 25% of Nil value	Positive*	*M. tuberculosis* infection likely
> 8.0†	Any	Indeterminate‡	Results are indeterminate for TB-Antigen responsiveness

* Where *M. tuberculosis* infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate in the QFT ELISA. If repeat testing of one or both replicates is positive, the individual should be considered test positive.

† In clinical studies, less than 0.25% of subjects had IFN-γ levels of > 8.0 IU/ml for the Nil value.

‡ Refer to the "Troubleshooting Guide" section for possible causes.

The magnitude of the measured IFN-γ level cannot be correlated to stage or degree of infection, level of immune responsiveness, or likelihood for progression to active disease.

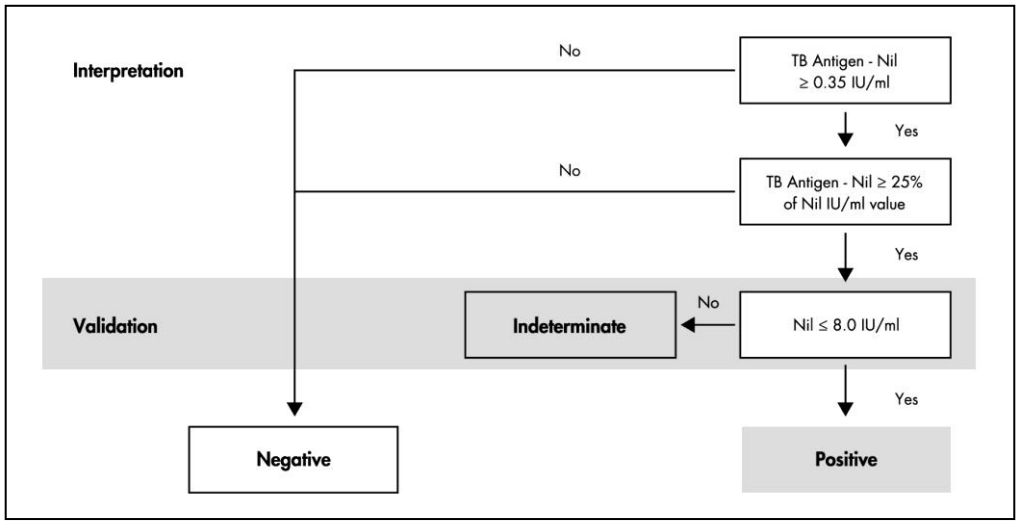


Figure 4. Interpretation flowchart when Nil and TB Antigen tubes are used.

8. Limitations

Results from QFT testing must be used in conjunction with each individual's epidemiological history, current medical status, and other diagnostic evaluations.

Individuals with Nil values greater than 8 IU/ml are classed as "indeterminate" because a 25% higher response to the TB antigens may be outside the assay measurement range.

Unreliable or indeterminate results may occur due to:

- Deviations from the procedure described in the *QuantiFERON-TB Gold (QFT) ELISA Package Insert*
- Excessive levels of circulating IFN-γ or presence of heterophile antibodies
- Longer than 16 hours between drawing the blood specimen and incubation at 37°C ± 1°C.

9. Performance Characteristics

Clinical Studies

As there is no definitive standard for latent tuberculosis infection (LTBI), an estimate of sensitivity and specificity for QFT cannot be practically evaluated. Specificity of QFT was approximated by evaluating false positive rates in the persons with low risk (no known risk factors) of tuberculosis infection. Sensitivity was approximated by evaluating groups of patients with culture-confirmed active TB disease.

Specificity

In a US study involving 866 volunteers, blood was drawn for QFT when a TST was placed. Demographic information and risk factors for TB were determined using a standard survey

at the time of testing. Of 432 volunteers with no known risk factors for *M. tuberculosis* infection, QFT and TST results were available for 391. None were BCG vaccinated. A second specificity study was performed with QFT in low-risk individuals in Japan, approximately 90% of whom had received BCG vaccination. Results from the 2 specificity studies are shown in Table 4.

Table 4. QFT specificity: Results for persons with no reported risk for *M. tuberculosis* infection.

Study	BCG status (% vaccinated)	Total tested	No. QFT indeterminate	No. QFT positive/ no. valid tests	QFT specificity (95% CI)	No. TST positive / no. tested	TST* specificity (95% CI)
US (unpublished)	0%	391	1	3/390	99.2% (98–100)	6/391	98.5% (97–99)
Japan (15)	~90%	168	6	2/162	98.8% (95–100)	–	–
Total	–	559	7/559 (1.3%)	5/552	99.1% (98–100)	–	–

* Using 10 mm TST cut off in non-BCG-vaccinated people. TST specificity estimate is 99.1% if using a 15 mm cut off.

Sensitivity for active TB

TB suspects from the US, Australia, and Japan who were subsequently confirmed to have *M. tuberculosis* infection by culture were tested to evaluate the sensitivity of QFT. While there is no definitive standard test for latent TB infection (LTBI), a suitable surrogate is the microbiological culture of *M. tuberculosis* because patients with disease are by definition infected. The patients had received less than 8 days of treatment prior to the collection of blood for QFT testing.

Table 5 summarizes findings from the 3 groups of *M. tuberculosis* culture-positive patients. The overall sensitivity of QFT for active TB disease was 89% (157/177).

Table 5. QFT: Subjects with culture-confirmed *M. tuberculosis* infection.

Study	No. QFT positive / no. valid tests	QFT sensitivity (95% CI)
Japan TB patients (15)	86/92	93% (86–97%)
Australian	24/27	89% (72–96%)
US	47/58	81% (69–90%)
Total	157/177	89% (83–93%)

Diagnosis of LTBI

A number of studies have been published which demonstrate the performance of QFT in various populations at risk of LTBI. The principle findings of some selected studies are shown in Table 6.

Table 6. Selected published studies on QFT in populations at risk of LTBI.

Study	Total tested	Outcomes and findings
Healthcare workers in India (Pai, et al 2005) (26)	726	Setting of very high TB rates. 40% QFT positive and 41% TST positive at 10 mm. High concordance with TST, no effect of BCG on either side. Both tests related to risk factors of age and period of work in healthcare.
Danish HIV+ patients (Brock, et al 2006) (5)	590	Overall prevalence of LTBI by QFT was 4.6% (27/590) in HIV+ persons. Positive results were associated with TB risks. Two QFT-positive subjects progressed to active TB within 1 year. Indeterminate responses (n=20. 3.4%) were significantly associated with a CD4 count <100/µl.
Hospitalized children in India (Dogra, et al 2006) (10)	105	Children in whom TB was suspected or who had a history of TB contact were tested with QFT and TST; 10.5% QFT positive and 9.5% TST positive at 10 mm. Agreement between tests was 95.2% overall and 100% in non-BCG vaccinated.
Contact investigations in Germany (Diel, et al 2006) (9)	309	Close contacts of 15 different index cases were tested: 51% were BCG vaccinated, 27% foreign born; 70% of BCG vaccinates and 18% of non-vaccinated were TST positive (5mm), whereas 9% and 11% were QFT positive, respectively. QFT was associated with TB risk. TST was only associated with BCG vaccination.

Many more publications describe the performance of the less-sensitive liquid antigen version of QuantiFERON-TB Gold (the precursor to QFT) and the QFT test. These studies include use of the test(s) in contacts of active TB cases (9, 11, 19, 25), children (6-10, 25, 28), HIV-positive patients (2, 5, 20), healthcare workers (13, 26, 32), immune suppressed patients (3, 4, 22, 23, 27, 30, 31), as well as TB suspects (7, 8, 10, 18), and low-risk individuals (15).

Repeatability and effect of TST on subsequent QFT testing

As part of the US specificity study, a subset of the volunteers was retested between 4 and 5 weeks after the original QFT test and TST. QFT results for 260 recruits were available at both time points and the level of agreement was 99.6% (259/260). A prior TST did not induce positive QFT responses.

10. Technical Information

Indeterminate Results

Indeterminate results should be uncommon and may be related to the immune status of the individual being tested, but may also be related to a number of technical factors:

- Longer than 16 hours between drawing of the blood and incubation at 37°C ± 1°C
- Storage of blood outside the recommended temperature range (17°C to 27°C) prior to 37°C ± 1°C incubation
- Insufficient mixing of blood collection tubes
- Incomplete washing of the ELISA plate

If technical issues are suspected with the collection or handling of the blood samples, repeat the entire QFT test with a new blood specimen. Repeating the ELISA testing of stimulated plasmas can be performed if inadequate washing or other procedural deviation with the ELISA test is suspected. Indeterminate tests that result from low Mitogen or high Nil values would not be expected to change on repeat unless there was an error with the ELISA testing. Indeterminate results should be reported as such. Physicians may choose to redraw a specimen or perform other procedures as appropriate.

Clotted Plasma Samples

Should fibrin clots occur with long-term storage of plasma samples, centrifuge the samples to sediment clotted material and facilitate pipetting of plasma.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the technical information provided at: **www.QuantiFERON.com**. For contact information, see the back cover.

ELISA troubleshooting

Non-specific color development

Possible cause	Solution
a) Incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
b) Cross-contamination of ELISA wells	Take care when pipetting and mixing sample to minimize risk.
c) Kit/components have expired	Ensure that the kit is used before the expiry date. Ensure reconstituted standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.
d) Enzyme Substrate Solution is contaminated	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.
e) Mixing of plasma in QFT tubes before harvesting	After centrifugation, avoid pipetting up and down or mixing plasma by any means prior to harvesting. At all times, take care not to disturb material on the surface of the gel.

Low optical density readings for standards

Possible cause	Solution
a) Standard dilution error	Ensure dilutions of the Kit Standard are prepared correctly as per the QFT ELISA Package Insert.
b) Pipetting error	Ensure pipets are calibrated and used according to manufacturer's instructions.
c) Incubation temperature too low	Incubation of ELISA should be performed at room temperature (22°C ± 5°C)
d) Incubation time too short	Incubation of the plate with the conjugate, standards and samples should be for 120 ± 5 minutes. The Enzyme Substrate Solution is incubated on the plate for 30 minutes.
e) Incorrect plate reader filter used	Plate should be read at 450 nm with a reference filter between 620 and 650 nm.

f) Reagents are too cold	All reagents, with the exception of the Conjugate 100X Concentrate, must be brought to room temperature prior to commencing the assay. This takes approximately one hour.
g) Kit/components have expired	Ensure that the kit is used before the expiry date. Ensure reconstituted standard and Conjugate 100X Concentrate are used within 3 months of the reconstitution date.

High background

Possible cause	Solution
a) Incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
b) Incubation temperature too high	Incubation of the ELISA should be performed at room temperature (22°C ± 5°C).
c) Kit/components have expired	Ensure that the kit is used before the expiry date. Ensure reconstituted standard and Conjugate 100X Concentrate are used within 3 months of the reconstitution date.
d) Enzyme Substrate Solution is contaminated	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.

Non-linear standard curve and duplicate variability

Possible cause	Solution
a) Incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
b) Standard dilution error	Ensure dilutions of the standard are prepared correctly as per the QFT ELISA Package Insert.
c) Poor mixing	Mix reagents thoroughly by inversion or gentle vortexing prior to their addition to the plate.
d) Inconsistent pipetting technique or interruption during assay set up	Sample and standard addition should be performed in a continuous manner. All reagents should be prepared prior to commencing the assay.

An assay procedure video and solutions to most technical problems can be found on Gnowee™ by registering directly at www.gnowee.net for online access. Product information and technical guides are available free of charge from QIAGEN, via your distributor, or by visiting www.QuantiFERON.com.

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A comprehensive list of QFT references is located on Gnowee — the QuantiFERON reference library, available at **www.gnowee.net**.

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26. Pai, M. et al. (2005) Mycobacterium tuberculosis infection in health care workers in rural India: comparison of a whole-blood, interferon-g assay with tuberculin skin testing. JAMA 293, 2746.

27. Ponce de Leon, D. et al. (2008) Comparison of an interferon-gamma assay with tuberculin skin testing for detection of tuberculosis (TB) infection in patients with rheumatoid arthritis in a TB-endemic population. J Rheumatol. 35, 776.

28. Richeldi, L. et al. (2008) Prior tuberculin skin testing does not boost QuantiFERON-TB results in paediatric contacts. Eur. Respir. J. 32, 524.

29. Rothel, J.S. and Andersen, P. (2005) Diagnosis of latent *Mycobacterium tuberculosis* infection: is the demise of the Mantoux test imminent? Expert Rev. Anti Infect. Ther. 3, 981.

30. Schoepfer, A.M. et al. (2008) Comparison of interferon-gamma release assay versus tuberculin skin test for tuberculosis screening in inflammatory bowel disease. Am. J. Gastroenterol. 103, 2799.

31. Silverman, M.S. et al. (2007) Use of an interferon-gamma based assay to assess bladder cancer patients treated with intravesical BCG and exposed to tuberculosis. Clin. Biochem. 40, 913.

32. Stebler, A. et al. (2008) Whole-blood interferon-gamma release assay for baseline tuberculosis screening of healthcare workers at a Swiss university hospital. Infect. Control Hosp. Epidemiol. 29, 681.

Symbols

 2 x 96	Sufficient for 2 x 96 sample preparations
	Legal manufacturer
	CE-IVD marked symbol
	For in vitro diagnostic use
	Batch code
	Catalog number
	Global Trade Item Number
	Use by date
	Temperature limitation
	Consult instructions for use
	Do not reuse
	Keep away from sunlight

12. Technical Service

For technical assistance and more information, please call toll-free 00800-22-44-6000, see our Technical Support Center at **www.qiagen.com/contact** or contact one of the QIAGEN Technical Service Departments (see back cover or visit **www.qiagen.com**).

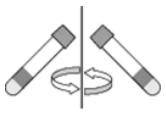
13. Abbreviated Test Procedure

Stage 1 — Blood Incubation

1. Collect patient blood into blood collection tubes and mix by shaking them ten (10) times just firmly enough to ensure that the entire inner surface of the tube has been coated with blood, to dissolve antigens on tube walls.

2. Incubate tubes upright at 37°C ± 1°C for 16 to 24 hours.

3. Following incubation, centrifuge tubes for 15 minutes at 2000 to 3000g RCF (g) to separate the plasma and the red cells.



4. After centrifugation, avoid pipetting up and down or mixing the plasma by any means prior to harvesting. At all times, take care not to disturb the material on the surface of the gel.

Stage 2 — IFN–γ ELISA

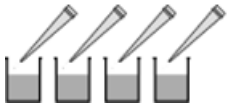
1. Equilibrate ELISA components, with the exception of the Conjugate 100X Concentrate, to room temperature (22°C ± 5°C) for at least 60 minutes.

2. Reconstitute the kit standard to 8.0 IU/ml with distilled or deionized water. Prepare four (4) standard dilutions.

3. Reconstitute freeze-dried Conjugate 100X Concentrate with distilled or deionized water.

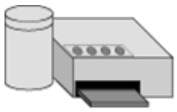
4. Prepare working strength conjugate in Green Diluent and add 50 µl to all wells.

5. Add 50 µl of test plasma samples and 50 µl standards to appropriate wells. Mix using shaker.

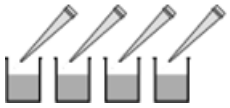


6. Incubate for 120 ± 5 minutes at room temperature.

7. Wash wells at least 6 times with 400 µl/well of wash buffer.

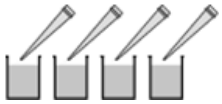


8. Add 100 µl Enzyme Substrate Solution to wells. Mix using shaker.



9. Incubate for 30 minutes at room temperature.

10. Add 50 µl Enzyme Stopping Solution to all wells. Mix using shaker.



11. Read results at 450 nm with a 620 to 650 nm reference filter.

12. Analyze results.

Significant Changes

Significant changes in this Version of the QFT ELISA Package Insert are summarized in the table below:

Section	Page	Change(s)
Precautions	10	New GHS information

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QFT™
QuantiFERON-TB Gold

QuantiFERON®-TB Gold Analysis Software (v2.62*) Instructional Guide

QuantiFERON-TB Gold Analysis Software is a PC-based program for calculating QuantiFERON-TB Gold (QFT™) test results.

The software may be downloaded from the Cellestis website. Alternatively, contact your authorised QuantiFERON distributor to obtain a copy via email or CD-ROM.

Customers will be advised by Cellestis or their QuantiFERON distributor as new editions of the software are made available.

This guide provides detailed step-by-step instructions on the use of QuantiFERON-TB Gold Analysis Software. It is recommended that you read these instructions before referring to the Software Quick Guide, available at www.cellestis.com

CONTENTS

Software features—

- Record test-related information.
- Automatically import, or manually enter, raw data.
- Highlight standards and samples to create an Analysis Format.
- Save Analysis Format for use with future tests.
- Assign subject's identity to each sample.
- Quality Control analysis of Standard Curve.
- Export data and results to other applications.
- Selection of reporting options.

Features addressed in this version—

- Correction of file loading error.

*For non-USA use only

cellestis
www.cellestis.com

Installation

FROM WEBSITE/EMAIL

- **Save** the **QFT_v2.62_setup.zip** file to an appropriate location on the computer's hard drive.

Alternatively, from the **Start Menu** select **Run** and then **Browse** to locate the Analysis Software .zip file. Ensure that **All Files** is chosen in the **Files of Type** field, then select **OK**.

If not currently installed, the program Winzip can be obtained from the website: **www.winzip.com**

- Using **My Computer**, locate the QFT_v2.62_setup.zip file and double-click on it. This will open the program Winzip®, which can be used to unzip the QFT Analysis Software **installation files**.

- Unzip the installation files to an appropriate location on the computer's hard drive—for example, the **temp** folder.

- Using My Computer, locate and run the file **QuantiFERON_Startup.exe**. An installation screen will appear, as described in the following section.

FROM SELF-INSTALLING CD-ROM

- Insert the QuantiFERON-TB Gold Analysis Software CD-ROM into the CD-ROM drive. An installation screen will automatically be displayed.

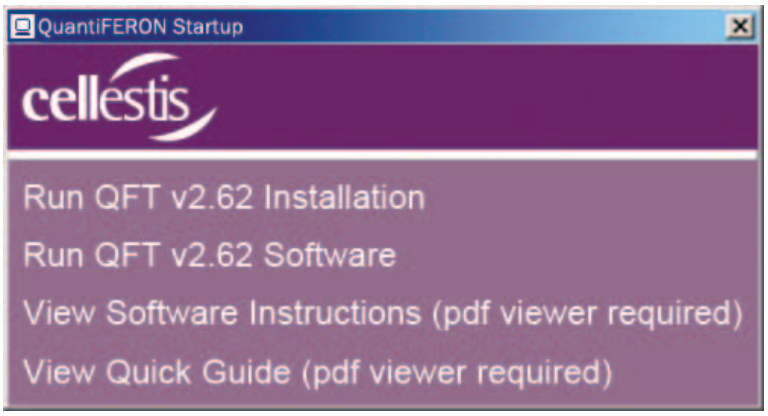


Figure 1 Installation screen.

The software and support files can also be accessed directly from the **CD-ROM** by selecting the other options on the installation screen.

- Select the option **Run QFT v2.62 Installation**. Follow the prompts to install the software and support files. A folder **Program Files\ QuantiFERON** is created for this purpose.

  Shortcuts to the Analysis Software are created on the desktop and in the **Start Menu.**

Getting Started

- Click on the **QFT v2.62 Software** shortcut to open the QuantiFERON-TB Gold Analysis Software.

  The program will open to the first of 4 screens that sequentially progress through the calculations. These 4 screens are

1. **Run Details**
   Enter general test details such as the **Run date**, **Run number**, **Kit batch number** and **Operator**.
2. **Raw Data**
   Enter Optical Density (OD) values and apply a format that defines the standards and samples.
3. **Standards Results**
   View Standard Curve results, which indicate the validity of the ELISA.
4. **Subject Results**
   View test results for each sample. Save, print and export data and results.

   The four screens are described in more detail on the following pages.

RUN DETAILS screen

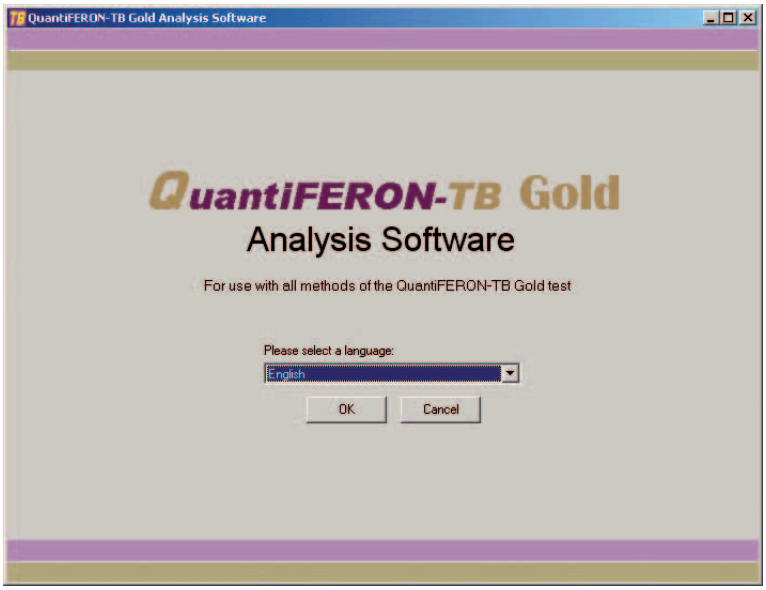


Figure 2 Language Selection screen.

- Select appropriate language.

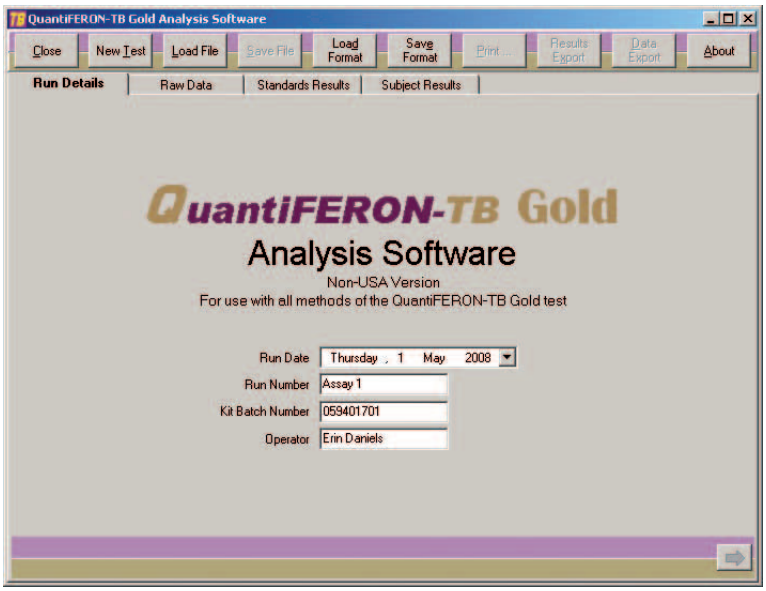


Figure 3 Run Details screen.

- Enter the following information in the fields provided:
  - Run Date (drop-down calendar)
  - Run Number
  - Kit Batch Number (shown on QuantiFERON ELISA box)
  - Operator

- Select the **Raw Data** tab to advance to the next screen.

RAW DATA screen

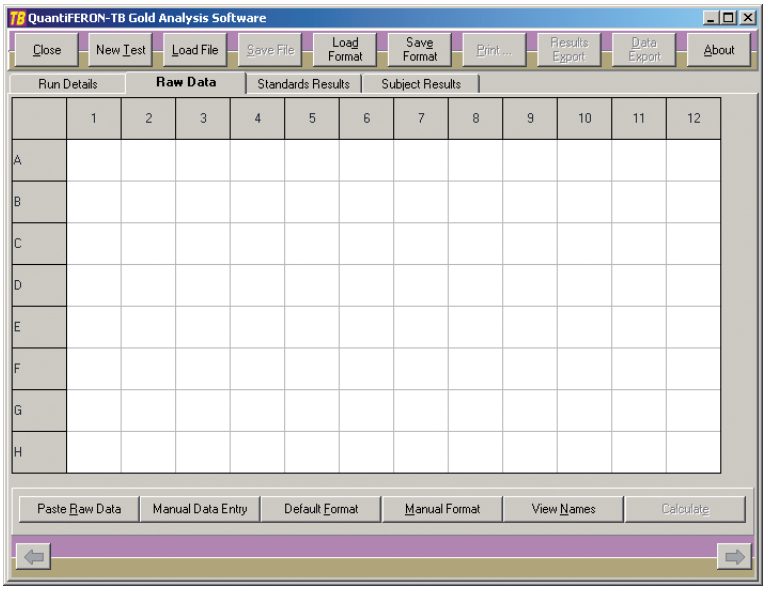


Figure 4 Raw Data screen.

DATA ENTRY

The QuantiFERON-TB Gold Analysis Software uses optical density (OD) values as the basis for all calculations. The user does not need to perform any calculations prior to using the software—simply enter the raw data from the plate reader into the software.

There are **two** methods of data entry

1. Automatic Data Entry

- **Copy** the raw data (OD values) to be analysed from the ELISA plate reader program. Some plate reader programs require the data to first be exported into a spreadsheet.

- Select the **Paste Raw Data** button—the data will be entered into the program's data cells.

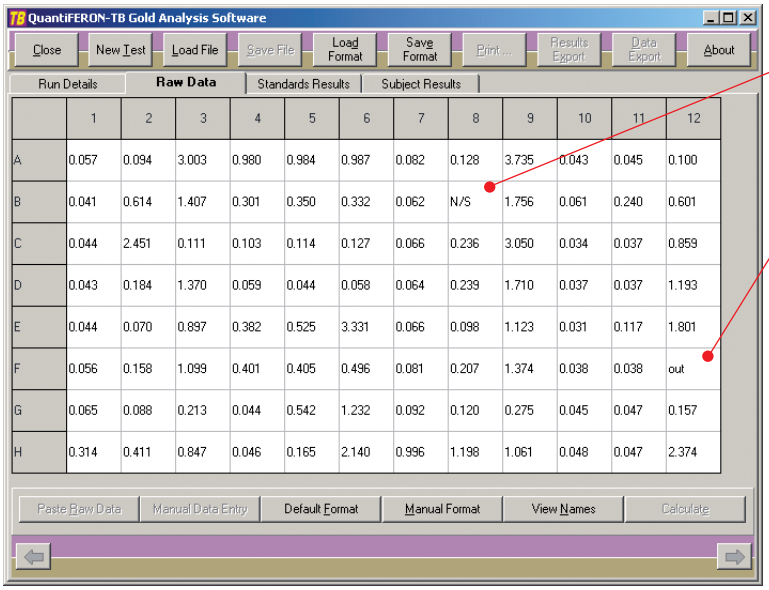


Figure 5 Raw Data screen after pasting raw data.

Data from plates with less than 12 strips can be analysed, however each strip of data pasted must contain 8 values (including empty cells, if necessary).

If for any reason data is **absent** from a cell, the cell is denoted by **N/S** (No Sample) and takes no further part in the analysis.

If a cell contains **text** (*****, out,** etc), the software interprets the OD value as being off-scale and the sample is given a final calculated value of **>10 IU/mL**.

Data cells for **standards** cannot be blank or contain text. If such a situation arises, the Analysis Software will report this as an Invalid ELISA.

Due to the logarithmic calculations performed by the software, **negative** OD values cannot be analysed. Negative OD values are not normally obtained for the QuantiFERON ELISA, and may indicate the need to service the plate reader.

Use ↑/↓ arrows—or the mouse—to navigate between cells.

RAW DATA screen

2. Manual Data Entry

- Select the **Manual Data Entry** button. Click on a cell to enter data manually, to three decimal places. Press **Enter**—or click on another cell—to store the value.

- When all data has been entered, select the **Complete** button on the **Manual Data Entry toolbar** to proceed.

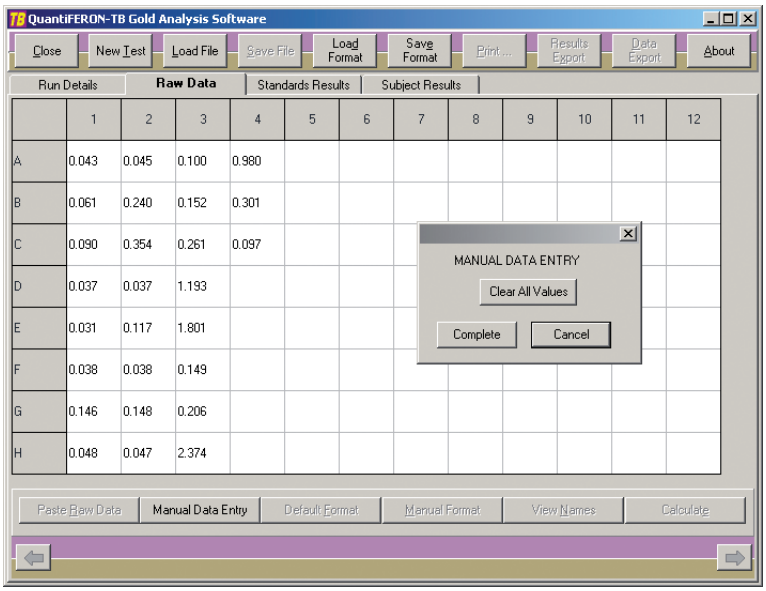


Figure 6 Raw Data screen while manually entering raw data.

RAW DATA screen

ANALYSIS FORMAT

Before data can be analysed, a **format** must be applied to nominate which cells are **samples** and which are **standards**.

- There are **two** methods for assigning a format

1. Default Format

- Select the **Default Format** button to automatically assign the relevant *Cellestis-recommended* testing layout to the data. The standards and samples will be set out in the same configuration as outlined in the relevant QFT Package Insert.

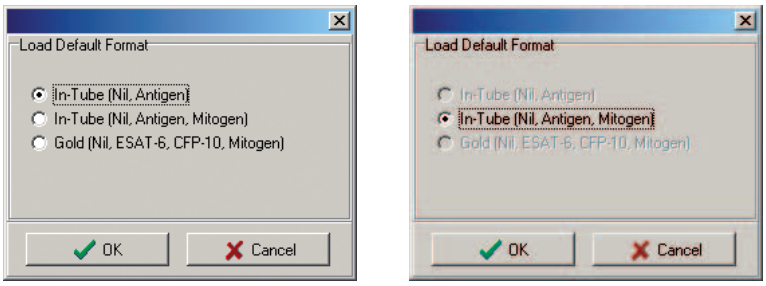


Figure 7 Load Default Format selection menu.

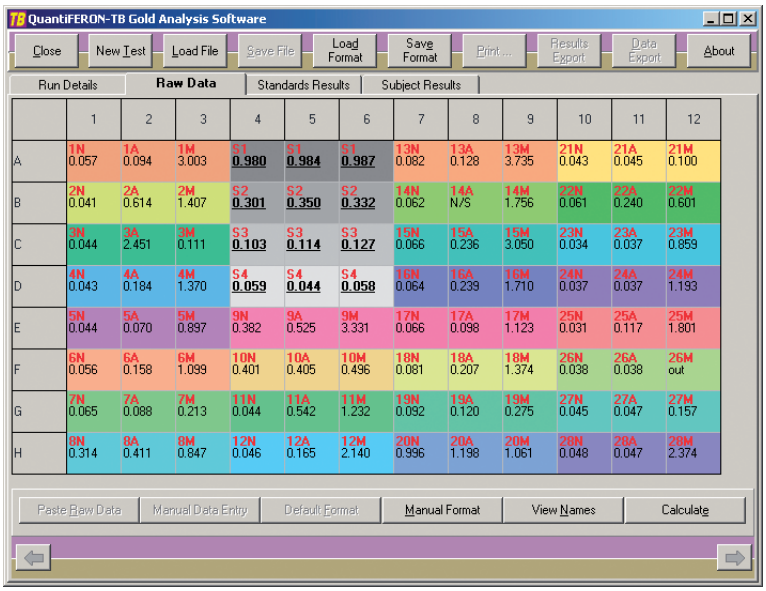


Figure 8 Raw Data screen after Default Format has been applied.

The format can be applied **either before or after** data entry. This allows formats to be prepared prior to obtaining the ELISA results.

Depending on the number of strips of data entered, the **Default Format** option may, or may not, be available—due to the location/orientation of samples and standards for each QFT test method (Gold, In-Tube).

The following table indicates the number of strips of data required for the Default Format option to be available.

QuantiFERON-TB Gold test method	Number of strips required
In-Tube (Nil, Antigen)	7+
In-Tube (Nil, Antigen, Mitogen)	6, 9, 12
Gold (Nil, ESAT-6, CFP-10, Mitogen)	7+

If the Default Format option is not available, the format can be applied manually.

Once the Default Format has been applied it can be edited by selecting the **Manual Format** button and following the instructions outlined below.

By **default**, the toolbar opens in **Standards** mode with standards ready to be assigned in a **vertical** orientation. These settings can be changed by selecting the appropriate radio buttons.

RAW DATA screen

2. Manual Format

- Select the **Manual Format** button to open the Manual Formatting Toolbar. This toolbar is used to manually assign both standards and subject samples to the data's format.

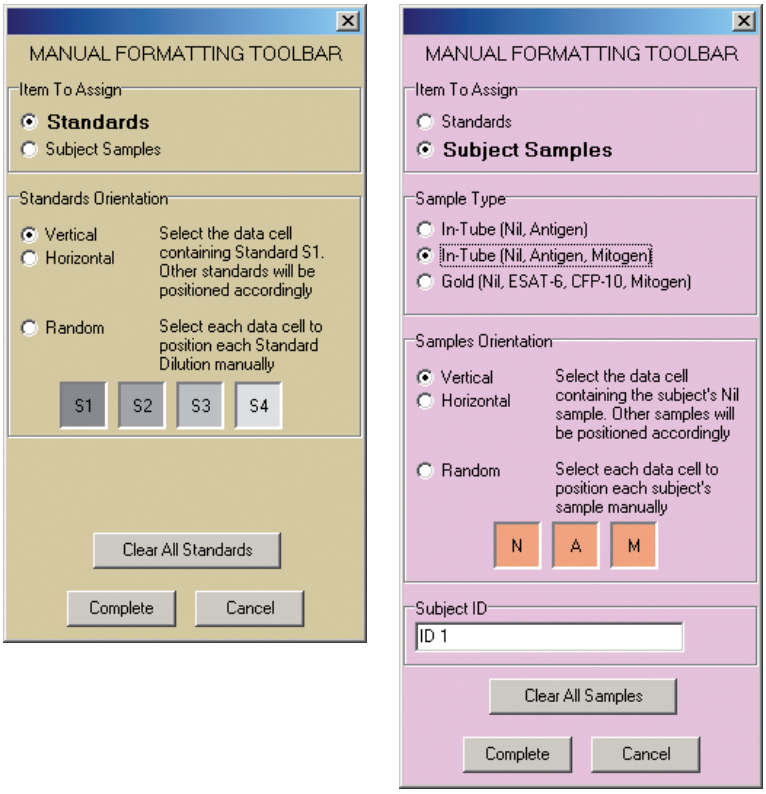


Figure 9 Manual Formatting Toolbar, in 'Standards mode' and 'Subject Samples mode'.

RAW DATA screen

Standards

- To assign a set of **standards** (S1, S2, S3, S4)—either **vertically** or **horizontally**—click on the cell that contains the data for standard **S1**. The chosen cell will be designated as S1, and the other standards will be appropriately positioned in adjacent cells, in order.

  To assign a set of standards in a **random** manner, each of the standards S1 to S4 must be positioned manually by clicking on the appropriate cells, in order.

Standard S1 is the highest standard, containing 4 IU/mL of IFN-γ. Standard S4 is the lowest standard, containing 0 IU/mL of IFN-γ.

Once the entire set of standards S1 to S4 has been assigned, the toolbar resets, ready to automatically assign another set of standards.

The **Standard Orientation** can be adjusted at any time, allowing replicates of standards to have different orientations in the one format.

- To **delete** a single set of standards, right-click on the coloured block and select **Delete Block** from the menu.

  Alternatively, to delete **all** standards, select the **Clear All Standards** button on the Manual Formatting Toolbar.

Subject Samples

- In order to assign **subject samples** to the data, select the **Subject Samples radio button** on the Manual Formatting Toolbar.

By default, **Subject Sample** mode opens with **Nil, Antigen and Mitogen** samples ready to be assigned in a **horizontal** orientation. Settings can be changed by selecting the appropriate radio buttons.

- To assign subject samples—either **vertically** or **horizontally**—click on the cell that contains the data for the subject's **Nil** sample. The chosen cell will be designated as Nil, and the other samples will be appropriately positioned in adjacent cells, in order.

  To assign subject samples in a **random** manner, each of the samples must be positioned manually by clicking on the appropriate cells.

Once the entire subject sample has been assigned, the toolbar is automatically ready to assign another sample of the **same type**. Subsequent subject samples are coloured differently in order to assist recognition of individual subjects.

The **Sample Type** and **Sample Orientation** can be adjusted at any time, in order to create a format containing a mixture of different QuantiFERON-TB Gold sample types.

- Prior to assigning a sample to the data, the subject's **name/ID** can be entered into the **Subject ID** field on the toolbar.

  Alternatively, subject naming can be performed according to the instructions in the next section.

- To **delete** a single subject sample, right-click on the coloured block and select **Delete Block** from the menu.

  Alternatively, to delete **all** subject samples, select the **Clear All Samples** button on the Manual Formatting Toolbar.

To **delete all** standards and subject samples, **right-click** on any coloured block and select **Clear Format** from the menu.

- Once the standards and subject samples have been applied, finish by selecting the **Complete** button.

  Upon completing a format, it can be **saved** as a file and reloaded for analysis of future data—allowing the user to create just a few format files for all of their analysis needs.

  Refer to the **Saving/Loading Files** section for further details.

Non-format information—such as run details and subject names—*is not* retained as part of the saved **format** file. These details *are*, however, retained as part of all saved **result** files.

RAW DATA screen

SUBJECT NAMES

As subject names can be up to 15 characters in length, they are not displayed on the Raw Data screen. Instead the stored subject names can be viewed via the **View Names** button.

- Subject names can be changed at any stage by left-clicking on the coloured block for each subject and typing the new name in the pop-up box.

- Alternatively, **multiple** Subject Names (IDs) can be changed more easily by selecting the **View Names** button. If all subject names are to begin with an identical prefix (eg. A009) these characters can be entered into the **ID Prefix** field. Afterwards, click on each subject's name in the list to add the remainder of the name manually.

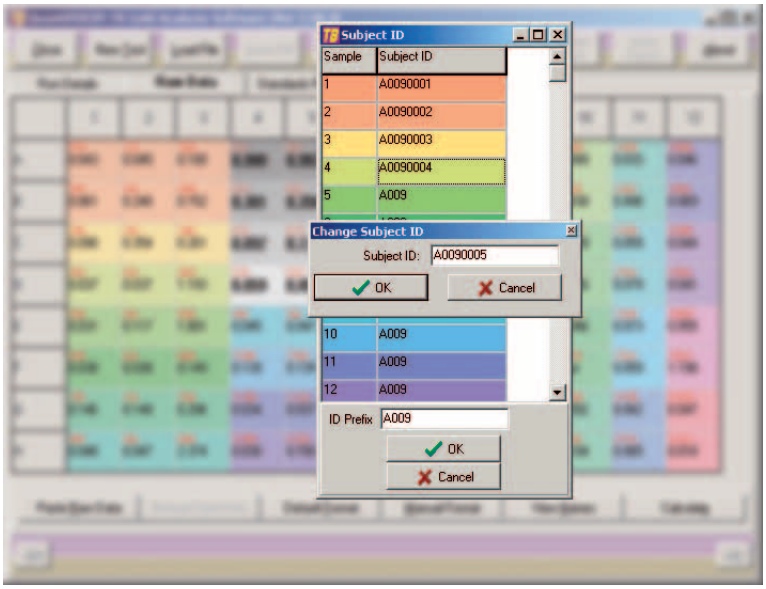


Figure 10 Renaming Subject Samples using the View Names button.

In order for the **Calculate** button to be enabled, *at least* **2 blocks of Standards** and **one Subject Sample** block must be assigned.

- Once the format has been generated, select the **Calculate** button. The standard curve for the assay will be automatically analysed and the **Standards Results** screen displayed.

STANDARDS RESULTS screen

The accuracy of test results is dependent on the accuracy of the standard curve. The software automatically performs Quality Control analysis of the standard curve prior to interpreting test sample results.

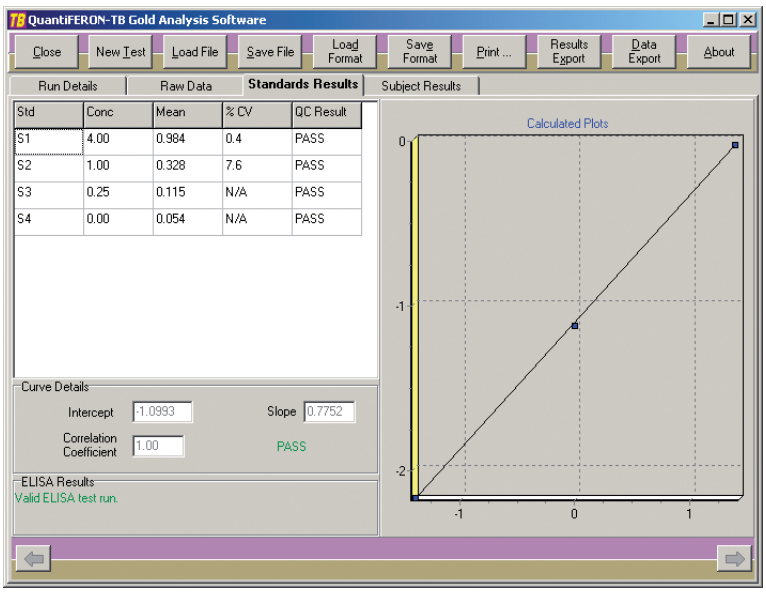


Figure 11 Standards Results screen.

- The **Standards Results** screen provides information that is directly related to the Acceptance Criteria of the ELISA:
  - Mean of the replicate standards
  - Coefficient of Variation of the replicate standards
  - Correlation Coefficient of OD values and known IFN-γ concentrations.

  The results of the Quality Control acceptance criteria for the Standard Curve are shown as **PASS** or **FAIL**.

For further details of the **acceptance criteria**, refer to the Package Insert.

- The following information is also displayed:
  - A graph of the Standard Curve, including Linear Regression line
  - Intercept and Slope of the linear regression.

- A statement indicating whether the ELISA test is **Valid** or **Invalid**—based on the QC criteria—is shown at the bottom of the screen. This statement is also displayed on all printed and PDF reports.

  If any of the QC criteria are not met, the ELISA test is INVALID and MUST be repeated.

- In the event that the Mean value of the **zero standard** (zero IFN-γ) is greater than **0.150 OD** units, a statement is displayed suggesting that ELISA plate washing procedures be investigated. This statement is also displayed on all printed and PDF reports.

The Package Insert contains additional information regarding **high background** results.

- Select the Subject Results tab to proceed to the next screen.

SUBJECT RESULTS screen

Refer to the Package Insert for more information on the calculation of QuantiFERON-TB Gold Results.

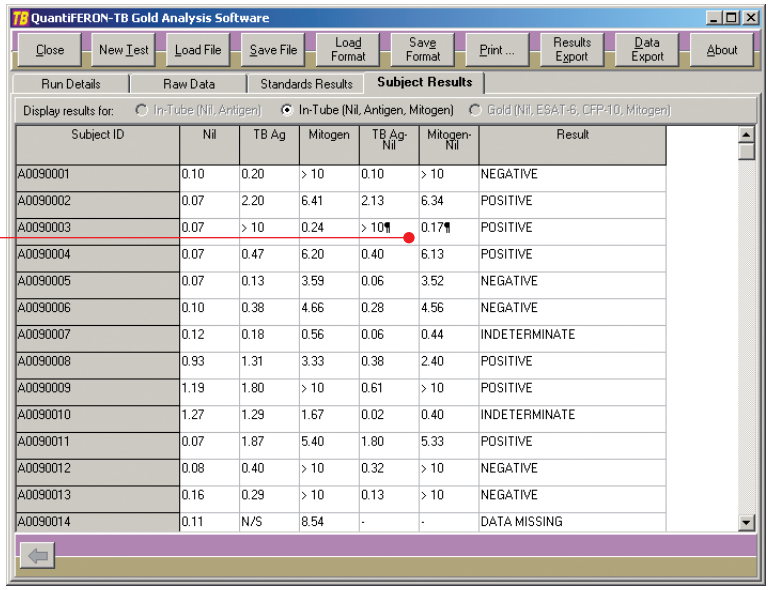
The Standard Curve is used to calculate a value (IU/mL of IFN-γ) for each subject's samples. The software **subtracts** the value of the **Nil** plasma sample from each of the other samples—based on these values, the **Result** for each Subject is reported.

- Subjects are grouped according to the QuantiFERON-TB Gold test method used for the samples.

  To view results for each QuantiFERON-TB Gold test method, select the relevant radio button. For example **In-Tube (Nil, Antigen)** in the example below..

The result **Data Missing** is reported if any of a Subject's plasma samples display the value **N/S** (No Sample).

In the unlikely event that a subject's result is reported as **positive** and their **Mitogen minus Nil result is less than 0.35 IU/mL**, the software will flag the result as a **possible sample mix-up** using the "¶" symbol. This warning helps to limit the possibility of a false positive result due to a mix-up of the TB antigen and Mitogen samples.



Subject ID	Nil	TB Ag	Mitogen	TB Ag-Nil	Mitogen-Nil	Result
A0090001	0.10	0.20	> 10	0.10	> 10	NEGATIVE
A0090002	0.07	2.20	6.41	2.13	6.34	POSITIVE
A0090003	0.07	> 10	0.24	> 10¶	0.17¶	POSITIVE
A0090004	0.07	0.47	6.20	0.40	6.13	POSITIVE
A0090005	0.07	0.13	3.59	0.06	3.52	NEGATIVE
A0090006	0.10	0.38	4.66	0.28	4.56	NEGATIVE
A0090007	0.12	0.18	0.56	0.06	0.44	INDETERMINATE
A0090008	0.93	1.31	3.33	0.38	2.40	POSITIVE
A0090009	1.19	1.80	> 10	0.61	> 10	POSITIVE
A0090010	1.27	1.29	1.67	0.02	0.40	INDETERMINATE
A0090011	0.07	1.87	5.40	1.80	5.33	POSITIVE
A0090012	0.08	0.40	> 10	0.32	> 10	NEGATIVE
A0090013	0.16	0.29	> 10	0.13	> 10	NEGATIVE
A0090014	0.11	N/S	8.54	-	-	DATA MISSING

Figure 12 Subject Results screen.

DATA EXPORT

This optional step is not required to obtain QuantiFERON-TB Gold results. It may be employed by the user for the purpose of pooling and trending data.

Care should be taken when pasting data into spreadsheet programs, due to the possibility of the spreadsheet's default formatting affecting the presentation of the data.

- If desired by the user, the **results** and/or **data** can be exported to external applications, such as Microsoft Excel.

  To export the results, select the **Results Export** button. The user can then choose to export the **assay details and results** to either the Windows Clipboard or a text file.

  Similarly, selecting the **Data Export** button offers the user the choice of exporting the **assay details, raw data and QC results** to either the Windows Clipboard or a text file.

Reports

Selecting the Print button will display a printing screen that is divided into two sections. The upper section displays the various printing options available, while the lower section displays a summary report of the ELISA details and results.

- The **Sample Type** options allow the user to print separate reports for all, or some, of the QuantiFERON-TB Gold test methods used.

  To print results for a specific test method, select the appropriate check box. Alternatively, select **Print All Reports** to print a separate report for all of the QuantiFERON-TB Gold test methods used.

If multiple test methods are selected for printing, the lower screen will display **only one** of the reports.

- The **Report Type** options allow the user to print various reports as follows:

  1. **All Subjects (Group Report)** prints the results for **all** subjects on **one** page.
  2. **All Subjects (Individual Report)** prints the results for **each** subject on a **separate** page.
  3. **Single Subject Report** prints the results for **one subject**, as selected from the drop-down box.

On the **All Subjects (Grouped)** report, the Raw OD values used to generate the Standard Curve are **highlighted** (bold and underlined).

- The **Print Standard Curve and Plate Formatting** option generates an additional report page containing the original raw data, plate layout and standard curve.

- Alternatively, reports can be saved as **PDF files**, as described in the **Saving/Loading Files** section.

  Once the desired type of summary report is selected, clicking on the **Print** button will print the report to the computer's **default** printer.

  Selecting the **Close Print Window** button will close the printing screen and return to the main software.

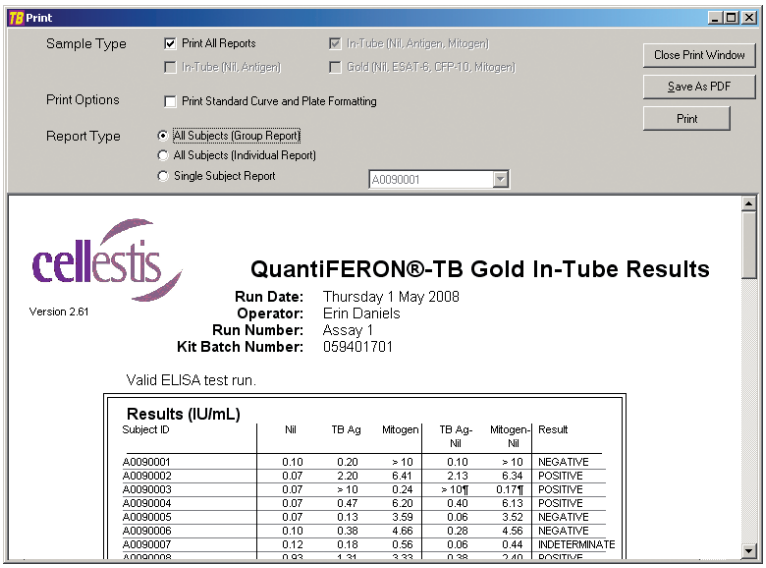


Figure 13 Summary report.

- The analytical range of the QuantiFERON-TB Gold ELISA is between zero and 10 IU/mL. Therefore, samples determined to have an IFN-γ concentration greater than this range are reported as **>10 IU/mL**.

Although values above 10 IU/mL are reported as **>10 IU/mL**, the calculations for subtracting the Nil control value are based on the **original** value. Therefore it is possible for a subject's TB antigen or Mitogen value to be reported as ">10 IU/mL", yet their "minus Nil" value be **less than** 10 IU/mL.

Saving / Loading Files

SAVING FILES

- Upon opening the QFT Analysis Software for the first time, a folder called **My Documents\QuantiFERON** is created. By default, all files are saved to sub-folders within this folder, and are given default file names as per the following table:

File Type	File Extension	Sub-Folder Name	Default File Name
Format	.qff	Format	OperatorDate
Results	.qdf	Save	Date_RunNumber
PDF Results	.pdf	PDF	Date_RunNumber

Run Details information *is not* retained within a saved **format** file.

- **Format files.** Select the **Save Format** button to save a completed format to file, which can be reloaded for use with future analysis.

Run Details information *is* retained within a saved **result** file.

- **Results files.** Select the **Save File** button to save a copy of the Results to file, which can be reloaded for further analysis.

- **PDF files.** Select the **Save As PDF** button to save the Results report in PDF format, for electronic viewing by others. It is recommended that PDF files be used for record keeping purposes.

  PDF files contain all of the information available in the printed report.

LOADING FILES

- **Format files** can be reloaded within the QFT Analysis Software by selecting the **Load Format** button.

After reloading a results file, the **Calculate** button must be selected in order to re-generate results.

- **Results files** can be reloaded by selecting the **Load File** button at any time.

END OF ANALYSIS

For convenience, the information previously entered into the Run Date, Kit Batch Details, and Operator fields on the Run Details screen is retained as default until the software is closed. These details can be modified as required.

- Selecting the **New Test** button clears all entered information, enabling new assay data to be analysed.

- Selecting the Close button will close the program.

Frequently Asked Questions

Q. *Why do I need to use the QuantiFERON-TB Gold Analysis Software? Can I use my own spreadsheet to calculate results instead?*

A. You can use your own spreadsheet to calculate QuantiFERON-TB Gold test results. However, the calculations required to obtain the correct IFN-γ values are logarithm based. Therefore, it is essential that your calculations, including Quality Control checks, are **validated** against the Analysis Software to ensure that the QuantiFERON-TB Gold test result obtained for each subject is correct.

The QFT software has already been rigorously validated to ensure that the Quality Control checks—and the results obtained—are accurate and reproducible.

The QFT software also has the added flexibility of simple one-click formatting of standards and samples, allowing for the format to be easily updated as changes to your ELISA test layout arise.

Q. *When a newer version of the software is available, should I uninstall the old version of the QFT Analysis Software? How do I do this?*

A. Yes, you should **always** uninstall obsolete versions of the software *before installing the new software*. The new version of the QFT software may contain changes to the test criteria, therefore it is essential that only the current version of the software be available for use.

To uninstall the old software, simply locate the default QuantiFERON folder in the Start Menu (Start>QuantiFERON) and select **Uninstall**.

Alternatively, locate and remove the software using Start>Control Panel> Add/Remove Programs.

Q. *I would like to contact Cellestis to discuss my data/results/technique. What information should I provide in order to obtain a prompt reply?*

A. It is best to provide the QFT software **results file** (.qdf) which by default is located in the folder My Documents\QuantiFERON\Save.

The easiest way to provide this information is via email, with a detailed outline of your enquiry, kit lot number and any other information you feel is relevant.

Q. *Why can't data cells for standards be blank or contain text?*

A. Because the standard curve is used to derive QuantiFERON-TB Gold In-Tube results, blank values or text may reduce the quality of the standard curve.

Q. *When I open the Analysis Software, some of the text appears to be missing, as though it is covered by other text. What is the problem?*

A. The computer's Display Settings may be incorrectly set up for the software. Under Start>Control Panel>Display>Settings>Advanced, make sure that the **Display DPI setting** is set to **Normal size (96 DPI)**.

Specifications

SOFTWARE SPECIFICATIONS

QuantiFERON-TB Gold Analysis Software

Version 2.62

Catalogue Number 05990026.

SYSTEM REQUIREMENTS

Intel® Pentium® processor, or equivalent

Microsoft® Windows® 98 or higher

16 MB RAM

5 MB available hard-disk space

Screen resolution set to 800 x 600 pixels, or higher.

Contact Information

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www.cellestis.com

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