

MANUAL ON SELECTION AND USE OF INTERFERON-GAMMA RELEASE ASSAYS (IGRAS) FOR TESTING FOR TB INFECTION



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Stop **TB** Partnership



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ABOUT THIS MANUAL

This manual provides an overview of the recommended use of interferon-gamma release assays (IGRAs) as a class of in-vitro tests for the detection of TB infection and describes the performance, steps for use and implementation considerations for specific IGRAs that are either approved by the WHO Global TB Programme or the Global Fund's Expert Review Panel for Diagnostics. This manual will be periodically updated as additional tests become approved. Please contact any of the authoring organizations to suggest any contributions to this manual.

This manual is intended to inform Ministry of Health officials, programme managers, testing site managers, quality assurance unit personnel, supervisory laboratory staff and technicians at national, state/provincial and testing site level, as well as technical partners and donors.

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
BCG	bacille Calmette-Guérin (vaccine)
BCT	blood collection tube
CFP-10	culture filtrate protein 10
ELISA	enzyme-linked immunosorbent assay
ESAT-6	early secretory antigenic target-6
HIV	human immunodeficiency virus
IFN-γ	interferon-gamma
IGRA	interferon-gamma release assay
LMICs	low- and middle-income countries
LTBI	latent tuberculosis infection
PLHIV	people living with HIV
POC	Point of care
PPD	purified protein derivative
QFT-Plus	QuantiFERON-TB Gold Plus
SOP	standard operating procedure
STBP/GDF	Stop TB Partnership's Global Drug Facility
TPT	tuberculosis preventive treatment
TST	tuberculin skin test
TB	tuberculosis
TBF	Standard E TB-Feron
WHO	World Health Organization

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BACKGROUND AND SCOPE

About one-fourth of the world's population is estimated to be infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis (TB). The vast majority of infected individuals show no signs or symptoms of TB and are not infectious, although they have an increased risk of progressing to active TB disease and becoming infectious. On average, about 5–10% of those infected will develop active TB disease over the course of their lives, most of them within the first five years after infection. The risk is particularly high among children under the age of 5 years and among people with compromised immunity (e.g. people living with HIV).

TB infection (also called latent TB infection or LTBI) is operationally defined as a state of persistent immune response to stimulation by *M. tuberculosis* antigens with no evidence of clinically manifest active TB disease. The WHO End TB Strategy has prioritized TB preventive treatment (TPT) for persons with TB infection who are at high risk of developing active TB disease as a key component under Pillar 1, and in September 2018 at the first-ever United Nations High-Level Meeting on Tuberculosis, Member States endorsed a political declaration committing to provide 30 million individuals with TPT by 2022 to protect them from the development of active TB disease.

Implementation and scale-up of TPT services require strengthening of each element in the cascade of diagnosis and care starting from identification of the target population to ruling out active TB disease to testing for TB infection to the provision of preventive treatment¹ (FIGURE 1).

Identification of target populations to test is critical because population-wide TB infection testing prior to TPT in low- to middle-income countries (LMICs) is not feasible or cost-effective. That is because population-wide testing for TB infection has a high cost and the public health impact is not well documented; the pooled prevalence of positive results to the WHO-recommended tests for TB infection among people eligible for testing was reported to be 61% in LMICs.² In addition, the available tests are imperfect and there is some risk of serious adverse drug reactions during TPT. On the other hand, the benefits of TPT are more likely to outweigh the harms in infected individuals belonging to population groups in whom the risk for progression to active disease significantly exceeds that of the general population. Children under the age of 5 and PLHIV are two of those groups in which the probability of progression is high and TPT benefits are very clear. Accordingly, WHO guidelines¹ on the detection and treatment of TB infection take into

account the probability of progression to active TB disease in a specific risk group, the epidemiology and burden of TB, the availability of resources, and the likelihood of a broad public health impact.

Testing for TB infection is desirable whenever feasible to identify persons at the highest risk for developing active TB. However, it is not required in people living with HIV/AIDS or in household contacts aged < 5 years, particularly in countries with high TB incidence, given that the benefits of treatment (even without testing) clearly outweigh the risks. For individuals or populations with a higher risk of harm due to TPT or (relatively) lower risk of progression to TB disease, the WHO recommendation states that confirmation of TB infection increases the certainty that individuals targeted for TPT would benefit from TPT. In particular, TB infection tests are recommended for HIV-negative children aged ≥5 years and adults who are contacts of persons with active TB disease as well as other high-risk adults identified for TPT. However, the current recommendations leave ambiguity as they state that unavailability of TB infection testing should not be a barrier to treat people who were judged to be at higher risk.

There is no gold standard test for TB infection. The current WHO-recommended tests for TB infection detection, skin tests (tuberculin skin test (TST) and antigen-based skin tests) and Interferon-Gamma Release Assays (IGRAs), are indirect and require the person to mount an immune response in order to work properly.² The tests measure the immune sensitization to mycobacterial protein antigens that occurs following *M. tuberculosis* infection. Whilst the earliest version of the TST measured a delayed hypersensitivity reaction to exposure to *Mycobacterium* purified protein derivative (PPD), newer, more specific versions have been developed that use *M. tuberculosis* (*M.tb*)-specific antigens CFP-10 and ESAT6. These new TB antigen-based skin tests include the Cy-Tb skin test (Serum Institute of India, India), Diaskintest (JSC Generium, Russian Federation), and C-TST (Anhui Zhifei, Longcom Biopharmaceutical Co. Ltd, China). These tests combine the low cost of the TST and the high specificity of the IGRA.

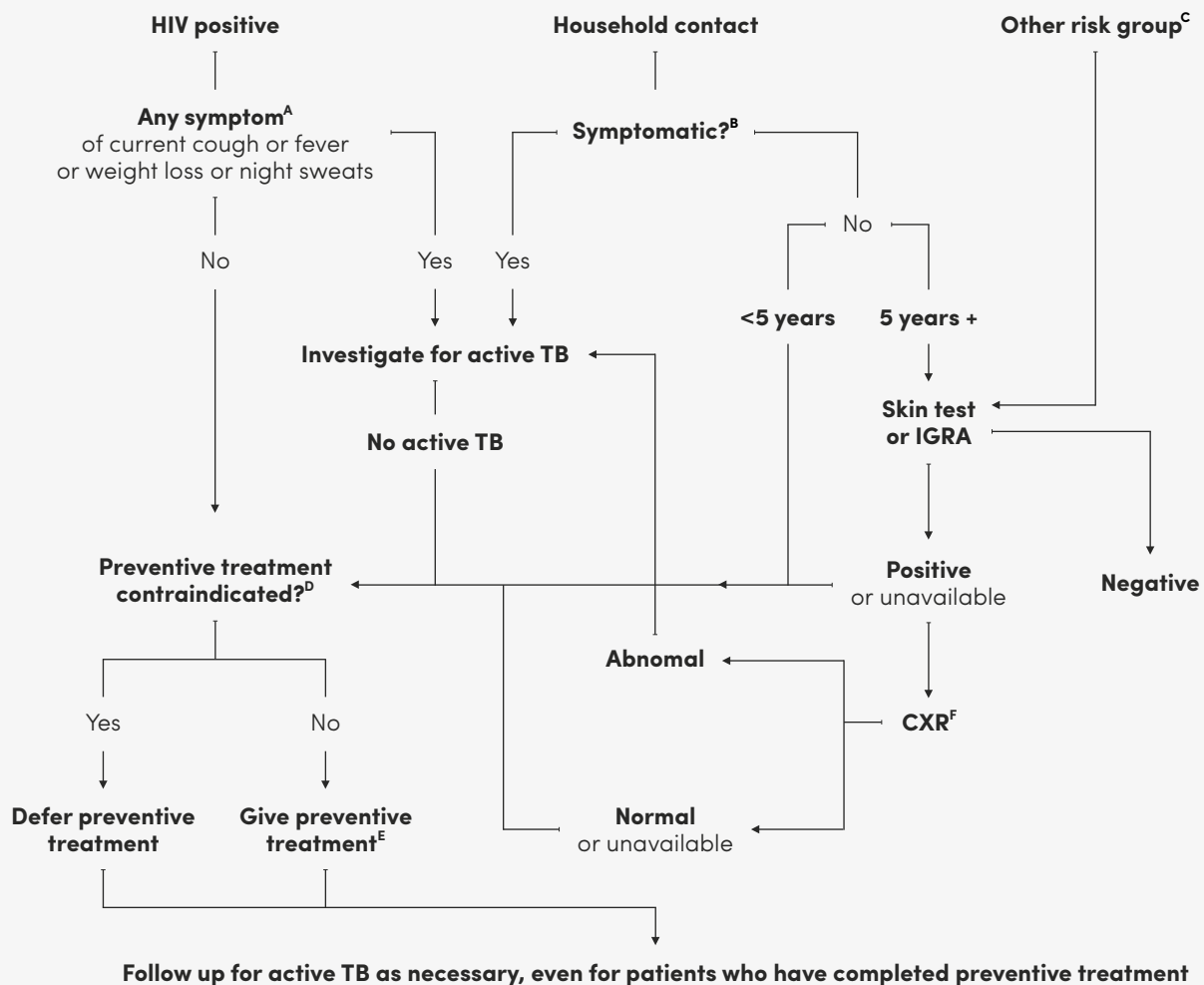
IGRAs measure the amount of interferon-gamma (IFN-γ) released in vitro by T-lymphocytes when mixed with *M. tuberculosis*-specific antigens ESAT6 and CFP-10 or the number of T-lymphocytes producing IFN-γ following stimulation with purified *M. tuberculosis*-specific antigens ESAT6 and CFP-10. The features of skin tests and IGRAs are compared in TABLE 1.

1. WHO consolidated guidelines on tuberculosis. Module 3: diagnosis. Tests for TB infection. Geneva: World Health Organization, 2022. <https://www.who.int/publications/i/item/9789240056084>


2. Alsdurf H, Hill PC, Matteelli A, Getahun H, Menzies D. The cascade of care in diagnosis and treatment of latent tuberculosis infection: a systematic review and meta-analysis. *Lancet Infect Dis.* 2016;16(11):1269–78

FIGURE 1

ALGORITHM FOR TB INFECTION TESTING AND TB PREVENTIVE TREATMENT IN INDIVIDUALS AT RISK



- A** If <10 years, any one of current cough or fever, or history of contact with TB or reported weight loss or confirmed weight loss >5% since last visit or growth curve flattening or weight for age <-2 Z-scores. Asymptomatic infants <1 year with HIV are only treated for LTBI if they are household contacts of TB. TST or IGRA may identify PLHIV who will benefit most from preventive treatment. Chest radiography may be used in PLHIV on ART, before starting LTBI treatment.
- B** Any one of the following symptoms: cough or fever or night sweats or hemoptysis or weight loss or chest pain or shortness of breath or fatigue. Children < 5 years, should also be free of anorexia, failure to thrive, not eating well, decreased activity or playfulness to be considered asymptomatic.
- C** Other risk groups include silicosis, dialysis, anti-TNF agent treatment, preparation for transplantation or as defined by national guidelines.
- D** Contraindications include acute or chronic hepatitis, peripheral neuropathy (if isoniazid used), and regular and heavy alcohol consumption. Pregnancy or a previous history of TB is not a contraindication.
- E** Regimen chosen based on considerations of age, strain (drug-susceptible or otherwise), risk of toxicity, availability, and preferences.
- F** CXR may have been carried out earlier on as part of intensified case finding.

 SOURCE: REPRODUCED FROM WHO CONSOLIDATED GUIDELINES ON TUBERCULOSIS¹ www.who.int/publications/i/item/9789240056084

1. WHO consolidated guidelines on tuberculosis. Module 3: diagnosis. Tests for TB infection. Geneva: World Health Organization, 2022. <https://www.who.int/publications/i/item/9789240056084>

TABLE 1

CHARACTERISTIC FEATURES OF SKIN TEST AND IGRA³

SKIN TEST	IGRA
TEST REQUIREMENTS	
<p>Requires intradermal administration of antigen into the volar surface of the forearm.</p> <p>Test must be read between 48–72 hours after the intradermal injection.</p> <p>Trained staff are required to administer and read skin induration.</p> <p>Can be administered in a community setting or health center.</p>	<p>Require fresh blood samples drawn by a trained phlebotomist or equivalent.</p> <p>Blood specimens must be incubated and tested within 8–30 hours after collection.</p> <p>Frequently IGRAs are performed in reference laboratories, (except newer tests that can be performed near-POC) which may require an efficient sample transport mechanism.</p>
POTENTIAL INACCURACY	
<p>False-positive TSTs can result from contact with nontuberculous mycobacteria or vaccination with bacille Calmette-Guérin (BCG).⁴</p> <p>Potential for inaccuracies and bias in reading skin induration.</p> <p>False-negative results may occur due to immunodeficiency conditions.</p>	<p>Inaccurate results may result from a delay in specimen transportation or errors in processing of blood specimen.</p> <p>False-negative results may occur due to immuno-suppressive conditions, faded immune memory, technical-operational variability, and in children below two years of age.</p>
ADVANTAGES	
<p>Antigen-based skin tests have similar specificity to IGRAs.</p> <p>Can be performed in the field.</p> <p>Significantly fewer resource needs compared to IGRA.</p> <p>No laboratory testing required.</p> <p>More familiar to practitioners in resource-constrained settings.</p>	<p>Single visit required to collect sample for conducting the test.</p> <p>Results possible within 24 hours.</p> <p>No booster effect.</p> <p>No false-positive results due to BCG vaccination.</p> <p>For ELISA-based IGRAs: Use of automated platforms can increase throughput and ensure greater test accuracy.</p> <p>For some IGRAs: Potential for connectivity, digital recording and transmission of results.</p>
CHALLENGES	
<p>Need for training in intradermal injection, reading and interpretation.</p> <p>Second visit (by individual or health care worker) required for test reading.</p> <p>Recurrent global shortages and stock-outs of quality assured PPD.</p> <p>Skin tests require a cold chain.</p> <p>Repeat test (two-step testing) for individuals whose immunity may have waned.</p>	<p>Higher test cost.</p> <p>For ELISA/ELISPOT assays: need for sophisticated laboratory equipment, skilled laboratory personnel.</p> <p>For near-POC IGRAs: run on specific proprietary equipment.</p> <p>Need for persons trained in phlebotomy.</p> <p>Second visit may be required to convey the test result to the patient and make clinical decisions.</p> <p>Potential for delays in sample transportation to laboratories that offer IGRA testing.</p> <p>If the laboratory SOP requires batching of tests to reduce costs there may be delays in reporting results.</p>

3. Adapted from WHO operational handbook on tuberculosis. Module 3: Diagnosis. Tests for tuberculosis infection. Geneva: World Health Organization, 2022. <https://www.who.int/publications/i/item/9789240058347>

4. Use of alternative interferon-gamma release assays for the diagnosis of TB infection: WHO policy statement. Geneva: World Health Organization, 2022. <https://apps.who.int/iris/bitstream/handle/10665/351191/9789240042346-eng.pdf>

WHO RECOMMENDATIONS ON TB INFECTION TESTING¹

Either a skin test or interferon-gamma release assay (IGRA) can be used to test for TB infection. There is no strong evidence that one test should be preferred over the other in terms of predicting progression from TB infection to TB disease.

Neither skin tests nor IGRAs should be used in persons having a low risk of TB infection and disease.

People living with HIV who have a positive test for TB infection benefit more from preventive treatment than those who have a negative TB infection test; TB infection testing can be used, where feasible, to identify such individuals.

TB infection testing by skin tests or IGRA is not a requirement for initiating preventive treatment in people living with HIV or child household contacts aged < 5 years.

TARGET POPULATION FOR SKIN TEST OR IGRA TESTING AND TPT

WHO recommends two broad groups of at-risk populations for systematic assessment of eligibility and provision of TPT:

→ **People with an elevated risk of progression from infection to TB disease**

- People living with HIV.
- Patients suffering from silicosis, patients starting or preparing for anti-tumor necrosis factor (TNF) treatment, patients receiving dialysis, and patients preparing for organ or haematologic transplantation.

→ **People with increased likelihood of exposure to TB disease**

- Household contacts of people with bacteriologically confirmed TB, usually subdivided into:
 - children below five years of age,
 - children five years and above, adolescents, and adults.
- Persons who live or work in institutional or crowded settings, such as prisoners, health workers, recent immigrants from countries with a high TB burden, homeless people and people who use drugs.

1. WHO consolidated guidelines on tuberculosis. Module 3: diagnosis. Tests for TB infection. Geneva: World Health Organization, 2022. <https://www.who.int/publications/i/item/9789240056084>

RECOMMENDATIONS FOR TB INFECTION TESTING BY GROUP*

PEOPLE WITH AN ELEVATED RISK OF PROGRESSION FROM INFECTION TO TB DISEASE

People living with HIV	TB infection testing desirable but not required for initiating TPT
Patients suffering from silicosis, starting anti-TNF treatment, receiving dialysis or preparing for transplantation	Systematic TB infection testing and treatment recommended

PEOPLE WITH INCREASED LIKELIHOOD OF EXPOSURE TO TB DISEASE

Children below five years of age who are household contacts of people with bacteriologically confirmed TB	TB infection testing desirable, but not required for initiating TPT
Children five years and above, adolescents and adults who are household contacts of people with bacteriologically confirmed TB	TB infection testing recommended, but not required for initiating TPT
Persons in institutional or crowded settings, such as prisoners or health workers, recent immigrants from countries with a high TB burden, homeless people and people who inject drugs.	Systematic TB infection testing and treatment may be considered

*Note that WHO does not recommend the use of skin tests or IGRA for the diagnosis of active TB disease.

SELECTION OF TB INFECTION TESTS

A positive test result by either method is not by itself a reliable indicator that the person will progress to TB disease, and may be followed by further diagnostic evaluation including chest X-ray or a mWRD test to exclude active TB as needed and available (FIGURE 1). Conversely, a negative skin test or IGRA test result does not rule out TB infection, given the possibility of a false-negative test result among at-risk groups, such as young children or among those recently infected. National health authorities need to decide how to

implement testing for TB infection, taking into account the benefit of preventive treatment, the risk of over or under treatment of contacts who are NOT tested against the costs and logistical difficulties to identify persons who are infected and would benefit most from TPT. While neither skin tests nor IGRAs have proven useful to predict, among those who test positive, who will develop active TB, the risk of progression is believed to 5-10% over a lifetime, and higher among individuals who are infected and immunocompromised.

SUMMARY

The initial WHO recommendations on the use of IGRAs were based on evidence from evaluations of the QuantiFERON®-TB Gold In-Tube (QIAGEN GmbH, Hilden, Germany) and T-SPOT®.TB IGRAs (Oxford Immunotec Ltd, Abingdon, UK).⁵ In 2021, WHO reviewed the evidence on the performance of the next-generation of QIAGEN's QuantiFERON-TB IGRA, QuantiFERON®-TB Gold Plus (QFT-Plus), and the WANTAI TB-IGRA (Beijing WANTAI Biological Pharmacy Enterprise Co., Ltd, Beijing, China), and in January 2022 updated its recommendations for the use of IGRAs for the detection of TB infection to include the use of the QIAGEN QFT-Plus and the Beijing WANTAI TB-IGRA.⁶

Based on available data at the time of WHO review in 2021, the QIAreacH® QuantiFERON®-TB (QIAreacH QFT) IGRA, T-SPOT®.TB 8 with T-Cell Select (Oxford Immunotec) and the Standard™ E TB-Feron (TBF) IGRA (SD Biosensor, Gyeonggi-do, Republic of Korea) could not be adequately compared with the WHO-recommended IGRAs. QIAreacH QFT and TBF have been reviewed and approved as Risk Category 2 by the Global Fund's Expert Review Panel for Diagnostics (ERPD), which acts as an interim approval mechanism

on the pathway to potential WHO endorsement.

This implementation manual describes the operational steps and implementation considerations for use of IGRAs approved by WHO or the Global Fund's Expert Review Panel for Diagnostics: as of the time of publication of this manual, this comprises the QFT-Plus IGRA, the QIAreacH QFT IGRA, the WANTAI TB-IGRA, the TB-Feron IGRA and the T-Spot.TB IGRA.

In October 2022, QIAGEN paused the commercialization of QIAreacH QFT, a near-POC IGRA. This manual will be updated when this product becomes available again from QIAGEN.

As of the time of publication of this manual, QFT-Plus reagents and T-Spot.TB reagents are available in the Stop TB Partnership GDF catalog.

A detailed discussion about the processes for implementing any new diagnostic test is available in Module 3 of the *WHO operational handbook on tuberculosis*.⁷ A detailed discussion about implementing a TPT program and TB infection testing is available in Module 1 of the *WHO operational handbook on tuberculosis*.⁵

ERPD APPROVAL FOR TB DIAGNOSTICS: WHAT IS IT?

The Global Fund's Expert Review Panel for Diagnostics (ERPD) is a group of independent experts who review the potential risks and benefits associated with the use of diagnostic products and make recommendations to the Global Fund and Unitaid on their use. The WHO Regulation and Prequalification Department hosts the ERPD.

ERPD approval of TB diagnostics is intended as an interim approval mechanism on the pathway to potential WHO endorsement, either as a WHO Global TB Programme recommendation or by WHO prequalification. ERPD approval allows countries to use Global Fund funding to procure products for a time-limited period, with possibility for renewal.

The current list of TB diagnostics approved by ERPD can be found on the Global Fund eligible products lists. ERPD approval of product is categorized as either Risk category 1 or 2. While products in both categories meet established ERPD standards around manufacturing site quality and risk management systems, and have adequate evidence of analytical performance, Risk category 2 products have limited clinical performance data in the settings of intended use and/or limited stability data to assign shelf-life.

5. WHO operational handbook on tuberculosis. Module 3: Diagnosis. Tests for TB infection. Geneva: World Health Organization, 2022. <https://www.who.int/publications/i/item/9789240058347>

6. Use of alternative interferon-gamma release assays for the diagnosis of TB infection: WHO policy statement. Geneva: World Health Organization, 2022. <https://apps.who.int/iris/bitstream/handle/10665/351191/9789240042346-eng.pdf>

7. WHO operational handbook on tuberculosis. Module 3: diagnosis. Rapid diagnostics for tuberculosis detection. Geneva: World Health Organization, 2021. <https://www.who.int/publications/i/item/who-operational-handbook-on-tuberculosis-module-3-diagnosis---rapid-diagnostics-for-tuberculosis-detection>

1.

QUANTIFERON-TB[®] GOLD PLUS (QFT-PLUS) IGRA

- 14 PRINCIPLES OF THE ASSAY
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- 16 HOW TO PERFORM THE ASSAY
- 19 OPERATIONAL CONSIDERATIONS

QUANTIFERON-TB® GOLD PLUS (QFT-PLUS) IGRA

The initial WHO 2018 recommendations for the use of IGRAs for the detection of TB infection were based, in part, on evidence from the QuantiFERON®-TB Gold In-Tube assay (QIAGEN GmbH, Hilden, Germany). However, the manufacturer discontinued the production of the QuantiFERON-TB Gold In-Tube assay and replaced it with the QuantiFERON®-TB Gold Plus (QFT-Plus) assay. In the 2022 recommendations, WHO concluded that the current recommendations for the use of IGRAs for the detection of TB infection are also valid for the QFT-Plus assay. However, there was insufficient evidence to compare the QIAreacH® QuantiFERON®-TB (QIAreacH QFT) with the WHO-recommended IGRAs.⁸ Only the QFT-Plus is discussed in this manual as QIAGEN has paused the commercialization of QIAreacH QFT. The QFT-Plus assay is available in the Stop TB Partnership GDF catalog.

The QuantiFERON-TB IGRAs are in vitro diagnostic tests that use peptides from the ESAT-6 and CFP-10 proteins to stimulate cells in heparinized whole blood followed by the detection of IFN- γ to measure in vitro responses to those peptide antigens. These IGRAs are indirect tests for *M. tuberculosis* infection and are intended for use in at-risk

populations in conjunction with risk assessment, radiography, and other medical and diagnostic evaluations. There are no known population restrictions for the use of the QuantiFERON IGRAs.

The tests rely on two main phases (FIGURE 2). In the first phase, whole blood is collected into specialized blood collection tubes (BCTs) containing antigens or controls, incubated at 37°C to elicit IFN- γ production by CD4 and CD8 lymphocytes and centrifuged to harvest plasma. In the second phase, the amount of IFN- γ present in the harvested plasma is measured to identify in vitro responses to the peptide antigens that are associated with *M. tuberculosis* infection.

The QFT-Plus assay collects whole blood into 4 BCTs. Two tubes contain antigenic peptides of ESAT-6 and CFP-10 (TB1 and TB2 tubes) and two tubes contain controls (Nil tube, Mitogen tube). An enzyme-linked immunosorbent assay (ELISA) is used to detect the amount of IFN- γ in the harvested plasma.

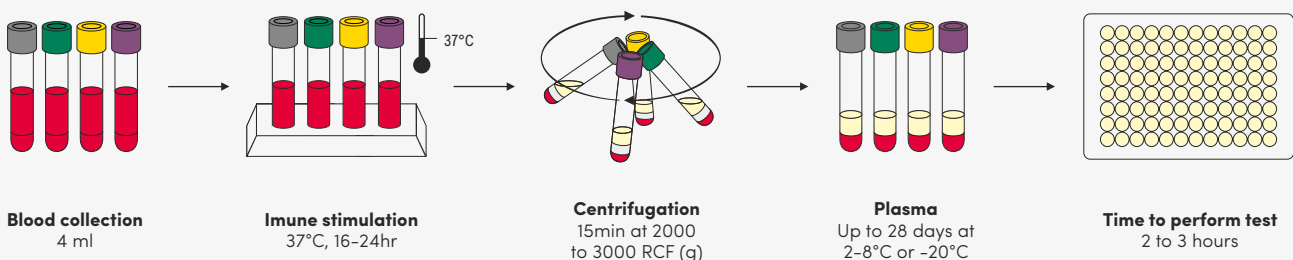
Details of the holding times and temperatures are described later in this document. The specimen transport must comply with the holding times and temperature requirements.

[www https://www.theglobalfund.org/media/9461/psm_productsdiagnosticstb_list_en.pdf](https://www.theglobalfund.org/media/9461/psm_productsdiagnosticstb_list_en.pdf)

FIGURE 2

SOURCE: REPRODUCED FROM QIAGEN KIT INSERT

WORKFLOW FOR THE QFT-PLUS



8. Use of alternative interferon-gamma release assays for the diagnosis of TB infection: WHO policy statement. Geneva: World Health Organization, 2022. <https://apps.who.int/iris/bitstream/handle/10665/351191/9789240042346-eng.pdf>

PRINCIPLES OF THE ASSAY

Whole blood specimens are collected into each of the four QFT-Plus blood collection tubes. The tubes differ by which antigens or controls have been dried onto the walls of the tube. Alternatively, blood may be collected in a single vacutainer tube that contains heparin as the anticoagulant and then transferred to the QFT-Plus blood collection tubes.

The Nil tube adjusts for background (e.g. excessive levels of circulating IFN- γ or presence of heterophile antibodies). The IFN- γ level of the Nil tube is subtracted from the IFN- γ level for the TB Antigen tubes and Mitogen tube.

The Mitogen tube is a positive control for the ability of the lymphocytes to produce IFN- γ and also serves as a control for correct blood handling and incubation.

The TB1 tube contains long peptides from ESAT-6 and CFP-10 that are designed to stimulate IFN- γ production from CD4+ T-helper lymphocytes.

The TB2 tube contains the same CD4 antigens of TB1 and an additional proprietary set of short peptides designed to stimulate IFN- γ production from CD8+ cytotoxic T lymphocytes. The detection of IFN- γ production by CD8 T cells appears to increase sensitivity for the detection of TB infection compared

to earlier versions of the test.

The contents of the QFT-Plus blood collection tubes must be thoroughly mixed with the collected blood to ensure that the antigens on the tube walls are completely dissolved. The tubes are then incubated in an upright position at 37°C for 16 to 24 hours, during which time the immune stimulation occurs. The samples are then centrifuged, the plasma is removed and the amount of IFN- γ (IU/ml) is measured by ELISA.

RESULT INTERPRETATION

A QFT-Plus assay is considered positive if the IFN- γ response to either TB antigen (TB1 or TB2) tube is significantly above the Nil IFN- γ IU/ml value; specifically, if the Nil value is ≤ 8.0 IU/ml and either TB antigen tube minus the Nil IFN- γ value is ≥ 0.35 IU/ml and at least 25% of the Nil value, irrespective of the mitogen minus Nil value. The plasma sample from the Mitogen tube serves as an IFN- γ positive control for each specimen tested. The test is considered indeterminate if the mitogen response is <0.5 IU/ml, together with both TB antigen responses <0.35 IU/ml or ≥ 0.35 IU/ml and $<25\%$ of Nil value, or the Nil response >8.0 IU/ml. (TABLE 1)

INTERPRETATION CRITERIA FOR QUANTIFERON-TB GOLD PLUS (QFT-PLUS)

RESULTS	NIL	TB RESPONSE	INTERPRETATION
Positive	≤ 8.0	TB1 and/or TB2 minus Nil ≥ 0.35 and $\geq 25\%$ of Nil	<i>M. tuberculosis</i> infection is likely
Negative	≤ 8.0	Mitogen minus Nil <0.5 , and TB1 and TB2 minus Nil <0.35 or ≥ 0.35 and $<25\%$ of Nil	<i>M. tuberculosis</i> infection is NOT likely
Indeterminate	>8.0	Any	Likelihood of <i>M. tuberculosis</i> infection cannot be determined
	≤ 8.0	TB1 and TB2 <0.35 or ≥ 0.35 and $<25\%$ of Nil and Mitogen minus Nil <0.5	

SOURCE: REPRODUCED FROM IMAGE PROVIDED BY SYLVIA M. LACOURSE⁹

9. Sylvia M et al. (2022). Latent Tuberculosis Infection. National HIV curriculum. <https://www.hiv.uw.edu/go/co-occurring-conditions/latent-tuberculosis/core-concept/all>

PERFORMANCE

Because there is no gold standard test to diagnose TB infection, direct measurement of the sensitivity and specificity of the skin tests and IGRAs to assess TB infection is not possible, therefore, surrogate measures have been used. The test's specificity is estimated by evaluating its performance in people at low risk of having TB infection. This frequently involves testing healthy individuals in settings with a very low prevalence of TB who have no known risk factors for TB infection. Sensitivity is approximated by evaluating the

performance of the test in those with microbiologically confirmed active disease.

Using this approach, the manufacturer showed that the QFT-Plus test has a sensitivity of 94.1% (398/423 individuals) for detecting persons known to have active TB disease and a specificity of 97.2% (713/733 individuals)¹⁰ for correctly identifying persons thought not to have TB infection. The indeterminate rate was less than 3% in these populations.

EQUIPMENT TO PERFORM THE ASSAY

MATERIALS REQUIRED BUT NOT PROVIDED

Phlebotomy materials

Calibrated variable volume pipettes for delivery of 10 µl to 1000 µl with disposable tips

Calibrated multichannel pipette capable of delivering 50 µl and 100 µl with disposable tips

Microplate shaker capable of speeds between 500 and 1000 rpm

Deionized or distilled water, 2 liters

Microplate washer (automated washer recommended)

Microplate reader fitted with 450 nm filter and 620 nm to 650 nm reference filter

37°C ± 1°C incubator (CO₂ not required)

Refrigerator 2°C–8°C

Timer

Centrifuge capable of centrifuging blood tubes at least to 3000RCF (g) for plasma separation

MATERIALS PROVIDED

QuantiFERON Nil Tubes (gray cap with white ring)

QuantiFERON TB1 Tubes (green cap with white ring)

QuantiFERON TB2 Tubes (yellow cap with white ring)

QuantiFERON Mitogen Tubes (purple cap with white ring)

QFT-Plus ELISA kit

NB: QFT-Plus can be automated on the Dynex DS2 platform or the DiaSorin LIAISON XL and XS platforms to simplify testing and interpretation of IGRA results (**FIGURE 3**).

¹⁰. QFTPlus-IGRA package insert.

HOW TO PERFORM THE ASSAY

(ADAPTED FROM MANUFACTURER'S INSTRUCTIONS FOR USE)

PHASE 1 : BLOOD COLLECTION, ANTIGEN STIMULATION AND HARVESTING OF PLASMA

1

SPECIMEN COLLECTION

Label each tube appropriately and record the date and time of sample collection.

For each patient, collect 1 ml of blood by venipuncture directly into each of the QFT-Plus BCTs.

This procedure should be performed by a trained phlebotomist or equivalent health worker competent in blood collection procedures. Alternatively, draw blood into a lithium/sodium-heparin tube (minimum volume 5 ml) and dispense 1 ml into each of the 4 QFT-Plus BCTs.

2

MIXING OF TUBE CONTENTS

Immediately after filling the QFT-Plus BCTs, gently mix by inverting them ten (10) times to make sure the entire inner surface of the tube is coated with blood. This will dissolve antigens on tube walls.

Transfer the QFT-Plus BCTs to a 37°C ± 1°C incubator as soon as possible and no more than 16 hours after sample collection. Prior to incubation at 37°C ± 1°, maintain the tubes at room temperature (22°C ± 5°C).

NB: blood collected in lithium or sodium heparin tubes must be maintained at room temperature for no more than 12 hours from the time of blood collection to transfer to BCTs and subsequent incubation.

Should the heparin tubes need to be refrigerated after sample collection, the following steps should be followed in the sequence specified:

The tubes must be kept at room temperature between 15 minutes and 3 hours after collection.

The blood sample can then be stored at 2–8°C for 16 to 48 hours.

Allow tubes to equilibrate at room temperature prior to transfer to BCTs.

Aliquoted BCTs should be placed in the 37°C incubator within 2 hours of blood transfer.

NB: The total time from blood draw into heparin tube to 37°C incubation must not exceed 53 hours.

3

INCUBATION AND HARVESTING OF PLASMA

Incubate QFT-Plus BCTs UPRIGHT at 37°C ± 1°C for 16 to 24 hours. The incubator does not require CO₂ or humidification.

After incubation, the tubes may be held between 4°C and 27°C for up to 3 days prior to harvesting of the plasma.

Harvesting of plasma is facilitated by centrifuging the tubes at 2000–3000 x g (RCF) for 15 minutes.

It is possible to harvest the plasma without centrifugation, but additional care is required to remove the plasma without disturbing the cells.

Harvest plasma samples using a pipette. 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. Plasma samples can be stored in centrifuged blood collection tubes for up to 28 days at 2°C to 8°C. Harvested plasma samples can also be stored below –20°C for extended periods.

HOW TO PERFORM THE ASSAY (CONTINUED)

(ADAPTED FROM MANUFACTURER'S INSTRUCTIONS FOR USE)

PHASE 2 : DETECTION OF IFN- γ AND GENERATION OF RESULTS

4

PREPARE IFN- γ STANDARDS

Reconstitute the IFN- γ Standard with the volume of deionized or distilled water indicated on the label of the vial.

Mix gently.

This generates a final concentration of 8.0 IU/ml.

Label 4 tubes "S1", "S2", "S3", and "S4".

Add 150 μ l of Green Diluent to S1, S2, S3, S4.

Add 150 μ l of the kit standard to S1 and mix thoroughly.

Transfer 50 μ l from S1 to S2 and mix thoroughly.

Green Diluent alone serves as the zero standard (S4).

5

PERFORM QFT-PLUS ELISA

Add 50 μ l of the working-strength conjugate to each well of the QFT-Plus ELISA plate.

Add 50 μ l of test plasma or standards to the appropriate wells of the QFT-Plus ELISA plate

Incubate for 120 \pm 5 minutes at room temperature (22°C \pm 5°C).

Wash the ELISA plate wells with 400 μ l wash buffer at least 6 times.

Add 100 μ l of substrate solution. Mix using a shaker set at a speed not more than 900 rpm.

Incubate for 30 minutes at room temperature.

Add 50 μ l of stop solution. Mix using shaker.

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

6

CALCULATE RESULTS

Calculate results using the most current version of the QuantiFERON-TB Gold Plus Analysis Software for your region.



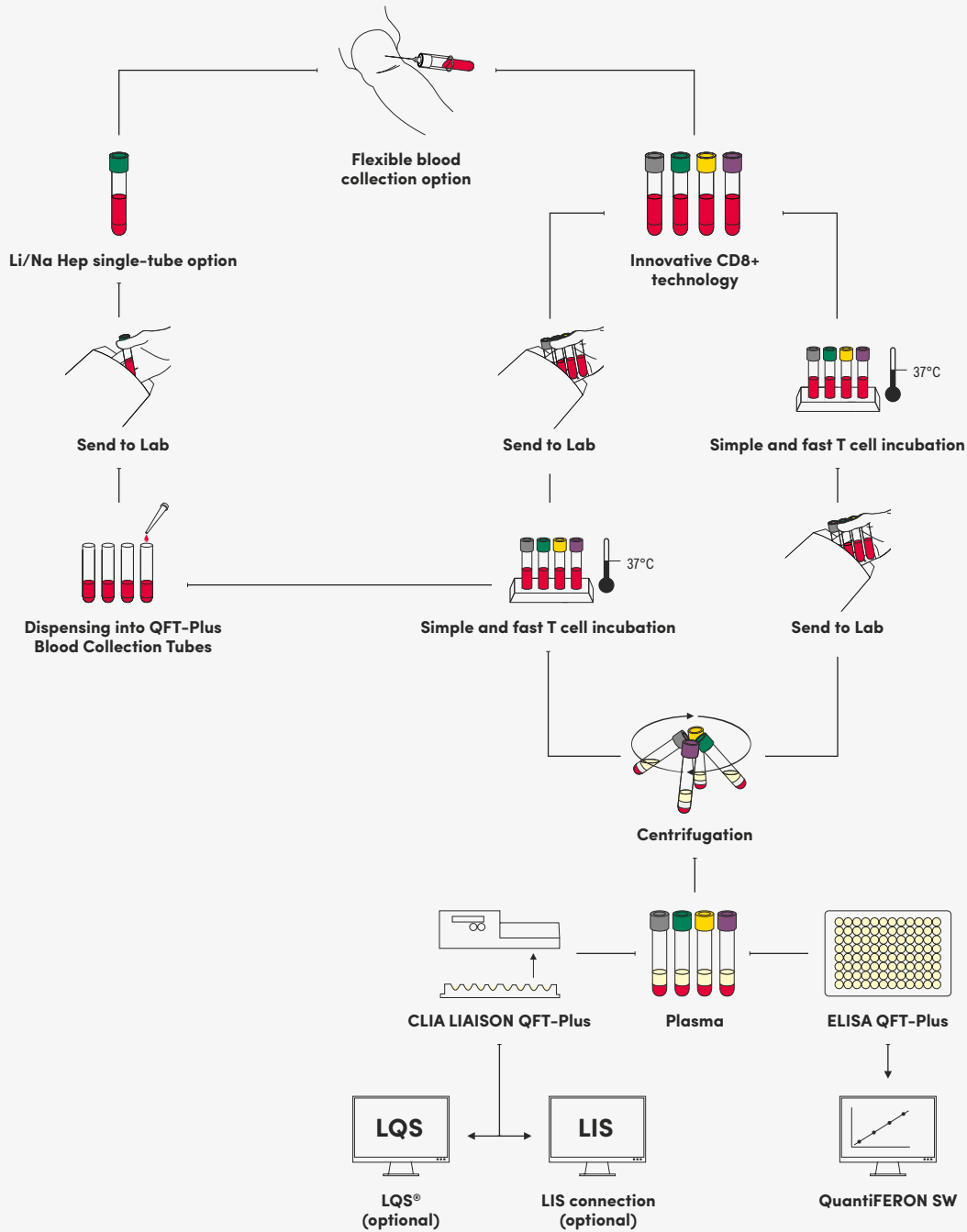
QuantiFERON-TB Gold Plus Analysis Software for your region, available for download at www.quantiferon.com

FIGURE 3

WORKFLOW OF THE QFT-PLUS IGRA

Following sample collection in QFT-Plus blood collection tubes, the sample should be transported to the testing lab and incubated within 16 hours of collection. Alternatively, stimulation can be performed at the sample collection site (37°C for 16-24h), then samples are transported

to the testing lab at 4-27°C within 3 days. Note that automation is possible on the DiaSorin LIAISON XL and XS platforms using the LIAISON QFT-Plus detection assay. Results can be viewed via the LIAISON Quantiferon Software (LQS) or the laboratory information system (LIS).



SOURCE: REPRODUCED FROM QIAGEN QUICK GUIDE
www.qiagen.com/dk/applications/tb-management/workflow

CASE STUDY: SELECTION OF AN AUTOMATED ELISA SYSTEM

The International Organization for Migration (IOM) performs IGRA testing for selected migrant and refugee populations as part of health assessments performed on behalf of destination governments. IOM currently operates 35 laboratories across Africa, Asia and the Middle East. Where the caseload for performing IGRA testing is high, the Qiagen QuantiFERON Gold Plus assay is used. In order to standardise testing across our laboratories, the Dynex DS2 automated ELISA system is used to perform the test.

Test accuracy depends on the generation of an accurate standard curve and the results derived from the standards must be examined before test sample results can be interpreted. The Dynex DS2 is one automated and programmable ELISA solution that allows for the automated preparation of a standard curve and the calculation of the in vitro responses to the peptide antigens that are associated with *M. tuberculosis* infection from individuals' blood samples.

One limitation with use of an automated ELISA system is the need for regular periodic maintenance. Although maintenance can be performed by QIAGEN and their local distributors, it is an additional cost that needs to be considered and budgeted for, when choosing the Dynex DS2 solution.

 www.dynextechnologies.com/our-products/ds2

OPERATIONAL CONSIDERATIONS

CONSIDERATIONS FOR SITE SELECTION

Because of the need for sophisticated laboratory equipment and highly skilled laboratory personnel to perform the ELISA, the test is best suited for implementation in intermediate or national reference laboratories.

Because of the use of ELISAs for other diseases, national programs may be able to leverage collaboration with other, non-TB-specific laboratories, having the capacity for blood draws and ELISA testing. The biosafety precautions for the QFT-Plus test are the same as most tests that are performed in BSL-2 laboratories.

If the test is performed at a centralized site, it will be necessary to establish efficient specimen transport systems to ensure that blood samples can be transported from peripheral sites to the IGRA testing laboratory within the recommended time frames. For QFT-Plus, blood samples may be transported at room temperature ($22 \pm 5^{\circ}\text{C}$) or transported under cold chain ($2-8^{\circ}\text{C}$) to the centralized site. The maximum time between blood collection in QFT-Plus tubes and processing at the testing laboratory for room temperature blood samples is 16 hours. For cold chain storage, blood must be collected and placed under cold chain in a single lithium heparin tube, and later aliquoted into the QFT-Plus BCTs. When under cold chain, this allows the maximum time between collection and processing to be extended to 53 hours.

TIME REQUIRED FOR PERFORMING ASSAY

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

→ **37°C incubation of blood tubes: 16 to 24 hours.**

→ **ELISA: Approx. 3-3.5 hours for one ELISA plate.**

- A 96-well ELISA plate can contain samples from up to 22 individuals.
 - <1 hour of labor.
 - Add 10 to 15 minutes for each extra plate.
-

STORAGE AND HANDLING

→ Blood collection tubes

- Store blood collection tubes at 2°C to 25°C.
- Minimum remaining shelf life at the time of order readiness as per GDF agreement with QIAGEN: 10 months.

→ Kit reagents

- Store kit reagents at 2°C to 8°C.
- Always protect Enzyme Substrate Solution from direct sunlight.
- Maximum remaining shelf life as per GDF agreement with QIAGEN: 24 months.

→ Reconstituted and unused reagents

- The reconstituted kit standard may be kept for up to 3 months if stored at 2°C to 8°C.
- Once reconstituted, unused Conjugate 100x Concentrate must be returned to storage at 2°C to 8°C and must be used within 3 months.
- 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.

FORECASTING AND ORDER PLANNING

The Stop TB Partnership's GDF has negotiated concessional pricing of US\$15.90 per patient sample for the QFT-Plus IGRA, for the following eligible countries:

→ **All low- and middle-income countries except Turkey.**

→ **Listed high-income countries:**

Antigua and Barbuda, Bahamas, Barbados, Chile, Estonia, Guam, Hong Kong SAR China, Israel, Latvia, Lithuania, Macao SAR China, New Caledonia, Palau, Panama, Seychelles, St. Kitts and Nevis, Taiwan China, Trinidad and Tobago, Uruguay.

SUPPLY PLANNING (SUGGESTED DELIVERY FREQUENCY)

The supply plan must account for the procurement and supplier lead times as well as the time required for country-specific importation processes; in total this may require a lead time of 4-6 months. The time required for in-country distribution must also be considered.

For planning of orders and shipments, the size of an order may include all of the product needs estimated for a year, though with multiple shipments.

For QFT-Plus Blood Collection Tubes, given their remaining shelf life is short (minimum of 10 months as per the GDF agreement with QIAGEN), shipments may be required at least twice a year in order to prevent stock-outs.

For QFT-Plus ELISA kits, given the relatively long shelf life (up to 24 months), shipments may be made annually.

QUALITY ASSURANCE, CONTROL, AND ASSESSMENT

For the blood handling and immune stimulation phases, the Mitogen tube serves as a positive control. A QFT-Plus Negative test must generate a Mitogen minus Nil value of ≥ 0.5 IU/ml.

A low response to Mitogen may result from insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, incorrect filling or mixing of the Mitogen tube, or inability of the patient's lymphocytes to generate IFN- γ .

The accuracy of the test results is dependent on the generation of an accurate standard curve. The reconstituted IFN- γ standard is used with each ELISA to construct the standard curve.

→ 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 $\leq 15\%$,
 - 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 .
- 5) 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.

These control parameters are calculated and reported by the QFT-Plus Analysis Software and must be reviewed before test results can be interpreted.

Each laboratory should determine appropriate types of control materials to be used and the frequency of testing in accordance with applicable accrediting organizations. For example, known positive/negative samples or the Quantiferon Control Panel vials¹¹ can be used to control performance between runs and operators. Proficiency testing programs for IGRAs are also available such as ones from the UK National External Quality Assessment Service (UK NEQAS), INSTAND e.V., Society for Promoting Quality Assurance in Medical Laboratories, and College of American Pathologists (CAP).

Key quality indicators that should be monitored monthly include the percentage of runs with invalid standard curves; percentage of indeterminate, errors or invalid results; percentage of positive results; distribution of IFN- γ concentrations; and turnaround times (ANNEX 2). Targets should be set for all indicators that are monitored, and any unexplained change in quality indicators, such as an increase in error rates or a change in positivity rate should be documented and investigated.

RECORDING AND REPORTING

Both the standard qualitative test interpretation (positive, negative, indeterminate), together with the criteria for test interpretation should be reported.

¹¹ <https://www.qiagen.com/de/products/diagnostics-and-clinical-research/transplant/quantiferon-transplant/quantiferon-control-panel>

COST OF KIT AND COST PER PATIENT SAMPLE

(ELISA KITS AND BLOOD COLLECTION TUBES ARE REQUIRED.)

PRODUCT	GDF ITEM NUMBER	COST OF KIT IN GDF CATALOG	UNITS PER KIT	PATIENT SAMPLES TESTED	COST PER PATIENT SAMPLE
QuantiFERON-TB Gold Plus (QFT-Plus) ELISA 2 x 96	106671	\$279.84	2 x 96 wells + reagents	44	\$6.36
QuantiFERON-TB Gold Plus (QFT-Plus) ELISA 20 x 96 (reference lab pack)	106672	\$2,798.40	20 x 96 wells + reagents	440	
QFT-Plus Blood Collection 200 Tubes	106673	\$477.00	200 tubes	50	\$9.54
QFT-Plus Blood Collection 200 Tubes - High Altitude ¹²	106674				
QFT-Plus Blood Collection 40 Tubes Single Patient	106675	\$95.40	40 tubes	10	
QFT-Plus Blood Collection 40 Tubes Single Patient High Altitude	106676				

ORDER SIZES AND DELIVERY PLANS FOR TESTING 5,000, 20,000 OR 100,000 PEOPLE IN ONE YEAR

(ELISA KITS AND BLOOD COLLECTION TUBES ARE REQUIRED.)

PRODUCT	GDF ITEM NUMBER	5,000 TESTS	20,000 TESTS	100,000 TESTS	SHELF LIFE	NUMBER OF DELIVERIES PER YEAR
QFT-Plus ELISA 2 x 96 OR QFT-Plus ELISA 20 x 96 (reference lab pack)	106671 106672	114 12	455 46	2,273 228	24 months	1
QFT-Plus Blood Collection 200 Tubes OR QFT-Plus Blood Collection 200 Tubes - High Altitude OR QFT-Plus Blood Collection 40 Tubes Single Patient OR QFT-Plus Blood Collection 40 Tubes Single Patient High Altitude	106673 106674 106675 106676	100 500	400 2,000	2,000 10,000	6 months	4*

*Second delivery after month 3, third after month 6, fourth after month 9.

¹² Standard QFT-Plus Blood Collection Tubes can be used up to an altitude of 810 meters above sea level. High Altitude QFT-Plus Blood Collection Tubes can be used between 1,020 meters above sea level to an altitude of 1,875 meters above sea level. *J Infect*, 2020. 80(5): p. 536-546. Doi: 10.1016/j.jinf.2020.02.009

2.

WANTAI TB-IGRA

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WANTAI TB-IGRA

The WANTAI TB-IGRA is operationally similar to the QFT-Plus IGRA in that there is a blood collection and antigen stimulation phase followed by detection of IFN- γ by ELISA. Two key differences are 1) the WANTAI IGRA uses one tube containing antigens for stimulation whereas the QFT-Plus IGRA uses two tubes (TB1 and TB2) and 2) the stimulating antigens used in the WANTAI TB-IGRA are recombinant- proteins (ESAT6 [first 80 amino acids] and the full-length CFP-10) whereas the stimulating antigens in the QFT-Plus IGRA are mixtures of synthetic peptides (ESAT6 and CFP-10). Also, IFN- γ concentrations are reported as pg/ml instead of IU/ml.

PRINCIPLES OF THE ASSAY

The basic principles of the WANTAI TB-IGRA are also similar to those of the QuantiFERON-TB assays in that an ELISA is used to measure the amount of IFN- γ released following stimulation of whole blood samples with *M. tuberculosis-specific* antigens. Briefly, whole blood is collected into a blood collection tube with lithium heparin as an anticoagulant and 1 ml dispensed into each of three tubes ('N', 'T', 'P').

The 'N' tube adjusts for background (e.g. excessive levels of circulating IFN- γ or presence of heterophile antibodies). The IFN- γ level of the N tube is subtracted from the IFN- γ level for the T tube and P tube.

The 'P' tube (positive control tube) is a positive control for the ability of the lymphocytes to produce IFN- γ and also serves as a control for correct blood handling and

incubation.

The 'T' tube (testing culture tube) contains TB-specific stimulating antigens (ESAT-6, CFP-10). These antigens are recombinant-produced proteins.

Following incubation at 37°C for 20 to 24 hours, the samples are then centrifuged, the plasma is removed and the amount of IFN- γ (pg/ml) is measured by ELISA. A WANTAI TB-IGRA is considered positive if the concentration of IFN- γ (pg/ml) in the T tube minus the concentration in the N tube is greater than or equal to 14 and is greater than or equal to the concentration in the N tube divided by 4 [i.e. $T-N \geq 14$ and $\geq N/4$]. A low response to the positive control ($P-N < 20$) indicates an indeterminate result when a blood sample also has a negative response to the TB antigens (**FIGURE 6**).

PERFORMANCE

Using the previously described approach relying on surrogate measures, the manufacturer showed that the WANTAI TB-IGRA has a sensitivity of 97.1% (68/70 test subjects) for detecting persons known to have a TB infection (i.e., persons with active TB disease) and a specificity of 94.8% (199/210 test subjects) for correctly identifying persons thought not to have a TB infection.¹³

In addition to the use of surrogate measures,

the manufacturer compared the performance of the WANTAI TB-IGRA with that of the QuantiFERON-TB Gold In-Tube assay and the T-Spot.TB assay. In two published studies, the agreement between the WANTAI TB-IGRA and the QFT-TB Gold In-Tube assay was good with a kappa statistic of 0.79 (95% CI: 0.60 to 0.99).

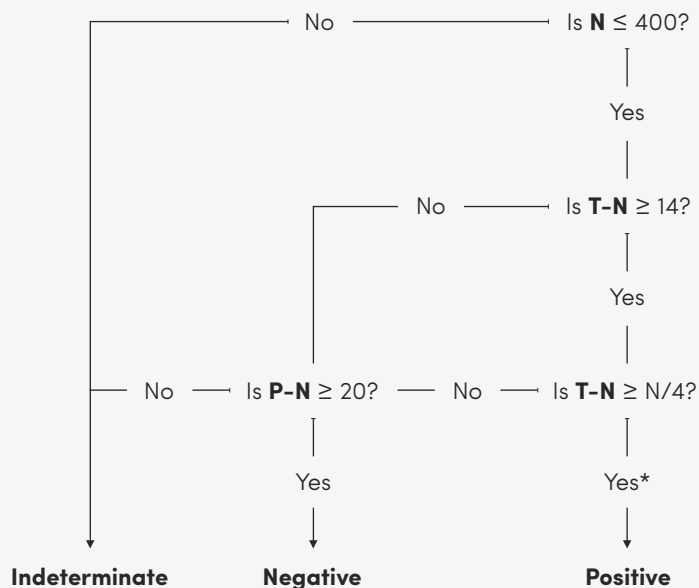
In three published studies the agreement with T-Spot.TB was very good with a kappa statistic of 0.87¹³

13. WANTAI TB-IGRA brochure: <https://www.ystwt.cn/wp-content/uploads/2018/04/Wantai-TB-IGRA.pdf>

FIGURE 6

ALGORITHM FOR INTERPRETING RESULTS OF THE WANTAI TB-IGRA

N = concentration of IFN- γ (pg/ml) in the Background Control Tube
P = concentration of IFN- γ (pg/ml) in the Positive Control Tube
T = concentration of IFN- γ (pg/ml) in the Testing Tube
* = P-N any value



EQUIPMENT TO PERFORM THE ASSAY

MATERIALS REQUIRED BUT NOT PROVIDED

- Phlebotomy materials include lithium heparin collection tube
- Calibrated variable volume pipettes with disposable tips
- Calibrated multichannel pipettes with disposable tips
- Microplate shaker
- Deionized or distilled water
- Microplate washer
- Microplate reader fitted with 450 nm filter and 620 nm to 650 nm reference filter
- 37°C ± 1°C incubator (CO2 not required)
- Refrigerator 2°C-8°C
- Centrifuge for separation of plasma
- Disposable gloves

MATERIALS PROVIDED

- Microwell plate
- Background Control Culture Tube
- Testing Culture Tube
- Positive Control Culture Tube
- Interferon gamma standard
- HRP-conjugate
- Specimen diluent
- Standard diluent
- Wash buffer
- Chromogen Solution A
- Chromogen Solution B
- Stop Solution

HOW TO PERFORM THE ASSAY

(ADAPTED FROM MANUFACTURER'S INSTRUCTIONS FOR USE)

PHASE 1 : BLOOD COLLECTION, ANTIGEN STIMULATION AND HARVESTING OF PLASMA

1	2	3	4
SPECIMEN COLLECTION	SPECIMEN DISPENSING AND MIXING	INCUBATION	CENTRIFUGATION AND HARVESTING PLASMA
<p>Label each tube appropriately.</p> <p>For each patient, collect 4 ml of blood by venipuncture directly into a blood collection tube with lithium heparin as the anticoagulant. Mix well.</p> <p>The tube may be stored at 20°C to 27°C for up to 16 hours before the specimen dispensing step.</p>	<p>Label each tube appropriately.</p> <p>Gently invert the tubes 3-5 times to mix the specimens.</p> <p>Dispense 1 ml of the whole blood specimen into each of the N, T and P tubes.</p> <p>Gently invert the tubes 5 times to mix the specimens.</p>	<p>Immediately after mixing, place the tubes in a 37°C ± 1°C incubator.</p> <p>Incubate the tubes in an upright position for 20 to 24 hours.</p>	<p>After incubation, centrifuge the specimens at 3000 to 5000 rpm for 10 minutes to separate plasma and red blood cells.</p> <p>Harvest the plasma using a pipette.</p> <p>The harvested plasma may be stored at 2°C to 8°C for up to 2 days or below -15°C for longer times.</p> <p>Store plasma in small volume aliquots to avoid multiple freeze-thaw cycles. The manufacturer recommends no more than 2 freeze-thaw cycles.</p>

HOW TO PERFORM THE ASSAY (CONTINUED)

(ADAPTED FROM MANUFACTURER'S INSTRUCTIONS FOR USE)

PHASE 2 : DETECTION OF IFN- γ AND GENERATION OF RESULTS

5

REAGENT PREPARATION

Allow reagents and specimens to reach room temperature (18°C to 30°C) for at least 15–30 minutes.

Dilute the concentrated wash buffer 1:20 with distilled or deionized water.

Add distilled or deionized water to the ampule according to the volume printed on the label of the ampule to reconstitute the freeze-dried standard. This generates the 400pg/ml standard.

Gently mix until it is homogeneously dissolved.

Prepare a series of two-fold dilutions using the Standard Diluent. The final concentrations of the standard samples should be 400pg/ml, 200pg/ml, 100pg/ml, 50pg/ml, 25pg/ml and 12.5pg/ml.

Set the strips needed in the strip holder.

Include 1 well for the plasma sample of each culture tube, two wells for each standard, and one well for a blank.

6

PERFORM THE TB-IGRA ELISA

Add 20 μ l of Specimen diluent to each well except the Blank well.

Add 50 μ l of the standards and 50 μ l of the specimen samples into their respective wells and mix by gently tapping the plate.

Cover the plate with the plate cover and incubate for 60 minutes at 37°C.

Add 50 μ l of the HRP-Conjugate Reagent to each well except the Blank well and mix by gently tapping the plate.

Cover the plate with the plate cover and incubate for 60 minutes at 37°C.

At the end of the incubation, wash each well 5 times with the diluted wash buffer. Each time allow the wells to soak for 30 to 60 seconds. After the final wash, turn the plate onto blotting paper and gently tap to remove any remaining wash buffer.

Add 50 μ l of Chromogen Solution A and 50 μ l of Chromogen Solution B to each well including the blank well and gently tap the plate to mix. Incubate the plate at 37°C in the dark for 15 minutes.

Add 50 μ l of Stop Solution to each well and gently mix.

Calibrate the plate reader using the Blank well and read the absorbance of each well at 450 nm. (NB: If the results will be determined by using a dual wavelength plate reader, the requirement for the use of a Blank well could be omitted. If a dual filter instrument is used, set the dual-wavelength at 450nm/600~650nm). Absorbance should be measured within 10 minutes of stopping the reaction.

7

CALCULATE RESULTS

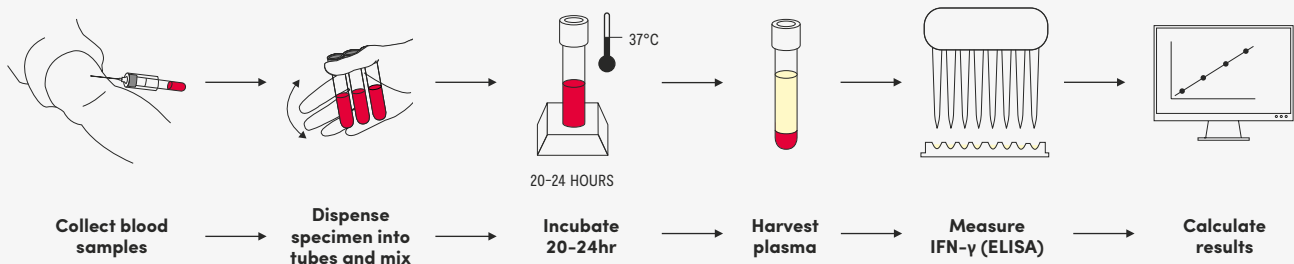
If the results are based on a single filter plate reader, subtract the absorbance of the Blank well from that of each test well. If a dual filter (450nm/600–650nm) plate reader is used, do not subtract the absorbance of the Blank well from that of the test wells.

Construct a standard curve based on the absorbance values of the wells containing the various solutions of the standard and determine the linear regression equation.

Enter the absorbance values of the wells containing the plasma samples into the linear regression equation and calculate the corresponding IFN- γ concentration.

FIGURE 7

WORKFLOW OF THE WANTAI TB



OPERATIONAL CONSIDERATIONS

CONSIDERATIONS FOR SITE SELECTION

Because of the need for sophisticated laboratory equipment and highly skilled laboratory personnel to perform the ELISA and interpret test results, the test is best suited for implementation in intermediate or national reference laboratories. Because of the use of ELISAs for other diseases, national programs may be able to leverage collaboration with other, non-TB-specific laboratories, having capacity for blood draws and ELISA testing. The biosafety precautions for the WANTAI TB-IGRA are the same as most ELISAs.

If the test is located in a centralized site, it will be necessary to establish efficient specimen transport systems to ensure that blood samples can be transported from peripheral sites to the IGRA testing laboratory within the recommended time frames. For the WANTAI TB-IGRA, the maximum time between blood collection and dispensing into the N, T, and P tubes at the testing laboratory is 16 hours.

THROUGHPUT AND TIME REQUIRED FOR PERFORMING ASSAY

The time required to perform the WANTAI TB-IGRA test is estimated below; the time of testing multiple samples when batched is also indicated:

→ **37°C incubation of blood tubes: 20 to 24 hours.**

→ **ELISA: Approx. 3 hours for one ELISA plate.**

- An ELISA plate can contain up to 28 patient samples.
- <1 hour of labor.
- Add 10 to 15 minutes for each extra plate.

**STORAGE
AND HANDLING****→ Kit reagents**

- Store kit reagents at 2°C to 8°C.
- Shelf life: 13 months (expiration date printed on each package).

→ Reconstituted and unused reagents

- Once opened, unused microwell strips may be stored at 2°C to 8°C in a plastic storage bag (provided) with desiccant for up to 3 weeks.
- Once reconstituted, the standards should be used on the same day.
- Once opened, the HRP-Conjugate is stable for 3 weeks at 2°C to 8°C.
- Working strength wash buffer may be stored at room temperature for up to 1 week or 2 weeks at 2°C to 8°C.
- Once opened, the Specimen Diluent is stable for 3 weeks at 2°C to 8°C.
- Once opened, the Standard Diluent is stable for 3 weeks at 2°C to 8°C.
- Once opened, the Chromogen Solution A is stable for 3 weeks at 2°C to 8°C.
- Once opened, the Chromogen Solution B is stable for 3 weeks at 2°C to 8°C.
- Once opened, the Stop Solution is stable for 3 weeks at 2°C to 8°C.

**INTERPRETATION OF RESULTS
AND QUALITY CONTROL****→ Result interpretation**

If the result reading is based on a single filter plate reader, the results should be calculated by subtracting the "A" value of the Blank well from the print report values of specimens and controls. For dual filter plate readers, do not subtract the A value of the Blank well from the print report values of specimens and controls. The limit of the blank (LOB) should not be higher than 3pg/ml. The linear range for the assay is 12.5pg/ml-400pg/ml. Should the IFN- γ concentration of a specimen be >400pg/ml; the specimen should be diluted using the standard diluent and the test repeated.

→ Quality control

For the blood handling and immune stimulation phases, the Positive Control tube ('T') serves as a positive control. The result should be considered invalid if either the correlation coefficient of the dose-response curve Correlation coefficient, $r > 0.9900$, or the mean of A values of 400pg/ml Standard < 1.0 . Samples with an invalid result should be repeated. A low response of positive control may result from insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, incorrect filling or mixing of the Positive Control Mitogen tube, or inability of the patient's lymphocytes to generate IFN- γ .

Important internal controls that are conducted with each ELISA are the known samples of IFN- γ used to construct the standard curve.

→ For the ELISA to be valid:

- the mean absorbance value for the 400pg/ml standards must be ≥ 1.0 ,
- the correlation coefficient (r) calculated from the mean absorbance values of the standards must be ≥ 0.99 .

Proficiency testing programs for IGRAs are available such as ones from the UK National External Quality Assessment Service (UK NEQAS), INSTAND e.V., Society for Promoting Quality Assurance in Medical Laboratories, and College of American Pathologists (CAP).

Key quality indicators that should be monitored monthly include the percentage of runs with invalid standard curves; percentage of indeterminate, errors or invalid results; percentage of positive results; distribution of IFN- γ concentrations; and turnaround times (ANNEX 2). Targets should be set for all indicators that are monitored, and any unexplained change in quality indicators, such as an increase in error rates or a change in positivity rate should be documented and investigated.

INTERPRETATION OF THE RESULTS

N	P-N	T-N	RESULT	INTERPRETATION
≤400	any value	≥ 14 and $\geq \frac{N}{4}$	positive	Infected with Mycobacterium tuberculosis (active, latent or inapparent infection)
	≥20	<14	negative	Not infected with Mycobacterium tuberculosis
	≥20	≥ 14 but $< \frac{N}{4}$	negative	
	<20	<14	indeterminate	
	<20	≥ 14 but <	indeterminate	Cannot determine whether Mycobacterium tuberculosis infection
≤400	any value	any value	indeterminate	

The concentration of Testing Culture Tube (T)=T, the concentration of Background control tube (N)=N, and the concentration of Positive control tube (P) = P(Unit=p/ml). The Background Control Tube ('N') assesses the level of circulating IFN-γ or presence of heterophile antibodies. A valid test must generate an 'N' value of ≤400pg/ml.

**RECORDING
AND REPORTING**

Both the standard qualitative test interpretation (positive, negative, indeterminate) and the quantitative assay measurements should be reported, together with the criteria for test interpretation.

3.

STANDARD™ E TB-FERON IGRA

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STANDARD™ E TB-FERON IGRA

The SD Biosensor TBF assay is operationally similar to the QFT-Plus IGRA in that there is a blood collection and antigen stimulation phase followed by detection of IFN- γ by ELISA. Two key differences are 1) the QFT-Plus IGRA uses two tubes (TB1 and TB2) whereas the TBF assay uses one tube containing antigen for stimulation and 2) the stimulating antigens in the QFT-Plus IGRA are mixtures of synthetic peptides (ESAT6 and CFP-10) whereas the stimulating antigens used in the TBF assay are recombinant-produced full-length proteins (ESAT6, CFP-10, and TB7.7).

PRINCIPLES OF THE ASSAY

For the TBF IGRA, whole blood specimens are collected into each of the three TBF tubes. The tubes differ by which antigens or controls have been dried onto the walls of the tube. Alternatively, blood may be collected in a single generic BCT that contains lithium heparin as the anticoagulant and then transferred to TBF tubes.

The Nil tube adjusts for background (e.g. excessive levels of circulating IFN- γ or presence of heterophile antibodies). The IFN- γ level of the Nil tube is subtracted from the IFN- γ level for the TB Antigen tubes and Mitogen tubes.

The Mitogen tube is a positive control for the ability of the lymphocytes to produce IFN- γ and also serves as a control for correct blood handling and incubation.

The TB antigen tube contains full-length recombinant-produced ESAT-6, CFP-10, and TB7.7 proteins.

The contents of the inoculated TBF tubes must be gently,

but thoroughly mixed with the collected blood to ensure that the antigens on the tube walls are completely dissolved. The tubes are then incubated in an upright position at 37°C for 16 to 24 hours, during which time the immune stimulation occurs. The samples are then centrifuged, the plasma is removed and the amount of IFN- γ (IU/ml) is measured by ELISA.

A TBF assay is considered positive if the IFN- γ response to the TB Antigen tube is significantly above the Nil IFN- γ IU/ml value. 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.

The manufacturer also describes the option of using only the Nil and TB antigen tubes. The testing protocol is the same (minus the use of the Mitogen tube). The interpretation of the results follows a similar algorithm.

PERFORMANCE

Performance was assessed in a study of 705 patients¹⁴ by comparing the results obtained using the TBF IGRA with the results obtained with the QFT-Plus assay. The overall positive agreement was 96.7% (119/123), the overall negative agreement was 95% (553/582) and the overall agreement was 95.3% (672/705).

The performance characteristics of the QFT-Plus assay

could thus be suggested to be extrapolated to the TBF IGRA.

Information on the performance of the two-tube assay (Nil and TB antigen tubes only) is lacking. In particular, it is unknown how the lack of a mitogen control in the two-tube assay affects the comparison with the QFT-Plus test, particularly in immunocompromised individuals.

	FREQUENCY		% (95% CI)
Negative Agreement	672/705		95.3% (93.5–96.8%)
Positive Agreement	119/123		96.7% (91.9–99.1%)
Overall Agreement	197/206		95.6% (92.9–98%)

TBF IGRA RESULTS	QFT-PLUS RESULTS		Total
	Negative	Positive	
Negative	553	4	557
Positive	29	119	148
Total	582	123	705

14. Standard E TB Feron ELISA kit insert.

FIGURE 8

ALGORITHM FOR INTERPRETING RESULTS OF THE STANDARD E TB-FERON IGRA (3 TUBE ASSAY)

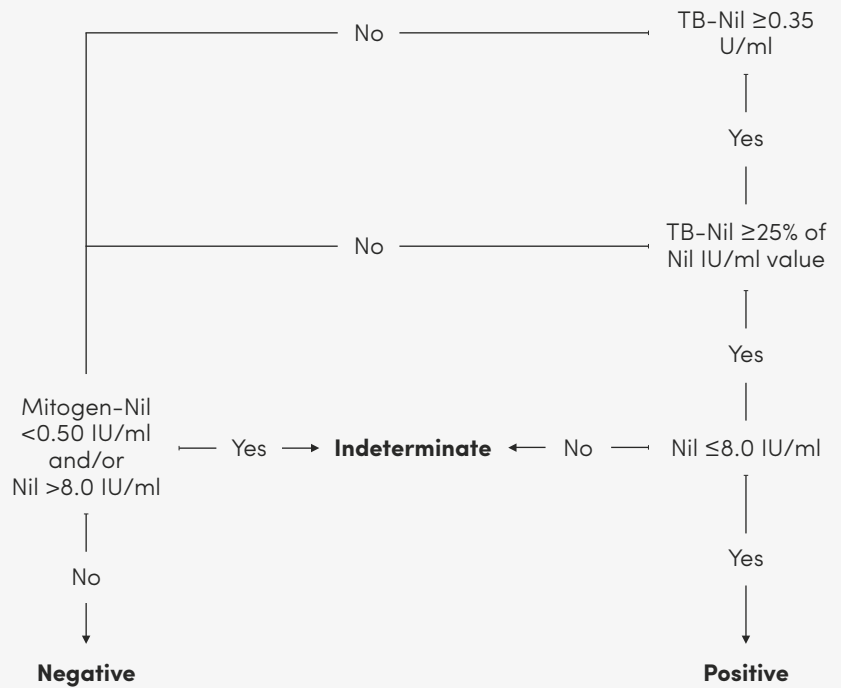
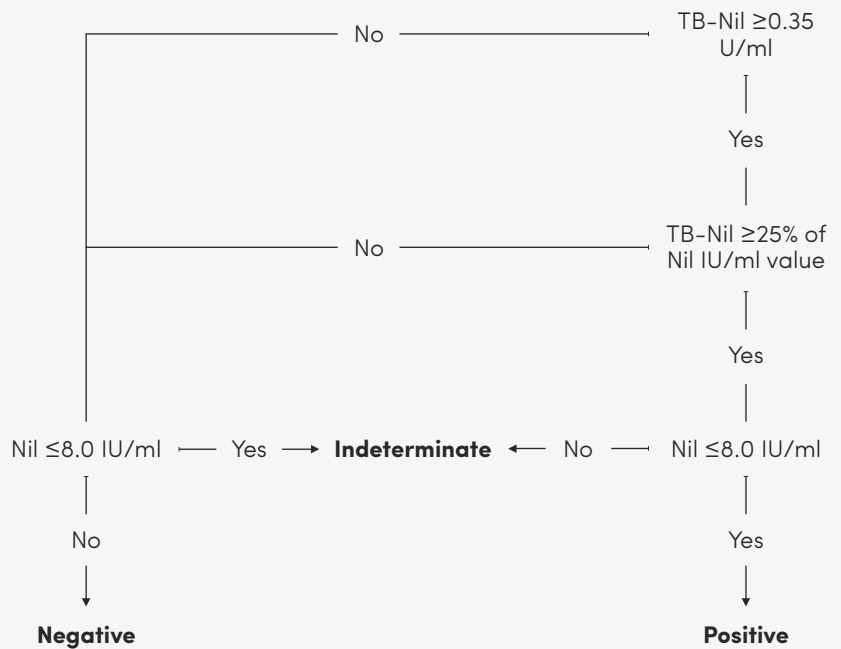


FIGURE 9

ALGORITHM FOR INTERPRETING RESULTS OF THE STANDARD E TB-FERON IGRA (2 TUBE ASSAY)



EQUIPMENT TO PERFORM THE ASSAY

MATERIALS REQUIRED BUT NOT PROVIDED

Phlebotomy materials

Calibrated variable volume pipettes with disposable tips

Calibrated multichannel pipettes with disposable tips

Microplate shaker

Deionized or distilled water

Microplate reader fitted with 450 nm filter and 620 nm to 650 nm reference filter

37°C ± 1°C incubator (CO₂ not required)

Refrigerator 2°C–8°C

Centrifuge for separation of plasma

Disposable gloves

MATERIALS PROVIDED

TB-Feron Nil Tubes (gray cap)

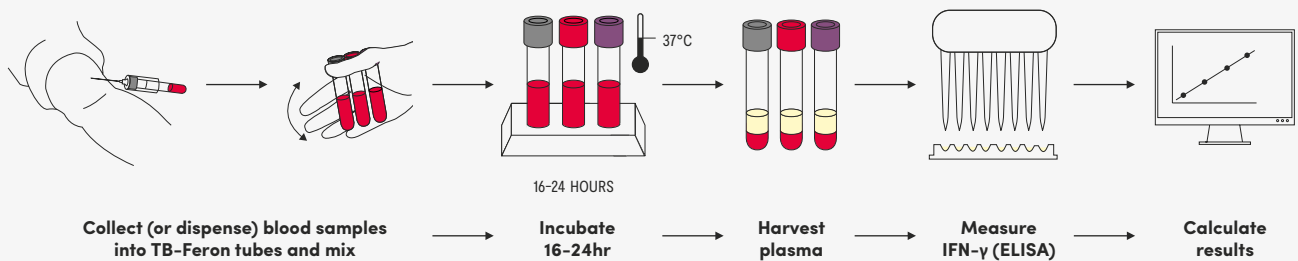
TB-Feron TB Antigen Tubes (red cap)

TB-Feron Mitogen Tubes (purple cap)

Standard E TB-Feron ELISA kit

FIGURE 10

WORKFLOW OF THE STANDARD E TB-FERON IGRA



HOW TO PERFORM THE ASSAY

(ADAPTED FROM MANUFACTURER'S INSTRUCTIONS FOR USE)

PHASE 1 : BLOOD COLLECTION, ANTIGEN STIMULATION AND HARVESTING OF PLASMA

1

SPECIMEN COLLECTION

Label each tube appropriately.
Allow tubes to come to room temperature (15–25°C).

For each patient, collect 1 ml of blood by venipuncture directly into each of the TB-Feron tubes. This procedure should be performed by a trained phlebotomist or equivalent.

Alternatively, draw blood into a lithium-heparin tube and dispense 1 ml into each of the three TB-Feron tubes. If necessary, blood may be stored in the lithium-heparin tube at 15°C to 25°C for up to 16 hours before dispensing into the TB-Feron tubes. The total time from blood collection to initiating the 37°C incubation should not exceed 16 hours.

2

MIXING OF TUBE CONTENTS

Immediately after filling the TB-Feron Tubes, shake them ten (10) times just firmly enough to make sure the entire inner surface of the tube is coated with blood. This will dissolve antigens on tube walls. Do not shake excessively.

Transfer the TB-Feron Tubes to a 37°C ± 1°C incubator as soon as possible and no more than 16 hours after blood collection. Prior to incubation at 37°C ± 1°, maintain the tubes at room temperature (20°C ± 5°C). The total time from blood collection to initiating the 37°C incubation should not exceed 16 hr.

3

INCUBATION AND HARVESTING OF PLASMA

Incubate the TB-Feron Tubes upright at 37°C ± 1°C for 16 to 24 hours. The incubator does not require CO₂ or humidification.

Harvesting of plasma is facilitated by centrifuging the tubes at 2200–2300 x g (RCF) for 15 minutes.

Harvest plasma samples using a pipette. 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. Separated plasma can be stored for up to one week at 2°C to 8°C. Separated plasma samples can also be stored below –20°C for extended periods.

HOW TO PERFORM THE ASSAY (CONTINUED)

(ADAPTED FROM MANUFACTURER'S INSTRUCTIONS FOR USE)

PHASE 2 : DETECTION OF IFN- γ AND GENERATION OF RESULTS

4

PREPARE IFN- γ STANDARDS

Label S1, S2, S3, and S4 on 4 empty tubes.

Add 300 μ l of ELISA Diluent to each tube.

Add 100 μ l of Reconstituted STANDARDS to STANDARD tube 1 (S1) and mix thoroughly (S1 contains 4 IU/ml).

Transfer 100 μ l of the STANDARD tube1 (S1) solution to STANDARD tube 2 (S2) (S2 contains 1 IU/ml).

Transfer 100 μ l of the STANDARD tube2 (S2) solution to STANDARD tube 3 (S3)

ELISA Diluent serves as a zero STANDARD (S4).

5

PERFORM STANDARD E TB-FERON ELISA

Add 50 μ l of prepared Working Detector solution into each of the wells

Add 50 μ l of STANDARD 1 to 4 and the specimens into the appropriate wells of the antibody-coated microplate.

Mix gently by tapping the plate.

Cover the plate with the attached plate sealer and incubate at 37 \pm 1 $^{\circ}$ C for 1 hour.

Wash the wells five times with 350 μ l of diluted wash buffer and aspirate all liquid from the wells. Or, wash the wells using an automatic washer with 350 μ l of diluted wash buffer.

Add 100 μ l of TMB substrate into each of the wells.

Incubate for 30 minutes at room temperature (15-25 $^{\circ}$ C) in the dark.

Add 100 μ l of stop solution into each of the wells. Mix by gentle shaking.

Read the absorbance at 450nm in an ELISA plate reader (with reference wavelength between 620 nm and 650 nm) within 30 minutes.

6

CALCULATE RESULTS

Calculate results using the STANDARD E ANALYSIS SOFTWARE. These calculations can also be performed using software available with microplate readers, standard spreadsheets, and statistical software (e.g. 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.

OPERATIONAL CONSIDERATIONS

CONSIDERATIONS FOR SITE SELECTION

Because of the need for sophisticated laboratory equipment and highly skilled laboratory personnel to perform the ELISA and interpret test results, the test is best suited for implementation in intermediate or national reference laboratories. Because of the use of ELISAs for other diseases, national programs may be able to leverage collaboration with other, non-TB-specific laboratories, having the capacity for blood draws and ELISA testing. The biosafety precautions for the TBF test are the same as most tests that require blood draws and ELISAs.

If the test is located in a centralized site, it will be necessary to establish efficient specimen transport systems to ensure that blood samples can be transported from peripheral sites to the IGRA testing laboratory within the recommended time frames. For the TBF test, the maximum time between blood collection in lithium-heparin tubes and processing at the testing laboratory is 16 hours.

THROUGHPUT AND TIME REQUIRED FOR PERFORMING ASSAY

The time required to perform the TB-Feron IGRA test is estimated below; the time of testing multiple samples when batched is also indicated:

→ **37°C incubation of blood tubes: 16 to 24 hours.**

→ **ELISA: Approx. 2.5 hours for one ELISA plate.**

- A 96-well ELISA plate can contain samples from up to 28 individuals (12 wells used for IFN- γ standards).
 - <1 hour of labor.
 - Add 10 to 15 minutes for each extra plate.
-

STORAGE AND HANDLING

→ **TB-Feron tubes**

- Store TB-Feron tubes at 2°C to 25°C.
- Shelf life: 15 months (expiration date printed in the package and in the label of each tube).

→ **Kit reagents**

- Store kit reagents at 2°C to 8°C.
- Always protect Enzyme Substrate Solution from direct sunlight.
- Shelf life: 15 months (expiration date printed in the package and in the label of each tube).
- Store STANDARD E TB-Feron Control at 2°C to 30°C.

→ **Reconstituted and unused reagents**

- The diluted wash buffer may be kept for up to 1 week if stored at 15°C to 25°C.
 - The working detector solution can be stored for up to 4 hours at 2°C to 8°C.
-

ORDER PLANNING

PRODUCT	SPECIMEN	PACK SIZE	CAT NO.
ETB-Feron Elisa (2 plates)	Plasma	192 wells/kit	07TBF10C
ETB-Feron Elisa Tube 100	WB	Mitogen tube x 100	07TBFA10
ETB-Feron Elisa Tube 200	WB	TB Antigen tube x 100	07TBFA20
	WB	Nile tube x 100	
ETB-Feron Elisa Control		Lv1 x 15 / Lv2 x 15 / Lv3 x 15	07TBFC10

QUALITY ASSURANCE, CONTROL, AND ASSESSMENT

For the blood handling and immune stimulation phases, the Mitogen tube serves as a positive control. A valid test must generate a Mitogen minus Nil value of ≥ 0.5 IU/ml. Low response to Mitogen may result from insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, incorrect filling or mixing of the Mitogen tube, or inability of the patient's lymphocytes to generate IFN- γ .

Important internal controls that are conducted with each ELISA are the known samples of IFN- γ used to construct the standard curve.

→ 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 $\leq 15\%$,
- replicate OD values for Standard 3 and Standard 4 must not vary by more than 0.040 optical density units from their mean,
- the mean O.D value for S4 must be 0.150 or less,
- the correlation coefficient (r) calculated from the mean absorbance values of the standards must be ≥ 0.98 .

Proficiency testing programs for IGRAs are available such as ones from the UK National External Quality Assessment Service (UK NEQAS), INSTAND e.V., Society for Promoting Quality Assurance in Medical Laboratories, and College of American Pathologists (CAP).

Key quality indicators that should be monitored monthly include the percentage of runs with invalid standard curves; percentage of indeterminate, errors or invalid results; percentage of positive results; distribution of IFN- γ concentrations; and turnaround times (**ANNEX 2**). Targets should be set for all indicators that are monitored, and any unexplained change in quality indicators, such as an increase in error rates or a change in positivity rate should be documented and investigated.

RECORDING AND REPORTING

Both the standard qualitative test interpretation (positive, negative, indeterminate) and the quantitative assay measurements should be reported, together with the criteria for test interpretation.

4.

T-SPOT[®].TB ASSAY

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T-SPOT®.TB ASSAY

The T-SPOT®.TB test is an in vitro diagnostic test for the detection of effector T cells that respond to stimulation by *M. tuberculosis* antigens ESAT-6 and CFP-10 by detecting the IFN- γ secreted in the vicinity of T cells by capturing the IFN- γ on an antibody-coated membrane.

PRINCIPLES OF THE ASSAY

The immune response to infection with *M. tuberculosis* is mediated predominantly through T cell activation. As part of this response, T cells are sensitized to *M. tuberculosis* antigens and the activated effector T cells, both CD4+ and CD8+, produce IFN- γ when stimulated by these antigens. The T-SPOT.TB test uses the enzyme-linked immunospot (ELISPOT) method to enumerate *M. tuberculosis*-sensitized T cells by capturing IFN- γ in the vicinity of the T cells from which it was secreted.

Peripheral blood mononuclear cells (PBMCs) are separated from a whole blood sample, washed, counted, and then placed into microtiter wells where they are exposed to:

A nil control which adjusts for background IFN- γ -producing PMBCs. The number of spots in the nil control is subtracted from the number of spots in the positive control well and antigen wells.

A positive control (phytohemagglutinin) which assesses cell functionality.

A mixture of peptides representing overlapping

sequences of the entire amino acid sequence of ESAT-6 (Panel A).

A mixture of peptides representing overlapping sequences of the entire amino acid sequence of CFP-10 (Panel B).

The PBMCs are incubated for 16 to 20 hours with the antigens to allow the stimulation of any sensitized T cells present. Any secreted IFN- γ is captured by specific antibodies on the surface of the membrane, which forms the base of the well. After incubation, the cells and other unwanted materials are removed by washing. A second antibody, conjugated to alkaline phosphatase and directed to a different epitope on the IFN- γ molecule, is added and binds to the IFN- γ captured on the membrane surface. Any unbound conjugate is removed by washing. A soluble substrate is added to each well; this is cleaved by bound enzymes to form a (dark blue) spot of insoluble precipitate at the site of the reaction.

Evaluating the number of spots obtained provides a measurement of the abundance of *M. tuberculosis* sensitive effector T cells in the peripheral blood.

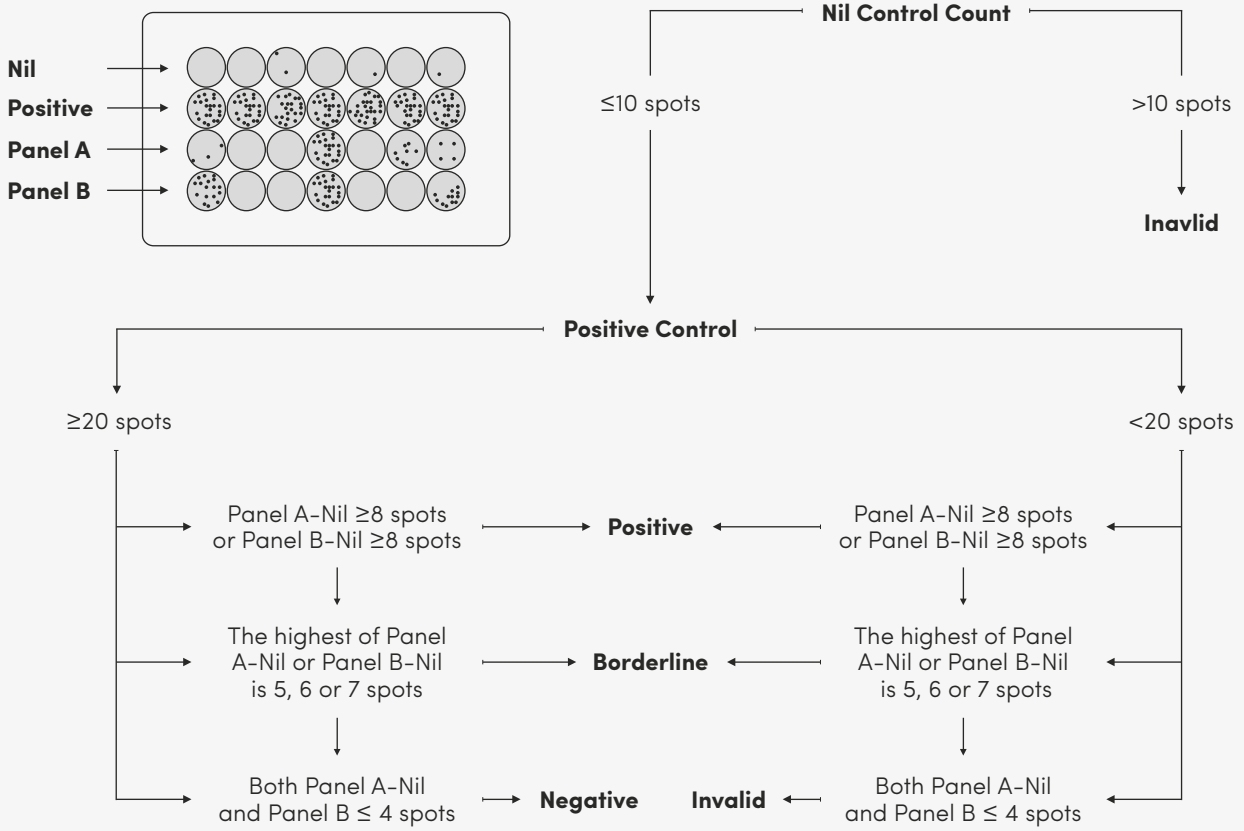
PERFORMANCE

Using the previously described approach relying on surrogate measures, the manufacturer showed that the T-SPOT.TB test has a sensitivity of 95.6% (175/183 test subjects) for detecting persons known

to have a TB infection (i.e. persons with active TB disease) and a specificity of 97.1% (297/306 test subjects) for correctly identifying persons thought not to have a TB infection.

FIGURE 11

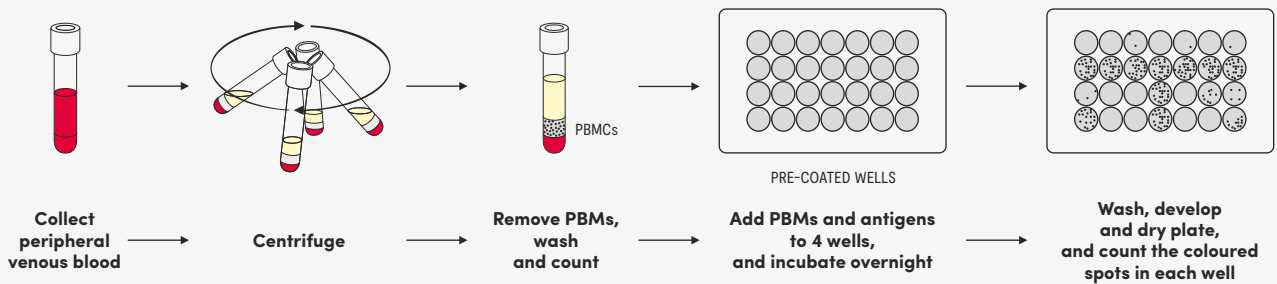
ALGORITHM FOR INTERPRETING RESULTS OF THE T-SPOT.TB ASSAY



SOURCE: REPRODUCED FROM T-SPOT.TB PACKAGE INSERT

FIGURE 12

WORKFLOW OF THE T-SPOT.TB TEST



SOURCE: REPRODUCED FROM T-SPOT.TB PACKAGE INSERT

EQUIPMENT TO PERFORM THE ASSAY

MATERIALS REQUIRED BUT NOT PROVIDED

Phlebotomy materials

Blood collection tubes, such as Vacutainer® CPT™ or heparinized tubes

Class II biosafety cabinet (recommended)

T-Cell *Xtend*® reagent (if used)

Ficoll® and 15 mL centrifuge tubes (if not using CPT tubes)

Centrifuge for preparation of PBMCs (capable of at least 1800 RCF (g) and able to maintain the samples at 18°C –25°C)

Equipment and reagents to enable counting of PBMCs: e.g., a hemocytometer on a microscope or a suitable hematology analyzer

Magnetic bead-based processing system, if T-Cell *Select*™ is used instead of Leucosep™ tubes

Humidified incubator capable of 37 ± 1°C with a 5% CO₂ supply

Sterile cell culture medium such as GIBCO AIM-VTM

Adjustable pipettes and sterile pipette tips

8-well strip plate frame

Automatic microtiter plate washer or an 8 channel or stepper pipette to manually wash plates

Sterile PBS solution

Distilled or deionized water

A means of visualizing the wells, or capturing a digital image of the well, such as a stereomicroscope, magnifying glass or plate imager to allow counting of spots

MATERIALS PROVIDED

Microtiter plate: supplied as 12x 8-well strips in a frame or as a solid 96-well plate, coated with a mouse

Monoclonal antibody to IFN-γ

Panel A: contains ESAT-6 antigens, bovine serum albumin, and antimicrobial agents

Panel B: contains CFP10 antigens, bovine serum albumin and antimicrobial agents

Positive Control: contains phytohemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents

200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN-γ conjugated to alkaline phosphatase

Substrate Solution: ready-to-use BCIP/NBT^{plus} solution

HOW TO PERFORM THE ASSAY

(ADAPTED FROM MANUFACTURER'S INSTRUCTIONS FOR USE)

1

SAMPLE COLLECTION AND PREPARATION

Collect a blood sample according to the instructions supplied with the collection device. Store collected blood at room temperature (18°C–25°C) or at 10°C–25°C if the T-Cell *Xtend* reagent is to be used.

Do not refrigerate or freeze.

Harvest PBMCs:

- When using CPT blood collection tubes, follow the manufacturer's instructions for the separation of PBMCs.
- When using blood collection vacutainers containing heparin or citrate, separate PBMCs by centrifugation through Ficoll-Paque Plus using published procedures.
- If Leucosep tubes, or the T-Cell *Xtend* reagent (available from Oxford Immunotec) are used, follow the protocols provided with these reagents.

Collect the white, cloudy band of PBMCs using a pipette and transfer it to a 15ml conical centrifuge tube. Make up the volume to 10ml with the cell culture medium. Alternatively, a cell washing centrifuge, e.g., DiaCent-CW (Bio-Rad), may be used to facilitate the cell washing stages. If this system is used then DPBS should be used to wash the cells.

Centrifuge at 600xg for 7 minutes.

Pour off the supernatant and resuspend the pellet in 1ml of the medium.

Make up the volume to 10mL with fresh medium and centrifuge at 350xg for 7 minutes.

Pour off the supernatant and resuspend the pellet in 0.7ml of AIM V culture medium.

Cell Counting and Dilution: The T-SPOT. *TB* test requires 2.5×10^5 viable PBMCs per well.

A total of four wells are required for each patient sample. Perform a viable cell count, e.g. manual counting using Trypan Blue and a haemocytometer or automated counting using an appropriate instrument. Calculate the concentration of viable cells present in the stock cell suspension.

Prepare 500µL of the final cell suspension at a concentration of 2.5×10^5 cells/100µL.

Ensure cells are thoroughly mixed before removing an aliquot for dilution.

2

PLATE SET UP AND INCUBATION

THE T-SPOT. *TB* TEST REQUIRES FOUR WELLS TO BE USED FOR EACH PATIENT SAMPLE

Remove the pre-coated 8-well strips from the packaging, clip them into a plate frame and allow them to equilibrate to room temperature. Or remove a solid 96-well plate from the packaging and allow it to equilibrate to room temperature.

Each patient sample requires the use of 4 individual wells:

- Add 50µl AIM V culture medium to each Nil Control well.
- Add 50µl Panel A solution to each well required.
- Add 50µl Panel B solution to each well required.
- Add 50µL Positive Control solution to each cell functionality control well.

To each of the 4 wells to be used for a patient sample, add 100µL of the patient's final cell suspension (containing 250,000 viable cells).

Incubate the plate in a humidified incubator at 37°C with 5% CO₂ for 16–20 hours.

HOW TO PERFORM THE ASSAY (CONTINUED)

(ADAPTED FROM MANUFACTURER'S INSTRUCTIONS FOR USE)

3

SPOT DEVELOPMENT AND COUNTING

Remove the plate from the incubator.

Discard the cell culture medium and add 200µl D-PBS solution to each well.

Discard the D-PBS solution.
Repeat the well washing a further 3 times with fresh D-PBS solution for each wash.

Dilute concentrated Conjugate Reagent 200-fold in PBS to create the working strength solution.

Add 50µl working strength Conjugate Reagent solution to each well and incubate at 2°C–8°C for 1 hour.

Discard the conjugate and perform four D-PBS washes as described in the 2nd and 3rd steps above.

Add 50µl Substrate Solution to each well and incubate at room temperature for 7 minutes.

Wash the plate thoroughly with distilled or deionised water to stop the detection reaction.

Allow the plate to dry by standing it in a well-ventilated area or in an oven at up to 37°C.

Count and record the number of distinct, dark blue spots on the membrane of each well.

4

RESULTS INTERPRETATION AND TEST CRITERIA

A Nil Control spot count in excess of 10 spots should be considered as 'Indeterminate'.

Another sample should be collected from the individual and tested.

A Positive Control spot count of <20 spots should be considered as 'Indeterminate' unless either Panel A or Panel B is 'Positive' as described in the next step, in which case the result is valid.

The test result is 'Positive' if (Panel A minus Nil Control) and/or (Panel B minus Nil Control) ≥ 6 spots.

The test result is 'Negative' if both (Panel A minus Nil Control) and (Panel B minus Nil Control) ≤ 5 spots. This includes values less than zero.

NB: If using the US version of the kit:

- The test result is 'Positive' if (Panel A minus Nil Control) and/or (Panel B minus Nil Control) ≥ 8 spots.
- The test result is 'Negative' if both (Panel A minus Nil Control) and (Panel B minus Nil Control) ≤ 4 spots.

Results where the highest of the Panel A or Panel B spot count is such that the (Panel minus Nil) spot count is 5, 6, or 7 spots can be considered Borderline (equivocal), and retesting by collecting another patient sample is recommended.

NB: a borderline result is a valid, reportable result.

OPERATIONAL CONSIDERATIONS

CONSIDERATIONS FOR SITE SELECTION

Because of the need for highly skilled laboratory personnel to perform and interpret the T-SPOT. TB test, it is best suited for implementation in intermediate or national reference laboratories. The biosafety precautions for the T-SPOT. TB tests are the same as most tests that require BSL-2 level laboratories.

If the test is located in a centralized site, it will be necessary to establish efficient specimen transport systems to ensure that blood samples can be transported from peripheral sites to the T-SPOT. TB testing laboratory within the recommended time frames. Blood samples must be stored at room temperature and tested within 8 hours of blood collection, or within 32 hours with storage at 10°C–25°C if the sample is treated with a means of removing granulocytes, such as the use of the T-Cell *Xtend* reagent. Using the T-Cell Select reagent extends the time from blood collection to processing to 54 hours.

TIME REQUIRED FOR PERFORMING ASSAY

- Blood collection and sample processing: 3 hours.
A 96-well plate can accommodate samples from up to 24 individuals.
- 37°C incubation of blood tubes: 16 to 20 hours.
- Wash, develop, and dry plate and count spots: 1.5 to 2 hours.

STORAGE AND HANDLING

→ Kit reagents

- Store unopened kit reagents at 2°C to 8°C.
- Always protect Enzyme Substrate Solution from direct sunlight.
- The T-SPOT. TB test kit has a minimum remaining shelf life of 14 months as per the Stop TB GDF agreement with Oxford Immunotec.
Always use before the expiration date printed on the kit label.

→ Reconstituted and unused reagents

- Store opened kit components at 2°C to 8°C. Components must be used within 8 weeks of opening.
- Working strength conjugate may be stored for up to six weeks at 2°C to 8°C prior to use.

FORECASTING AND ORDER PLANNING

Eligible countries for T-SPOT. TB concessional prices through GDF include:

→ All low- and middle-income countries except:

Azerbaijan, Brazil, Bulgaria, China, Democratic People's Republic of Korea, India, Indonesia, Iran, Jordan, Kazakhstan, Lebanon, Malaysia, Maldives, Morocco, Myanmar, Peru, Philippines, Russian Federation, Thailand, Turkey, Tunisia, Ukraine, Vietnam.

→ Listed high-income countries:

Antigua and Barbuda, Bahamas, Barbados, Brunei Darussalam, New Caledonia, Palau, Seychelles, St. Kitts and Nevis, Trinidad and Tobago.

To perform T-SPOT. TB IGRAs, products from the T-SPOT. TB test kit and AIM-V culture medium are required. Furthermore, either Leucosep tubes or T-Cell Select (plus generic blood collection tubes) are required. T-Cell *Xtend*, allowing blood samples to be processed up to 32 hours after venipuncture, is optional. The concessional prices reflect significant discounts from market pricing in high-income countries. **The least expensive option to perform T-SPOT. TB IGRAs is when Leucosep tubes are used and T-Cell *Xtend* is not used; this results in a cost per patient sample of \$10.32. If T-Cell *Xtend* is used, the cost per patient sample is \$13.20. If T-Cell *Select* is used instead of Leucosep tubes, the cost per patient sample is \$13.35.** The above costs per patient sample do not take into consideration the costs of ancillary consumables.

PRODUCT	GDF ITEM NUMBER	COST OF KIT IN GDF CATALOG	UNITS PER KIT	PATIENT SAMPLES TESTED	COST PER PATIENT SAMPLE
T-SPOT. <i>TB</i> test kit	106665	\$168.00	1 x 96 wells	24	\$7.00
T-Cell Xtend ¹⁵	106666	\$115.00	3 x 2 ml	40	\$2.88
T-Cell Select ¹⁶	106667	\$693.00	1 kit	144	\$4.81
Leucosep tubes ¹⁷	106668	\$89.00	50 tubes	50	\$1.78
AIM-V culture medium ¹⁸ (50 mL)	106669	\$23.00	50 ml	2.4	N/A
AIM-V culture medium (500 mL)	106670	\$37.00	500 ml	24	\$1.54

15. T-Cell Xtend allows for the sample storage time to be extended from 8 to 32 hours.

16. T-Cell Select reagent is used for isolation of peripheral blood mononuclear cells from whole blood.

17. Leucosep blood collection tubes facilitate separation of peripheral blood mononuclear cells from whole blood.

18. AIM-V sterile cell culture medium is used for the incubation step.

SUPPLY PLANNING (SUGGESTED DELIVERY FREQUENCY)

The supply plan must account for the procurement and supplier lead times as well as the time required for country-specific importation processes; in total this may entail 4–6 months. The time required for in-country distribution must also be considered.

For the planning of orders and shipments, the size of an order may include all of the product needs estimated for a year, though with multiple shipments.

For the AIM-V culture medium, given the maximum shelf life of 9 months, shipments are required at least 2–3 times a year in order to prevent stock-outs.

For T-Cell Select and T-SPOT. *TB* test kits, given the maximum shelf lives of 12 and 14 months, respectively, shipments may also be required twice a year.

For the remaining products, including Leucosep tubes and T-Cell Xtend, given the maximum shelf life of 18 months and 38 months, respectively, shipments may be made annually.

QUALITY ASSURANCE, CONTROL, AND ASSESSMENT

For the blood handling and immune stimulation phases, the Positive Control sample tube serves to assess cell functionality. A valid test must generate a Positive Control spot count of ≥ 20 spots. A Positive Control spot count of < 20 spots should be considered as 'Indeterminate' unless either Panel A or Panel B is 'Positive' as described above, in which case the result is valid.

A low response to the Positive Control may result from insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, or the inability of the patient's lymphocytes to generate IFN- γ .

Proficiency testing programs for IGRAs are available such as ones from the UK National External Quality Assessment Service (UK NEQAS), INSTAND e.V., Society for Promoting Quality Assurance in Medical Laboratories and College of American Pathologists (CAP).

Key quality indicators that should be monitored monthly include the percentage of runs with invalid standard curves; percentage of indeterminate, errors or invalid results; percentage of positive results; distribution of IFN- γ concentrations; and turnaround times (ANNEX 2). Targets should be set for all indicators that are monitored, and any unexplained change in quality indicators, such as increase in error rates or a change in positivity rate should be documented and investigated.

RECORDING AND REPORTING

Both the standard qualitative test interpretation (positive, negative, borderline, invalid) and the quantitative assay measurements should be reported, together with the criteria for test interpretation.

**SUMMARY TABLE:
COMPARISON OF IGRAS FOR THE DETECTION OF TB INFECTION**

	QFT-PLUS	WANTAI	STANDARD E TBF	T-SPOT.TB
Number of tubes used	4 (Nil, Mitogen, TB1, TB2)	3 (Nil, Mitogen, TB antigen)	3 (Nil, Mitogen, TB antigen)	1
Correction for background IFN- γ level	Yes, by subtracting Nil tube IFN- γ value	Yes, by subtracting Nil tube IFN- γ value	Yes, by subtracting Nil tube IFN- γ value	Yes, by subtracting Nil tube IFN- γ value
Positive control	Yes, Mitogen tube	Yes, Mitogen tube	Yes, Mitogen tube	Yes, Mitogen tube
Stimulating antigens	ESAT6, CFP-10 TB1: long peptides TB2: short peptides	ESAT6, CFP-10 Recombinant proteins	ESAT6, CFP-10, TB7.7 Recombinant proteins	ESAT6, CFP-10 A: ESAT6 peptides B: CFP-10 peptides
Antigen stimulation	16–24 hr	20–24 hr	16–24 hr	16–20 hr
IFN- γ detection	ELISA	ELISA	ELISA	ELISPOT, single cell resolution
Hands-on time	<1 hr	<1 hr	<1 hr	3.9 hr (manual system) 1.5 hr (automated system)
Total processing time	3 hr for one 96-well plate plus antigen stimulation 1–2 hr hands on time with automated ELISA systems and results available within 24 hours	3 hr for one 96-well plate plus antigen stimulation	2 hr for one 96-well plate plus antigen stimulation	5.5 hr plus antigen stimulation plus 4–16 hr for plate drying
Test capacity (each patient test requires 3 or 4 wells)	Up to 22 tests per 96-well plate or 2 tests per 8-well strip (8 wells or one strip used for IFN- γ standards) Up to 100 samples/run with automated ELISA systems	28 tests per 96-well plate or 3 tests per 8-well strip or 4 tests per 12-well strip (12 wells or one strip used for IFN- γ standards)	28 tests per 96-well plate (12 wells used for IFN- γ standards)	24 tests per 96-well plate or 2 tests per 8-well strip
Computer required to calculate results	Yes, calculation of standard curve and results using QFT-Plus Analysis Software or other software	Yes, calculation of standard curve and results	Yes, calculation of standard curve and results	No
Result interpretation	Positive, negative, indeterminate, invalid	Positive, negative, indeterminate, invalid	Positive, negative, indeterminate, invalid	Positive, negative, borderline, invalid
Standard curve	Yes, required for calculation of IFN- γ concentration	Yes, required for calculation of IFN- γ concentration	Yes, required for calculation of IFN- γ concentration	No
Type of laboratory	Reference laboratory	Reference laboratory	Reference laboratory	Reference laboratory
Operating temperature	2°C to 8°C	2°C to 8°C	2°C to 8°C	2°C to 8°C
Reagent Storage temperature	17°C to 27°C	18°C to 30°C	15°C to 25°C	18°C to 25°C
Required equipment	<ul style="list-style-type: none"> • 37°C \pm 1°C incubator • Calibrated pipettes • Microplate shaker • Microplate washer (automated washer recommended) • Microplate reader fitted with 450 nm filter and 620 nm to 650 nm reference filter • Optional but recommended: centrifuge for harvesting plasma 	<ul style="list-style-type: none"> • 37°C \pm 1°C incubator • Calibrated pipettes • Microplate shaker • Microplate washer (automated washer recommended) • Microplate reader • Centrifuge • Refrigerator 	<ul style="list-style-type: none"> • 37°C \pm 1°C incubator • Calibrated pipettes • Microplate shaker • Microplate washer (automated washer recommended) • Microplate reader • Centrifuge • Refrigerator 	<ul style="list-style-type: none"> • Calibrated pipettes • Class II biosafety cabinet (recommended) • Centrifuge • Equipment to count PBMCs • Humidified 37 \pm 1°C incubator with a 5% CO₂ • automatic microtiter plate washer • stereomicroscope, magnifying glass or plate imager
Maintenance and calibration	Microplate reader, incubator, pipette, shaker requires maintenance and calibration	Microplate reader requires maintenance and calibration	Microplate reader requires maintenance and calibration	Biosafety cabinet requires maintenance and certification
Cost	Stop TB GDF catalog: \$15.90 per test	Global access pricing not yet announced	Global access pricing not yet announced	Stop TB GDF catalog: \$10.32–\$13.35 per test

ANNEXES

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ANNEX 1

IGRA IMPLEMENTATION - HIGH LEVEL CHECKLIST

→ Policy and planning

- Have roles and responsibilities for coordinating the implementation process been clearly defined?
- Which national guidelines, policies and other materials will need to be updated to include the use of IGRAs (consider NTP policies and guidelines; screening, diagnostic and preventative treatment algorithms, TB/HIV policies and guidelines, etc.)?
- Has a stakeholder mapping process been conducted, including all key internal (within government) and external stakeholders (local and international)?
- What support can partners provide for the implementation process?===
- Has the intended use of IGRAs been decided? Have projections been made for the number of samples to be tested per year or per site?
- Has a costed implementation plan been developed?
- Have adequate financial resources for capital investments, implementation and projected on-going costs been secured?

→ Regulatory

- What are the importation requirements for instruments, reagents and supplies for IGRAs?
- What is the regulatory process required for IGRAs?
- Is verification of IGRAs needed for regulatory approval?
- If so, what type of protocol and number of samples are required? Timeline? Where will verification studies be conducted?
- Is the designated authority (NTP, procurement agency or partners) engaged with manufacturers to support regulatory processes?

→ Site readiness

- Are adequate laboratory facilities, space and infrastructure available?
- Do facilities, equipment, policies and practices meet biosafety standards?
- Are appropriately trained and competent staff available to conduct IGRAs?
- Which IGRA instruments have been selected and what are the requirements for installation and maintenance?
- Are adequate specimen referral and results reporting systems available?

→ Procurement and supply chain

- Which partners support IGRAs in the country, and what is their scope of activities (how can they contribute to the transition)?
- Which partners procure instruments and consumables?
- Have manufacturers or distributors been identified who can support implementation, equipment maintenance (warranties or service contracts) and commodities?
- Is a procurement system available to ensure the availability of reagents and supplies that accounts for procurement times, consumption rates and shelf-life of reagents?
- What is the planned procurement by MOH and partners for this year?

IGRA IMPLEMENTATION - HIGH LEVEL CHECKLIST (CONTINUED)

→ Procedures

- Which SOPs and forms will need to be updated or developed?

→ Quality assurance

- Are the essential elements of a quality assurance system in place at the testing site?
- Are protocols in place to conduct and document quality checks of each step of the IGRA process and ensure the use of positive and negative controls?
- Is an external quality assessment programme in place?
- Which partners can assist with proficiency testing, supervisory visits and re-checking of samples?
- Have quality (performance) indicators been defined and appropriate data collection tools developed?

→ Recording and reporting

- Are revisions of the current data collection form and request for laboratory testing form required?
- Is revision of forms for reporting the results of laboratory testing needed?
- Is a revision of laboratory, clinical or surveillance registers needed?
- If an electronic laboratory information system is in use, what updates will be required?
- If an electronic recording and reporting system is in place, what updates will be required?

→ Training

- Have terms of reference and competency-based job descriptions been developed for key staff?
- Is a national approved training curriculum available?
- Who is responsible and what is the process for updating training materials for laboratory, clinical and programme staff?
- Is the approved curriculum used for all trainings, including those delivered by partners?
- Are standard procedures used to assess and document the competence of all staff involved in IGRAs?

→ Monitoring the transition

- What changes to M&E tools and processes would be required to enable monitoring of additional indicators (i.e. progress indicators and laboratory indicators)?
- What support can partners provide in monitoring of new algorithms and adherence to guidelines at sites?

ANNEX 2

QUALITY INDICATORS FOR INTERFERON-GAMMA RELEASE ASSAYS (IGRAS)

Targets should be set for all indicators that are monitored, and any unexplained change in quality indicators, such as increase in error rates or a change in positivity rate should be documented and investigated. Targets may need to be adjusted as information on the use of these tests in routine diagnostic settings become available.

INDICATOR	DESCRIPTION	TARGET
Number of samples received per month		
Number and proportion of tests performed per month		
Number and proportion of rejected samples ^A	Number of rejected samples / Total number of specimens received	<5%
Number and proportion of samples with positive results	Number of samples with positive results / Number of samples tested	Dependent on population tested
Number and proportion of samples with borderline results (T-Spot. <i>TB</i> only)	Number of samples with borderline results / Number of samples tested	Dependent on population tested
Number and proportion of test runs with valid IFN- γ standard curves (ELISA-based tests)	Number of test runs with valid standard curves / Number of test runs	>95%
Number and proportion of samples with indeterminate results	Number of samples with indeterminate results / Number of samples tested	<5%
Number and proportion of samples with errors ^B	Number of specimens with errors/ Total number of samples tested	<3% (Xpert)
Number and proportion of samples with invalid results	Number of samples with invalid results / Total number of samples tested	<1% (Xpert)
Number and proportion of samples tested with an IGRAs for which a result was reported within the target turnaround time (i.e. time from blood collection to reporting of results) ^C	Number and proportion of samples tested with an IGRAs for which a result was reported within the target turnaround time / Total number of samples tested	>90%

A Stratify by reason rejected (insufficient volume, hemolyzed, incorrect collection tube, received past cutoff) to enable troubleshooting.

B Errors should be stratified by type to enable troubleshooting.

C Target turnaround time should be set by the program taking into consideration sample transport, testing schedules and test characteristics.

For troubleshooting, analyse time from blood collection to receipt in the laboratory, time from blood collection to initiation of the immune stimulation, and time for the IFN- γ detection step (e.g., ELISA or ELISPOT).

ANNEX 3

IMPACT MEASURES FOR IGRAS

For each of the below indicators, stratification by target population and risk of exposure (e.g. household contacts and children under 5 years) or risk of progression (e.g. PLHIV) will allow for optimal assessment of the impact of activities:

- Number and proportion of eligible persons that are tested for TB infection using an IGRAS
- Number and proportion of tested persons with a positive IGRAS result
- Number and proportion of tested persons with a positive IGRAS result placed on TPT
(proportion = number of tested persons with a positive IGRAS placed on TPT) / number of persons with a positive IGRAS)
- Number and proportion of persons placed on TPT that had a positive IGRAS test
(proportion = number of persons placed on TPT that had a positive IGRAS result / number of persons placed on TPT)

SUGGESTED READING

1. WHO consolidated guidelines on tuberculosis. Module 3. Diagnosis tests for TB infection. Geneva: World Health Organization, 2022. <https://www.who.int/publications/i/item/9789240056084>
2. WHO operational handbook on tuberculosis. Module 3. Diagnosis tests for TB infection. Geneva: World Health Organization, 2022. <https://www.who.int/publications/i/item/9789240058347>
3. WHO operational handbook on tuberculosis. Module 3: diagnosis. Rapid diagnostics for tuberculosis detection. Geneva: World Health Organization, 2021. <https://www.who.int/publications/i/item/who-operational-handbook-on-tuberculosis-module-3-diagnosis---rapid-diagnostics-for-tuberculosis-detection>
4. Framework for the evaluation of new tests for tuberculosis infection. Geneva: World Health Organization, 2020. <https://apps.who.int/iris/rest/bitstreams/1288536/retrieve>
5. Use of alternative interferon-gamma release assays for the diagnosis of TB infection: WHO policy statement. Geneva: World Health Organization, 2022. <https://apps.who.int/iris/bitstream/handle/10665/351191/9789240042346-eng.pdf>
6. Alsdurf H, Hill PC, Matteelli A, Getahun H, Menzies D. The cascade of care in diagnosis and treatment of latent tuberculosis infection: a systematic review and meta-analysis. *Lancet Infect Dis.* 2016;16(11):1269–78.



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