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QuantiFERON®-TB Gold Plus ELISA Kit Instructions for Use

Σ 2 x 96 (622120)

Σ 20 x 96 (622822)

Version 1

IVD

For In Vitro Diagnostic Use

For use with QuantiFERON®-TB Gold Plus Blood Collection Tubes

CE 0197

REF 622120, 622822

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Sample to Insight

Contents

Intended Use

The QuantiFERON®-TB Gold Plus (QFT®-Plus) assay is an *in vitro* diagnostic test using a peptide cocktail simulating ESAT-6 and CFP-10 proteins to stimulate cells in heparinized whole blood. Detection of Interferon-γ (IFN-γ) by Enzyme-Linked Immunosorbent Assay (ELISA) is used to identify *in vitro* responses to those peptide antigens that are associated with *Mycobacterium tuberculosis* infection.

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

Intended User

This kit is intended for professional use.

The QuantiFERON-TB Gold Plus (QFT-Plus) assay is to be used by trained personnel in a laboratory environment.

Description and Principle

Pathogen information

Tuberculosis is a communicable disease caused by infection with *M. tuberculosis* complex organisms (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canetti*, and *M. caprae*), which typically spreads to new hosts via airborne droplet nuclei from patients with pulmonary tuberculosis disease. A newly infected individual can become ill from tuberculosis within weeks to months, but most infected individuals remain well. Latent tuberculosis infection (LTBI), a non-communicable asymptomatic condition, persists in some, who might develop tuberculosis disease months or years later. The main purpose of diagnosing LTBI is to consider medical treatment for preventing tuberculosis disease. For more than 100 years, the tuberculin skin test (TST) was the only available method for diagnosing LTBI (4). Cutaneous sensitivity to tuberculin develops from 2 to 10 weeks after infection. However, some infected individuals, including those with a wide range of conditions hindering immune functions, but also others without these conditions, do not respond to tuberculin. Conversely, some individuals who are unlikely to have *M. tuberculosis* infection exhibit sensitivity to tuberculin and have positive TST results after vaccination with Bacille Calmette-Guérin (BCG) or infection with mycobacteria other than *M. tuberculosis* complex, or undetermined other factors.

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

Summary and explanation

QuantiFERON-TB Gold Plus (QFT-Plus) test is the fourth generation in QuantiFERON-TB testing technology assessing cell-mediated response through a quantitative measurement of IFN-γ in a whole blood sample. QFT-Plus is a qualitative test that measures the cell-mediated immune (CMI) responses to peptide antigens that simulate mycobacterial proteins. These proteins, ESAT-6 and

CFP-10, are absent from all BCG strains and from most non-tuberculosis mycobacteria with the exception of *M. kansasii*, *M. szulgai,* and *M. marinum* (1). Individuals infected with *M. tuberculosis* complex organisms usually have lymphocytes in their blood that recognize these and other mycobacterial antigens. This recognition process involves the generation and secretion of the cytokine, IFN-γ. The detection and subsequent quantification of IFN-γ forms the basis of this test.

Tuberculin skin tests and IGRA tests are helpful but insufficient for diagnosing *M. tuberculosis* complex infection in sick patients – a positive result can support the diagnosis of tuberculosis disease; however, infections by other mycobacteria (e.g., *M. kansasii*) could also cause positive results. Other medical and diagnostic evaluations are necessary to confirm or exclude tuberculosis disease.

The antigens used in QFT-Plus are a peptide cocktail simulating the proteins ESAT-6 and CFP-10. Numerous studies have demonstrated that these peptide antigens stimulate IFN-γ responses in T cells from individuals infected with *M. tuberculosis* but generally not from uninfected or BCG-vaccinated persons without disease or risk for LTBI (1,2,6,9). However, medical treatments or conditions that impair immune functionality can potentially reduce IFN-γ responses. Patients with certain other mycobacterial infections might also be responsive to ESAT-6 and CFP-10, as the genes encoding these proteins are present in *M. kansasii*, *M. szulgai*, and *M. marinum* (1, 3,7).

The testing population for QFT-Plus testing are patients with clinically confirmed active tuberculosis and patients with risk for tuberculosis infection or latent tuberculosis infection (LTBI). No age, gender or other limitations apply.

In Mycobacterium tuberculosis (MTB) infection, $CD4^+$ T cells play a critical role in immunological control through their secretion of the cytokine IFN-γ. Evidence now supports a role for $CD8^+$ T cells participating in the host defense to MTB by producing IFN-γ and other soluble factors, which activate macrophages to suppress growth of MTB, kill infected cells, or directly lyse intracellular MTB. IFN-γ producing MTB-specific $CD8^+$ cells have been detected in subjects with LTBI and with active TB. Moreover, ESAT-6 and CFP-10 specific $CD8^+$ T lymphocytes are described as being more frequently detected in subjects with active TB disease versus LTBI and may be associated with a recent MTB exposure (8,10–12). In addition, MTB-specific $CD8^+$ T cells producing IFN-γ have also been detected in active TB subjects with HIV co-infection (13, 14) and in young children with TB disease (15).

QFT-Plus has two distinct TB antigen tubes: TB Antigen Tube 1 (TB1) and TB Antigen Tube 2 (TB2). Both tubes contain peptide antigens from the MTB-complex-associated antigens, ESAT-6 and CFP-10. Both the TB1 and TB2 tubes contain peptides from ESAT-6 and CFP-10 that are designed to elicit CMI responses from $CD4^+$ T-helper lymphocytes; the TB2 tube contains an additional set of peptides targeted to the induction of CMI responses from $CD8^+$ cytotoxic T lymphocytes.

Risk factors for *M. tuberculosis* infection include historical, medical, or epidemiological predictors for tuberculosis disease or exposure to tuberculosis. Refer to the most recent WHO guidance **https://www.who.int/publications/i/item/who-consolidated-guidelines-on-tuberculosis-module-1-prevention-tuberculosis-preventive-treatment** for detailed recommendations about diagnosing *M. tuberculosis* infection (including disease) and selecting persons for testing (16). QFT-Plus has been tested in some patient groups indicated for screening for TB infection according to current WHO guidance (16), including: persons who have tested positive for human immunodeficiency virus (HIV), contacts of recent TB patients and residents in high congregate settings who have been exposed to adults at high- risk of TB (5).

Principles of the assay

QFT-Plus is a qualitative assay that uses specialized blood collection tubes, containing peptide antigens that simulate *M. tuberculosis* proteins, which are used to collect whole blood. Incubation of the blood occurs in the tubes for 16 to 24 hours, after which, plasma is harvested and tested for the presence of IFN-γ produced in response to the peptide antigens.

First, whole blood is collected into each of the QFT-Plus Blood Collection Tubes, which include a Nil tube, TB1 tube, TB2 tube, and a Mitogen tube. Alternatively, blood may be collected in a single blood collection tube that contains lithium- or sodium heparin as the anticoagulant, and then transferred to QFT-Plus Blood Collection Tubes.

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

Heterophile (e.g., human anti-mouse) antibodies in serum or plasma of certain individuals are known to cause interference with immunoassays. The effect of heterophile antibodies in the QFT-Plus ELISA is minimized by the addition of normal mouse serum to the Green Diluent and the use of F(ab')2 monoclonal antibody fragments as the IFN-γ capture antibody coated to the microplate wells.

A QFT-Plus assay is considered positive for an IFN-γ response to either TB antigen tube that 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. This pattern may occur with insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, filling/mixing of the Mitogen tube, or inability of the patient's lymphocytes to generate IFN-γ. Elevated levels of IFN-γ in the Nil sample may occur with the presence of heterophile antibodies, or to intrinsic IFN-γ secretion. The Nil tube adjusts for background (e.g., elevated 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 the Mitogen tube. The measuring range of the QFT-Plus ELISA is up to 10 IU/ml.

Materials Provided

Kit contents

ELISA components	2-plate kit	Reference Lab Pack
Catalog no.	**622120**	**622822**
Microplate strips (12 x 8 wells) coated with murine anti- human IFN-γ monoclonal antibody	2 sets of 12 x 8 Microplate Strips	20 sets of 12 x 8 Microplate Strips
IFN-γ Standard, lyophilized (contains recombinant human IFN-γ, bovine casein, 0.01% w/v Thimerosal)	1 x vial (8 IU/ml when reconstituted)	10 x vials (8 IU/ml when reconstituted)
Green Diluent (contains bovine casein, normal mouse serum, 0.01% w/v Thimerosal)	1 x 30 ml	10 x 30 ml
Conjugate 100x Concentrate, lyophilized (murine anti- human IFN-γ HRP, contains 0.01% Thimerosal)	1 x 0.3 ml (when reconstituted)	10 x 0.3 ml (when reconstituted)
Wash Buffer 20x Concentrate (pH 7.2, contains 0.05% v/v ProClin® 300)	1 x 100 ml	10 x 100 ml
Enzyme Substrate Solution (contains H_2O_2, 3,3′,5,5′ Tetramethylbenzidine)	1 x 30 ml	10 x 30 ml
Enzyme Stopping Solution (contains 0.5 M H_2SO_4)	1 x 15 ml	10 x 15 ml
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Components of the kit

Controls and calibrators

The QFT-Plus ELISA uses a recombinant human IFN-γ standard, which has been assayed against a reference IFN-γ preparation (NIH Ref: Gxg01-902-535).

Platform and software

QFT-Plus Analysis Software is optional for use and can be used to analyze raw data and calculate results. It is available for download at **www.qiagen.com**.

Materials Required but Not Provided

Additional reagents

- QuantiFERON-TB Gold Plus Blood Collection Tubes
- Deionized or distilled water, 2 liters

Consumables

- Plate lid for a 96 well plate
- **Optional:** 1 ml microtubes with caps in 96-well format racks or uncoated microplates with plastic seals for plasma storage (22 patients/rack or plate)
- Reagent reservoirs

Equipment*

- 37°C ± 1°C incubator (with or without CO_2)
- Calibrated variable volume pipets for delivery of 10 µl to 1000 µl with disposable tips
- Calibrated multichannel pipet capable of delivering 50 µl and 100 µl with disposable tips
- Microplate shaker capable of speeds between 500 and 1000 rpm
- Microplate washer (for safety in handling plasma samples, an automated plate washer is recommended)
- Microplate reader fitted with 450 nm filter and 620 nm to 650 nm reference filter
- Variable speed vortex
- Centrifuge capable of centrifuging the blood collection tubes at least to 3000 RCF (g)
- Graduated cylinder, 1 liter or 2 liters

* Prior to use, ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Warnings and Precautions

Please be aware that you may be required to consult your local regulations for reporting serious incidents that have occurred in relation to the device to the manufacturer and/or its authorized representative and the regulatory authority in which the user and/or the patient is established.

For in vitro diagnostic use.

Safety information

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

- Specimens and samples are potentially infectious. Discard sample and assay waste according to your local safety procedures.
- A negative QFT-Plus result does not preclude the possibility of *M. tuberculosis* infection or tuberculosis disease: false-negative results can be due to stage of infection (e.g., specimen obtained prior to the development of cellular immune response), incorrect handling of the blood collection tubes following venipuncture, incorrect performance of the assay, or other individual immunological variables including those related to any comorbidities. Heterophile antibodies or non-specific IFN-γ production from other inflammatory conditions may mask specific responses to ESAT-6 or CFP-10 peptides.
- A positive QFT-Plus result should not be the sole or definitive basis for determining infection with *M. tuberculosis*. Incorrect performance of the assay may cause false-positive QFT-Plus results.

- A positive QFT-Plus result should be followed by further medical evaluation for active tuberculosis disease (e.g., Acid Fast Bacilli smear and culture, chest X-ray).
- While ESAT-6 and CFP-10 are absent from all BCG strains and from most known nontuberculous mycobacteria, it is possible that a positive QFT-Plus result may be due to infection by *M. kansasii*, *M. szulgai*, or *M. marinum*. If such infections are suspected, alternative tests should be performed.
- A false-negative QFT-Plus result can be caused by incorrect blood sample collection or improper handling of the specimen affecting lymphocyte function. Please refer to "Protocol: Performing the ELISA" section, page 20, for correct handling of the blood specimens. Delay in incubation may cause false negative or indeterminate results, and other technical parameters may affect ability to detect a significant IFN-γ response.

Emergency information

CHEMTREC
Outside USA & Canada +1 703-527-3887

Precautions

CAUTION 	Handle human blood as if potentially infectious. Observe relevant blood handling guidelines. Dispose of samples and materials in contact with blood or blood products in accordance with federal, state, and local regulations.

QuantiFERON Enzyme Stopping Solution

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

QuantiFERON Enzyme Substrate Solution

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

QuantiFERON Green Diluent

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

QuantiFERON Wash Buffer 20x Concentrate

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

Further information

Safety Data Sheets: **www.qiagen.com/safety**

- Thimerosal is used as a preservative in some QFT-Plus reagents. It may be toxic upon ingestion, inhalation or skin contact.
- Deviations from the *QuantiFERON-TB Gold Plus (QFT-Plus) Instructions for Use* may yield erroneous results. Please read the instructions carefully before use.
- Do not use kit if any reagent bottle shows signs of damage or leakage prior to use.
- **Important:** Inspect vials prior to use. Do not use Conjugate or IFN-γ Standard vials that show signs of damage or if the rubber seal has been compromised. Do not handle broken vials. Take the appropriate safety precautions to dispose of vials safely. It is recommended to use a vial de-crimper to open the Conjugate or IFN-γ Standard vials to minimize risk of injury from the metal crimp cap.
- Do not mix or use the Microplate strips, IFN-γ Standard, Green Diluent, or Conjugate 100x Concentrate from different QFT-Plus kit batches. Other reagents (Wash Buffer 20x Concentrate, Enzyme Substrate Solution, and Enzyme Stopping Solution) can be interchanged between kits providing the reagents are within their expiration periods and lot details recorded.
- Discard unused reagents and biological samples in accordance with local, state, and federal regulations.
- Do not use the QFT-Plus ELISA kit after the expiration date.
- Correct laboratory procedures should be adhered to at all times.
- Make sure that laboratory equipment such as plate washers and readers have been calibrated/validated for use.

Reagent Storage and Handling

Attention should be paid to expiration dates and storage conditions printed on the box and labels of all components. Do not use expired or incorrectly stored components.

In-use stability

- Store ELISA kit at 2–8°C (36–46°F).
- Always protect Enzyme Substrate Solution from direct sunlight.

Reconstituted and unused reagents

- For instructions on how to reconstitute the reagents, refer to "Protocol: Performing the ELISA", page 20.
- The reconstituted kit standard may be kept for up to 3 months if stored at 2–8°C.

  Note the date the kit standard was reconstituted.
- The reconstituted Conjugate 100x Concentrate must be returned to storage at 2–8°C and must also be used within 3 months.

  Note the date the conjugate was reconstituted.
- Working strength conjugate must be used within 6 hours of preparation.
- Working strength wash buffer may be stored at room temperature for up to 2 weeks.
- Microplate strips are for single-use only. Unused strips can be removed from the plate frame and store for future use.

Specimen Storage and Handling

Refer to the *QuantiFERON-TB Gold Plus (QFT-Plus) Blood Collection Tubes Instructions for Use* (1123668) for details on the blood collection workflow for the QFT-Plus test.

Protocol: Performing the ELISA

Important points before starting

Setting up (Time required for performing the assay)

- In order to obtain valid results from the QFT-Plus assay, the operator needs to perform specific tasks within set times. Prior to use of the assay it is recommended that the operator plan each stage of the assay carefully to allow adequate time to perform each stage. The time required is estimated below; the time of testing multiple samples when batched is also indicated.

    - Approximately 3 hours for one ELISA Plate
    - <1 hour labor
    - Add 10 to 15 minutes for each extra plate

IFN-γ ELISA

- Refer to "Kit contents", page 11 and "Materials Required but Not Provided", page 13 for materials required to perform ELISA.

Procedure

1. All plasma samples and reagents, except for Conjugate 100x Concentrate, must be brought to room temperature (22°C ± 5°C [71.6°F ± 9°F]) before use. Allow at least 60 minutes for equilibration.
2. Remove ELISA plate strips that are not required from the frame, reseal in the foil pouch, and return to the refrigerator for storage until required.

3. Allow at least 1 strip for the QFT-Plus standards and sufficient strips for the number of subjects being tested (refer to Figure 2 for recommended plate format). After use, retain frame and lid for use with remaining strips.

   3a. Reconstitute the IFN-γ Standard with the volume of deionized or distilled water indicated on the label of the vial. Mix gently to minimize frothing and ensure that the entire content of the vial is completely dissolved. Reconstitution of the IFN-γ standard to the correct volume will produce a solution with a concentration of 8.0 IU/ml.

   3b. Using the reconstituted standard, prepare a dilution series of 4 IFN-γ concentrations (refer to Figure 1).

   3c. A standard curve should be generated with the following IFN-γ concentrations:
   - S1 (Standard 1) contains 4.0 IU/ml
   - S2 (Standard 2) contains 1.0 IU/ml
   - S3 (Standard 3) contains 0.25 IU/ml
   - S4 (Standard 4) contains 0 IU/ml (Green Diluent [GD] alone).

   3d. The standards must be assayed at least in duplicate.

   3e. Prepare fresh dilutions of the kit standard for each ELISA session.

	Procedure
A	Label 4 tubes: S1, S2, S3, S4
B	Add 150 µl of GD to S1, S2, S3, S4
C	Add 150 µl of the kit standard to S1 and mix thoroughly
D	Transfer 50 µl from S1 to S2 and mix thoroughly
E	Transfer 50 µl from S2 to S3 and mix thoroughly
F	GD alone serves as the zero standard (S4)

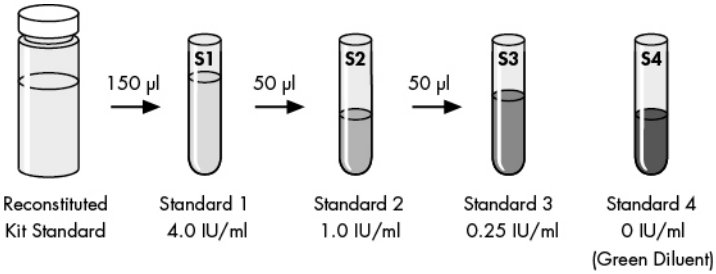

Figure 1. Preparation of standard curve dilution series.

4. Reconstitute lyophilized Conjugate 100x Concentrate with 0.3 ml of deionized or distilled water. Mix gently to minimize frothing and ensure that the entire content of the vial is completely dissolved.

   4a. Working strength conjugate is prepared by diluting the required amount of reconstituted Conjugate 100x Concentrate in Green Diluent (Table 1).

   4b. Working strength conjugate should be used within 6 hours of preparation.

   4c. Return any unused Conjugate 100x Concentrate to 2°C to 8°C immediately after use.

Table 1. Conjugate preparation (working strength)

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

5. For plasma samples harvested from blood collection tubes and subsequently stored (refrigerated or frozen), thoroughly mix the stored sample before addition to the ELISA well. Plasma samples can be stored in centrifuged QFT-Plus Blood Collection Tubes for up to 28 days at 2–8°C. Or harvested plasma samples can be stored for up to 28 days at 2–8°C. Harvested plasma samples can also be stored below –20°C (preferably less than –70°C) for extended periods.

   Plasma samples can be loaded/used directly from centrifuged blood collection tubes for measurement in the QFT-Plus ELISA plate.

   Important: If plasma samples are to be transferred directly from the centrifuged QFT-Plus Blood Collection Tubes, any mixing of the plasma should be avoided. At all times take care not to disturb material on the surface of the gel.

6. Add 50 µl of freshly prepared working strength conjugate to each ELISA plate well.
7. Add 50 µl of test plasma sample to appropriate wells (refer to recommended ELISA plate layout in Figure 2).

8. Finally, add 50 µl each of the Standards 1 to 4 to the appropriate plate wells (refer to recommended ELISA plate layout in Figure 2). The standards should be assayed in at least duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 N	3 N	5 N	7 N	9 N	S1	S1	13 N	15 N	17 N	19 N	21 N
B	1 TB1	3 TB1	5 TB1	7 TB1	9 TB1	S2	S2	13 TB1	15 TB1	17 TB1	19 TB1	21 TB1
C	1 TB2	3 TB2	5 TB2	7 TB2	9 TB2	S3	S3	13 TB2	15 TB2	17 TB2	19 TB2	21 TB2
D	1 M	3 M	5 M	7 M	9 M	S4	S4	13 M	15 M	17 M	19 M	21 M
E	2 N	4 N	6 N	8 N	10 N	11 N	12 N	14 N	16 N	18 N	20 N	22 N
F	2 TB1	4 TB1	6 TB1	8 TB1	10 TB1	11 TB1	12 TB1	14 TB1	16 TB1	18 TB1	20 TB1	22 TB1
G	2 TB2	4 TB2	6 TB2	8 TB2	10 TB2	11 TB2	12 TB2	14 TB2	16 TB2	18 TB2	20 TB2	22 TB2
H	2 M	4 M	6 M	8 M	10 M	11 M	12 M	14 M	16 M	18 M	20 M	22 M

Figure 2. Recommended ELISA plate layout. S1 (Standard 1), S2 (Standard 2), S3 (Standard 3), S4 (Standard 4). 1N (Sample 1. Nil Control plasma), 1 TB1 (Sample 1. TB1 plasma), 1 TB2 (Sample 1. TB2 plasma), 1M (Sample 1. Mitogen plasma).

9. Cover ELISA plate and mix the conjugate and plasma samples/standards thoroughly using a microplate shaker for 1 minute at 500 to 1000 rpm. Avoid splashing.
10. Cover ELISA plate and incubate at room temperature (22°C ± 5°C [71.6°F ± 9°F]) for 120 ± 5 minutes. The ELISA plate should not be exposed to direct sunlight during incubation. Deviation from specified temperature range can lead to erroneous results.
11. During the ELISA plate incubation prepare working strength wash buffer. Dilute one part Wash Buffer 20x Concentrate with 19 parts deionized or distilled water and mix thoroughly. Sufficient Wash Buffer 20x Concentrate has been provided to prepare 2 liters of working strength wash buffer.
12. When ELISA plate incubation is complete, wash ELISA plate wells with 400 µl of working strength wash buffer. Perform wash step at least 6 times. An automated plate washer is recommended for safety reasons when handling plasma samples.

    Thorough washing is very important to the performance of the assay. Make sure each well is completely filled with wash buffer to the top of the well for each wash cycle. A soak period of at least 5 seconds between each cycle is recommended.

Standard laboratory disinfectant should be added to the effluent reservoir, and established procedures followed for the decontamination of potentially infectious material.

13. Tap ELISA plate face down on absorbent (low-lint) towel to remove residual wash buffer. Add 100 µl of Enzyme Substrate Solution to each plate well, cover the plate and mix thoroughly for 1 minute at 500–1000 rpm using a microplate shaker.
14. Cover ELISA plate and incubate at room temperature (22°C ± 5°C [71.6°F ± 9°F]) for 30 minutes. The ELISA plate should not be exposed to direct sunlight during incubation.
15. Following the 30 minute incubation, add 50 µl of Enzyme Stopping Solution to each plate well in the same order as the substrate was added and mix thoroughly at 500 to 1000 rpm using a microplate shaker.
16. Measure the Optical Density (OD) of ELISA plate wells within 5 minutes of stopping the reaction using a microplate reader fitted with a 450 nm filter and with a 620 nm to 650 nm reference filter. OD values are used to calculate results.

Results (Calculations)

QFT-Plus Analysis Software can be used to analyze raw data and calculate results. It is available at **www.qiagen.com**. Please make sure that the latest version of the QFT-Plus Analysis Software is used.

The software performs a Quality Control assessment of the assay, generates a standard curve and provides a test result for each subject, as detailed in "Interpretation of Results ", page 30. The software reports all concentrations greater than 10 IU/ml as ">10" as such values fall beyond the validated linear range of the ELISA.

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

Generation of standard curve and sample values

If QFT-Plus Analysis Software is not used

Determination of the standard curve and determination of sample IU/ml values require a spreadsheet program, such as Microsoft® Excel®, if the QFT-Plus Analysis Software is not used.

Using a spreadsheet program

1. Determine the mean OD values of the kit standard replicates on each plate.
2. Construct a $\log_{(e)}$–$\log_{(e)}$ standard curve by plotting the $\log_{(e)}$ of the mean OD (y axis) against the $\log_{(e)}$ of the IFN-γ concentration of the standards in IU/ml (x axis), omitting the zero standard from these calculations. Calculate the line of best fit for the standard curve by regression analysis.
3. Use the standard curve to determine the IFN-γ concentration (IU/ml) for each of the test plasma samples, using the OD value of each sample.

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

Sample calculation

If the following OD readings were obtained for the standards, the calculations using – log(e) – would follow those in Table 2.

Table 2. Standard curve

Standard	IU/ml	OD values a and b	Mean OD	%CV	$Log_{(e)}$ IU/ml	$Log_{(e)}$ Mean (OD)
Standard 1	4	1.089, 1.136	1.113	3.0	1.386	0.107
Standard 2	1	0.357, 0.395	0.376	7.1	0.000	–0.978
Standard 3	0.25	0.114, 0.136	0.125	NA	–1.386	–2.079
Standard 4	0	0.034, 0.037	0.036	NA	NA	NA

The equation of the curve is y = 0.7885(X) – 0.9837, where "m" = 0.7885 and "c" = –0.9837. These values are used in the equation X = (Y–c)/m to solve for X. Based on the standard curve, the calculated correlation coefficient (*r*) = 1.000. NA: Not applicable.

Using the criteria specified in "Quality control of the test", page 28, the validity of the assay is determined.

The standard curve (Table 2) is used to convert the Antigen OD responses to International Units (IU/ml).

Table 3. Sample calculation

Antigen	OD value	$Log_{(e)}$ OD value	X	e^X (IU/ml)	Antigen –Nil (IU/ml)
Nil	0.037	–3.297	–2.934	0.05	–
TB1	1.161	0.149	1.437	4.21	4.16
TB2	1.356	0.305	1.634	5.12	5.07
Mitogen	1.783	0.578	1.981	7.25	7.20

IFN-γ values (in IU/ml) for the TB1, TB2, and Mitogen are corrected for background by subtracting the IU/ml value obtained for the respective Nil control. These corrected values are used for interpretation of the test results.

Quality control of the test

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

For the ELISA to be valid:

- The mean OD value for Standard 1 must be ≥0.600.
- The %CV for Standard 1 and Standard 2 replicate values must be ≤15%.
- Replicate OD values for Standard 3 and Standard 4 must not vary by more than 0.040 optical density units from their mean.
- The correlation coefficient (*r*) calculated from the mean absorbance values of the standards must be ≥0.98.
- If the above criteria are not met, the run is invalid and must be repeated.
- The mean OD value for the Zero Standard (Green Diluent) should be ≤0.150. If the mean OD value is > 0.150, the plate washing procedure should be investigated.

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

Each laboratory should determine appropriate types of control materials and frequency of testing in accordance with local, state, federal, or other applicable accrediting organizations. External quality assessment and alternative validation procedures should be considered.

Note: Plasmas spiked with recombinant IFN-γ have shown reductions of up to 50% in concentration when stored at either 2–8°C and –20°C. Recombinant IFN-γ is not recommended for establishing control standards.

Interpretation of Results

QFT-Plus results are interpreted using the following criteria (Table 4).

Important: Diagnosing or excluding tuberculosis disease, and assessing the probability of LTBI, requires a combination of epidemiological, historical, medical, and diagnostic findings that should be taken into account when interpreting QFT-Plus results. See general guidance on the diagnosis and treatment of TB disease and LTBI: (**https://www.cdc.gov/tb/publications/guidelines/default.htm**).

Table 4. Interpretation of QFT-Plus Test Results

Nil (IU/ml)	TB1 minus Nil (IU/ml)	TB2 minus Nil (IU/ml)	Mitogen minus Nil (IU/ml)*	QFT-Plus result	Report/Interpretation
≤8.0	≥0.35 and ≥25% of Nil	Any	Any	Positive†	*M. tuberculosis* infection likely
	Any	≥0.35 and ≥25% of Nil			
	<0.35 or ≥0.35 and <25% of Nil	<0.35 or ≥0.35 and <25% of Nil	≥0.50	Negative	*M. tuberculosis* infection NOT likely
	<0.35 or ≥0.35 and <25% of Nil	<0.35 or ≥0.35 and <25% of Nil	<0.50	Indeterminate‡	Likelihood of *M. tuberculosis* infection cannot be determined
>8.0§	Any				

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

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

‡ Refer to "Troubleshooting Guide", page 66 for possible causes.

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

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

response in persons who are negative to Mitogen is rare but has been seen in patients with TB disease. This indicates the IFN-γ response to TB antigens is greater than that to Mitogen, which is possible as the level of Mitogen does not maximally stimulate IFN-γ production by lymphocytes.

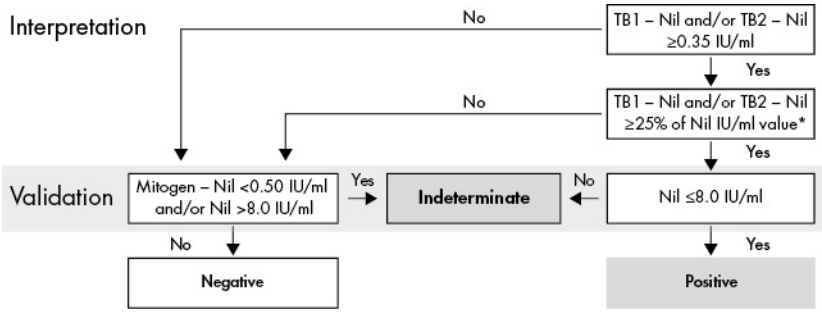

Figure 3. QFT-Plus test interpretation. *For TB1 minus Nil or TB2 minus Nil value to be valid, the ≥25% of Nil IU/ml value must be from the same tube as the original ≥0.35 IU/ml result.

Limitations

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

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

- The predictive value of a positive QFT-Plus result in diagnosing *M. tuberculosis* infection depends on the probability of infection, which is assessed by historical, epidemiological, diagnostic, and other findings.
- A diagnosis of LTBI requires that tuberculosis disease must be excluded by medical evaluation including an assessment of current medical and diagnostic tests for disease as indicated.
- A negative result must be considered with the individual's medical and historical data relevant to probability of *M. tuberculosis* infection and potential risk of progression to tuberculosis disease, particularly for individuals with impaired immune function.

Unreliable or indeterminate results may occur due to:

- Deviations from the procedure described in the Instructions for Use
- Incorrect transport/handling of blood specimen
- Elevated levels of circulating IFN-γ or presence of heterophile antibodies
- Exceeding validated blood times from blood specimen draw to incubation. Refer to the *QFT-Plus Blood Collection Tubes Instructions for Use* (1123668).

Performance Characteristics

Clinical Studies

As there is no definitive standard test for confirming or excluding the diagnosis of LTBI, an estimate of sensitivity and specificity for QFT-Plus cannot be practically evaluated. Specificity of QFT-Plus was approximated by evaluating false-positive rates in persons with low risk (no known risk factors) of tuberculosis infection. Sensitivity was approximated by evaluating groups of study subjects with culture-confirmed active TB disease. In addition, assay performance was evaluated for positive and negative rates in a population of healthy subjects with identified risk factors for tuberculosis infection (a mixed-risk population).

Specificity

A multi-center study evaluating the clinical specificity of QFT-Plus was performed including 733 study subjects who were considered to have either low risk of *M. tuberculosis* infection or no risk factors for exposure to infection or disease. Demographic information and risk factors for TB exposure were determined using a standardized survey at the time of testing. The study was conducted at four independent sites, including one in the United States, two in Japan, and one in Australia. The QFT-Plus test was compared to the QuantiFERON-TB Gold-In-Tube (QFT) test. A summary of the clinical specificity performance data, stratified by study site and region, is provided in Table 5. The performance results are based on the total number of valid tests. There were no indeterminate results.

Table 5. QFT-Plus specificity in a low-risk population

		Positive		Negative		Indeterminate		Specificity (95% CI)	
Site	**N**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**
United States									
(#1) USA-4	212	2	4	210	208	0	0	99.06% (210/212) (96.63–99.74)	98.11% (208/212) (95.25–99.26)
Japan									
(#2) JPN-3	106	1	2	105	104	0	0	99.06% (105/106) (94.85–99.83)	98.11% (104/106) (93.38–99.48)
(#3) JPN-1	216	3	5	213	211	0	0	98.61% (213/216) (96.00–99.53)	97.69% (211/216) (94.70–99.01)
Total Japan	322	4	7	318	315	0	0	98.76% (318/322) (96.85–99.52)	97.83% (315/322) (95.6–98.9)
Australia									
(#4) AU-3	199	8	9	191	190	0	0	95.98% (191/199) (92.27–97.95)	95.48% (190/199) (91.63–97.60)

The specificity of QFT-Plus was 98.11% in the US, 97.83% in Japan, and 95.48% in Australia. The overall specificity of the QFT-Plus was 97.27% (713/733). The specificity of QFT was 99.06% in the US, 98.76% in Japan, and 95.98% in Australia. The overall specificity of QFT was 98.09% (719/733).

A breakdown of the results by TB antigen tube type and combinations thereof is shown to provide an example of expected results in a low-risk population (Table 6).

Table 6. QFT-Plus Specificity Study Results by TB Antigen Tube

Interpretation based on TB Antigen-Nil IU/ml in	TB1	TB2	QFT-Plus (positive by TB1 and/or TB2)*	Concordant positive TB1 and TB2 (alternate analysis)†
Positive	10	18	20	8
Negative	723	715	713	725
Indeterminate	0	0	0	0
Specificity (95% CI)	–	–	97.3% (713/733) (95.8–98.2)	–
Negativity rate (95% CI)	98.6% (723/733) (97.5–99.3)	97.5% (715/733) (96.2–98.4)	–	98.9% (725/733) (97.9–99.5)

* Interpretation based on a TB antigen – Nil value ≥0.35 IU/ml in both (TB1 and TB2) or either TB tube to fit the interpretation criteria for the QFT-Plus (TB1 or TB2) to be determined positive.

† Alternate analysis provided for information only.

In the subjects with low risk for TB infection, a total of 20/733 subjects returned a positive result. Of these, only 8 subjects returned a value of >0.35 IU/ml in both TB1 and TB2 tubes. A comparison of the QFT versus QFT-Plus assays was performed in the low-risk study cohort, and showed an overall concordance of 97.5% (715/733), and a negative percent agreement of 98.3% (707/719).

Sensitivity

While there is no definitive standard test for LTBI, a suitable surrogate is microbiological culture of *M. tuberculosis* because infection with TB is a necessary precursor to disease.

A multi-center study evaluating the clinical sensitivity of QFT-Plus was performed including 434 study subjects who presented with signs and symptoms of active *M. tuberculosis* disease

confirmed by culture and/or PCR, and were not on TB treatment or with ≤14 days of treatment prior to blood collection. The study was performed at 7 independent sites including three sites in the United States, three sites in Japan, and one site in Australia. The QFT-Plus test was compared to the QuantiFERON-TB Gold in Tube (QFT) test. A summary of the clinical sensitivity performance data, stratified by study site and country is provided in Table 7. The performance results are based on the total number of valid tests. The frequency of indeterminate results for the QFT and QFT-Plus was 2.3% (10/434) and 2.5% (11/434), respectively.

Table 7. Clinical sensitivity study performance summary stratified by site, country, and overall

		Positive		Negative		Indeterminate		Sensitvity (95% CI)	
Site	**N**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**
United States									
(#1) USA-5	15	13	13	2	2	0	0	86.67% (13/15) (62.12–96.26)	86.67% (13/15) (62.12–96.26)
(#2) USA-1	33	29	29	4	4	0	0	87.88% (29/33) (72.67–95.18)	87.88% (29/33) (72.67–95.18)
(#3) USA-4	5	5	5	0	0	0	0	100.0% (5/5) (56.55–100.0)	100.0% (5/5) (56.55–100.0)
Total United States	53	47	47	6	6	0	0	88.7% (47/53) (77.4–94.7)	88.7% (47/53) (77.4–94.7)
Japan									
(#4) JPN-2	76	72	67	1	3	3	6	98.63% (72/73) (92.64–99.76)	95.71% (67/70) (88.14–98.53)
(#5) JPN-3	99	97	98	2	1	0	0	97.98% (97/99) (92.93–99.44)	98.99% (98/99) (94.50–99.82)

Table continued on next page

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Table 7. Clinical sensitivity study performance summary stratified by site, country, and overall (cont'd)

		Positive		Negative		Indeterminate		Sensitivity (95% CI)	
Site	**N**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**	**QFT**	**QFT-Plus**
(#6) JPN-1	177	159	157	12	15	6	5	92.98% (159/171) (88.14–95.94)	91.28% (157/172) (86.11–94.64)
Total Japan	352	328	322	15	19	9	11	95.63% (328/343) (92.91–97.33)	94.43% (322/341) (91.5–96.4)
Australia									
(#7) AU-2	29	27	29	1	0	1	0	96.43% (27/28) (82.29–99.37)	100.0% (29/29) (88.30–100.0)

The analysis in the table above does not include indeterminate results.

The sensitivity of QFT-Plus was 88.7% in the US, 94.43% in Japan, and 100.0% in Australia. The overall sensitivity of QFT-Plus was 94.09% (398/423). The sensitivity of QFT was 88.7% in the US, 95.63% in Japan, and 96.43% in Australia. The overall sensitivity of QFT was 94.81% (402/424).

A breakdown of the results by TB antigen tube type and combinations of tubes is shown to provide an example of expected results in a confirmed TB infected population (Table 8).

Table 8. QFT-Plus sensitivity study results by TB antigen tube

Interpretation based on TB Antigen-Nil in IU/ml	TB1	TB2	QFT-Plus (positive by TB1 and/or TB2)
Positive	388	397	398
Negative	32	26	25
Indeterminate	14	11	11
Sensitivity* (95% CI)	–	–	94% (398/423) (91.4–96.0)
Positivity rate* (95% CI)	92.4% (388/420) (89.4–94.6)	93.9% (397/423) (91.1–95.8)	–

* Excluding indeterminate values.

A comparison of the QFT and QFT-Plus assays was assessed in the culture confirmed active TB cohort (sensitivity study cohorts) and showed an overall concordance of 95.9% and a positive percent agreement of 97.3% (391/402).

Table 9. QFT-Plus likelihood ratios

Site*	Sensitivity	Specificity	LR+	LR-
Australia	100.00%	95.48%	22.11	0.00
Japan	94.43%	97.83%	43.44	0.06
United States	88.68%	98.11%	47.00	0.12

* Total

Performance in subjects with identified risk factors for a MTB infection (mixed-risk individuals)

A cohort of 601 individuals with mixed risk factors for TB infection (e.g., HIV positivity, history of treatment for active or latent TB, exposure to active TB case, HCW status, etc.) was assessed with both the QFT and QFT-Plus tests. Risk factors were identified using a standardized survey and individuals displayed no symptoms associated with active TB at the time of recruitment. Demographics and risk factors are reported in Table 10. .. In this population, 68/601 (11.3%) subjects returned a positive QFT-Plus result, with a positive percent agreement (PPA) and negative percent agreement (NPA) of 98.44% and 99.07%, respectively (Table 11). In this cohort of 68 QFT-Plus positive subjects, a total of 62 subjects were positive by both TB1 and TB2 tubes, 2 subjects were positive by TB1 only, and 4 subjects were positive by TB2 only. No indeterminate results (0/601) were observed.

Table 10. Demographics and factors associated with risk of TB infection in a mixed cohort

Total subjects (601)		Number	Percentage
Gender	Male	539	89.7%
	Female	62	10.3%
Age (years)	Range	18–70	–
	Mean	46.7	
BCG vaccinated	Yes	15	2.5%
	No	586	97.5%
HIV positive or tested positive for HTLV viruses	Yes	12	2.0%
	No	589	98%
Previously diagnosed with active TB	Yes	11	1.8%
	No	590	98.2%
Had a positive Tuberculin Skin Test (TST)/Mantoux test for TB	Yes	47	7.8%
	No	554	92.2%
Ever been treated for active or latent TB	Yes	35	5.8%
	No	566	94.2%
Lived, worked or volunteered (>1 month) in a jail or prison	Yes	373	62.1%
	No	228	37.9%
Lived, worked or volunteered (>1 month) in a homeless shelter	Yes	525	87.4%
	No	76	12.6%
Healthcare worker	Yes	8	1.3%
	No	593	98.7%
Close contact of someone with or suspected of having active TB disease	Yes	9	1.5%
	No	592	98.5%

Table 11. Summary performance of QFT-Plus versus QFT in subjects with known risk factors for latent TB infection

		QFT		
		Positive (+)	Negative (–)	Total
QFT-Plus	Positive (+)	63	5*	68
	Negative (–)	1*	532	533
	Total	64	537	601

*All 6 discordant samples had IFN-γ levels of the TB Antigen tubes that were close to the assay cut-off.

The positive percent agreement (PPA) and negative percent agreement (NPA) between the results of QFT and QFT-Plus were as follows:

- PPA: 98.44% (63/64), 95%CI (91.67, 99.72)
- NPA: 99.07% (532/537), 95% CI (97.84, 99.60)

Table 12 below illustrates the performance of QFT-Plus as compared to the QFT test in BCG vaccinated study subjects.

Table 12. Performance of QFT-Plus as compared to the QFT Test in BCG-vaccinated study subjects (combined data from sensitivity, specificity, and LTBI study subjects)

		QFT		
		Positive (+)	Negative (–)	Total
QFT-Plus	Positive (+)	66	5	71
	Negative (–)	3	268	271
	Total	69	273	342*

* Two sensitivity study subjects were excluded from the analysis due to indeterminate results

- PPA = 95.6% (66/69), 95%CI (87.98, 98.51)

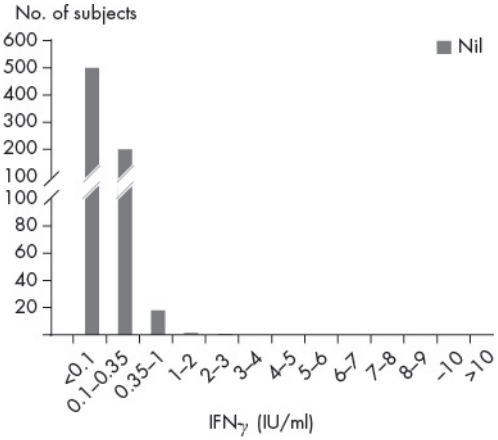
NPA = 98.2% (268/273), 95%CI (95.79, 99.22)

Expected values

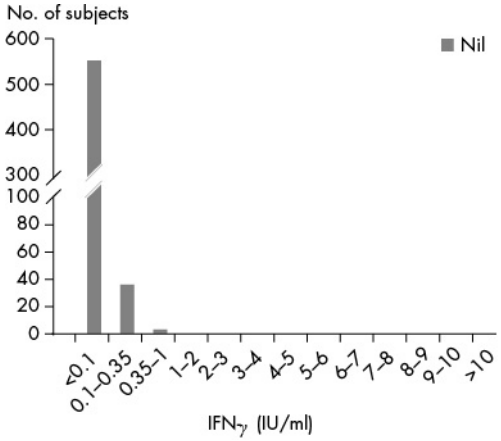
Observed response distributions — risk stratified

A range of IFN-γ responses to TB1, TB2, and control tubes were observed in clinical trials and stratified by risk of *M. tuberculosis* infection (Figure 4 through Figure 7). The mixed risk group consists of subjects representative of a general testing population, including subjects with and without risk factors for TB exposure, and where active TB is unlikely (i.e., LTBI).

A



B



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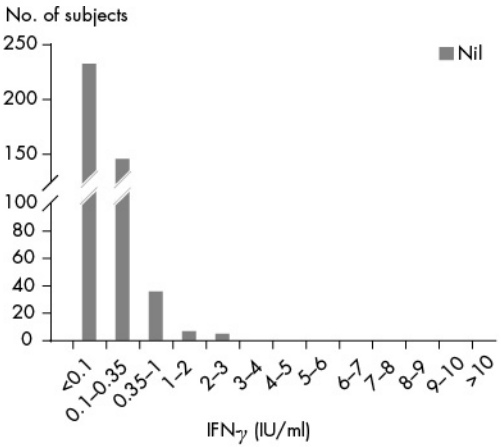
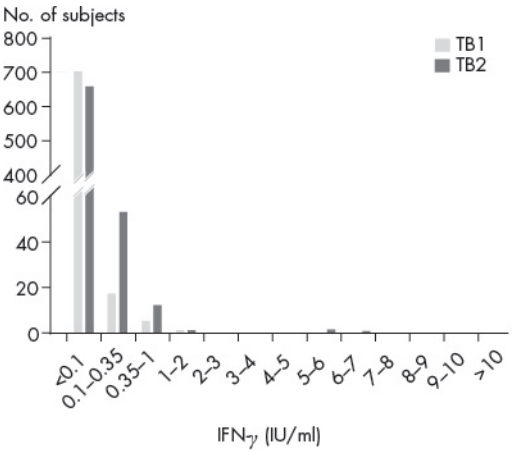
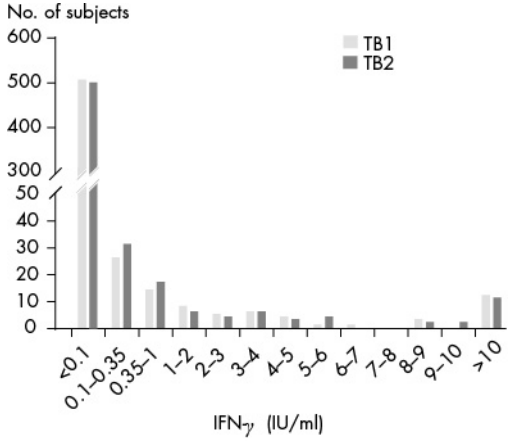


Figure 4. Distribution of Nil. **A** Distribution of Nil values in a low-risk population (n=744). **B** Distribution of Nil values in a mixed-risk population (n=601). **C** Distribution of Nil values in a population with culture-confirmed *M. tuberculosis* infection (n=416).

A


No. of subjects
800
700
600
500
400
60
40
20
0
TB1
TB2
<0.1
0.1–0.35
0.35–1
1–2
2–3
3–4
4–5
5–6
6–7
7–8
8–9
9–10
>10
IFN-γ (IU/ml)

B


No. of subjects
600
500
400
300
50
40
30
20
10
0
TB1
TB2
<0.1
0.1–0.35
0.35–1
1–2
2–3
3–4
4–5
5–6
6–7
7–8
8–9
9–10
>10
IFN-γ (IU/ml)

C

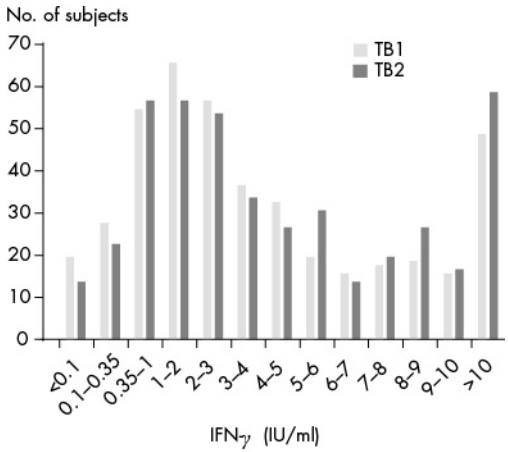
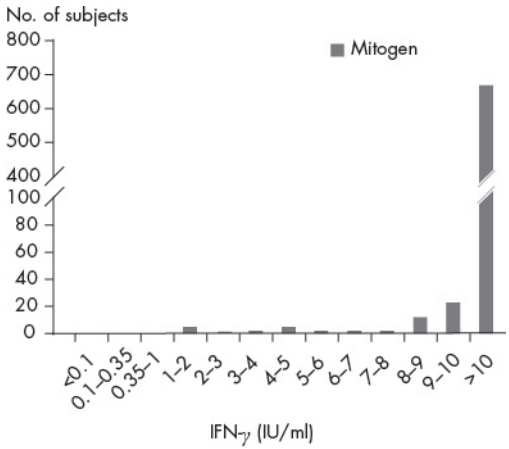
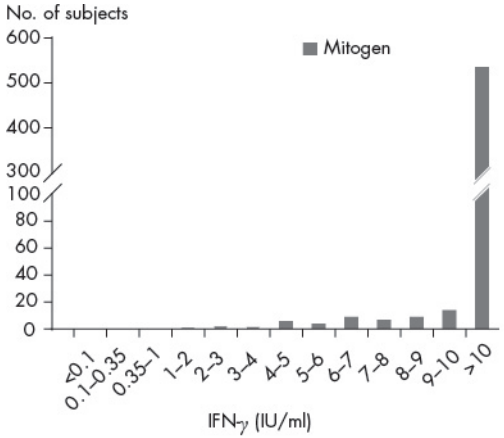


Figure 5. Distribution of TB1 and TB2 (Nil Subtracted). **A** Distribution of TB1 and TB2 (Nil subtracted) values in a low-risk population (n=744). **B** Distribution of TB1 and TB2 (Nil subtracted) values in a mixed-risk population (n=601). **C** Distribution of TB1 and TB2 (Nil subtracted) values in a population with culture-confirmed *M. tuberculosis* infection (n=416).

A


No. of subjects
800
700
600
500
400
100
80
60
40
20
0
Mitogen
<0.1
0.1–0.35
0.35–1
1–2
2–3
3–4
4–5
5–6
6–7
7–8
8–9
9–10
>10
IFN-γ (IU/ml)

B


No. of subjects
600
500
400
300
100
80
60
40
20
0
Mitogen
<0.1
0.1–0.35
0.35–1
1–2
2–3
3–4
4–5
5–6
6–7
7–8
8–9
9–10
>10
IFN-γ (IU/ml)

C

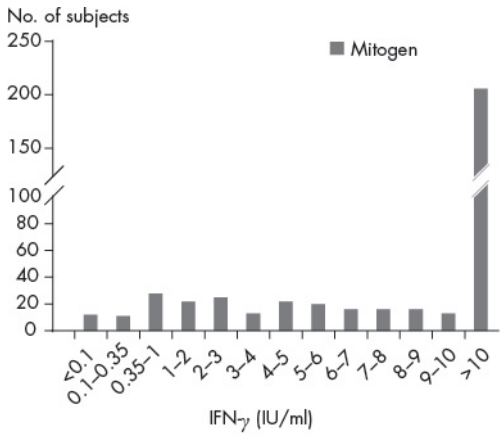


Figure 6. Distribution of Mitogen (Nil Subtracted). **A** Distribution of Mitogen (Nil subtracted) values in a low-risk population (n=744). **B** Distribution of Mitogen (Nil subtracted) values in a mixed-risk population (n=601). **C** Distribution of Mitogen (Nil subtracted) values in a population with culture-confirmed *M. tuberculosis* infection (n=415).

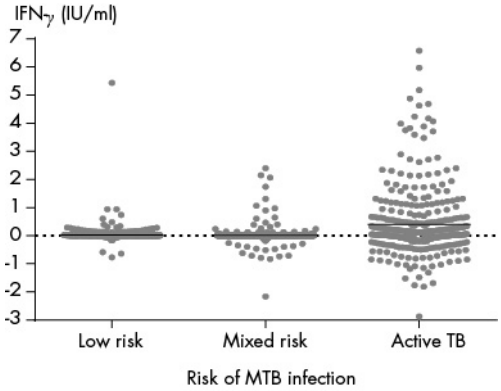


Figure 7. Observed difference between TB1 and TB2 values (Nil Subtracted), stratified by risk. Includes data from the mixed-risk cohort study to show differences between low risk, active risk and mixed-risk cohorts. This data analysis included a mixed-risk cohort with known risk factors. Therefore, from the low risk cohort n=733, from the mixed-risk cohort n=588 and from the active TB cohort n=357. The quantitative difference in IU/ml for each subject was obtained by subtracting the TB1 value from the TB2 value.

Summary of Safety and Performance

The summary of safety and performance can be found on the EUDAMED website.

Assay Performance Characteristics

Analytical performance

Assay cut-off

The QFT-Plus assay cut-off was determined using data from 216 subjects with no identified risk factors for TB exposure, who had been BCG vaccinated and assumed to be free of infection and 118 subjects with culture confirmed *M. tuberculosis* infection. The sensitivity and specificity data was combined and analyzed by Receiver Operator Characteristic (ROC) curve analysis. The sensitivity and specificity data analyzed using the ROC analysis demonstrated that the optimal ELISA cut-off was 0.35 IU/mL (see Figure 8).

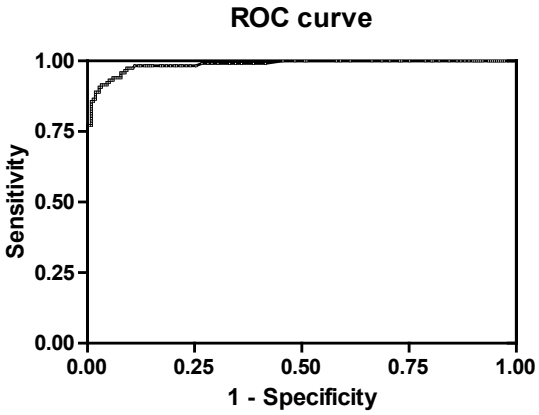


Figure 8. ROC Curve for the ESAT-6 and CFP-10 Responses.

Table 13. Sensitivity and Specificity Values for the ELISA at Various Cut-Offs

Cutoff IU/ml IFN-γ	Sensitivity %	95% CI	Specificity %	95% CI	Sensitivity + Specificity
0.20	91.53	84.97% to 95.86%	96.31	92.87% to 98.40%	187.84
0.23	91.53	84.97% to 95.86%	96.77	93.47% to 98.69%	188.30
0.26	90.68	83.93% to 95.25%	96.77	93.47% to 98.69%	187.45
0.28	90.68	83.93% to 95.25%	97.24	94.08% to 98.98%	187.92
0.30	89.83	82.91% to 94.63%	97.24	94.08% to 98.98%	187.07
0.31	88.98	81.90% to 94.00%	97.24	94.08% to 98.98%	186.22
0.33	88.98	81.90% to 94.00%	97.70	94.71% to 99.25%	186.68
0.35	**88.98**	**81.90% to 94.00%**	**98.16**	**95.35% to 99.50%**	**187.14**
0.39	88.14	80.90% to 93.36%	98.16	95.35% to 99.50%	186.3
0.42	87.29	79.90% to 92.71%	98.16	95.35% to 99.50%	185.45
0.43	86.44	78.92% to 92.05%	98.16	95.35% to 99.50%	184.6
0.45	86.44	78.92% to 92.05%	98.62	96.01% to 99.71%	185.06

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Table 13. Sensitivity and Specificity Values for the ELISA at Various Cut-Offs

Cutoff IU/ml IFN-γ	Sensitivity %	95% CI	Specificity %	95%CI	Sensitivity + Specificity
0.47	85.59	77.94% to 91.38%	99.08	96.71% to 99.89%	184.67
0.48	84.75	76.97% to 90.70%	99.08	96.71% to 99.89%	183.83
0.50	83.90	76.00% to 90.02%	99.08	96.71% to 99.89%	182.98

Linearity

The QFT-Plus ELISA has been demonstrated to be linear by placing 5 replicates of 11 plasma pools of known IFN-γ concentrations randomly on the ELISA plate. The linear regression line has a slope of 1.002 ± 0.011 and a correlation coefficient of 0.99 (Figure 9).

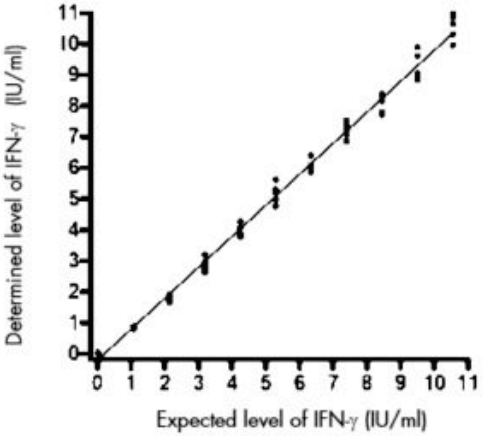

Figure 9. Illustration of Linearity Study Regression Analysis – High Pool Mean = –0.24 +0.9964•Expected.

Reproducibility

A multi-center study reproducibility study was conducted to evaluate performance of QFT-Plus across study sites with multiple operators. This was a prospective study conducted at three external testing sites and one collection site. A total of 32 positive and 34 negative (determined by the QFT test) study subjects were enrolled. The study subjects were comprised of healthcare workers in the United States. The study subjects represented groups with mixed risk for TB exposure due to their occupation or as foreign born healthcare workers originating from a location with a TB rate exceeding 50/100,000.

Three lithium-heparin blood collection tubes were obtained from each study subject at the collection site. The lithium-heparin blood collection tubes were then transferred to each of three testing sites where they were aliquoted into two sets of QFT-Plus Blood Collection Tubes (QFT-Plus TB1, TB2, Mitogen, and Nil) then tested in accordance with the QFT-Plus assay procedure. At each site at least two operators ran the two tests per study subject independently. Each operator was blinded to the results obtained by the other operator and blinded to the QFT test result of the study subject.

There were six results generated across all three testing sites per each of the 66 study subjects, resulting in a total of 396 data points. A summary of the reproducibility summary results are provided in Table 14.

Table 14. Reproducibility study results summary – within site % agreement of qualitative results between operators; N = 66 patient samples

Site 1 – 2 Operators	Site 2 – 2 Operators	Site 3 – 3 Operators
64/66 = 96.97%	64/66 = 96.97%	59/66 = 89.39%
Agreement of Qualitative Results of Tube Set 1 and Tube Set 2	Agreement of Qualitative Results of Tube Set 1 and Tube Set 2	Agreement of Qualitative Results of Tube Set 1 and Tube Set 2

The qualitative percent agreement across all study sites is 94.7% (375/396). In this calculation, the total number of test results in agreement (375) includes those instances where

there is agreement of all 6 results, agreement of 5 out of 6 results, agreement of 4 out of 6 results, and agreement of 3 out 6 results, combined.

Inter-lot repeatability

A study was conducted to determine the inter-lot variability of QFT-Plus Blood Collection Tubes when compared to the QFT tubes. A total of 30 subjects (15 confirmed TB positive and 15 confirmed TB negative determined by the QFT test) were tested. Three separate lots each of the QFT-Plus TB1, TB2, and QFT TB Blood Collection Tubes were included in this study. Three replicates per donor per blood collection tube lot were tested. Nil and Mitogen tubes were tested with one replicate each.

Blood from each subject was collected into lithium-heparin blood collection tubes and then 1 ml of blood was transferred to each of the QFT-Plus and QFT Blood Collection Tubes and tested in accordance with the assay procedure. For each positive and negative sample group, the total variance of the QFT-Plus tube results must not have been significantly greater than the total variance of the QFT tube results. This was determined from the p-value given by the Levene's Homogeneity of Variance (HOV) test. If the p-value was not significant ($p>0.05$) and/or the variation of the QFT-Plus TB tubes was lower than that of the QFT TB tube, then there was variance between the QFT-Plus and QFT TB tubes.

Table 15. Comparison of Variances between QFT-Plus and QFT TB Blood Collection Tubes using Levene's HOV Test

Sample type	Difference	Effect	Dependent	P-value	Significant
Positive	TB2 vs QFT	Sub_Type	Residual	0.0378	Yes
Positive	TB2 vs QFT	Sub_Type	Residual	0.0540	No
Negative	TB2 vs QFT	Sub_Type	Residual	0.1025	No
Negative	TB2 vs QFT	Sub_Type	Residual	0.6344	No

The variation between the QFT-Plus and QFT TB Blood Collection Tubes was not significant with the exception of the QFT-Plus TB2 tube when tested with positive subjects. When the

estimate of standard deviation was analyzed, the variation seen in the QFT-Plus TB2 tube was smaller (0.06089) than the QFT TB tube (0.07641) as shown in Table 16. Therefore, the variance of the QFT-Plus TB1 and TB2 Blood Collection Tubes was no greater than the QFT TB Blood Collection Tube.

Table 16. Standard Deviation for Residual and 95% Confidence Interval for Positive Subjects

Sample type	Sub type	Standard Deviation Estimate	95% LCL	95% UCL
Positive	QFT	0.07641	0.06826	0.08680
Positive	TB1	0.06275	0.05605	0.07127
Positive	TB2	0.06089	0.05439	0.06917

Intra-lot repeatability

A study was conducted to assess the intra-lot reproducibility of the QFT-Plus Blood Collection Tubes by comparing the IFN-γ concentration from replicates of QFT-Plus TB Blood Collection Tubes of blood.

Six aliquots of one blood sample from the same subjects with a confirmed TB infection were run in 6 repeat blood collection tubes from one lot each of both QFT-Plus tubes (TB1 and TB2). The testing was performed in 13 subjects. The %CV was calculated for each donor and across all donors to generate a mean %CV as shown in Table 17.

Table 17. The %CV for Mean, Standard Deviation, Minimum, Median and Maximum in each QFT-Plus TB Blood Collection Tube in TB Positive Subjects

QFT-Plus Tube	Sample size	Mean(%CV)	Standard Deviation	Minimum	Median	Maximum
TB1	13	13.31	6.88	4.17	12.87	29.56
TB2	13	13.04	7.48	4.86	10.75	29.44

The results demonstrated that the mean %CV for TB1 and TB2 was ~13%, meeting the ≤ 30% acceptance criteria and demonstrating intra-lot repeatability.

Limit of Blank (LoB)

The Limit of Blank (LoB) was evaluated for the QFT-Plus assay. Two replicates each of 14 individual normal human plasma samples (as the blanks) were tested with 2 lots of the QFT-Plus ELISA by 3 operators on 3 testing days, one operator per testing day for a total of 84 replicates from each ELISA kit lot.

The LoB values (IU/mL) for the 2 ELISA kit lots were calculated separately as shown in Table 18.

Table 18. LoB Values (IU/mL) for the 2 QFT-Plus ELISA Kit Lots

QFT-Plus ELISA Kit	LoB estimated (IU/ml)
Kit 1	0.030
Kit 2	0.040

The larger LoB value, 0.040 IU/mL, across both QFT-Plus ELISA kit lots, was reported as the final LoB value.

Limit of Detection (LoD)

The Limit of Detection (LoD) was evaluated for the QFT-Plus assay. A TB negative human plasma pool was generated by combining 14 individual plasma samples. Each of the 3 operators prepared an IFN-γ reference standard stock at 1.0 IU/mL diluted in buffer. A dilution series of 8 concentrations were made. The study was conducted over 3 days, by 3 alternating operators using 2 QFT-Plus ELISA kit lots. For each testing day, 5 replicates of each concentration within each set of the serial dilution series were tested for a total of 45 replicates for each dilution of IFN-γ concentration for each QFT-Plus ELISA kit lot.

The LoD value for each of the QFT-Plus ELISA kit lots tested was calculated separately as shown in Table 19.

Table 19. Estimated LoD Values (IU/mL) for the 2 QFT-Plus ELISA Kit Lots

QFT-Plus ELISA Kit	Probability	Concentration estimate (IU/ml)	Lower 95% Confidence Limit for Estimate	Upper 95% Confidence Limit for Estimate
Kit 1	0.95	0.063	0.060	0.067
Kit2	0.95	0.065	0.060	0.073

The larger LoD value calculated across both QFT-Plus ELISA kit lots, 0.065 IU/mL, was reported as the final LoD value.

Interfering substances

A study was conducted to determine the effects of potential interfering substances on the performance of the QFT-Plus ELISA detection of IFN-γ. The interferents included in this testing were: triglycerides (Total), hemoglobin, protein (total serum), bilirubin (conjugated), bilirubin (unconjugated), Abacavir sulfate, Cyclosporine and Prednisolone. Five plasma pools with known concentrations of IFN-γ were prepared using different interferent concentrations. The base pool IFN-γ level was previously prepared with a pre-determined amount of IFN-γ present (approximately 0.21, 0.45 and 1.4 IU/mL). This pool was then used to prepare the interferent pools. The interferent concentrations tested were 0 mg/dL, 5 mg/dL, 10 mg/dL, 15 mg/dL and 20 mg/dL. The target interferent concentrations were based on reference intervals, pathological values, therapeutic ranges, and toxic ranges or as recommended by vendor or general clinical levels. Six replicates were tested for each interferent sample concentration level.

For each sample concentration, a two-sample t-test was performed, comparing the difference in mean log10 (IU/mL) of the primary interferent level compared to the control (i.e. interferent-free level) as shown in Table 20 and 21. The estimated difference in mean response, along with the corresponding two-sided 95% confidence limits and p-value were also reported.

Table 20. Log10 IU/mL: T-Test Summary Table for Differences in Means between Control and Primary Interferent Level for each Interferent and IFN-γ Concentration Level

Interferent	Interferent level	Sample concentration (IU/ml)	Variances	Mean Difference	Lower 95% CI	Upper 95% CI	P-value	Pass
Triglycerides	High	1.4	Equal	0.019	–0.040	0.077	0.491	Yes
		0.45	Equal	0.004	–0.022	0.030	0.732	Yes
		0.21	Equal	0.006	–0.035	0.047	0.759	Yes
Hemoglobin	High	1.4	Equal	–0.005	–0.42	0.032	0.784	Yes
		0.45	Equal	–0.000	–0.023	0.023	0.981	Yes
		0.21	Equal	0.000	–0.034	0.035	0.980	Yes
Protein	High	1.4	Equal	0.004	–0.034	0.042	0.836	Yes
		0.45	Equal	0.001	–0.38	0.040	0.962	Yes
		0.21	Equal	–0.008	–0.076	0.060	0.809	Yes
Bilirubin Conjugated	High	1.4	Equal	–0.011	–0.057	0.034	0.589	Yes
		0.45	Equal	–0.002	–0.058	0.053	0.923	Yes
		0.21	Equal	–0.014	0.074	0.046	0.625	Yes
Bilirubin Unconjugated	High	1.4	Equal	–0.008	–0.041	0.026	0.614	Yes
		0.45	Equal	–0.000	–0.042	0.041	0.982	Yes
		0.21	Equal	–0.000	–0.048	0.048	0.989	Yes
Abacavir	High	1.4	Equal	0.008	–0.025	0.041	0.601	Yes
		0.45	Equal	0.012	–0.019	0.044	0.412	Yes
		0.21	Equal	–0.006	–0.052	0.040	0.770	Yes

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Table 20. Log10 IU/mL: T-Test Summary Table for Differences in Means between Control and Primary Interferent Level for each Interferent and IFN-γ Concentration Level

Interferent	Interferent level	Sample concentration (IU/ml)	Variances	Mean difference	Lower 95% CI	Upper 95% CI	P-value	Pass
Cyclosporine	High	1.4	Equal	0.014	–0.020	0.047	0.383	Yes
		0.45	Equal	0.005	–0.035	0.045	0.773	Yes
		0.21	Equal	0.024	–0.008	0.056	0.131	Yes
Prednisolone	High	1.4	Equal	0.017	–0.017	0.050	0.293	Yes
		0.45	Equal	0.000	–0.036	0.036	0.979	Yes
		0.21	Equal	0.015	–0.035	0.065	0.524	Yes

Table 21. Log10 IU/mL: T-Test Summary Table for Differences in Means between Control and High Interferent Level for each Interferent and IFN-γ Concentration Level

Interferent	Interferent level	Sample concentration (IU/ml)	Variances	Mean Difference	Lower 95% CI	Upper 95% CI	P-value	Pass
Triglycerides	High	1.4	Equal	0.053	–0.004	0.110	0.063	Yes
		0.45	Equal	0.039	–0.021	0.058	<.001	Yes
		0.21	Equal	0.034	–0.002	0.071	0.061	Yes
Hemoglobin	High	1.4	Equal	–0.001	–0.042	0.040	0.967	Yes
		0.45	Equal	0.016	–0.007	0.040	0.152	Yes
		0.21	Equal	0.014	–0.030	0.059	0.489	Yes
Protein	High	1.4	Equal	–0.030	–0.071	0.011	0.136	Yes
		0.45	Equal	0.000	–0.046	0.046	0.992	Yes
		0.21	Equal	–0.045	–0.103	0.012	0.109	Yes
Bilirubin Conjugated	High	1.4	Equal	0.001	–0.046	0.048	0.961	Yes
		0.45	Equal	0.012	–0.043	0.067	0.639	Yes
		0.21	Equal	0.015	–0.044	0.074	0.586	Yes
Bilirubin Unconjugated	High	1.4	Equal	0.015	–0.011	0.042	0.231	Yes
		0.45	Equal	0.015	–0.023	0.052	0.411	Yes
		0.21	Equal	0.012	–0.033	0.057	0.566	Yes
Abacavir	High	1.4	Equal	0.013	–0.015	0.040	0.322	Yes
		0.45	Equal	0.015	–0.014	0.044	0.283	Yes
		0.21	Equal	0.008	–0.034	0.050	0.677	Yes

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Table continued from previous page

Table 21. Log10 IU/mL: T-Test Summary Table for Differences in Means between Control and High Interferent Level for each Interferent and IFN-γ Concentration Level

Interferent	Interferent level	Sample concentration (IU/ml)	Variances	Mean difference	Lower 95% CI	Upper 95% CI	P-value	Pass
Cyclosporine	High	1.4	Equal	0.002	–0.019	0.024	0.816	Yes
		0.45	Equal	0.007	–0.030	0.043	0.682	Yes
		0.21	Equal	0.015	–0.007	0.038	0.155	Yes
Prednisolone	High	1.4	Equal	0.007	–0.016	0.030	0.518	Yes
		0.45	Equal	–0.001	–0.034	0.033	0.964	Yes
		0.21	Equal	0.021	–0.025	0.068	0.334	Yes

The results showed no significant differences between the primary interference level and control (interferent-free level) and for the high interferent level except for the Triglyceride 0.45 IU/mL concentration level. The mean difference was determined to be within the +/- 2 standard deviation range. This demonstrates that the difference is within the expected variability of the assay and that Triglyceride did not have an interfering effect on the QFT-Plus ELISA.

Disposal

Observe relevant blood handling guidelines. Dispose of samples and materials in contact with blood or blood products in accordance with federal, state, and local regulations.

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Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support** (for contact information, visit **www.qiagen.com**).

Comments and suggestions

ELISA troubleshooting

Nonspecific color development

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

Low optical density readings for standards

	Comments and suggestions
a) Standard dilution error	Ensure dilutions of the Kit Standard are prepared correctly as per this Instructions for Use.
b) Pipetting error	Ensure pipets are calibrated and used according to manufacturer's instructions.
c) Incubation temperature too low	Incubation of the ELISA should be performed at room temperature (22°C ± 5°C).
d) Incubation time too short	Incubation of the plate with the conjugate, standards, and samples should be for 120 ± 5 minutes. The Enzyme Substrate Solution should be incubated on the plate for 30 minutes.
e) Incorrect plate reader filter used	Plate should be read at 450 nm with a reference filter of between 620 and 650 nm.
f) Reagents are too cold	All reagents, with the exception of the Conjugate 100x Concentrate, must be brought to room temperature prior to commencing the assay. This takes approximately 1 hour.
g) Kit/components have expired	Ensure that the kit is used before the expiry date. Ensure that the reconstituted Standard and Conjugate 100x Concentrate are used within 3 months of the reconstitution date.

High background

a) Incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required. A soak time of at least 5 seconds between cycles should be used.
b) Incubation temperature too high	Incubation of the ELISA should be performed at room temperature (22°C ± 5°C).
c) Kit/components have expired	Ensure that the kit is used within the expiry date. Ensure reconstituted standard and Conjugate 100x Concentrate are used within three months of the reconstitution date.

	Comments and suggestions
d) Enzyme Substrate Solution is contaminated	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.

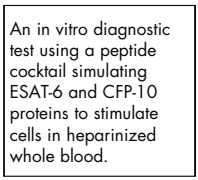
Nonlinear standard curve and duplicate variability

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

Symbols

The following symbols appear in the instructions for use or on the packaging and labeling:

Symbol	Symbol definition
 <N>	Contains reagents sufficient for <N> reactions
	Use by
CE 0197	This product fulfills the requirements of the European Regulation 2017/746 for in vitro diagnostic medical devices.
EC REP	Authorized representative in the European Community / European Union
IVD	In vitro diagnostic medical device
REF	Catalog number
LOT	Lot number
MAT	Material number (i.e., component labeling)
COMP	Components
CONT	Contains
NUM	Number
GTIN	Global Trade Item Number
Rn	R is for revision of the Instructions for Use and n is the revision number
	Temperature limitation

Symbol	Symbol definition
	Manufacturer
	Consult instructions for use
	Protect from light
	Warning/caution or Cautions, consult accompanying documents
	An in vitro diagnostic test using a peptide cocktail simulating ESAT-6 and CFP-10 proteins to stimulate cells in heparinized whole blood
	Contains biological material of animal origin
	Contains biological material of human origin
	Unique Device identifier

Symbol	Symbol definition
	Contains Tartrazine
	Contains sulfuric acid

Appendix A: Technical Information

Indeterminate results

Indeterminate results are uncommon and may relate to the immune status of the individual being tested (5) but may also be related to a number of technical factors (e.g., inappropriate handling/storage of blood collection tubes, incomplete ELISA plate washing) if the above instructions for use are not followed.

If technical issues are suspected with the reagent storage, blood collection, or handling of the blood samples, repeat the entire QFT-Plus test with new blood specimens. Repeating the ELISA testing of stimulated plasmas can be performed if inadequate washing or other procedural deviation with the ELISA test is suspected. Physicians may choose to redraw a specimen or perform other procedures as appropriate.

Clotted plasma samples

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

Lipemic plasma samples

Care should be exercised when pipetting lipemic samples as fatty deposits can block pipet tips.

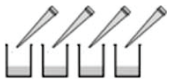
Appendix B: Abbreviated ELISA Test Procedure

1. Equilibrate ELISA components, with the exception of the Conjugate 100x Concentrate, to room temperature for at least 60 minutes.

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

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

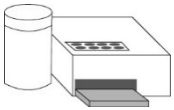
4. Prepare working strength conjugate in Green Diluent and add 50 µl to all wells.



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

6. Incubate for 120 minutes at room temperature.

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

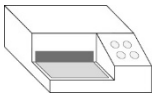


8. Add 100 µl Enzyme Substrate Solution to wells. Mix using shaker.

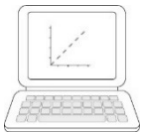
9. Incubate for 30 minutes at room temperature.

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

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



12. Analyze results.



Ordering Information

Product	Contents	Cat. no.
QuantiFERON-TB Gold Plus (QFT-Plus) ELISA Kit	2-plate ELISA kit	622120
QuantiFERON-TB Gold Plus (QFT-Plus) Reference Lab Pack	20-plate ELISA kit	622822
Related products		
QuantiFERON-TB Gold Plus Blood Collection Tubes	200 tubes (50 each Nil, TB1, TB2 and Mitogen)	622526
QuantiFERON-TB Gold Plus Blood Collection Tubes Dispenser Pack	100 tubes (25 each Nil, TB1, TB2 and Mitogen)	622423
QuantiFERON-TB Gold Plus Single Patient Pack	40 tubes (1 each Nil, TB1, TB2 and Mitogen/pack), pack of 10	622222
QuantiFERON-TB Gold Plus High Altitude Blood Collection Tubes	200 tubes (50 each Nil, TB1, TB2 and Mitogen)	623526
QuantiFERON-TB Gold Plus High Altitude Blood Collection Tubes Dispenser Pack	100 tubes (50 each Nil, TB1, TB2 and Mitogen)	623423
QuantiFERON-TB Gold Plus High Altitude Single Patient Pack	40 tubes (1 each Nil, TB1, TB2 and Mitogen/pack), pack of 10	623222

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit Instructions for Use. QIAGEN kit Instructions for Use are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
R2, June 2021	Included information about Single Patient Pack Revised Tables 10 and 11 to distinguish QFT-GIT vs. QFT-Plus data Updated Description and Principle section to add information about testing population and measuring range Added Table 9 to add data about QFT-Plus likelihood ratio
R3, October 2021	Reverted catalog number back to the original catalog numbers Added single-use statement for Microplate strips in Kit contents
R4, March 2023	Formatting fixes

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Limited License Agreement for QuantiFERON®-TB Gold Plus (QFT-Plus) ELISA Kit

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