

	QuantiFERON® – TB Gold-In-Tube	IOS EBP-ID 002	
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Destinatari: Coordinatore, Tecnici e Studenti del Settore Immunodiagnosi - EBP

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1. SCOPE

This instruction describes the detection of interferon- γ (IFN- γ) by Enzyme-Linked Immunosorbent Assay (ELISA) in order to identify *in vitro* responses to certain peptide antigens that are associated with *Mycobacterium tuberculosis* infection.

2. APPLICATION

QuantiFERON®-TB Gold IT is an indirect test for *M. tuberculosis* infection and is intended for use in conjunction with risk assessment, radiography and other medical and diagnostic evaluations. The present instruction is applicable within the Immunodiagnosis Area and it reflects the package insert of the commercially available kit QuantiFERON® - TB Gold-In-Tube (*Cellestis Limited* – Carnegie, Victoria, Australia). The Section 6.3 of the present instruction is to be considered part of the procedure, although it takes place at the enrolment centres. The main points of the Section 6.3 are summarised in a leaflet handed out to enrolment centres (Annex 1)

3. DEFINITIONS AND ABBRAVIATIONS

TB	Tuberculosis
ESAT-6	Early Secretory Antigenic Target – 6
CFP-10	Culture Filtrate Protein 10
ELISA	Enzyme-Linked Immunosorbent Assay
LTBI	Latent Tuberculosis Infection
TST	Tuberculin Skin Test
IFN- γ	Interferon - gamma
IU	International Unit
OD	Optical Density

4. RESPONSIBILITIES

The supervision and the correct application of the following instruction is the responsibility of the area coordinator. The execution of the test is responsibility of area technicians, master students and coordinator.

5. EQUIPMENT AND MATERIALS

5.1 Reagents provided with the Kit

Tuberculosis and Control Antigen Blood Collection Tubes (**Cat. No: 0590 0301**)

- √ Nil Control (Grey cap) 100 x tubes
- √ TB Antigen (Red cap) 100 x tubes
- √ Mitogen Control (Purple cap) 100 x tubes

NOTE: Tubes are also available in other configurations:

100 x Nil Control, 100 x TB Antigen tubes (Cat. No. 0590 0201)

100 x Mitogen Control tubes (Cat. No. 0593 0201)

ELISA Components (**Cat. No: 0594 0201**)

- √ Microplate strips 24 x 8 well
- √ Human IFN- γ Standard, lyophilised 1 x vial
- √ Green Diluent 1 x 30mL
- √ Conjugate 100X Concentrate, lyophilised 1 x 0.3mL
- √ Wash Buffer 20X Concentrate 1 x 100mL
- √ Enzyme Substrate Solution 1 x 30mL
- √ Enzyme Stopping Solution 1

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5.2 Materials and Reagents not provided

- √ 37°C incubator. CO2 not required.
- √ Calibrated variable-volume pipettes for delivery of 10µL to 1000µL with disposable tips.
- √ Calibrated multichannel pipette capable of delivering 50µL and 100µL with disposable tips.
- √ Microplate shaker.
- √ Deionised or distilled water - 2L.
- √ Microplate washer (automated washer recommended).
- √ Microplate reader fitted with 450nm filter and 620nm to 650nm reference filter.

5.3 Storage

Blood Collection Tubes

- √ Store blood collection tubes at 4°C to 25°C (The shelf life of the QuantiFERON®-TB Gold blood collection tubes is 15 months from the date of manufacture when stored at 4°C to 25°C)

Kit Reagents

- √ Store kit refrigerated at 2°C to 8°C
- √ Always protect Enzyme Substrate Solution from direct sunlight. (The shelf life of the QuantiFERON®-TB Gold ELISA kit is 3 years from the date of manufacture when stored at 2°C to 8°C.)

Reconstituted and Unused Reagents

- √ The reconstituted Kit Standard may be kept for up to 3 months if stored at 2°C to 8°C (*Note the date the Kit Standard was reconstituted*)
- √ Once reconstituted, unused Conjugate 100X concentrate must be returned to storage at 2°C to 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

6. PROCEDURES

6.1 Explanation of the test

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

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

The QuantiFERON®-TB Gold IT test is a test for Cell Mediated Immune (CMI) responses to peptide antigens that simulate mycobacterial proteins. These proteins, ESAT-6, CFP-10 and TB7.7(p4), are absent from all BCG strains and from most nontuberculosis mycobacteria with the exception of *M. kansasii*, *M. szulgai* and *M. marinum*. Individuals infected with *M. tuberculosis* complex organisms usually have lymphocytes in their blood that recognise these and other mycobacterial antigens. This recognition process involves the generation and secretion of the cytokine, IFN- γ . The detection and subsequent quantification of IFN- γ forms the basis of this test. The antigens used in QuantiFERON®-TB Gold IT are a peptide cocktail simulating the proteins ESAT-6, CFP-10 and TB7.7(p4). Numerous studies have demonstrated that these peptides antigens stimulate IFN- γ responses in T-cells from individuals infected with *M. tuberculosis* but generally not from uninfected or BCG vaccinated persons without disease or risk for LTBI.1-34 However, medical treatments or conditions that impair immune functionality can potentially reduce IFN- γ responses. Patients with certain other mycobacterial infections might also be responsive to ESAT-6, CFP-10 and TB7.7(p4) as the genes encoding these proteins are present in *M. kansasii*, *M. szulgai* and *M. marinum*.1,22 The QuantiFERON®-TB Gold IT test is both a test for LTBI and a helpful aid for diagnosing *M. tuberculosis* complex infection in sick patients. A positive result supports the diagnosis of tuberculosis disease; however, infections by other mycobacteria (e.g., *M. kansasii*) could also lead to positive results. Other medical and diagnostic evaluations are necessary to confirm or exclude tuberculosis disease.

6.2 Principle of the Assay

The QuantiFERON®-TB Gold IT system uses specialised blood collection tubes, 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. The QuantiFERON®-TB Gold IT test is performed in two stages. First, whole blood is collected into each of the QuantiFERON®-TB Gold blood collection tubes, which include a Nil Control tube, TB Antigen tube, and an optional Mitogen tube. The Mitogen tube can be used with the QuantiFERON®-TB Gold IT test as a positive control. This may be especially warranted where there is doubt as to the individual's immune status. The Mitogen tube also serves as a control for correct blood handling and incubation. The tubes should be incubated at 37°C as soon as possible, and within 16 hours of collection. Following a 16 to 24 hour incubation period, the tubes are centrifuged, the plasma is removed and the amount of IFN- γ (IU/mL) measured by ELISA.

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

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6.3 Specimen Collection and Handling

QuantiFERON®-TB Gold IT uses the following collection tubes:

1. Nil Control (Grey cap).
2. TB Antigen (Red cap).
3. Mitogen Control (Purple cap) (optional).

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

The following procedures should be followed for optimal results:

1. For each subject collect 1mL of blood by venepuncture directly into each of the QuantiFERON®-TB Gold IT blood collection tubes. (As 1mL tubes draw blood relatively slowly, keep the tube on the needle for 2-3 seconds once the tube appears to have completed filling, to ensure that the correct volume is drawn).

The black mark on the side of the tubes indicates the 1mL fill volume. QuantiFERON®-TB Gold blood collection tubes have been validated for volumes ranging from 0.8 to 1.2mL. If the level of blood in any tube is not close to the indicator line, it is recommended to obtain another blood sample. (If a “butterfly needle” is being used to collect blood, a “purge” tube should be used to ensure that the tubing is filled with blood prior to the QuantiFERON®-TB Gold tubes being used.

2. Mix by vigorously shaking the tubes up and down 10 times to ensure that the entire inner surface of the tube has been coated with blood. (Thorough mixing is required to ensure complete mixing of the blood with the tube’s contents).

3. Label tubes appropriately.

4. The tubes must be transferred to a 37°C incubator as soon as possible, and within 16 hours of collection. Do not refrigerate or freeze the blood samples (If the blood is not incubated immediately after collection, mixing of the tubes must be repeated immediately prior to incubation).

6.4 Stage 1: Incubation of Blood and Harvesting of Plasma

1. If the blood is not incubated immediately after collection, mixing of the tubes must be repeated immediately prior to incubation.

2. Incubate the tubes upright at 37°C for 16 to 24 hours. The incubator does not require CO₂ or humidification.

3. After incubation at 37°C, blood collection tubes may be held between 2°C and 27°C for up to 3 days prior to centrifugation.

4. After incubation of the tubes at 37°C, harvesting of plasma is facilitated by centrifuging tubes for 15 minutes at 2000 to 3000 RCF (g). The gel plug will separate the cells from the plasma. If this does not occur, the tubes should be re-centrifuged at a higher speed.

5. Plasma samples can be loaded directly from blood collection tubes into the QuantiFERON®-TB Gold ELISA plate,

6. Alternatively, plasma samples can be stored prior to ELISA, either in the centrifuged tubes or collected into 1.5 ml Eppendorf tubes. (Plasma samples can be stored for up to 4 weeks at 2°C to 8°C or below –20°C for extended periods).

6.5 Stage 2: Human IFN-γ ELISA

1. All plasma samples and reagents, except for Conjugate 100X Concentrate, must be brought to room temperature (22°C ± 5°C) before use. Allow at least 60 minutes for equilibration.

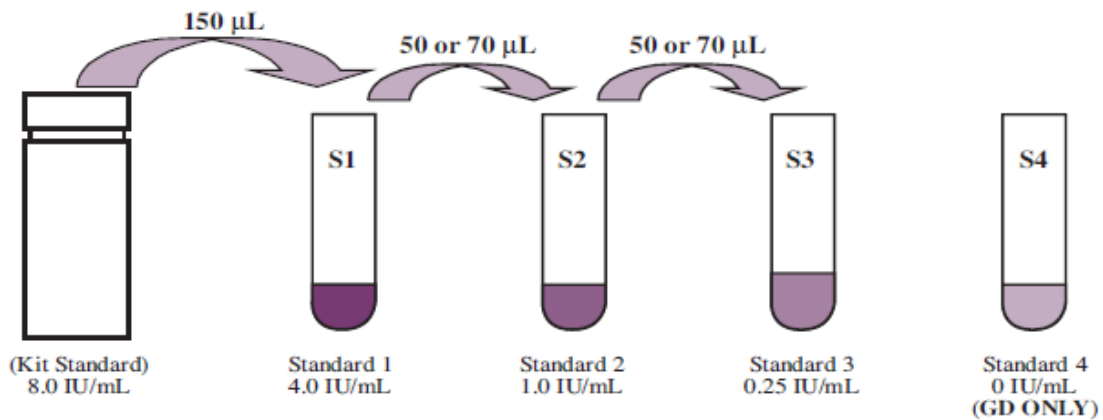
2. Remove strips that are not required from the frame, reseal in the foil pouch, and return to the refrigerator for storage until required. Allow at least one strip for the QuantiFERON®-TB Gold Standards and sufficient strips for the number of subjects being tested (refer to Figures 2A and 2B for 2-tube and 3-tube formats, respectively). After use, retain frame and lid for use with remaining strips.

3. Reconstitute the freeze dried Kit Standard with the volume of deionised or distilled water indicated on the label of the Standard vial. Mix gently to minimise frothing and ensure complete solubilisation. Reconstitution of the Standard to the stated volume will produce a solution with a concentration of 8.0 IU/mL.

Note: The reconstitution volume of the Kit Standard will differ between batches.

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

FIGURE 1. Preparation of Standard Curve



RECOMMENDED PROCEDURE FOR DUPLICATE STANDARDS
<ul style="list-style-type: none"> a. Label 4 tubes "S1", "S2", "S3", "S4". b. Add 150µL of GD to S1, S2, S3, S4. c. Add 150µL of the Kit Standard to S1 and mix thoroughly. d. Transfer 50µL from S1 to S2 and mix thoroughly. e. Transfer 50µL from S2 to S3 and mix thoroughly. f. GD alone serves as the zero standard (S4).

RECOMMENDED PROCEDURE FOR TRIPPLICATE STANDARDS
<ul style="list-style-type: none"> a. Label 4 tubes "S1", "S2", "S3", "S4". b. Add 150µL of GD to S1. c. Add 210µL of GD to S2, S3, S4. d. Add 150µL of the Kit Standard to S1 and mix thoroughly. e. Transfer 70µL from S1 to S2 and mix thoroughly. f. Transfer 70µL from S2 to S3 and mix thoroughly. g. GD alone serves as the zero standard (S4).

Note: Prepare fresh dilutions of the Kit Standard for each ELISA session.

4. Reconstitute freeze dried Conjugate 100X Concentrate with 0.3mL of deionised or distilled water. Mix gently to minimize frothing and ensure complete solubilisation of the Conjugate. Working Strength conjugate is prepared by diluting the required amount of reconstituted Conjugate 100X Concentrate in Green Diluent as set out in Table 1 - Conjugate Preparation.

TABLE 1. Conjugate Preparation

NUMBER OF STRIPS	VOLUME OF CONJUGATE 100X CONCENTRATE	VOLUME OF GREEN DILUENT
2	10µL	1.0mL
3	15µL	1.5mL
4	20µL	2.0mL
5	25µL	2.5mL
6	30µL	3.0mL
7	35µL	3.5mL
8	40µL	4.0mL
9	45µL	4.5mL
10	50µL	5.0mL
11	55µL	5.5mL
12	60µL	6.0mL

Mix thoroughly but gently to avoid frothing.

Return any unused Conjugate 100X Concentrate to 2°C to 8°C immediately after use.

5. Prior to assay, plasmas should be mixed to ensure that IFN-γ is evenly distributed throughout the sample.

6. Add 50µL of freshly prepared Working Strength conjugate to the required ELISA wells using a multichannel pipette.

7. Add 50µL of test plasma samples to appropriate wells using a multichannel pipette (Refer to recommended plate layout below – Figures 2A and 2B). Finally, add 50µL each of the Standards 1 to 4.

FIGURE 2A. Recommended Sample Layout for Nil & TB Antigen Tubes (44 tests per plate)

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

- S1 (Standard 1), S2 (Standard 2), S3 (Standard 3), S4 (Standard 4).

- 1N (Sample 1. Nil Control plasma); 1A (Sample 1. TB Antigen plasma).

FIGURE 2B. Recommended Sample Layout for Nil, TB Antigen & Mitogen Tubes (28 tests per plate)

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

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

8. Mix the conjugate and plasma samples/standards thoroughly using a micro plate shaker for 1 minute.

9. Cover each plate with a lid and incubate at room temperature (22°C ± 5°C) for 120 ± 5 minutes (Plates should not be exposed to direct sunlight during incubation).

10. During the incubation, dilute one part Wash Buffer 20X Concentrate with 19 parts deionised or distilled water and mix thoroughly. Sufficient Wash Buffer 20X Concentrate has been provided to prepare 2L of Working Strength wash buffer.

Wash wells with 400µL of Working Strength wash buffer for at least 6 cycles with a multichannel pipette (Thorough washing is very important to the performance of the assay. Ensure each well is completely filled with wash buffer to the top of the well for each wash cycle. A soak period of at least 5 seconds between each cycle is recommended. Standard laboratory disinfectant should be added to the effluent reservoir, and established procedures followed for the decontamination of potentially infectious material).

11. Tap plates face down on absorbent towel to remove residual wash buffer. Add 100µL of Enzyme Substrate Solution to each well and mix thoroughly.

12. Cover each plate with a lid and incubate at room temperature (22°C ± 5°C) for 30 minutes (Plates should not be exposed to direct sunlight during incubation).

13. Following the 30 minute incubation, add 50µL of Enzyme Stopping Solution to each well and mix (Enzyme Stopping Solution should be added to wells in the same order and at approximately the same speed as the substrate in step 11).

14. Measure the Optical Density (OD) of each well within 5 minutes of stopping the reaction using a microplate reader fitted with a 450nm filter and with a 620nm to 650nm reference filter. OD values are used to calculate results.

6.6 Calculations and Test Interpretation

QuantiFERON®-TB Gold IT Analysis Software, used to analyse raw data and calculate results, is available from Cellestis. The software performs a Quality Control assessment of the assay, generates a standard curve and provides a test result for each subject.

QuantiFERON®-TB Gold IT results are interpreted using the following criteria:

- When only Nil and TB Antigen tubes are used

Nil [IU/mL]	TB Antigen minus Nil [IU/mL]	QuantiFERON®-TB [IU/mL]	Report/Interpretation
≤ 8.0	< 0.35	Negative	<i>M. tuberculosis</i> infection NOT likely
	≥ 0.35 and < 25% of Nil value		
	≥ 0.35 and ≥ 25% of Nil value	Positive¹	<i>M. tuberculosis</i> infection likely
> 8.0 ²	Any	Indeterminate³	Results are indeterminate for TB Antigen responsiveness

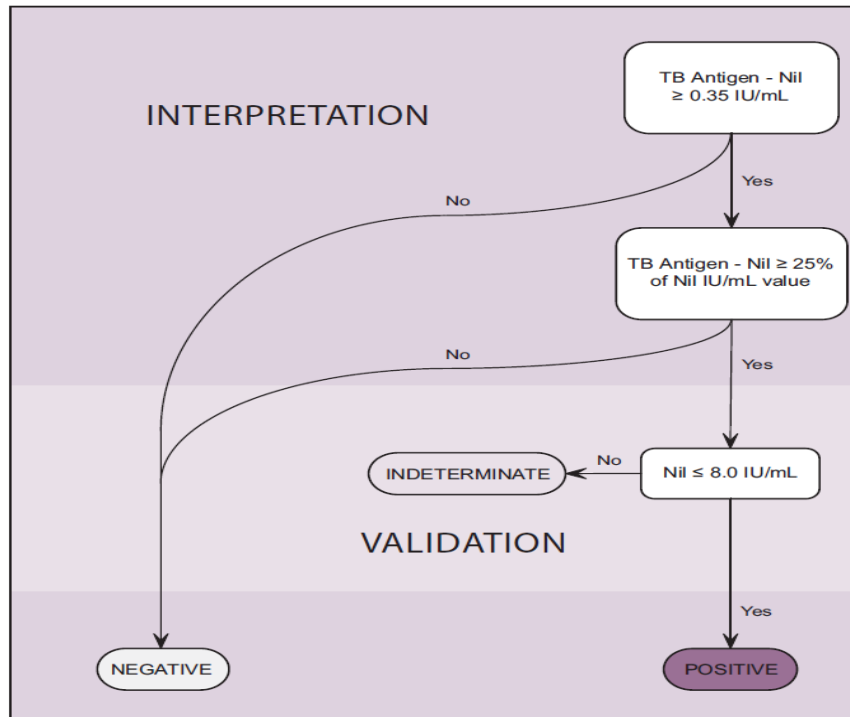
¹ Where *M. tuberculosis* infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate by TSPOT.TB8®. If repeat testing of one or both replicates is positive, the individual should be considered test positive.

² In clinical studies, less than 0.25% of subjects had IFN-γ levels of > 8.0 IU/mL for the Nil Control.

³ Results are indeterminate for TB Antigen responsiveness

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

FIGURE 3. Interpretation Flow Diagram when NIL & TB ANTIGEN tubes used



- When Nil, TB Antigen and Mitogen tubes used:

Nil [IU/mL]	TB Antigen minus Nil [IU/mL]	Mitogen minus Nil [IU/mL] ¹	QuantiFERON®-TB [IU/mL]	Report/Interpretation
≤ 8.0	< 0.35	≥ 0.5	Negative	<i>M. tuberculosis</i> infection NOT likely
	≥ 0.35 and < 25% of Nil value	≥ 0.5		
	≥ 0.35 and ≥ 25% of Nil value	Any	Positive ²	<i>M. tuberculosis</i> infection likely
≤ 8.0	< 0.35	< 0.5	Indeterminate ³	Results are indeterminate for TB-Antigen responsiveness
	≥ 0.35 and < 25% of Nil value	< 0.5		
> 8.0 ⁴	Any	Any		

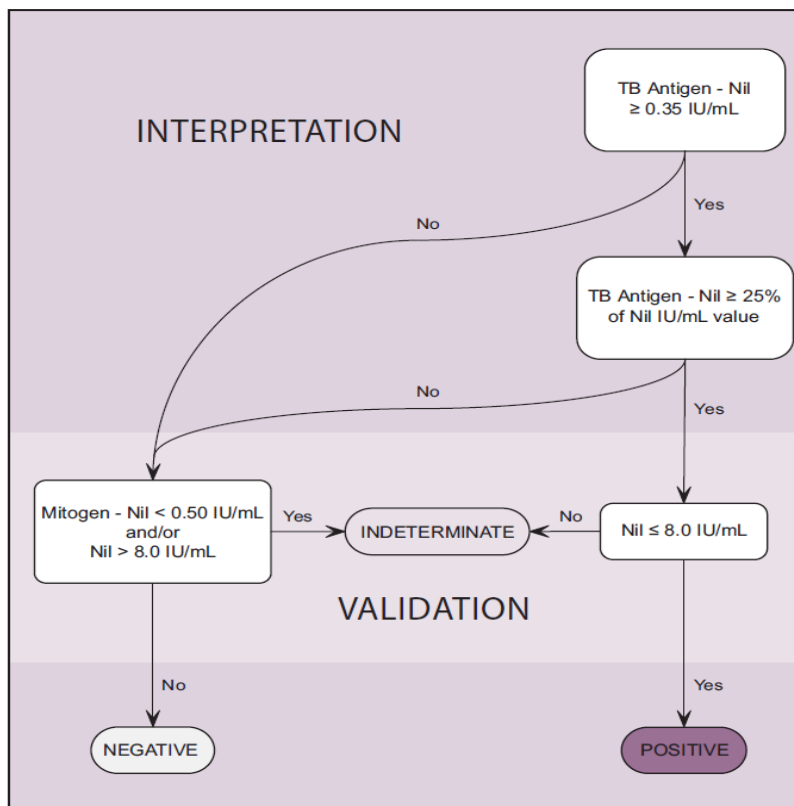
¹ Responses to the Mitogen positive control (and occasionally TB Antigen) can be commonly outside the range of the microplate reader. This has no impact on test results.

² Where *M. tuberculosis* infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate with TSPOT.TB8®. If repeat testing of one or both replicates is positive, the individual should be considered test positive.

³ Results are indeterminate for TB Antigen responsiveness.

⁴ In clinical studies, less than 0.25% of subjects had IFN-γ levels of > 8.0 IU/mL for the Nil Control. The magnitude of the measured IFN-γ level cannot be correlated to stage or degree of infection, level of immune responsiveness, or likelihood for progression to active disease.

FIGURE 4. Interpretation Flow Diagram when NIL, TB ANTIGEN & MITOGEN tubes used



7. RECORDING AND REPORTING

Results coming from the QuantiFERON®-TB Gold IT Analysis Software (Cellestis) are reported and printed out according to these two options:

- one page including all samples tested and results in case the individuals were referred by the same clinician or centre.
- single report in case the samples tested and results in case the individuals were referred by different clinicians or centre.

Tests included in a specific study protocol should be recorded into the specific Study Database.

8. RELATED DOCUMENTS

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9. ANNEXES

Annex 1. Conservazione delle provette per QuantiFERON-TB GIT

1. controllo nullo (tappo grigio)
2. antigene TB (tappo rosso)
3. controllo mitogeno – facoltativo (tappo viola)

Conservare le provette ad una temperatura compresa tra i 4 ed i 25 °C prima del prelievo.

Per ciascun soggetto in esame prelevare 1 ml di sangue per venopuntura direttamente in ognuna delle provette dedicate.

Dato che nelle provette da 1 ml il sangue fluisce con relativa lentezza, mantenere la provetta sull'ago per altri 2-3 secondi dopo che sembra riempita completamente, per accertarsi di aver prelevato il volume corretto.

La tacca nera sul lato delle provette indica il volume di riempimento di 1 ml. Le provette per il prelievo del sangue QuantiFERON®-TB Gold sono state validate per volumi che variano da 0,8 a 1,2 ml. Se il livello di sangue in una qualunque delle provette non raggiunge la linea indicata, si consiglia di prelevare un altro campione di sangue.

Miscelare il contenuto delle provette scuotendole energicamente per 5 secondi (o 10 volte) per assicurarsi che l'intera superficie interna della provetta sia ricoperta di sangue.

Le provette devono essere trasferite in un incubatore a 37°C il prima possibile e comunque entro 16 ore dal prelievo dei campioni.

Non refrigerare né congelare i campioni di sangue.