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Abstract

Worldwide there are about 1.7 billion individuals with latent tuberculosis infection (LTBI) and only 5% to 15% will develop active tuberculosis (TB). It is recommended to treat only those most at risk of developing active TB to avoid problems of drug resistance. LTBI diagnosis involves reviewing the individual's medical history, physical examination, and biological tests. Interferon gamma release assays (IGRA) can yield 'indeterminate' or 'uncertain' results which makes clinical management decisions difficult. We assessed an ultra-sensitive immunoassay prototype based on single molecule array (SiMoA) technology to evaluate its overall performance, and in particular, its performance for indeterminate and uncertain positive or negative samples, as classified by results from the current ELISA used for IFNγ quantification. We analyzed samples from hospitalized or consulting patients and healthcare workers from three hospitals in Paris previously classified as negative (n=30), positive (n=35), uncertain negative (n=25), uncertain positive (n=31) and indeterminate (n=30). We observed that with the SiMoA assay 83.3% of the indeterminate samples became interpretable and could be classified as negative, while 74% of uncertain positive samples were classified as positive. Most uncertain negative samples (72%) were re-classified as uncertain positive (68%) or positive (4%). The results suggest that ultra-sensitive SiMoA IFNγ assay could represent a useful tool for the identification of true positive and negative samples among those giving indeterminate or uncertain results with the TB IGRA assay currently used.

INTRODUCTION

Tuberculosis (TB) is a bacterial infection caused by *Mycobacterium tuberculosis* complex that affects the lungs in about 70-80% of cases [1, 2]. When people with pulmonary TB cough, sneeze or spit the bacteria is expelled into the air and other people can become infected by inhalation of only a few of the bacteria [1]. TB infection can be either active (disease state) or latent. TB is a major public health threat that has important medical and economic consequences. In 2017, about 10 million people were reported to have active TB and it was among the top ten causes of death worldwide, with 1.6 million deaths [1]. TB is responsible for about 40% of deaths in co-infected HIV patients [1].

The WHO (World Health Organization) defines latent tuberculosis infection (LTBI) as the persistence of an immune response to *M. tuberculosis* antigens, coupled with the absence of clinical signs suggestive of tuberculosis [3]. In 2014 it was estimated that there were about 1.7 billion individuals with LTBI, i.e. 23% of the global population [4]. About 5% to 15% of those with LTBI will develop active TB during their lifetime [5].

To prevent active TB from developing, individuals with LTBI can receive treatment, which is less intensive than treatment for active TB [6]. However, it is not recommended to treat everyone with LTBI to avoid problems of drug resistance, particularly multi-drug resistant strains. The priority, therefore, is to identify those who are most at risk of developing active TB, i.e., those with an incompetent immune system, either due to disease, e.g. HIV or immunosuppressive therapy, such as anti-tumor necrosis factor alpha (anti-TNF-α) therapies for the management of certain pathologies (e.g. Crohn's disease and rheumatoid arthritis). The growing number of people with LTBI that could potentially evolve to active TB underlines the need for reliable means to diagnose LTBI [7].

LTBI is diagnosed using the patient’s medical history, physical examination, and biological tests. These tests assess the capacity of a patient's immune system to recognize mycobacterial

antigens, thereby providing indirect proof of infection [8, 9]. The first test, developed in the early 20th century by Robert Koch, involved an intradermal injection of mycobacterial antigen extracts (tuberculin skin test, TST) that triggers an *in vivo* delayed hypersensitivity reaction, indicating the presence of a more or less specific immune response [10]. Recently, two more specific assays, i.e., interferon gamma release assays (IGRA), based on an *in vitro* immune response and subsequent secretion of interferon gamma (IFNγ) in whole blood samples have been developed, the QuantiFERON®-TB Gold in-tube and T-SPOT.TB® [11-13]. These assays have significantly improved the diagnosis of LTBI because, unlike the TST, they do not cross-react with BCG (bacillus Calmin Guérin). The most widely used TB IGRA tests is the QuantiFERON-TB test (Qiagen, Hilden, Germany). The QuantiFERON®-TB Gold in-Tube assay (QFT®-GIT) is being replaced by the new generation QuantiFERON®-TB Gold Plus (QFT®-Plus). These assays involve an *in vitro* stimulation of lymphocytes specific to the bacterial *tuberculosis* complex by a cocktail of peptides from two *M. tuberculosis*-specific proteins (ESAT-6 and CFP-10). This is followed by the quantification of IFNγ produced in response to this stimulation (expressed as international units/mL (IU/mL)) using the same microplate QFT IFNγ ELISA kit. Both QFT-GIT and QFT-Plus rely on a differential interpretation of different *in vitro* stimulations, based on the quantity of detected IFNγ. The negative control (NIL) provides information on the background level of IFNγ in the sample at the time of testing and a positive control (MIT) validates the IFNγ secretion capacity of T lymphocytes in the whole blood sample. In the QFT-GIT assay there is a single *M. tuberculosis* specific stimulation (TB), compared with two (TB1 & TB2) in the QFT-Plus assay. The test result is considered positive if the difference between the *M. tuberculosis* specific stimulations (TB1 or TB2) and the negative control exceeds a predefined threshold. However, the QFT results are considered ‘indeterminate’ when the positive control result is low or there is a high background response to the negative control. Indeterminate results are

often associated with immunodeficiency due to lymphopenia, HIV, or immunosuppressive treatments, for example [14-19]. This immunodeficiency reduces the T lymphocyte population and, consequently, the amount of IFNγ secreted is decreased. However, other factors can reduce the number of viable T-cells, e.g. delayed reception of samples in the laboratory and result in lower levels of secreted IFNγ for the positive control [20-22]. High results for negative controls, either due to excessive levels of circulating IFNγ or heterophilic antibodies, can also result in indeterminate results. The incidence of indeterminate results is usually low in healthy individuals (<2%) but can increase significantly in immunosuppressed populations due to the lower secretion of IFNγ and the level of the IGRA detection threshold [23, 24].

'Uncertain' results are linked to an additional uncertainty zone around the QFT manufacturer's predefined threshold. An 'uncertainty zone' has been proposed to deal with variations occurring in the interpretation of results from serial QFT assays. In one study assessing serial testing of samples from healthcare workers in a low-incidence setting using the QFT-GIT assay, the use of an uncertainty zone from 0.2 to 0.7 IU/mL instead of a strict threshold of 0.35 IU/mL resulted in a lower percentage of conversions and reversions (2.6% vs. 6.1% and 15.4% vs. 32.6%, respectively) [25]. Several sources of variability that can have an impact on serial QFT testing were identified in a systematic review, with the QFT IFNγ ELISA kit variability itself being the most important [26]. More recently, results from a study investigating the pre-analytical, analytical and inter-assay variability of the QFT-GIT assay demonstrated that the variability could be improved by implementing optimized procedures, some of which were linked to the combined effects of blood volume and incubation times in the QFT stimulation tubes and some linked to the QFT IFNγ ELISA kit itself [27].

Overall, these observations suggest that a more sensitive, automated assay could provide a clearer clinical interpretation of the results for these specific patient populations. Recently, a

highly-sensitive SiMoA prototype for IFNγ quantification, based on single molecule array (SiMoA) technology and digital ELISA signal detection, has been developed. This technology enables detection of lower concentrations of proteins than with conventional tests such as microplate ELISA assays; the detection threshold can be 100-fold lower or more, compared with current analog methods [28, 29].

The aim of this study was to assess the analytical performance of this new IFNγ immunoassay prototype based on SiMoA technology and to compare it with the performance of the QFT Plus IFNγ ELISA kit. In particular, we investigated if SiMoA IFNγ assay can improve the clinical interpretation of samples collected using QFT-Plus tubes and classified as 'indeterminate' and 'uncertain' using the QFT IFNγ ELISA kit.

MATERIALS AND METHODS

QFT-TB Gold Plus assay

The QuantiFERON TB-Gold Plus (QFT-Plus) assay (Qiagen) measures cell-mediated immune responses to two different peptide cocktails (TB1 and TB2, see below) from two *M. tuberculosis* proteins (ESAT-6 and CFP10). The assay is performed in two stages. In the first stage, blood samples are transferred to the laboratory in collection tubes containing the peptide cocktail where they are incubated at 37°C for 16-24 hours. In the second stage, the plasma is harvested and the secreted IFNγ is measured with the QFT ELISA kit in a 50µL aliquot.

The routine QFT-Plus assay was performed according to manufacturer's recommendations except the whole blood samples that had been collected in lithium heparin tubes could be received up to 24h hours after collection at the Pitié-Salpêtrière Hospital in the Cellular and Tissue Immunology laboratory, whereas the manufacturer recommends up to 16h. For each patient, blood samples were transferred into the four specialized collection QFT-Plus tubes: (1) a negative control tube i.e. NIL (without antigen); (2) a positive control tube i.e. mitogen

(MIT; PHA); (3) a tube containing ESAT-6 & CFP10 long peptides i.e. TB antigen 1 (TB1); (4) a tube containing ESAT-6 & CFP10 short & long peptides i.e. TB antigen 2 (TB2).

A log-log standard curve was generated by plotting the log of the mean optical density (OD) on the y-axis against the log of the IFNγ concentration of the four standards (0, 0.25, 1.0 and 4.0 IU/mL) supplied in the kit on the x-axis. The line of best fit for the standard curve was then determined with regression analysis and used to determine the IFNγ concentration in IU/mL for each of the tested plasma samples, using the OD value of each sample. The results were calculated as MIT minus NIL and TB1 (or TB2) minus NIL. When the TB1-NIL (δTB1) and TB2-NIL (δTB2) results were discordant, the highest value was taken into account for interpretation. The results were interpreted as summarized in Table 1.

SiMoA IFNγ immunoassay prototype

The SiMoA IFNγ immunoassay prototype is a fully automated three-step sandwich immunoassay that quantifies IFNγ in plasma and cell culture supernatants using the HD-1 Analyzer. In this assay, the IFNγ is captured onto antibody-coated paramagnetic beads and detected with a biotin-labeled antibody and an enzyme-conjugated streptavidin. The individual beads are then isolated and sealed in arrays of femtoliter-sized wells in the presence of a fluorogenic enzyme substrate. The fluorescence emitted is captured by a charged coupled device (CCD) camera, allowing the number of wells containing an enzyme-labelled bead and the level of emitted fluorescence to be ascertained. Both the fraction of beads associated with at least one enzyme and the fluorescence intensity from each well are determined, enabling the instrument to detect ultra-low IFNγ concentrations (digital readout mode). The assay was calibrated with native IFNγ antigen obtained by stimulation of peripheral blood mononuclear cells (PBMCs) and diluted in the sample diluent. Standards were calibrated in IU/mL, based on determinations with the QFT ELISA assay. The calibration curve was established using seven standards tested in duplicate (0, 0.0023, 0.0056,

0.029, 0.13, 0.61 and 2.34 IU/mL) using a four parameter logistic (4PL) regression model [30]. Two duplicate control samples were included; one with a concentration within the digital range and the other with a concentration within the analog range for the SiMoA IFNγ assay.

The carboxy-paramagnetic microbeads (2.7-µm, provided by Agilent Technologies) coated with a mouse monoclonal anti-human IFNγ antibody (developed by bioMérieux) were mixed with 75 µL of the pre-diluted sample (1/4) and incubated for 15 min. An additional dilution (1/20) was analyzed when saturation of TB1 or TB2 occurred. The antibody-coated beads were diluted to obtain a concentration of $2x10^7$ beads/mL in Tris buffered saline with 0.05% tween 20 and 0.05% bovine serum albumin (BSA). The capture microbeads were collected into a pellet using a magnet, washed and then incubated for 5 minutes with biotinylated anti-human IFNγ–detector monoclonal antibody (also developed by bioMérieux) at 0.1 µg/mL in phosphate-buffered saline containing 0.05% tween 20 (PBST) and 0.05% BSA. After pelleting and washing, the beads were incubated with streptavidin-β-galactosidase (SβG; enzymatic conjugate) compound for five minutes. The SβG compound was prepared at bioMérieux by covalent conjugation of purified streptavidin (Thermo Scientific) and βG (Sigma), and diluted to 150 ng/mL in PBST and 0.05% BSA. The beads were then pelleted and washed and finally incubated with the fluorogenic substrate, resorufin β-D-galactopyranoside (RGP). The HD-1 analyzer processed the substrate incubation, bead transfer onto the disk and the CCD camera reading and image acquisition in about 3 minutes.

Assessment of assay reproducibility and limits of quantification

The reproducibility of the SiMoA IFNγ and QFT IFNγ ELISA assays was assessed using samples that were within their specific detection ranges: from 0.0023 to 2.34 IU/mL and from 0.065 IU/mL (LOD) to 10.0 IU/mL (extrapolated highest standard), respectively [31].

The reproducibility of the QFT ELISA assay was assessed using 13 blood samples that were tested in duplicate over three days. Two technicians performed the assays in one laboratory, using the same QFT ELISA assay batch (n = 12 for each sample). The limit of quantification (LOQ) at 20% CV was estimated using concentrations of the 13 blood samples ranging from 0.02 IU/mL to 4.0 IU/mL, with 8 of the samples in the low range (0.02-0.37 IU/mL).

The reproducibility of the SiMoA IFNγ assay was performed using one HD-1 instrument. Seven plasma samples and two controls in duplicate (one digital and one analog) with concentrations ranging from 0.02 to 1.0 IU/mL were tested twice-a-day for three days (n = 12 for each sample). Five of the samples, obtained by dilution in the sample diluent, were used specifically to assess the assay LOQ at 20% CV.

Linearity of the SiMoA IFNγ and QFT assays

To assess the linearity of the SiMoA IFNγ assay two plasma samples, containing 0.64 and 2.51 IU/mL of IFNγ, were serially diluted with the sample diluent from 3/4 to 1/20 for sample A, and from 3/5 to 1/20 for sample B, giving concentrations from 0.48 IU/mL to 0.032 IU/mL and from 1.51 IU/mL to 0.13 IU/mL, respectively. Each dilution was tested in duplicate to assess the linearity of SiMoA IFNγ assay. The IFNγ concentrations were determined using an in-house standard curve.

We did not assess the linearity of the QFT IFNγ ELISA here because the manufacturer had documented its linearity previously [31].

Samples tested

A total of 1,717 fresh blood samples from hospitalized or consulting patients from three hospital in Paris, France (Pitié Salpêtrière, Saint –Antoine and Tenon Hospital), were assessed routinely in our laboratory using the QFT Plus assay from 19 June to 10 October 2017. There were 1,387 negative (81%), 188 positive (11%), 50 uncertain negative (3%), 56 uncertain positive (3%) and 36 indeterminate (2%) samples. A total of 151 of these samples that were

frozen after the QFT IFNγ ELISA test were later assessed with the SiMoA IFNγ assay: 30 negative, 25 uncertain negative, 35 positive, 31 uncertain positive and 30 indeterminate samples. The samples were a convenience selection of samples that had a sufficient volume for the SiMoA IFNγ assay to obtain about 30 in each category. The majority of the samples came from hospitalized patients and 21 were from healthcare professionals (**Table 2**). Lymphocyte counts were available for 41% of the samples.

These samples were used to assess if the higher sensitivity of the SiMoA IFNγ assay could confirm the positive and negative results and improve the clinical interpretation of samples classified as indeterminate or uncertain positive or negative based on the results from the QFT IFNγ ELISA assay. The indeterminate samples were mainly from immunosuppressed patients, with a MIT - NIL <0.5 IU/mL. The thresholds for the negative and positive δTB1and δTB2 samples were <0.2 IU/mL and >0.7 IU/mL, respectively. The results for the uncertain negative and uncertain positive samples fell into the uncertainty zones around the cut off of 0.35 IU/mL; 0.2 to 0.35 IU/mL and 0.35 to 0.7 IU/mL, respectively [27].

Statistical analysis

Analyses were performed using the GraphPad Prism (version 7.03), SAS-Add in (version 4.3) and Analyse-it (version 3.70) software. Passing and Bablok regression analysis, a non-parametric statistical method, was used to estimate the agreement between the assays and detect any systematic bias between them [32].

RESULTS

Analytical performance of the SiMoA IFNγ assay and the QFT IFNγ ELISA

Reproducibility and limits of quantification

The intra-assay CVs for the SiMoA IFNγ assay were below 4% for samples ranging from 0.045 to 1.037 IU/mL compared with CVs from 4.4% to 14.1% for the QFT ELISA assay for samples ranging from 0.595 to 3.998 IU/mL (**Table 3**). The inter-assay CVs for the SiMoA

IFNγ assay ranged from 3.7% to 8.2% compared with from 10.5% to 21.3% for the QFT ELISA.

The precision profiles for both assays were plotted using the results for the lowest concentration samples tested in the repeatability study to determine the LOQ, corresponding to 20% of the CV (**Figure 1**). The LOQ for the QFT ELISA was 0.169 IU/mL compared with 0.002 IU/mL for the SiMoA IFNγ assay.

Linearity of the SiMoA IFNγ assay

For the assessment of the linearity of the SiMoA IFNγ assay the recovery rate ranged from 99.2% to 115.7% for sample A (initial IFNγ concentration 0.64 IU/mL) and from 103.3% to 111.0% for sample B (initial IFNγ concentration 2.51 IU/mL), with slopes and a R^2 close to 1.0 for both samples (**Figure 2**).

Verification of the metrological traceability between the QFT IFNγ ELISA and SiMoA IFNγ assay

The correlation of the results from the SiMoA and QFT IFNγ ELISA assays was assessed using the 35 positive samples, i.e. where δTB1 and/or δTB2 were ≥0.7 IU/mL, to ensure that the poorer precision of the QFT IFNγ ELISA assay in the low range of concentrations would not bias the results. The IFNγ concentrations obtained with SiMoA IFNγ assay were, on average, 17% higher than those obtained with the QFT ELISA assay for δTB1 and 24% higher for δTB2 (95% CI: 0.99-1.42 and 1.01-1.41, respectively). The correlation equations were $y = 1.17x + 0.16$ for δTB1 and $y = 1.24x + 0.09$ for δTB2 (**Figure 3**). The Pearson correlation coefficient R^2 was 0.923 for both δTB1 and δTB2.

SiMoA IFNγ assay results for non-positive QFT Plus results

Comparison of indeterminate results

A total of 30 samples with indeterminate MIT - NIL results (<0.5 IU/mL) from the QFT Plus assay were also analyzed with the SiMoA assay. The SiMoA assay results for the majority of

NIL tubes were above the LOQ of 0.002 IU/mL (median value 0.026 IU/mL, interquartile range (IQR) 0.008 to 0.055 IU/mL), compared with none of the QFT ELISA results (median value 0.055 IU/mL, IQR 0.033 to 0.080 IU/mL) (**Figure 4A**). Only two of the samples were not measurable by the SiMoA assay, but they were both detectable and lower than the LOQ (0.0014 IU/mL). The median MIT results from the SiMoA and the QFT assays were 0.393 IU/mL with IQR 0.198 to 0.690 IU/mL and 0.230 IU/mL with IQR 0.089 to 0.300 IU/mL, respectively (**Figure 4B**). The MIT-NIL values for the SiMoA assay were significantly different from those for the QFT IFNγ ELISA assay, resulting in a MIT–NIL value >0.5 IU/mL for 11/30 samples making interpretation possible according to the manufacturer's recommendations (**Table 1; Figure 4C**).

The lymphocyte count was within the normal range (1,500-4,000 lymphocytes/mm^3) for 3 of the 17 QFT Plus indeterminate samples for which lymphocyte counts were available. For the remaining 14, the counts were below the normal range (mean: 698 lymphocytes/mm^3, SD: 332 lymphocytes/mm^3). No link between lymphopenia and the MIT – NIL value was observed.

The δTB1 and δTB2 results for the 11/30 samples with MIT – NIL values >0.5 IU/mL in the SiMoA assay (maximum=0.027 and 0.016, respectively) were all below the uncertain positivity thresholds of 0.2 IU/mL and 0.35 IU/mL for the SiMoA and QFT IFNγ ELISA assays, respectively and they were interpreted as negative. Since the LOQ for the SiMoA assay is lower than that for the QFT ELISA, a MIT – NIL threshold lower than 0.5 IU/mL could be considered. The results with lower MIT - NIL thresholds on the clinical interpretation of the indeterminate samples are summarized in **Table 4**. The lowest MIT - NIL threshold, >0.1 IU/mL, enabled the results for 25 of the 30 indeterminate samples to be interpreted; all were negative. Four of the five samples that remained indeterminate had high NIL values (data not shown).

Comparison of uncertain positive, uncertain negative and negative results

Passing-Bablok regression analyses were used to compare the results for samples that were classified as uncertain (positive and negative) with the QFT Plus assay with those from the SiMoA assay (**Figure 5**). We also used the QFT Plus manufacturer's criteria for the uncertainty categories to compare the interpretation of the results from both assays.

Unlike the analyses with positive samples, where the Passing Bablok analyses gave slopes of 1.17 and 1.24 for the δTB1 and δTB2 results, respectively, higher slopes were observed with the uncertain categories (**Figures 3 and 5**). In these analyses the slopes for the δTB1 and δTB2 results for uncertain negative samples were 2.79 and 3.37, respectively, and 2.17 and 2.30, respectively, for uncertain positive samples (**Figure 5**).

When the uncertainty thresholds and the QFT Plus manufacturer's thresholds were used to interpret the results, discordant results were observed for 43 and 19 samples, respectively (**Table 5**). The δTB1 and δTB2 SiMoA assay results were classified in a higher category than the QFT Plus assay results for all 19 discordant samples, using the manufacturer's thresholds (data not shown).

Passing Bablok regression analyses were not performed for the negative samples because most of the QFT δTB1 and δTB2 were close to 0 or negative.

DISCUSSION

The WHO recommends screening for LTBI in certain at-risk populations [3]. Biological exploration with TB IGRA assays is an important element for the diagnosis of LTBI since there are no clinical symptoms. However, the results for transplanted patients or individuals living with HIV may be indeterminate due to lymphopenia or immunosuppressive therapy [23, 33-35]. A more sensitive test, such as the SiMoA IFNγ assay, could provide more definite results confirming or refuting the diagnosis of LTBI in these patients.

The LOQ for the SiMoA IFNγ assay was lower than that for the QFT ELISA assay (0.002 IU/mL vs. 0.169 IU/mL) confirming that the IFNγ assay based on digital SiMoA technology is about 100-fold more sensitive than the QFT ELISA assay.

We obtained inter-assay CVs ranging from 10.5% to 21.3% for samples containing IFNγ concentrations ranging from 0.6 to 4.0 IU/mL for the QFT ELISA, which is similar to those indicated in the manufacturer's package insert and those previously reported; inter-assay CVs of 13% around an individual mean of ±0.47 IU/mL for all values (irrespective of the initial IFNγ value) and CVs of 30% around an individual mean of ±0.26 IU/ml for individuals with an initial borderline IFNγ response (in the range of 0.25–0.80 IU/mL) [26, 27]. The inter-assay CVs were smaller for the SiMoA IFNγ assay, ranging from 3.7% to 8.2% for samples containing IFNγ concentrations ranging from 0.05 to 1.04 IU/mL. This variability for samples with low IFNγ concentrations ranging from 0.01 to 0.40 IU/mL with the QFT assay has an important impact on the clinical interpretation of assay results and therefore the management of these patients. In addition, the manufacturer recommends only one assay per sample, which could result in a higher risk of false positive and negative results due to the poor precision.

We found good agreement for the clinical interpretation of results from the two assays, when we checked the metrological traceability of the SiMoA assay to the QFT IFNγ ELISA assay by regression analysis using the 35 positive samples, i.e. with a δTB1 and/or δTB2 $\geq$0.7 IU/mL.

In clinical practice, samples that have either an uncertain positive, uncertain negative or indeterminate result are retested with the T-SPOT-TB assay, which is a more sensitive TB IGRA assay that the QFT assay, to attempt to provide a clear clinical interpretation [36]. The advantage of T-SPOT-TB assay is that a standard number of PBMCs is used, which can correct for a patient's immune status, but a second blood sample has to be taken, which can be inconvenient for both clinicians and patients. The SiMoA IFNγ assay results, using the

original frozen samples, were consistent with the T-SPOT-TB assay results using the second blood samples from 6/14 patients (data not shown). Among the remaining eight samples, four that gave uncertain positive results with the QFT assay and negative results with T-SPOT-TB assay, were positive with the SiMoA IFNγ assay. This may be explained by the higher sensitivity of the SiMoA IFNγ assay and its measurement precision at low IFNγ concentrations, since in samples with low levels of secreted IFNγ, the SiMoA technology enables to distinguish between no response and a weak response to the TB antigens. Thus the SiMoA IFNγ assay could help to provide a clearer interpretation to guide clinical decisions about LTBI without the need for a second blood sample. Although, it has been reported that IGRA assays are sensitive to blood lymphocyte counts, we observed no link between lymphopenia and MIT - NIL values, although lymphocyte counts were only available for 41% of the samples [34, 37, 38].

The main limitation of this study relates to heterogeneity of the patient population since the cohort included immunosuppressed patients as well as immunocompetent patients and healthcare workers. However, this heterogeneity is representative of patients in a real-world setting that had undergone TB testing for a variety of reasons.

Our results suggest that the ultra-sensitive SiMoA IFNγ assay could be a useful tool for the identification of true positive and negative samples among those giving indeterminate or uncertain results with the currently used TB IGRA assay and therefore allow appropriate clinical management of the patients. Future studies should be conducted on defined populations at risk, such as individuals living with HIV, patients receiving anti-TNF therapy and those who have undergone organ transplant, to confirm the potential advantages of using a more sensitive detection method such as the SiMoA IFNγ assay.

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Conflicts of interests

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References

[1] World Health Organization. Tuberculosis 2018 [Last accessed 22 September 2018]. Available from: http://www.who.int/news-room/fact-sheets/detail/tuberculosis.

[2] Centres for Disease Control and Prevention. Tuberculosis 2018 [Last accessed 22 September 2018]. Available from: https://wwwnc.cdc.gov/travel/yellowbook/2018/infectious-diseases-related-to-travel/tuberculosis.

[3] World Health Organization. Guidelines on the management of latent tuberculosis infection. Spain: World Health Organization; 2015.

[4] Houben RM, Dodd PJ. The global burden of latent tuberculosis infection: a re-estimation using mathematical modelling. PLoS Med. 2016;13:e1002152.

[5] World Health Organization. Global tuberculosis report 2017 2017 [Last accessed 22 September 2018]. Available from: http://www.who.int/tb/publications/global_report/gtbr2017_main_text.pdf.

[6] Borisov AS, Bamrah Morris S, Njie GJ, Winston CA, Burton D, Goldberg S, et al. Update of recommendations for use of once-weekly isoniazid-rifapentine regimen to treat latent *Mycobacterium tuberculosis* infection. 2018;67:723–726. MMWR Morb Mortal Wkly Rep. 2018;67:723-6.

[7] Esmail H, Barry CE, 3rd, Young DB, Wilkinson RJ. The ongoing challenge of latent tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2014;369:20130437.

[8] Huebner RE, Schein MF, Bass JB, Jr. The tuberculin skin test. Clin Infect Dis. 1993;17:968-75.

[9] Brock I, Munk M E, Kok-Jensen A, Andersen P. Performance of whole blood IFN-gamma; test for tuberculosis diagnosis based on PPD or the specific antigens ESAT-6 and CFP-10. Int J Tuberc Lung Dis. 2001;5:462-7.

401 [10] Centres for Disease Control and Prevention. Centennial: Koch's discovery of the
402 tubercle bacillus. MMWR Morb Mortal Wkly Rep. 1982;31:121-3.
403 [11] Mazurek GH, LoBue PA, Daley CL, Bernardo J, Lardizabal AA, Bishai WR, et al.
404 Comparison of a whole-blood interferon gamma assay with tuberculin skin testing for
405 detecting latent Mycobacterium tuberculosis infection. JAMA. 2001;286:1740-7.
406 [12] Meier T, Eulenbruch HP, Wrighton-Smith P, Enders G, Regnath T. Sensitivity of a
407 new commercial enzyme-linked immunospot assay (T SPOT-TB) for diagnosis of
408 tuberculosis in clinical practice. Eur J Clin Microbiol Infect Dis. 2005;24:529-36.
409 [13] Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma
410 interferon release assays for detection of Mycobacterium tuberculosis Infection. Clin
411 Microbiol Rev. 2014;27:3-20.
412 [14] Strickland N, Muller TL, Berkowitz N, Goliath R, Carrington MN, Wilkinson RJ, et
413 al. Characterization of Mycobacterium tuberculosis-specific cells using MHC class II
414 tetramers reveals phenotypic differences related to HIV infection and tuberculosis disease. J
415 Immunol. 2017.
416 [15] Kobashi Y, Mouri K, Obase Y, Fukuda M, Miyashita N, Oka M. Clinical evaluation of
417 QuantiFERON TB-2G test for immunocompromised patients. Eur Respir J. 2007;30:945-50.
418 [16] Aichelburg MC, Tittes J, Breitenecker F, Reiberger T, Kohrgruber N, Rieger A.
419 Prognostic value of indeterminate IFN-gamma release assay results in HIV-1 infection. J Clin
420 Microbiol. 2012;50:2767-9.
421 [17] Huson MA, Hoogendijk AJ, de Vos AF, Grobusch MP, van der Poll T. The impact of
422 HIV infection on blood leukocyte responsiveness to bacterial stimulation in asymptomatic
423 patients and patients with bloodstream infection. J Int AIDS Soc. 2016;19:20759.

[18] Syed Ahamed Kabeer B, Sikhamani R, Swaminathan S, Perumal V, Paramasivam P, Raja A. Role of interferon gamma release assay in active TB diagnosis among HIV infected individuals. PloS One. 2009;4:e5718.

[19] Bao L, Li T, Diao N, Shen Y, Shao L, Zhang Y, et al. Fluctuating behavior and influential factors in the performance of the QuantiFERON-TB Gold In-Tube assay in the diagnosis of tuberculosis. PloS One. 2015;10:e0103763.

[20] Banaei N, Gaur RL, Pai M. Interferon gamma release assays for latent tuberculosis: what are the sources of variability? J Clin Microbiol. 2016;54:845-50.

[21] Herrera V, Yeh E, Murphy K, Parsonnet J, Banaei N. Immediate incubation reduces indeterminate results for QuantiFERON-TB Gold in-tube assay. J Clin Microbiol. 2010;48:2672-6.

[22] Yun JW, Chung HS, Koh WJ, Chung DR, Kim YJ, Kang ES. Significant reduction in rate of indeterminate results of the QuantiFERON-TB Gold In-Tube test by shortening incubation delay. J Clin Microbiol. 2014;52:90-4.

[23] Fabre V, Shoham S, Page KR, Shah M. High proportion of indeterminate QuantiFERON-TB Gold In-Tube results in an inpatient population Is related to host factors and preanalytical steps. Open Forum Infect Dis. 2014;1:ofu088.

[24] Qiagen. Gnowee – A QuantiFERON reference guide 2018 [Last accessed 22 September 2018]. Available from: http://us-tb.gnowee.net/technical-support.

[25] Schablon A, Harling M, Diel R, Ringshausen FC, Torres Costa J, Nienhaus A. Serial testing with an interferon-gamma release assay in German healthcare workers. GMS Krankenhhyg Interdiszip. 2010;5.

[26] Tagmouti S, Slater M, Benedetti A, Kik SV, Banaei N, Cattamanchi A, et al. Reproducibility of interferon gamma (IFN-gamma) release assays. A systematic review. Ann Am Thorac Soc. 2014;11:1267-76.

[27] Nemes E, Rozot V, Geldenhuys H, Bilek N, Mabwe S, Abrahams D, et al. Optimization and interpretation of serial QuantiFERON testing to measure acquisition of Mycobacterium tuberculosis infection. Am J Respir Crit Care Med. 2017;196:638-48.

[28] Rissin DM, Fournier DR, Piech T, Kan CW, Campbell TG, Song L, et al. Simultaneous detection of single molecules and singulated ensembles of molecules enables immunoassays with broad dynamic range. Anal Chem. 2011;83:2279-85.

[29] Wilson DH, Rissin DM, Kan CW, Fournier DR, Piech T, Campbell TG, et al.The Simoa HD-1 Analyzer: A novel fully automated digital immunoassay analyzer with single molecule sensitivity and multiplexing. J Lab Autom. 2016 Aug;21(4):533-47.

[30] O'Connell MA, Belanger BA, Haaland PD. Calibration and assay development using the four-parameter logistic model. Chemometr Intell Lab Syst. 1993;20:97-114.

[31] Qiagen. QuantiFERON®-TB Gold Plus (QFT®-Plus) package insert 2017 [Last accessed 22 September]. Available from: http://www.quantiferon.com/wp-content/uploads/2017/10/QFT-Plus-ELISA-IFU-L1095849-R02.pdf.

[32] Bilic-Zulle L. Comparison of methods: Passing and Bablok regression. Biochem Med (Zagreb). 2011;21:49-52.

[33] Jung HJ, Kim TJ, Kim HS, Cho YN, Jin HM, Kim MJ, et al. Analysis of predictors influencing indeterminate whole-blood interferon-gamma release assay results in patients with rheumatic diseases. Rheumatol Int. 2014;34:1711-20.

[34] Woo KS, Kim BG, Choi JL, Kim BR, Kim KH. Neutrophil-to-lymphocyte ratio Is associated with impaired interferon-gamma release to phytohemagglutinin. PloS One. 2015;10:e0125794.

[35] Gonzalez-Moreno J, Garcia-Gasalla M, Losada-Lopez I, Cifuentes Luna C, Mir Viladrich I, Fernandez-Baca V, et al. IGRA testing in patients with immune-mediated inflammatory diseases: which factors influence the results? Rheumatol Int. 2018;38:267-73.

[36] Person AK, Pettit AC, Sterling TR. Diagnosis and treatment of latent tuberculosis infection: an update. Curr Respir Care Rep. 2013;2:199-207.

[37] Komiya K, Ariga H, Nagai H, Teramoto S, Kurashima A, Shoji S, et al. Impact of peripheral lymphocyte count on the sensitivity of 2 IFN-gamma release assays, QFT-G and ELISPOT, in patients with pulmonary tuberculosis. Intern Med. 2010;49:1849-55.

[38] Lange B, Vavra M, Kern WV, Wagner D. Indeterminate results of a tuberculosis-specific interferon-gamma release assay in immunocompromised patients. Eur Respir J. 2010;35:1179-82.

484 **Table 1**: Rules for interpreting the results from the QuantiFERON-TB Gold Plus assay

Categories of results	**MIT - NIL**	δTB1 and δTB2	
		Manufacturer's recommendation	Uncertainty-categories
Indeterminate	<0.5 IU/mL	Not interpretable	Not interpretable
Negative	≥0.5 IU/mL	<0.35 IU/mL	<0.2 IU/mL
Uncertain negative	≥0.5 IU/mL	Not considered	[0.2 – 0.35[IU/mL*
Uncertain positive	≥0.5 IU/mL	Not considered	[0.35 – 0.7[IU/mL*
Positive	all	≥ 0.35 IU/mL*	≥0.7 IU/mL*

485 *MIT: positive control tube or mitogen; NIL: negative control tube: δTB1: TB1 - NIL; δTB2: TB2 - NIL;*
486 ** δTB1 or δTB2 >25% of NIL*
487

488 **Table 2:** Origin of the 151 samples assessed with the SiMoA IFNγ assay by classification

489 based on QFT IFNγ ELISA results

Classification of sample (N)	Total	Internal medicine	Rheumatology / Gastroenterology	Occupational medicine	Others**	Lymphocyte count available
Positive	35*	6	2	5	20	10
Negative	30	5	6	6	13	14
Uncertain positive	31*	8	3	6	12	11
Uncertain negative	25*	4	4	4	11	10
Indeterminate	30	10	5	0	15	17
Total	151	33	20	21	71	62 (41%)

490 * information missing for two samples in each category; ** from infectious medicine, pulmonology, neurology,
491 ophthalmology, nephrology and organ transplantation departments
492

493

Table 3: Reproducibility of QFT and SiMoA IFNγ assays

IFNγ sample mean (IU/mL)	Intra-assay CV (%)	Inter-assay CV (%)
QFT IFNγ ELISA assay (n=12)		
0.595	4.4	10.5
1.471	14.1	21.3
2.211	12.0	12.4
3.998	7.2	11.6
SiMoA IFNγ assay (n=12)		
0.045	2.7	8.2
0.080	3.2	3.7
0.484	3.7	4.5
1.037	3.4	7.4

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495

496 **Table 4:** Impact of lower MIT-NIL thresholds with the SiMoA assay on the classification of

497 30 samples from the QFT PLUS 'indeterminate' category

MIT - NIL threshold	Indeterminate	Interpretable	Interpretation based on δTB1 and δTB2
> 0.5 IU/mL	19/30		Not considered
		11/30	Negative
> 0.35 IU/mL	15/30		Not considered
		15/30	Negative
> 0.2 IU/mL	10/30		Not considered
		20/30	Negative
> 0.1 IU/mL	5/30		Not considered
		25/30	Negative

498

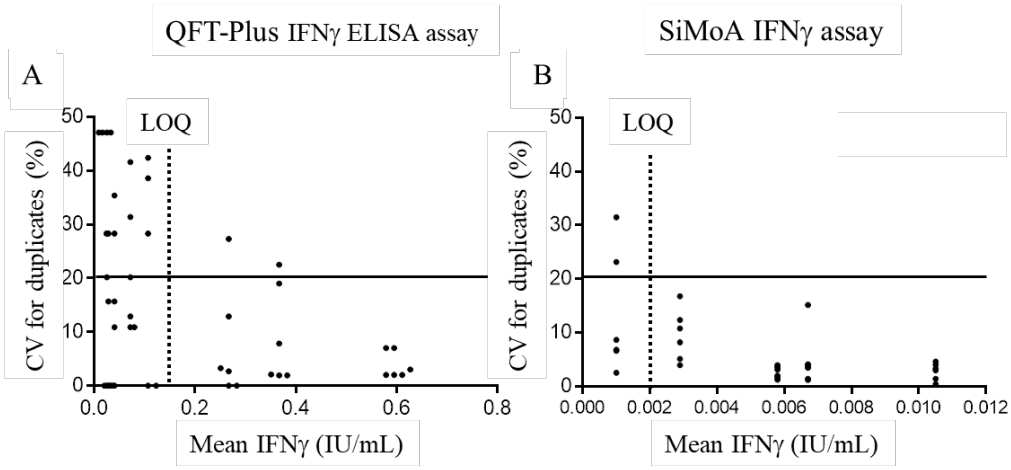
499

Table 6: Classification of the samples based on A) uncertainty categories and B) QFT Plus manufacturer's categories. The numbers of concordant samples for each classification are indicated in bold.

A		QFT IFNγ ELISA				
	Uncertainty categories	Negative (δTB1 or δTB2 < 0.2 IU/ml)	Uncertain negative (δTB1 or δTB2 [0.2 – 0.35[IU/ml)	Uncertain positive (δTB1 or δTB2 [0.35 – 0.7[IU/ml)	Positive (δTB1 or δTB2 > 0.7 IU/ml)	*Total*
SiMoA IFNγ	Negative (δTB1 or δTB2 < 0.2 IU/ml)	**28**	0	0	0	*28*
	Uncertain negative (δTB1 or δTB2 [0.2 – 0.35[IU/ml)	1	**7**	0	0	*8*
	Uncertain positive (δTB1 or δTB2 [0.35 – 0.7[IU/ml)	1	17	**8**	0	*26*
	Positive (δTB1 or δTB2 > 0.7 IU/ml)	0	1	23	**35**	*59*
	Total	*30*	*25*	*31*	*35*	*121*

B		QFT IFNγ ELISA		
	QFT Plus categories	Negative (δTB1 or δTB2 < 0.35 IU/ml)	Positive (δTB1 or δTB2 ≥ 0.35 IU/ml)	*Total*
SiMoA IFNγ	Negative (δTB1 or δTB2 < 0.35 IU/ml)	**36**	0	*36*
	Positive (δTB1 or δTB2 ≥ 0.35 IU/ml)	19	**66**	*85*
	Total	*55*	*66*	*121*

508 **Figure 1**: Limits of quantification (LOQ) for (A) QFT IFNγ ELISA and (B) SiMoA IFNγ
509 assays determined using the 20% CV for the lowest concentration samples in the repeatability
510 study



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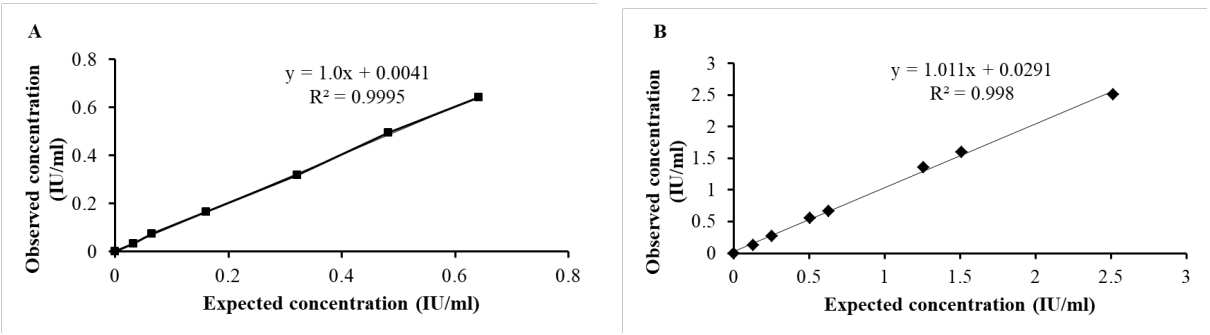
513 **Figure 2**: Linear regression curves with regression equations and R^2 values for SiMoA IFNγ
514 assay for (A) sample A, initial INFγ concentration 0.64 IU.mL and (B). sample B, initial INFγ
515 concentration 2.51 IU/mL



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519 **Figure 3**: Passing-Bablok correlation between (A) δTB1 and (B) δTB2 IFNγ concentrations
520 (IU/mL) obtained with the QFT IFNγ ELISA and SiMoA IFNγ assays using 35 positive blood
521 samples (δTB1 or δTB2 ≥0.7 IU/mL, as determined with the QFT IFNγ ELISA assay). The
522 grey dashed line indicates 100% agreement (slope =1.00).

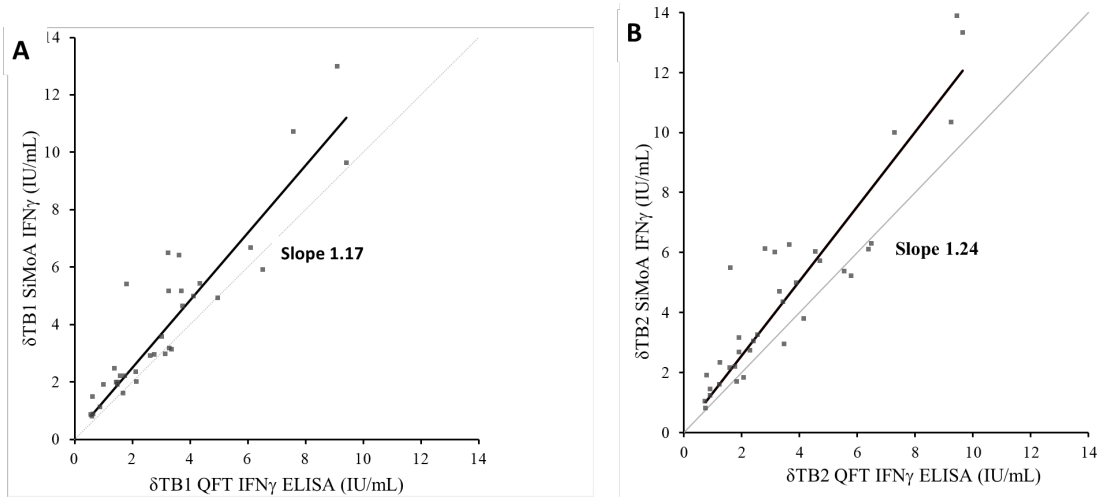

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Figure 4: Box and whisker plots of (A) NIL and (B) MIT responses (IU/mL) with the QFT ELISA and SiMoA IFNγ assays for samples with indeterminate results with the QFT Plus assay (n=30) showing median, interquartile range and the minimum and maximum values. P values were calculated by Wilcoxon test. The dashed lines denote the means. (C) Pairwise comparison for the MIT - NIL results from the QFT Plus and SiMoA IFNγ assays for the 30 samples with indeterminate results with the QFT Plus assay.

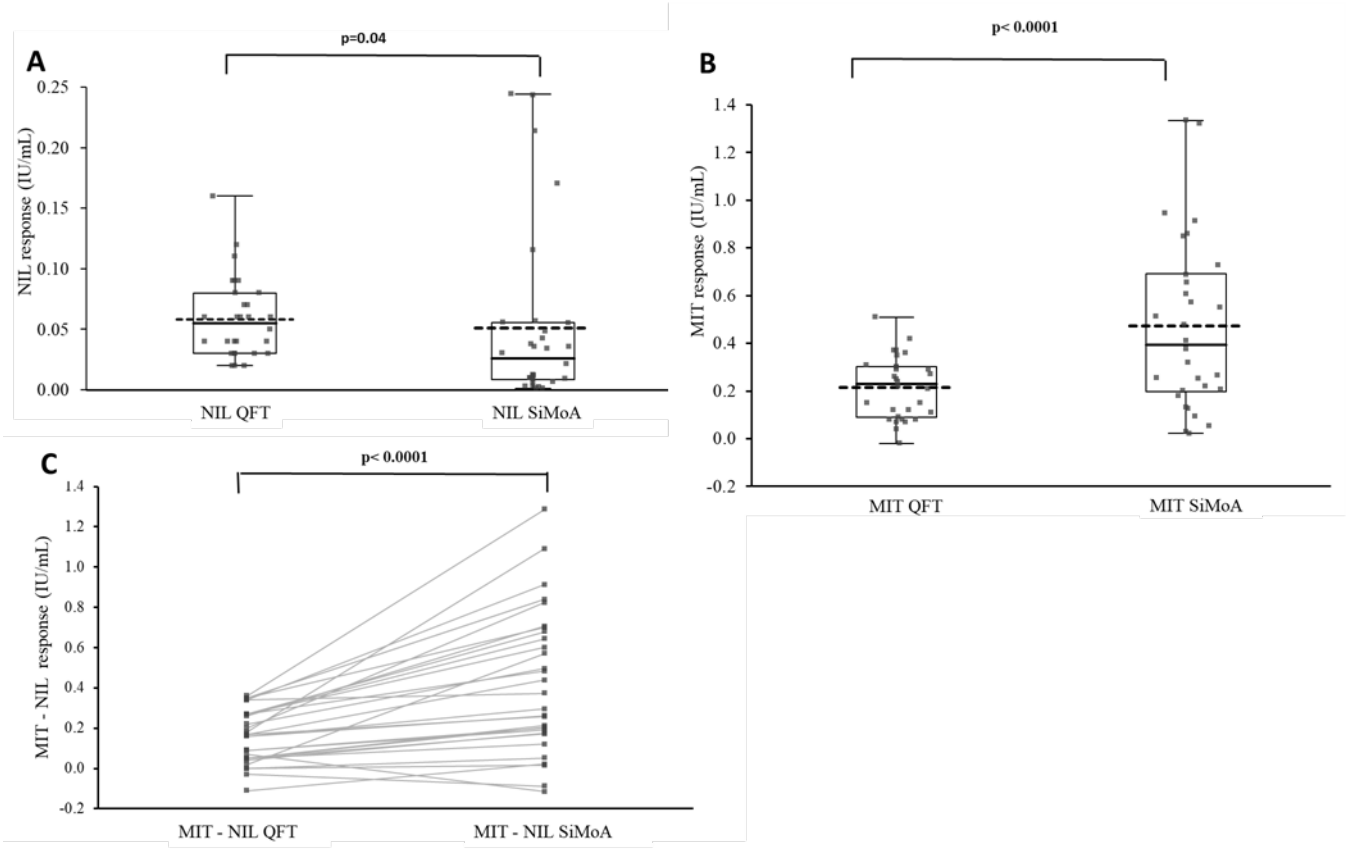

Figure 5: Passing-Bablok regression analyses of results from the QFT Plus δTB1 and δTB2 versus SiMoA δTB1 and δTB2. (A) QFT Plus δTB1 vs SiMoA δTB1 and (B) QFT Plus δTB2 vs SiMoA δTB2 using 25 uncertain negative blood samples (δTB1 or δTB2 with the QFT assay [0.2 – 0.35[IU/mL). (C) QFT Plus δTB1 vs SiMoA δTB1 and (D) QFT Plus δTB2 vs SiMoA δTB2 using 31 uncertain positive blood samples (δTB1 or δTB2 with the QFT Plus assay [0.35 – 0.7[IU/mL). The grey line indicates 100% agreement.