**APPENDIX**

**Supplementary panel: Measures of diagnostic accuracy and test performance**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| |  |  |  | | --- | --- | --- | |  | **Diseased** | **Non-diseased** | | **Test positive** | *a (true-positive)* | *b (false-positive)* | | **Test negative** | *c (false-negative)* | *d (true-negative)* |   **Sensitivity** is the probability that a patient with the target disease has a positive test result. Sensitivity is expressed as a percentage and calculated as follows:  *Sensitivity = (a / (a + c)) \* 100*  A test of high sensitivity will give high true-positive and low false-negative rates. Conversely, a test of low sensitivity will give low true-positive and high false-negative rates.  **Specificity** is the probability that a patient without the target disease has a negative test result. Specificity is expressed as a percentage and calculated as follows:  *Specificity = (d / (b + d)) \* 100*  A test of high specificity will give high true-negative and low false-positive rates. Conversely, a test of low specificity will give low true-negative and high false-positive rates.  **Positive likelihood ratio (PLR)** is the likelihood that a positive test result wold be expected in a patient with the target disease compared to the likelihood that a positive test result would be expected in a patient without the target disease. It is calculated as follows:  *PLR = (a / (a + c)) / (b / (b + d))*  A PLR of >1 indicates the positive test result is associated with the presence of disease; a PLR of <1 indicates that the positive test result is associated with absence of disease. The further the PLR from 1, the stronger the association.  **Negative likelihood ratio (NLR)** is the likelihood that a negative test result wold be expected in a patient with the target disease compared to the likelihood that a negative test result would be expected in a patient without the target disease. It is calculated as follows:  *NLR = (c / (a + c)) / (d / (b + d))*  A NLR of >1 indicates the negative test result is associated with the presence of disease; a NLR of <1 indicates that the negative test result is associated with absence of disease. The further the PLR from 1, the stronger the association.  **Positive predictive value (PPV)** is the probability that a patient with a positive test result actually has the target disease. PPV is often expressed as a percentage and calculated as follows:  *PPV = (a / (a + b)) \* 100*  **Negative predictive value (NPV)** is the probability that a patient with a negative test result truly does not have the target disease. NPV is often expressed as a percentage and calculated as follows:  *NPV = (d / (c + d)) \* 100* |

**Supplementary checklist: STARD**

|  |  |  |  |
| --- | --- | --- | --- |
| **Section & Topic** | **No** | **Item** | **Reported on page #** |
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| **TITLE OR ABSTRACT** |  |  |  |
|  | **1** | Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC) | 1, 5 |
| **ABSTRACT** |  |  |  |
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|  | **7** | On what basis potentially eligible participants were identified  (such as symptoms, results from previous tests, inclusion in registry) | 9 |
|  | **8** | Where and when potentially eligible participants were identified (setting, location and dates) | 9 |
|  | **9** | Whether participants formed a consecutive, random or convenience series | 9 |
| *Test methods* | **10a** | Index test, in sufficient detail to allow replication | 11 & Appendix pg 4-5 |
|  | **10b** | Reference standard, in sufficient detail to allow replication | 10, 26 (table 1) |
|  | **11** | Rationale for choosing the reference standard (if alternatives exist) | 10, 26 (table 1 footnote a) |
|  | **12a** | Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory | Appendix pg 4-5 |
|  | **12b** | Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory | 10, 26 (table 1) |
|  | **13a** | Whether clinical information and reference standard results were available to the performers/readers of the index test | 11 |
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| **RESULTS** |  |  |  |
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|  | **21a** | Distribution of severity of disease in those with the target condition | 27-28 (table 2), 29 (table 3), Appendix pg 6 (S.table 1) |
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|  | **24** | Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals) | 13-15, 30-31 (table 4) |
|  | **25** | Any adverse events from performing the index test or the reference standard | Not applicable |
| **DISCUSSION** |  |  |  |
|  | **26** | Study limitations, including sources of potential bias, statistical uncertainty, and generalisability | 18-19 |
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| **OTHER INFORMATION** |  |  |  |
|  | **28** | Registration number and name of registry | Not applicable |
|  | **29** | Where the full study protocol can be accessed | 9 |
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**Supplementary methods: Laboratory procedures**

QFT-GIT was carried out according to the manufacturer’s instructions (<http://www.quantiferon.com/wp-content/uploads/2018/03/L1075115_QFT_EU_ROW_>Rev005.pdf) and as described in Whitworth et al.6 In brief, blood was incubated overnight (16-24 hours) at 37°C in collection tubes containing a pool of Mtb-specific antigens (ESAT-6, CFP-10 and Rv2654) and positive (mitogen) and negative controls. Plasma was separated and stored at 4˚C prior to measurement of IFN-γ released in response to antigen stimulation by enzyme-linked immunosorbent assay (ELISA). Optical density readings were determined using a microplate reader (Elx800 Absorbance reader, VIC, Australia), and interferon-gamma (IFN-γ) levels calculated against a series of standard concentrations. The test was considered positive if the IFN-γ level for TB-specific antigens was ≥0.35 IU/mL after subtracting the negative control reading. It was considered indeterminate (invalid) if the negative control reading was >8.0 IU/ml and/or the positive control was <0.5 IU/ml.

T-SPOT.TB was carried out by enzyme-linked immunospot (ELISPOT) assay on PBMCs isolated from heparinised whole-blood (using the Ficoll Paque density centrifugation method), as per the manufacturer’s instructions (http://www.tspot.com/wp-content/uploads/2017/07/PI-TB-US-V6.pdf) and as described in Whitworth et al.6 The second-generation and ESAT-6-free IGRAs used the same platform and methodology as T-SPOT.TB. In brief, freshly-isolated PBMCs were suspended in serum-free AIM-V at a concentration of 2.5 million cells per ml. Cells were incubated overnight (18 hours; 37°C) with T-SPOT.TB antigens (ESAT-6, CFP-10), novel antigens (Rv3615c, Rv3879c), and positive (phytohemagglutinin (PHA)) and nil (RPMI medium) controls in a 96-well plate, pre-coated with IFN-γ specific monoclonal capture antibodies. For the novel antigens, peptide pools comprised 15-mer peptides overlapping their adjacent peptides by 10 amino acids representing the full sequence of Rv3615c (n=19 peptides) and previously defined selected sequences from Rv3879c (n=17 peptides; covering amino acid residues 1-95),1,13 as shown below.

|  |  |  |  |
| --- | --- | --- | --- |
| Rv3879c/1 | MSITRPTGSYARQML | Rv3615c/1 | MTENLTVQPERLGVL |
| Rv3879c/2 | PTGSYARQMLDPGGW | Rv3616c/2 | TVQPERLGVLASHHD |
| Rv3879c/3 | ARQMLDPGGWVEADE | Rv3615c/3 | RLGVLASHHDNAAVD |
| Rv3879c/4 | DPGGWVEADEDTFYD | Rv3615c/4 | ASHHDNAAVDASSGV |
| Rv3879c/5 | VEADEDTFYDRAQEY | Rv3615c/5 | NAAVDASSGVEAAAG |
| Rv3879c/6 | DTFYDRAQEYSQVLQ | Rv3615c/6 | ASSGVEAAAGLGESV |
| Rv3879c/7 | RAQEYSQVLQRVTDV | Rv3615c/7 | EAAAGLGESVAITHG |
| Rv3879c/8 | SQLVQRVTDVLDTCR | Rv3615c/8 | LGESVAITHGPYCSQ |
| Rv3879c/9 | RVTDVLDTCRQQKGH | Rv3615c/9 | AITHGPYCSQFNDTL |
| Rv3879c/10 | LDTCRQQKGHVFEGG | Rv3615c/10 | PYCSQFNDTLNVYLT |
| Rv3879c/11 | QQKGHVFEGGLWSGG | Rv3615c/11 | FNDTLNVYLTAHNAL |
| Rv3879c/12 | VFEGGLWSGGAANAA | Rv3615c/12 | NVYLTAHNALGSSLH |
| Rv3879c/13 | LWSGGAANAANGALG | Rv3615c/13 | AHNALGSSLHTAGVD |
| Rv3879c/14 | AANAANGALGANINQ | Rv3615c/14 | GSSLHTAGVDLAKSL |
| Rv3879c/15 | NGALGANINQLMTLQ | Rv3615c/15 | TAGVDLAKSLRIAAK |
| Rv3879c/16 | ANINQLMTLQDYLAT | Rv3615c/16 | LAKSLRIAAKIYSEA |
| Rv3879c/17 | LMTLQDYLATVITWH | Rv3615c/17 | RIAAKIYSEADEAWR |
|  |  | Rv3615c/18 | IYSEADEAWRKAIDG |
|  |  | Rv3615c/19 | DEAWRKAIDGLFT |

For each peptide, identity was confirmed by mass spectrometry, and purity exceeded 80%. Pooled peptides were diluted firstly in DMSO (25mg/ml) and secondly in RPMI. Each T-SPOT.TB antigen, novel antigen peptide pool or control was added to an individual well of the plate, with a final concentration for each T-SPOT.TB antigen or novel antigen peptide of 10μg/ml. The final concentration of DMSO per well ranged from 0.68% (for ESAT-6 and Rv3879) to 0.76% (for Rv3615c). After incubation, wells were washed with phosphate-buffered saline and an alkaline phosphatase-conjugated secondary IFN-γ specific monoclonal antibody was added. For a visible representation of the spots (spot-forming cells; SFCs) on the membrane, an alkaline-phosphatase chromogen substrate was added. SFCs were enumerated using an ELISPOT plate reader (AID ELISpot read system ELRIFL04, Advanced Imaging Devices GmbH, Straßberg, Germany).

The test was considered positive if the number of SFCs for the TB antigen minus the negative control was ≥8. Where this difference was 5, 6 or 7 SFCs, the assay was deemed borderline. Results were classified as indeterminate (invalid) if the positive control produced <20 SFCs and/or the negative control produced >10 SFCs.

All samples were processed within eight hours of blood collection.

Supplementary table 1: Medication history. Column percentages for each medication are shown.

| **Medication, n (%)** | **Diagnosis as per Reference Standard1** | | | | **Total**  **N = 845** |
| --- | --- | --- | --- | --- | --- |
| **Culture-confirmed TB**  **N = 261** | **Highly-probable TB**  **N = 102** | **Clinically indeterminate**  **N = 43** | **Active TB excluded**  **N = 439** |
| None | 63 (24.1) | 35 (34.3) | 13 (30.2) | 203 (46.2) | 314 (37.2) |
| Chemotherapy | 0 | 0 | 0 | 1 (0.2) | 1 (0.1) |
| Corticosteroids ≥15 mg/day | 20 (7.7) | 5 (4.9) | 5 (11.6) | 20 (4.6) | 50 (5.9) |
| Corticosteroids <15 mg/day | 13 (5.0) | 7 (6.9) | 1 (2.3) | 19 (4.3) | 40 (4.7) |
| Corticosteroids unknown | 1 (0.4) | 1 (1.0) | 0 | 0 | 2 (0.2) |
| Other immune suppressants | 1 (0.4) | 0 | 0 | 11 (2.4) | 12 (1.4) |
| Other | 191 (73.2) | 64 (62.7) | 30 (69.8) | 233 (53.1) | 518 (61.3) |
| Unknown | 1 (0.4) | 0 | 0 | 1 (0.2) | 2 (0.2) |

**Supplementary table 2: Symptoms at presentation.** Column percentages for each symptom are shown.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Symptom** | **Diagnosis as per Reference Standard1** | | | | **Total**  **N = 827a** |
| **Culture-confirmed TB**  **N = 256** | **Highly-probable TB**  **N = 99** | **Clinically indeterminate**  **N = 43** | **Active TB excluded**  **N = 429** |
| Cough, n (%) | 174 (68.0) | 53 (53.5) | 23 (53.5) | 326 (76.0) | 576 (69.6) |
| Fever, n (%) | 126 (49.2) | 49 (49.5) | 14 (32.6) | 195 (45.5) | 384 (46.4) |
| Night sweats, n (%) | 129 (50.4) | 53 (53.5) | 20 (46.5) | 215 (50.1) | 417 (50.4) |
| Weight loss, n (%) | 154 (60.2) | 54 (54.5) | 21 (48.8) | 211 (49.2) | 440 (53.2) |
| Haemoptysis, n (%) | 31 (12.1) | 8 (8.0) | 3 (7.0) | 65 (15.2) | 107 (12.9) |
| Lethargy, n (%) | 133 (52.0) | 56 (56.6) | 23 (53.5) | 222 (51.7) | 434 (52.5) |
| Other, n (%) | 163 (63.7) | 59 (59.46) | 25 (58.1) | 202 (47.1) | 449 (54.3) |
| Median no. of symptoms (range) | 4 (1–10) | 4 (1–8) | 3 (1–7) | 3 (1–10) | 4 (1–10) |

aEighteen participants were recruited on the basis of abnormal clinical signs rather than symptoms.

**Supplementary table 3: Cross-tabulation of T-SPOT.TB and QFT-GIT results for patients with active TB (active TB positive) and non-TB diagnoses (active TB-negative)**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **ACTIVE TB POSITVE** | | **T-SPOT.TB** | | | | | |
|  |  | **Positive** | **Negative** | **Borderline** | **Indeterminate** | **Missing** | **Total** |
| **QFT-GIT** | **Positive** | 187 | 13 | 6 | 9 | 5 | 220 |
| **Negative** | 49 | 41 | 8 | 7 | 2 | 107 |
| **Indeterminate** | 16 | 4 | 3 | 1 | 2 | 26 |
| **Missing** | 1 | 0 | 0 | 0 | 9 | 10 |
| **Total** | 253 | 58 | 17 | 17 | 18 | 363 |
| **ACTIVE TB NEGATIVE** | | **T-SPOT.TB** | | | | | |
|  |  | **Positive** | **Negative** | **Borderline** | **Indeterminate** | **Missing** | **Total** |
| **QFT-GIT** | **Positive** | 37 | 30 | 3 | 3 | 1 | 74 |
| **Negative** | 12 | 250 | 12 | 26 | 4 | 304 |
| **Indeterminate** | 2 | 36 | 1 | 8 | 0 | 47 |
| **Missing** | 0 | 3 | 0 | 0 | 11 | 14 |
| **Total** | 51 | 319 | 16 | 37 | 16 | 439 |

For patients with a definitive final diagnosis (categories 1, 2 and 4), there was 73% concordance in QFT-GIT and T-SPOT.TB positivity, and 77% concordance in negativity.

**Supplementary table 4: Cross-tabulation of T-SPOT.TB and second-generation IGRA results for patients with active TB (active TB positive) and non-TB diagnoses (active TB-negative)**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **ACTIVE TB POSITVE** | | **Second-generation IGRA** | | | | | |
|  |  | **Positivea** | **Negative** | **Borderline** | **Indeterminate** | **Missing** | **Total** |
| **T-SPOT.TB** | **Positive** | 253 | 0 | 0 | 0 | 0 | 253 |
| **Negative** | 16 | 33 | 9 | 0 | 0 | 58 |
| **Borderline** | 4 | 0 | 13 | 0 | 0 | 17 |
| **Indeterminate** | 0 | 0 | 0 | 17 | 0 | 17 |
| **Missing** | 0 | 0 | 0 | 0 | 18 | 18 |
| **Total** | 273 | 33 | 22 | 17 | 18 | 363 |
| **ACTIVE TB NEGATIVE** | | **Second-generation IGRA** | | | | | |
|  |  | **Positive** | **Negative** | **Borderline** | **Indeterminate** | **Missing** | **Total** |
| **T-SPOT.TB** | **Positive** | 51 | 0 | 0 | 0 | 0 | 51 |
| **Negative** | 19 | 296 | 4 | 0 | 0 | 319 |
| **Borderline** | 4 | 0 | 12 | 0 | 0 | 16 |
| **Indeterminate** | 0 | 0 | 1 | 36 | 0 | 37 |
| **Missing** | 0 | 0 | 0 | 0 | 16 | 16 |
| **Total** | 74 | 296 | 17 | 36 | 16 | 439 |

aOf 20 additional TB cases detected by second-generation IGRA (compared to T-SPOT.TB), only three responses to ESAT-6 and one to CFP-10 were borderline.

**Supplementary table 5: Response magnitudes to individual antigens included in T-SPOT.TB and second-generation IGRA**

|  | **Dosanjh category** | | | | | | | | **Total** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **1** | **2** | **3** | **4A** | **4B** | **4C** | **4D** | **4A-D** |  |
| Spot-forming cells, median (IQR) |  |  |  |  |  |  |  |  |  |
| ESAT-6 | 14 (3–40) | 13 (1–46) | 0 (0–5) | 0 (0–1) | 2 (0–10) | 0 (0–1) | 0 (0–0) | 0 (0–1) | 1 (0–14) |
| CFP-10 | 18 (4–64) | 13 (1–70) | 1 (0–12) | 0 (0–0) | 1 (0–12) | 0 (0–1) | 0 (0–0) | 0 (0–1) | 1 (0–17) |
| Rv3615c | 25 (6–59) | 19 (2–60) | 1 (0–23) | 0 (0–1) | 1 (0–9) | 0 (0–2) | 0 (0–1) | 0 (0–2) | 2 (0–27) |
| Rv3879c | 2 (0–10) | 1 (0–10) | 0 (0–1) | 0 (0–0) | 0 (0–1) | 0 (0–1) | 0 (0–1) | 0 (0–1) | 0 (0–2) |

IQR: Interquartile range

**Supplementary table 6: Diagnostic accuracy of current and second-generation IGRAs for diagnosis of active TB among patients with HIV-infection.** Sensitivity and specificity are presented as percentages.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Test performance** | **T-SPOT.TBa** | | **QFT-GITa** | | **ESAT+ CFP10 + Rv3615ca** | | **CFP10 + Rv3615c + Rv3879ca** | | | |
| **n/N** | **Estimate (95% CI)** | **n/N** | **Estimate (95% CI)** | **n/N** | **Estimate (95% CI)** | **n/N** | **Estimate (95% CI)** | | |
| Sensitivity for active TB |  |  |  |  |  |  |  |  | | |
| All TB | 12/19 | 63.2 (41.0–80.9) | 13/23 | 56.5 (36.8–74.4) | 12/17 | 70.6 (46.9–86.7) | 11/16 | | 68.8 (44.4–85.8) |
| Culture-confirmed TBb | 7/11 | 63.6 (35.4–84.8) | 8/13 | 61.5 (35.5–82.3) | 7/9 | 77.8 (45.3–93.7) | 7/9 | 77.8 (45.3–93.7) | | |
| Specificity for active TB |  |  |  |  |  |  |  |  | | |
| Active TB excluded | 71/76 | 93.4 (85.5–97.2) | 80/87 | 92.0 (84.3–96.1) | 70/76 | 92.1 (83.8–96.3) | 67/77 | 87.0 (77.7–892.8) | | |
| Active TB excluded, TST-negative, no risk factors for LTBI | 28/29 | 96.6 (82.8–99.4) | 36/38 | 94.7 (82.7–98.5) | 27/28 | 96.4 (82.3–99.4) | 27/29 | 93.1 (78.0–98.1) | | |

LTBI, latent tuberculosis infection; TST, tuberculin skin test.

aOne QFT-GIT, and two T-SPOT.TB and second-generation IGRA results were missing due to blood draw difficulties, samples being unsuitable for testing, or samples being destroyed for laboratory reasons. Missing results were spread across all diagnostic categories.

bThirty-three HIV-positive patients had indeterminate T-SPOT.TB results, and 22 had indeterminate QFT-GIT results. Indeterminate and borderline IGRA results were excluded from analyses.

**Supplementary table 7: Diagnostic accuracy of current and second-generation IGRAs for diagnosis of active TB among patients with Diabetes.** Sensitivity and specificity are presented as percentages.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Test performance** | **T-SPOT.TBa** | | **QFT-GITa** | | **ESAT+ CFP10 + Rv3615ca** | | **CFP10 + Rv3615c + Rv3879ca** | | | |
| **n/N** | **Estimate (95% CI)** | **n/N** | **Estimate (95% CI)** | **n/N** | **Estimate (95% CI)** | **n/N** | **Estimate (95% CI)** | | |
| Sensitivity for active TB |  |  |  |  |  |  |  |  | | |
| All TB | 16/24 | 66.7 (46.7–82.0) | 15/27 | 55.6 (37.3–72.4) | 18/21 | 85.7 (65.4–95.0) | 18/21 | | 85.7 (65.4–95.0) |
| Culture-confirmed TBb | 13/20 | 65.0 (43.3–81.9) | 12/22 | 54.5 (34.7–73.1) | 14/17 | 82.4 (59.0–93.8) | 14/17 | 82.4 (59.0–93.8) | | |
| Specificity for active TB |  |  |  |  |  |  |  |  | | |
| Active TB excluded | 38/47 | 80.9 (67.5–89.6) | 37/47 | 78.7 (65.1–88.0) | 34/48 | 70.8 (56.8–81.8) | 34/47 | 72.3 (58.2–83.1) | | |
| Active TB excluded, TST-negative, no risk factors for LTBI | 6/7 | 85.7 (48.7–97.4) | 4/5 | 80.0 (37.6–96.4) | 6/7 | 85.7 (48.7–97.4) | 6/7 | 85.7 (48.7–97.4) | | |

LTBI, latent tuberculosis infection; TST, tuberculin skin test.

aTwo QFT-GIT,and four T-SPOT.TB and second-generation IGRA results were missing due to blood draw difficulties, samples being unsuitable for testing, or samples being destroyed for laboratory reasons. Missing results were spread across all diagnostic categories.

bTwo diabetic patients had indeterminate T-SPOT.TB results, and four had indeterminate QFT-GIT results. Indeterminate and borderline IGRA results were excluded from analyses.

**Supplementary table 8: Diagnostic accuracy of T-SPOT.TB and second-generation IGRAs for diagnosis of active TB using ≥5 vs ≥8 SFC cut-off criteria for scoring positive results.** Sensitivity and specificity are presented as percentages (95% CI).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Test performance** | **T-SPOT.TB** | | **ESAT+ CFP10 + Rv3615c** | | **CFP10 + Rv3615c + Rv3879c** | |
| **Borderline excludeda**  **(cut-off ≥8)** | **Borderline includedb**  **(cut-off ≥5)** | **Borderline excludeda**  **(cut-off ≥8)** | **Borderline includedb**  **(cut-off ≥5)** | **Borderline excludeda**  **(cut-off ≥8)** | **Borderline includedb**  **(cut-off ≥5)** |
| Sensitivity for active TB |  |  |  |  |  |  |
| All TB | 81.4 (76.6–85.3) | 82.3 (77.8–86.1) | 89.2 (85.2–92.2) | 89.9 (86.2–92.7) | 88.0 (83.8–91.2) | 89.0 (85.1–91.9) |
| Culture-confirmed TB | 84.9 (79.5–89.0) | 85.9 (80.9–89.8) | 94.0 (90.0–96.4) | 94.4 (90.7–96.7) | 93.4 (89.2–96.0) | 94.0 (90.2–96.4) |
| Specificity for active TB excluded | 86.2 (82.3–89.4) | 82.6 (78.6–86.1) | 80.0 (75.6–83.8) | 76.5 (72.0–80.4) | 79.6 (75.2–83.4) | 76.3 (71.8–80.3) |

aAnalyses *exclude* borderline IGRA results, giving a cut-off for a positive test result of *≥8* SFCs after subtraction of negative control.

bAnalyses *include* borderline IGRA results, giving a cut-off for a positive test result of *≥5* SFCs after subtraction of negative control.

*NB: References referred to in these Supplementary Materials are listed in the References section of the primary manuscript.*