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QuantiFERON-TB Gold Plus with Chemiluminescence Immunoassay: Do We Need a Higher Cutoff?

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(Article begins on next page)

1     **Quantiferon-TB Gold Plus by CLIA: Do we need a higher cut-off?**

2

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4

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7

8     **Running head:** Higher cut-off for QFT-Plus by CLIA?

9

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12

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14 **ABSTRACT**

15

16 QuantiFERON-TB Gold Plus (QFT-Plus) is the most widely used interferon- $\gamma$  release assay (IGRA) for  
17 the diagnosis of latent tuberculosis infection (LTBI). The aim of this study was to compare QFT-Plus  
18 results by enzyme-linked immunoassay (ELISA) on the SKYLAB system with those obtained with  
19 chemiluminescence immunoassay (CLIA) on the LIAISON XL analyzer.

20 Agreement between the two assays was evaluated on 419 QFT-Plus blood samples, and was found to  
21 be substantial (75.4%): higher agreement was found for positive (95.4%) and negative (80.4%)  
22 results, while most discordances were due to ELISA-indeterminate/CLIA-determinate results.

23 According to the Italian Clinical Microbiologist Association recommendations, in samples (n=79)  
24 with a borderline result in ELISA (0.20-0.70 IU/ml), CLIA median values statistically increased  
25 (from 0.29 to 0.59 IU/ml for TB1 and from 0.32 to 0.60 IU/ml for TB2), but remained in the borderline  
26 range.

27 Linear regression analysis indicated a substantial correlation between ELISA and CLIA for antigen  
28 tubes TB1 (Pearson's  $r=0.8666$ ) and TB2 (Pearson's  $r=0.8728$ ), but CLIA produced higher values  
29 than ELISA. ROC analysis showed that the optimal cut-off value in CLIA was 0.45 IU/ml for TB1  
30 and 0.46 IU/ml for TB2.

31 In conclusion, automated QFT-Plus with CLIA is comparable to QFT-Plus performed by ELISA.  
32 Within the linearity range of the test, CLIA detects higher quantitative values than ELISA, resulting  
33 in a higher number of determinate results, and the conversion of samples that were close to the cut-  
34 off into positive borderline results. A higher cut-off for QFT-CLIA needs to be defined, based on  
35 clinical diagnostic criteria.

## 36 INTRODUCTION

37 Latent tuberculosis infection (LTBI) is defined as a state of persistent immune response to  
38 *Mycobacterium tuberculosis* complex (MTBc) without clinically manifested evidence of active  
39 tuberculosis (TB) disease. About 1.7 billion people worldwide are estimated to have LTBI and 5–  
40 10% of them are at risk of developing active TB during their lifetime (1-5).

41 Two tests are available for the identification of LTBI: the tuberculin skin test (TST) and interferon  
42 gamma (IFN- $\gamma$ ) release assays (IGRAs). These are indirect markers of MTBc exposure and indicate  
43 a cellular immune response to MTBc. One IGRA, Quantiferon-TB Gold Plus (QFT-Plus, Qiagen)  
44 measures IFN- $\gamma$  released by T-cells following stimulation by MTBc-specific antigens (6). QFT-Plus  
45 contains two MTBc-specific antigen tubes, called TB1 and TB2: TB1 contains ESAT-6- and CFP-  
46 10-derived long peptides, designed to elicit cell-mediated immune responses from CD4+T-helper  
47 lymphocytes; TB2 contains the same long peptides as TB1, in addition to shorter peptides able to  
48 stimulate CD8 T-cells (6-8). As CD8+ T-cell response seems to play a role in the early phase of  
49 MTBc infection and in reactivation from LTBI, the QFT-Plus test might be useful in identifying  
50 recent and remote LTBI, facilitating the decision to start LTBI treatment (9,10). IFN- $\gamma$  detection with  
51 QFT-Plus assay is almost exclusively performed with enzyme-linked immunosorbent assay (ELISA),  
52 which has some disadvantages in clinical laboratories, such as labour-intense and time-consuming  
53 steps and requiring standard serial dilutions for each microplate.

54 Recently, new chemiluminescence immunoassays (CLIA) have been developed to detect IFN- $\gamma$  in  
55 human plasma samples. However, to date only few studies have been published comparing QFT-Plus  
56 and the previous version QFT-TB Gold In-Tube by ELISA to CLIA (11-13). Among these, the study  
57 with the most relevant sample size (341 samples) reports a high degree of agreement (99.1%) between  
58 the two methods, using the AdvaSure I3 platform for CLIA (13).

59 A new fully-automated CLIA detection system to measure IFN- $\gamma$  in human plasma has recently been  
60 developed on the LIAISON XL analyzer (DiaSorin, Italy). CLIA repeatability and reproducibility on  
61 this platform were studied by Brantestig, who found that the imprecision of the method is within an

62 acceptable range and analysis of linearity showed acceptable recovery (12). Furthermore, a recent  
63 paper by De Maertaelere et al. conducted on 92 samples showed that CLIA gave significantly higher  
64 values for TB1 and TB2 than ELISA (11).

65 The aim of this study was the head-to-head comparison of IFN- $\gamma$  detection by ELISA on SKYLAB  
66 system and CLIA on the LIAISON XL analyzer in a large number of plasma samples. Furthermore,  
67 we compared quantitative IFN- $\gamma$  responses to MTBc antigens (TB1 and TB2) and Mitogen detected  
68 by both methods.

69

70

## 71 MATERIAL AND METHODS

72

### 73 *Samples*

74 In this study, 419 clinical specimens which had been submitted to the Microbiology Unit of S. Orsola-  
75 Malpighi University Hospital (Bologna, Italy) for QFT-Plus test by ELISA were also analysed by  
76 CLIA.

77 Sample selection was based on the 3 categories of ELISA QFT-Plus results (positive, negative,  
78 indeterminate) according to the manufacturer's cut-off and having a sufficient volume to perform  
79 CLIA. Furthermore, an additional category of samples defined borderline was included according to  
80 Italian Clinical Microbiologist Association recommendations. Sample size for each category was  
81 chosen to ensure a large enough number to perform the analysis on ELISA indeterminate and  
82 borderline results since they could be greatly influenced by different methods of measurement.

83 Samples were anonymized with an alphanumeric code according to the ELISA qualitative result.

84 Clinical data were not collected for this study. Informed consent was not required as the data were  
85 analysed anonymously. The study was conducted in accordance with the Declaration of Helsinki.

86

87

88 ***Quantiferon-TB Gold Plus (QFT-Plus)***

89 QFT-Plus samples (Qiagen, Germany) were analyzed by ELISA on SKYLAB automated system  
90 (DASIT, Italy) and by CLIA on the LIAISON XL instrument (DiaSorin, Italy), according to the  
91 standard procedures recommended by the manufacturers (6,14).

92 The clinical samples were handled according to the standard procedure for QFT-Plus assay, i.e.  
93 incubation at 37°C for 16–24 h within 16 hours of sampling, followed by centrifugation at 2700 g at  
94 room temperature for 15 minutes, and IFN- $\gamma$  detection by ELISA. For this study selected samples  
95 were promptly frozen at -20°C after ELISA to assure IFN- $\gamma$  stability (15,16). Before CLIA testing,  
96 frozen samples (range 1-101 days) were re-centrifuged at 2700g for 15 minutes to sediment the fibrin  
97 clots that can form during storage.

98 In accordance to the manufacturer's interpretation, positive results were defined as background (Nil)-  
99 corrected MTBc antigens (TB1 and/or TB2) values of  $\geq 0.35$  IU IFN- $\gamma$ /ml; if the Nil-corrected  
100 Mitogen value was  $< 0.50$  IFN- $\gamma$  IU/ml and/or if the Nil value was  $> 8.0$  IFN- $\gamma$  IU/ml the test was  
101 considered indeterminate.

102 Furthermore, according to the Italian Clinical Microbiologist Association recommendations, the  
103 category borderline was defined as Nil-corrected MTBc antigens (TB1 and/or TB2) values within the  
104 range 0.20-0.70 IFN- $\gamma$  IU/ml (17).

105

106 ***Statistical analysis***

107 Cohen's  $\kappa$  statistics were used to assess agreement between ELISA and CLIA QFT Plus results as  
108 well as agreement between TB1 and TB2 results for each assay.

109 The Mann-Whitney test was used to compare medians of Nil-corrected IFN- $\gamma$  responses to TB1 and  
110 TB2 and Mitogen. Since the ELISA QFT-Plus test cannot accurately determine IFN- $\gamma$  values  $> 10$   
111 IU/ml, a value of 10 IU/ml was attributed to plateau values in all the analyses by convention, as  
112 already adopted in the literature (18).

113 Samples with TB1 and TB2 IFN- $\gamma$  levels within the analytical range of each assay ( $<10$  IU/ml),  
114 excluding indeterminate results, were used to assess the correlation between ELISA and CLIA.  
115 Correlation was expressed by Pearson's correlation coefficient ( $r$ ). For this group, the optimal cut-off  
116 values of CLIA for TB1 and TB2 were determined from receiver-operator characteristic (ROC) curve  
117 analysis assuming the positive result of the ELISA method as true LTBI or TB.  
118 Statistical analysis was performed using GraphPad Prism version 8.0.1 (USA). Statistical significance  
119 was set at  $p < 0.05$ .

120

121

## 122 RESULTS

123

### 124 *Agreement between ELISA and CLIA QFT-Plus results*

125 A total of 419 QFT-TB Plus samples analyzed by ELISA were included in this study with the  
126 following results: 153 (36.5%) positive, 168 (40.1%) negative, 97 (23.2%) indeterminate due to low  
127 Nil-corrected Mitogen value ( $<0.50$  IFN- $\gamma$  IU/ml) and 1 (0.2%) indeterminate due to high Nil value  
128 ( $>8.0$  IFN- $\gamma$  IU/ml) according to the manufacturer's cut-off. The same QFT-Plus samples were then  
129 processed by CLIA and produced the following results: 182 (43.4%) positive, 197 (47.0%) negative,  
130 34 (8.1%) indeterminate due to low Nil-corrected Mitogen value and 6 (1.5%) indeterminate due to  
131 high Nil value.

132 The comparison of the results obtained by both assays is reported in Table 1. Concordant results were  
133 obtained for 316 out of 419 samples (agreement 75.4%,  $\kappa=0.61$ , 95% CI 0.55-0.67). The agreement  
134 between TB1 and TB2 results was 95.7% for ELISA ( $\kappa=0.91$ , 95% CI 0.86-0.95), and 95.0% for  
135 CLIA ( $\kappa=0.90$ , 95% CI 0.85-0.94).

136 Of the 103 (24.6%) samples with discordant results, 63 (61.2%) were due to indeterminate ELISA  
137 results, which were determinate with CLIA (60 negative and 3 positive). Median mitogen IFN- $\gamma$  value  
138 in these samples was 0.34 IU/ml in ELISA and 0.94 IU/ml in CLIA. In contrast, median mitogen

139 IFN- $\gamma$  value in 34 samples which remained indeterminate in CLIA was 0.16 IU/ml in ELISA and 0.27  
140 IU/ml in CLIA, excluding 1 indeterminate case due to high Nil value. These differences were  
141 statistically significant ( $p < 0.0001$ ).

142 The discordant cases with a determinate ELISA result were: 2 ELISA-positive/CLIA-negative (both  
143 with only 1 MTBc antigen tube positive in ELISA), 5 ELISA-positive/CLIA-indeterminate (all due  
144 to high Nil values in ELISA with a median value of 6.78 IU/ml), 33 ELISA-negative/CLIA-positive  
145 (median values of 0.21 and 0.24 IU/ml with ELISA statistically lower than median values of 0.49 and  
146 0.51 IU/ml with CLIA for TB1 and TB2, respectively,  $p < 0.0001$ ).

147 Results interpreted according to the Italian Clinical Microbiologist Association recommendations by  
148 introducing the category “borderline” (TB1 and/or TB2 values within 0.20-0.70 IFN- $\gamma$  IU/ml), are  
149 reported in Table 2. Concordant results were obtained for 295 of the 419 samples (70.4%,  $\kappa = 0.59$ ,  
150 95% CI 0.54-0.65). In samples with a borderline result in ELISA ( $n = 79$ ), CLIA median values  
151 statistically increased from 0.29 to 0.59 IU/ml for TB1 and from 0.32 to 0.60 IU/ml for TB2  
152 ( $p < 0.0001$ ).

#### 154 ***Correlation between ELISA and CLIA TB1 and TB2 IFN- $\gamma$ levels***

155 For MTBc antigen tubes results within the linearity range 0-10 IU/ml and excluding indeterminate  
156 results ( $n = 301$ ), linear regression analysis showed that there was substantial correlation between the  
157 two tests, both for TB1 (Pearson's  $r = 0.8666$ ) and TB2 (Pearson's  $r = 0.8728$ ) (Figure 1A, 1B).  
158 Furthermore, the regression slopes (1.094 for TB1, 1.177 for TB2) and the intercepts (+0.2606 for  
159 TB1, +0.2521 for TB2) indicated that CLIA produces significantly higher values both for TB1 and  
160 TB2 than ELISA ( $p < 0.0001$ ). In fact, in this group median IFN- $\gamma$  values of MTBc antigen tubes were  
161 statistically higher in CLIA than in ELISA both for TB1 (0.42 vs. 0.21 IU/ml,  $p = 0.0039$ ) and TB2  
162 (0.40 vs. 0.22 IU/ml,  $p = 0.0047$ ).

163 Area Under the Curve (AUC) results for TB1 and TB2 are reported in Figure 2A and 2B respectively.  
164 For TB1 AUC was 0.978 (95% CI 0.962-0.994,  $< 0.0001$ ) and the cut-off value with the maximal sum

165 of sensitivity and specificity was  $>0.45$  IFN- $\gamma$  IU/ml (sensitivity 97.6%, specificity 85.9%). Using the  
166 manufacturer's suggested cut-off value of 0.35 IFN- $\gamma$  IU/ml, the results showed a comparable  
167 sensitivity 99.2%, but a lower specificity 81.9%.

168 For TB2 AUC was 0.980 (95% CI 0.964-0.996,  $<0.0001$ ) and the cut-off value with the maximal sum  
169 of sensitivity and specificity was  $>0.46$  IFN- $\gamma$  IU/ml (sensitivity 99.2%, specificity 88.7%). Using the  
170 manufacturer's suggested cut-off value of 0.35 IFN- $\gamma$  IU/ml, the results showed the same sensitivity  
171 99.2%, but a lower specificity 81.9%.

172

173

## 174 DISCUSSION

175 In this study we compared the QFT-Plus routinely performed by ELISA in our laboratory with the  
176 automated CLIA performed on the LIAISON XL instrument on a large number of selected samples  
177 ( $n=419$ ). In particular, we focused our analysis on ELISA indeterminate and borderline results since  
178 they could be greatly influenced by different methods of measurement.

179 We found substantial agreement (75.4%) between the assays interpreted according to the  
180 manufacturer's cut-off. Most discordant results (61.2%,  $n=63$ ) were due to indeterminate ELISA  
181 which were determinate in CLIA. In literature only a few studies have been published regarding this  
182 comparison, on a smaller number of samples, reporting higher agreement between the two tests: De  
183 Maertelaere et al. described a population of 92 samples with 4.3% of indeterminate ELISA and found  
184 an overall agreement of 95% (11); Brantestig et al. analyzed 125 samples with 8% indeterminate  
185 ELISA and found an agreement of 96.8% (12); Kim and colleagues reported an overall agreement of  
186 99.12% on 341 samples (13). The near perfect agreement obtained by Kim et al. was probably due to  
187 the lack of indeterminate cases (0.3%) in their sample population. In contrast, in our population  
188 indeterminate ELISA due to low Mitogen value ( $<0.50$  IFN- $\gamma$  IU/ml) accounted for 23.2%.

189 The high number of indeterminate results selected for this study allowed us to show that ELISA  
190 median Mitogen IFN- $\gamma$  value in samples which converted to a determinate result in CLIA was 0.34  
191 IU/ml, significantly higher than those which remained indeterminate in CLIA (0.16 IU/ml).  
192 Further discordant results (32%, n=33) were ELISA-negative/CLIA-positive, confirming that CLIA  
193 detects higher quantitative values than ELISA. However, in this group ELISA median TB1 and TB2  
194 values were close to the cut-off (0.21 and 0.24 IFN- $\gamma$  IU/ml for TB1 and TB2, respectively), as were  
195 the corresponding CLIA values (0.49 and 0.51 IFN- $\gamma$  IU/ml for TB1 and TB2, respectively).  
196 The Italian Clinical Microbiologist Association recently suggested defining borderline results as TB1  
197 and/or TB2 values within the range 0.20-0.70 IFN- $\gamma$  IU/ml and recommended re-testing borderline  
198 samples. According to this recommendation, agreement between the two assays was moderate  
199 (70.4%), lower than the agreement observed when the manufacturer's cut-off was used. Similarly,  
200 Brantestig et al. found a lower agreement (88%) using the Swedish National recommendations, that  
201 define a broad borderline range (0.20-0.99 IFN- $\gamma$  IU/ml), than applying the manufacturer's cut-off  
202 (96.8%) (12). However, in our study among the ELISA-borderline samples (n=79) the increased  
203 CLIA values remained in the borderline range (0.29 vs. 0.59 IU/ml for TB1 and 0.32 vs. 0.60 IU/ml  
204 for TB2), suggesting that this range may not need to be revised for CLIA.  
205 Linear regression analysis indicated substantial correlation between ELISA and CLIA despite the two  
206 different methods of measurement; however, CLIA produced significantly higher values both for TB1  
207 and TB2 than ELISA. This is in agreement with previous data on CLIA performance in a smaller  
208 study population (11). In our opinion, this difference is not due to pre-analytical factors, but rather to  
209 the intrinsic chemistry of the assay based on chemiluminescence technology with paramagnetic  
210 microparticle solid phase, allowing the detection of very low levels of IFN- $\gamma$  (13,19).  
211 AUC analysis indicated that cut-off values of 0.45 IU/ml for TB1 and 0.46 IU/ml for TB2 returned  
212 the maximal sum of sensitivity and specificity, suggesting the need of a higher cut-off for QFT-Plus  
213 with CLIA compared to ELISA.

214 The limitation of our study is the lack of clinical data; further studies on a larger sample size with  
215 medical records available should be performed to more clearly define the CLIA threshold on the  
216 LIAISON XL system.

217 In conclusion, QFT-Plus performed with CLIA showed substantial agreement with ELISA. The  
218 LIAISON XL analyzer has several advantages such as rapid turn-around time, high analytical  
219 measurement ranges and good precision. Within the linearity range of the test, CLIA detects higher  
220 quantitative values than ELISA, resulting in a higher number of determinate results, and the  
221 conversion of negative samples close to the cut-off into positive borderline results. A higher cut-off  
222 for QFT-CLIA needs to be defined, based on clinical diagnostic criteria.

223

224

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229

230

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285 **FIGURE LEGENDS**

286

287 **FIG 1: Regression analysis of TB1 (A) and TB2 (B) IFN- $\gamma$  levels between ELISA and CLIA**  
288 **QFT Plus.** Regression line (solid) and 95% confidence intervals (dotted) for the Nil-subtracted  
289 antigen tubes, within the range 0-10 IU/ml, are plotted;  $r$ =Pearson's correlation coefficient.

290

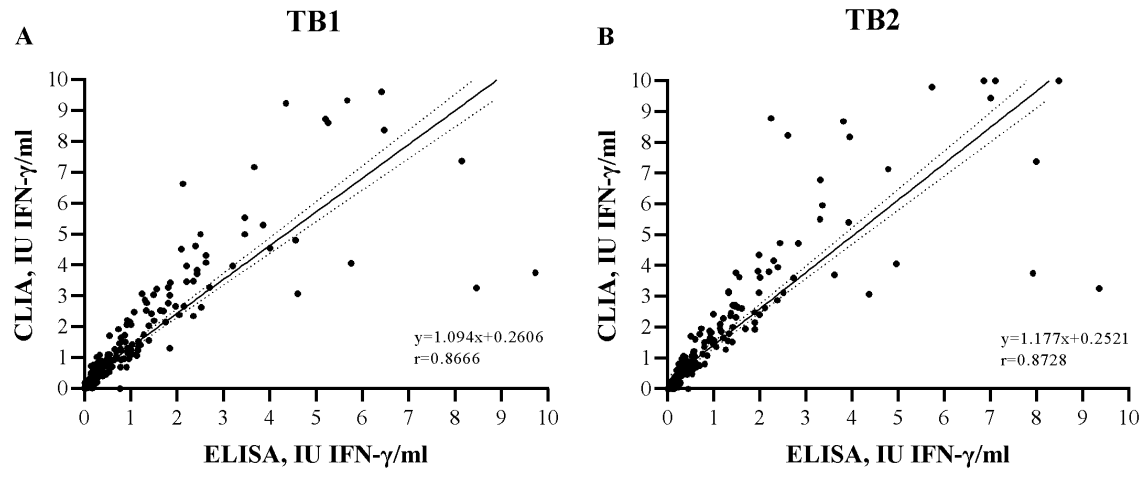
291 **FIG 2: ROC curve of the CLIA QFT-Plus TB1 (A) and TB2 (B) to diagnose latent tuberculosis**  
292 **infection.** Sensitivity and specificity according to manufacturer's cut-off and to the cut-off defined  
293 by AUC analysis are reported. Infection was assessed based on the results of ELISA QFT-Plus.

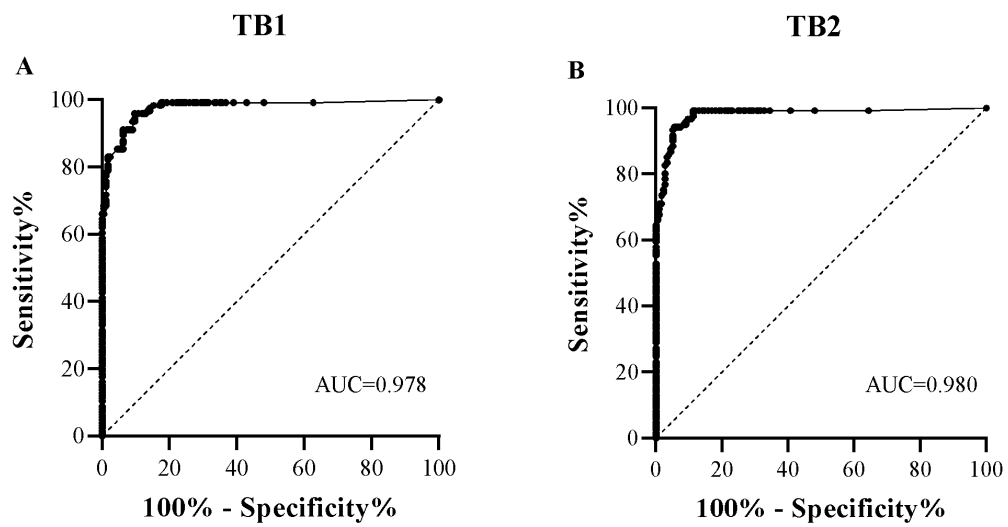
		QFT Plus ELISA			Total
		Positive, n	Negative, n	Indeterminate, n	
QFT Plus CLIA	Positive, n	146	33	3	182
	Negative, n	2	135	60	197
	Indeterminate, n	5	0	35	40
	Total	153	168	98	419
Agreement, %		95.4	80.4	35.7	75.4

**TABLE 1** Results and agreement of QFT Plus assay performed by ELISA and CLIA according to the manufacturer's cut-off.

		QFT Plus ELISA				
		Positive, n	Borderline, n	Negative, n	Indeterminate, n	Total
QFT Plus CLIA	Positive, n	104	41	0	0	145
	Borderline, n	2	34	8	4	48
	Negative, n	1	4	122	59	186
	Indeterminate, n	5	0	0	35	40
	Total	112	79	130	98	419
Agreement, %		92.8	43.0	93.8	35.7	70.4

**TABLE 2** Results and agreement of QFT Plus performed by ELISA and CLIA according to the Italian Clinical Microbiologist Association recommendations.





TB1 cut-off	Sensitivity (95% CI)	Specificity (95% CI)
>0.35	99.2 (95.57-99.96)	81.9 (75.59-86.89)
>0.45	97.6 (93.13-99.34)	85.9 (79.98-90.25)

TB2 cut-off	Sensitivity (95% CI)	Specificity (95% CI)
>0.35	99.2 (95.47-99.46)	81.9 (75.59-86.89)
>0.46	99.2 (95.47-99.96)	88.7 (83.19-92.57)