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Journal

Journal of Clinical Microbiology, 59(5)

ISSN

0095-1137

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Publication Date

2021-04-20

DOI

10.1128/jcm.00120-21

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IMMUNOASSAYS

Impact of T-Cell Xtend on T-SPOT.*TB* Assay in High-Risk Individuals after Delayed Blood Sample Processing

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ABSTRACT T-SPOT.*TB* (T-SPOT) is an interferon gamma release assay (IGRA) used to detect infection with *Mycobacterium tuberculosis* based on the number of spot-forming T cells; however, delays in sample processing have been shown to reduce the number of these spots that are detected following laboratory processing. Adding T-Cell Xtend (XT) into blood samples before processing reportedly extends the amount of time allowed between blood collection and processing up to 32 h. In this study, paired blood samples from 306 adolescents and adults at high risk for latent tuberculosis (TB) infection (LTBI) or progression to TB disease were divided into three groups: (i) early processing (~4.5 h after collection) with and without XT, (ii) delayed processing (~24 h after collection) with and without XT, and (iii) early processing without XT and delayed processing with XT. The participants' paired samples were processed at a local laboratory and agreement of qualitative and quantitative results was assessed. The addition of XT did not consistently increase or decrease the number of spots. In groups 1, 2, and 3, samples processed with XT had 13% (10/77), 28.0% (30/107), and 24.6% (30/122), respectively, more spots, while 33.8% (26/77), 26.2% (28/107), and 38.5% (47/122) had fewer spots than samples processed without XT. The findings suggest that XT does not reliably mitigate the loss of spot-forming T cells in samples with processing delay.

KEYWORDS T.SPOT.*TB*, T-Cell Xtend, *Mycobacterium tuberculosis*, interferon gamma release assays, latent tuberculosis infection

Since 2013, the annual tuberculosis (TB) incidence in the United States has remained steady at approximately 3 cases/100,000 persons (1–3). The Centers for Disease Control and Prevention (CDC) has established a goal of TB elimination, defined as <1 case per million population (4). Because an estimated 80% of TB cases are caused by reactivation of *Mycobacterium tuberculosis* infection (5), identification and treatment of persons with latent TB infection (LTBI) constitute the most effective way to accelerate progress toward TB elimination (6). The Food and Drug Administration has approved three cellular immune response tests for TB infection: the tuberculin skin test (TST) and

Citation Feng P-J, Wu Y, Ho CS, Chinna L, Whelen AC, Largen A, Brostrom R, Reves R, Belknap R, Cattamanchi A, Banaei N. 2021. Impact of T-Cell Xtend on T-SPOT.*TB* assay in high-risk individuals after delayed blood sample processing. J Clin Microbiol 59:e00120-21. https://doi.org/10.1128/JCM.00120-21.

Editor Brad Fenwick, University of Tennessee at Knoxville

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Received 22 January 2021
Returned for modification 15 February 2021
Accepted 23 February 2021
Accepted manuscript posted online 3 March 2021
Published 20 April 2021

two interferon gamma release assays (IGRAs), QuantiFERON (Qiagen, Venlo, Netherlands; acquired by Thermo Fisher Scientific Inc.) and T-SPOT.*TB* (T-SPOT) (Oxford Immunotec Ltd. [OI], Abingdon, United Kingdom) (7). Unlike TST results, IGRA results are not affected by prior bacillus Calmette-Guérin vaccination and require only one clinic visit, as opposed to two visits for TST—one to administer the test and a second to read the result (8).

T-SPOT is an enzyme-linked immunosorbent spot (ELISpot) assay, in which the numbers of stimulated effector T cells producing interferon gamma after exposure to *M. tuberculosis* antigens—ESAT-6 and CFP-10—are counted as spots (9). Although OI recommends that blood samples be processed with T-SPOT $<$8 h after blood draw, this is often not practical in the field. Prior studies using ELISpot assay have shown that a delay in processing, which entails stimulating peripheral blood mononuclear cells (PBMC) with *M. tuberculosis* antigens, results in markedly lower numbers of detectable spot-forming T cells (10). To address this issue, the manufacturer has developed the T-Cell Xtend (XT) reagent to remove inhibitory granulocytes prior to isolation of PBMC. XT works by cross-linking granulocytes with red blood cells and thereby separating them from the PBMC layer during centrifugation. According to the XT package insert, the separation step allows the T-SPOT assay to be performed on blood samples stored at 18°C to 25°C up to 32 h after blood draw without compromising the accuracy of T-SPOT (11).

Few studies have investigated the effectiveness of XT, and it remains unclear whether XT can prevent a decrease in spot-forming T cells in samples with delayed processing time. We investigated the impact of XT on the performance of T-SPOT in participants at high risk for LTBI or progression to TB disease.

MATERIALS AND METHODS

Study population. The Tuberculosis Epidemiologic Studies Consortium (TBESC), funded by the CDC, is a partnership with academic and public health programs in 11 states. TBESC-affiliated clinics recruited individuals at high risk for LTBI or progression to TB disease to assess the ability of TST and IGRAs to predict progression to TB disease.

All participants had $\geq$1 of the following risk factors for LTBI or progression to TB disease: (i) close contact with a person with infectious TB disease, (ii) recent immigration ($\leq$5 years) from a country whose U.S. population had a moderate (10 to 99 per 100,000 persons) rate of TB (see Table S1a in the supplemental material) (15), (iii) immigration at any time from a country whose U.S. population had a high ($\geq$100 per 100,000 persons) rate of TB (Table S1b) (15), and (iv) recent ($\leq$5 years) residence for $\geq$30 days in a country whose U.S. population had a high rate of TB. All participants provided written informed consent, assent, or parental permission. The study was approved by CDC's institutional review board and was registered at clinicaltrials.gov (identifier NCT01622140).

The population for this specific TBESC study included all TBESC participants who were recruited and enrolled from October 2015 through August 2016 at the Lanakila Health Center in Hawaii, were at least 15 years old, and were HIV negative. Participants identified with TB disease during the enrollment process were excluded from this analysis.

Study procedures. Trained study personnel interviewed each participant to collect information on demographic and LTBI-related risk factors using a standardized questionnaire at enrollment. Participants had a total of 12 ml of blood drawn into two lithium heparin BD Vacutainer plastic blood collection tubes (BD Diagnostics, Franklin Lakes, NJ). Each tube had 6 ml of blood. All blood samples were taken to and processed at the Hawaii State Laboratories (HSL). Paired samples for each participant were used to limit sources of variation, so that differences in T-SPOT results would be attributed solely to the effect of XT.

In order to assess the effect of XT on time between sample collection and processing, paired samples were divided into 3 groups (Fig. 1) based on convenience sampling. Paired samples in group 1 were transported to HSL ~4.5 h after collection and then processed with or without XT. Paired samples in group 2 were first stored at 23°C overnight (i.e., ~24 h) and then transported to HSL for processing with or without XT. For group 3, one sample from each pair was transported to HSL ~4.5 h after collection and processed without XT, while the other sample was stored at 23°C overnight, then transported to HSL, and processed with XT.

Laboratory procedures. All testing procedures for T-SPOT, with the exception of the use of XT, were conducted according to the manufacturer's instructions. Upon arrival at the laboratory, XT (25 μl/ml of whole blood) was added to one sample of each pair before processing, in accordance with the study protocol. Samples were then left for 20 min at room temperature, followed by PBMC isolation. The final spot count for each specimen was calculated by taking the greater of the spot difference between each panel and the nil (i.e., panel A $-$ nil and panel B $-$ nil); accordingly, it is possible for the final spot count to be less than zero. Spot counts of "$<$50" were considered "50." In accordance with FDA regulations, samples with four spots or less were considered negative, those with five to seven spots were

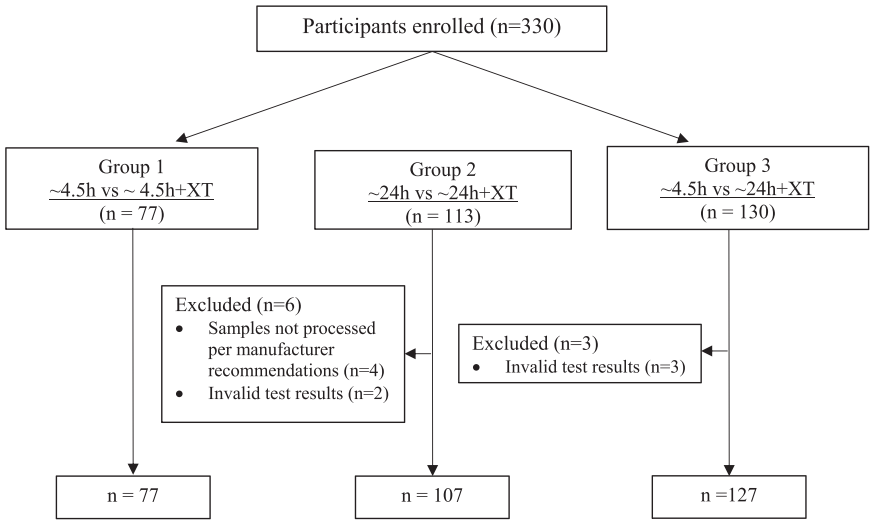

FIG 1 Study flow of participants. XT, T-Cell Xtend (Oxford Immunotec, Ltd., Abingdon, United Kingdom).

borderline, and those with eight spots or more were positive. An invalid result was defined as the nil control having >10 spots or the positive control having <20 spots.

Statistical analysis. To assess the agreement between T-SPOT results with and without XT, Cohen's kappa value was calculated with 95% confidence intervals (CIs) using SAS version 9.3 (SAS Institute, Inc., Cary, NC). We computed the difference in spot counts between the paired specimens for each group by subtracting the number of spots of the samples without XT from that of the samples with XT added. Because of the large number of zero differences in paired samples, we used bootstrapping methods, with replacement, in R (version 3.3.3; R Foundation, Vienna, Austria) to create 5,000 simulated data sets, in order to calculate 95% CIs of the median spot count differences of each group. The 95% CIs were calculated using the percentile method. We reported the median and 95% CIs of the median spot count differences for each group with the bootstrapped data. We performed sensitivity analyses by recoding differences of zero as both −1 and 1; because results and conclusions remained the same, we report only our main results from bootstrapping.

RESULTS

Participants. A total of 320 participants were enrolled in our substudy. Five participants with invalid T-SPOT results (group 2, $n = 2$; group 3, $n = 3$) and 9 participants who had samples without XT that were not processed according to manufacturer guidelines (group 2, $n = 4$; group 3, $n = 5$) were excluded from the analysis, resulting in 77, 107, and 122 participants in groups 1, 2, and 3, respectively (Fig. 1).

Each group had approximately equal numbers by gender; approximately half were ≥45 years old, and almost all (98.0%) were non-U.S. born, most from East Asia (Table 1).

Group 1: ~4.5-h delay without XT versus ~4.5-h delay with XT. The majority of pairs (69/77 [89.6%]) had concordant test results, of which 55 (71.4%) were negative, 13 (16.9%) were positive, and 1 (1.3%) was borderline. Of the 8 pairs with discordant test results, 3 were borderline on the sample without XT and either positive ($n = 1$) or negative ($n = 2$) on the sample with XT. Similarly, four pairs were borderline with XT and either positive ($n = 3$) or negative ($n = 1$) without XT. One pair was positive without XT and negative with XT (Table 2). The Kappa value of agreement was 0.75 (95% CI, 0.60 to 0.89). Figure 2A plots the spot counts of samples processed with XT against the paired sample processed without XT. About half (41/77) of the pairs had no difference in spot counts between the two samples (Table 3 and Fig. 3A). Of the pairs with a difference of 0, 29 (70.7%) had a spot count of 0 on both samples and 4 (9.8%) had a spot count of 50 on both (Fig. 2A). Thirty-six pairs had a difference in spot counts. Of these, 10 (27.8%) had a greater number of spots with XT and 26 (72.2%) had a lower number of spots with XT (Table 3). Using the bootstrapped data, the median of the median spot count difference was zero (95% CI, 0 to 0).

TABLE 1 Demographic, medical, and social characteristics of study participants, by group

Characteristic	All participants		Group 1[a]		Group 2[b]		Group 3[c]	
	n	%	*n*	%	*n*	%	*n*	%
All	306		77		107		122	
Gender								
Male	152	49.7	39	50.6	56	52.3	57	46.7
Female	154	50.3	38	49.4	51	47.7	65	53.3
Age group (yrs)[d]								
15–24	47	15.4	11	14.3	13	12.1	23	18.9
25–44	103	33.7	31	40.3	32	29.9	40	32.8
>45	155	50.7	34	44.2	62	57.9	59	48.4
Non-U.S. born	300	98.0	75	97.4	105	98.1	120	98.4
Country of birth								
Philippines	245	80.1	62	80.5	83	77.6	100	82.0
Vietnam	16	5.2	5	6.5	4	3.7	7	5.7
China	13	4.2	3	3.9	4	3.7	6	4.9
Federal States of Micronesia	11	3.6	2	2.6	6	5.6	3	2.5
United States	6	2.0	2	2.6	2	1.9	2	1.6
Other	15	4.9	3	3.9	8	7.5	4	3.3
Self-reported medical conditions								
Liver disease	6	2.0	1	1.3	4	3.7	1	0.8
Chronic kidney failure	1	0.3	1	1.3	0	0.0	0	0.0
Diabetes	20	6.5	4	5.2	10	9.3	6	4.9
Immunosuppressive therapy	3	1.0	0	0.0	1	0.9	2	1.6

[a]Both samples processed with an ~4.5-h delay, one sample with Xtend and one without.
[b]Both samples processed with an ~24-h delay, one sample with Xtend and one without.
[c]One sample processed with an ~4.5-h delay without Xtend and one sample processed with an ~24-h delay with Xtend.
[d]For one participant in group 1, age was not reported.

Group 2: ~24-h delay without XT versus ~24-h delay with XT. The majority of pairs (93/107 [86.9%]) had concordant test results, of which 68 (63.6%) were negative, 24 (22.4%) were positive, and 1 (0.9%) was borderline on both samples. Of the 14 pairs with discordant test results, 5 (35.7%) were borderline on the sample without XT and either positive ($n = 2$) or negative ($n = 3$) on the sample processed with XT, while 8 (57.1%) were borderline with XT and either positive ($n = 3$) or negative ($n = 5$) without XT. One pair (7.1%) was negative without XT and positive when processed with XT (Table 4). The kappa value of agreement was 0.73 (95% CI, 0.60 to 0.85). Figure 2B shows the paired spot counts plotted against one another in group 2; 45.8% (49/107) had no difference in spot counts between the two samples (Table 3 and Fig. 3B). Of the pairs with a difference of 0, 39 (79.6%) had a spot count of 0 on both samples and 7 (14.3%) had a spot count of 50 on both. Fifty-eight pairs had a difference in spot

TABLE 2 Frequency of test result combinations between samples with and without T-Cell Xtend for group 1 (~4.5-h delay without Xtend versus ~4.5-h delay with Xtend)

No Xtend	Xtend: Positive		Xtend: Borderline		Xtend: Negative		Total
	n	%[a]	*n*	%[a]	*n*	%[a]	
Positive	13	16.9	3	3.9	1	1.3	17
Borderline	1	1.3	1	1.3	2	2.6	4
Negative	0	0.0	1	1.3	55	71.4	56
Total	14		5		58		77

[a]Of grand total.

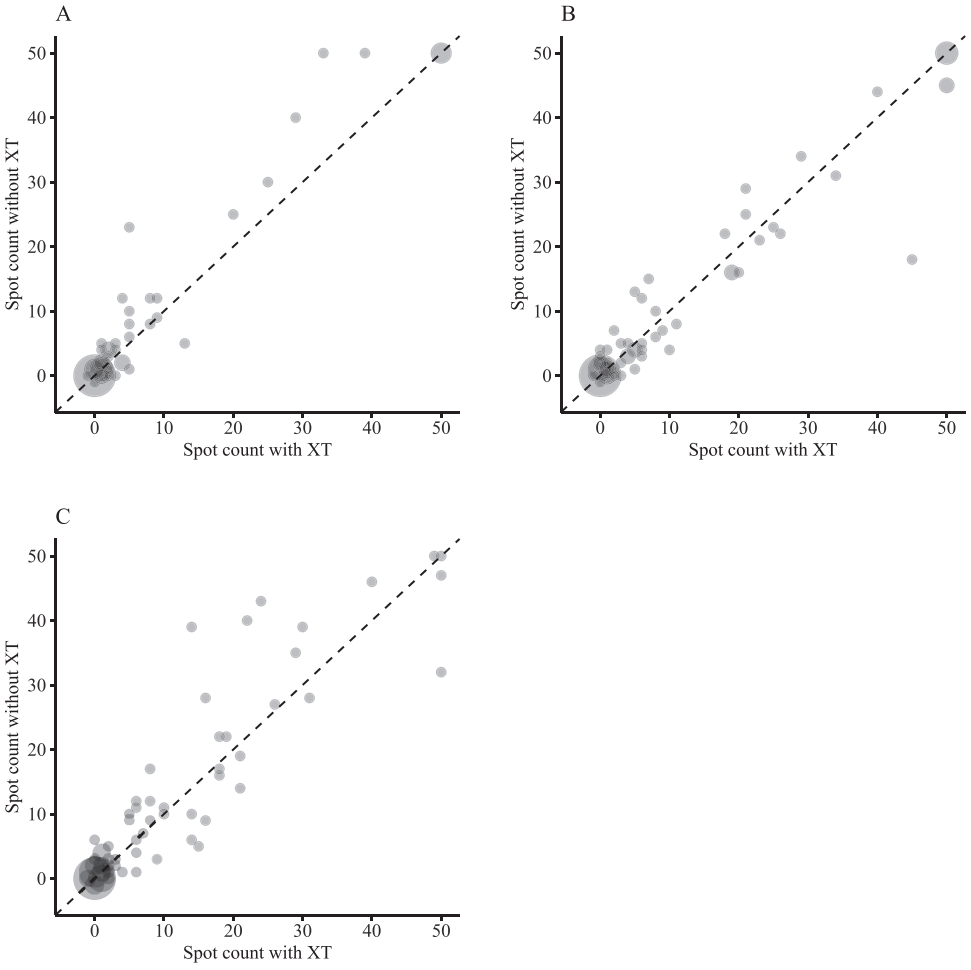

FIG 2 Spot counts of samples processed with XT plotted against samples processed without XT within each pair, by group. (A) Group 1, ~4.5-h delay without XT versus ~4.5-h delay with XT; (B) group 2, ~24-h delay without XT versus ~24-h delay with XT; (C) group 3, ~4.5-h delay without XT versus ~24-h delay with XT. The frequency of each unique spot count pair is represented by the size of the circles. The diagonal line on the plot represents spot count differences of zero.

counts. Of these, 30 (51.2%) had a greater number of spots and 28 (48.3%) had a lower number of spots on the sample processed with XT (Table 3). Like for group 1, the median of the bootstrapped median differences in spot counts was zero (95% CI, 0 to 0).

Group 3: ~4.5-h delay without XT versus ~24-h delay with XT. The majority of pairs (111/122 [91.0%]) had concordant test results, of which 83 (68.0%) were negative, 26 (21.3%) were positive, and 2 (1.6%) were borderline on both samples. Of the 11 pairs with discordant test results, 4 (36.4%) were borderline on the sample without XT and either positive ($n = 2$) or negative ($n = 2$) on the sample processed with XT, while 6

TABLE 3 Effect of T-Cell Xtend on spot count

	More spots with Xtend		Fewer spots with Xtend		Same		
Group[a]	*n*	%[b]	*n*	%[b]	*n*	%[b]	Total
1	10	13.0	26	33.8	41	53.2	77
2	30	28.0	28	26.2	49	45.8	107
3	30	24.6	47	38.5	45	36.9	122

[a]Group 1, both samples processed with an ~4.5-h delay, one sample with Xtend and one without; group 2, both samples processed with an ~24-h delay, one sample with Xtend and one without; group 3, one sample processed with an ~4.5-h delay without Xtend and one sample processed with an ~24-h delay with Xtend.
[b]Of row total.

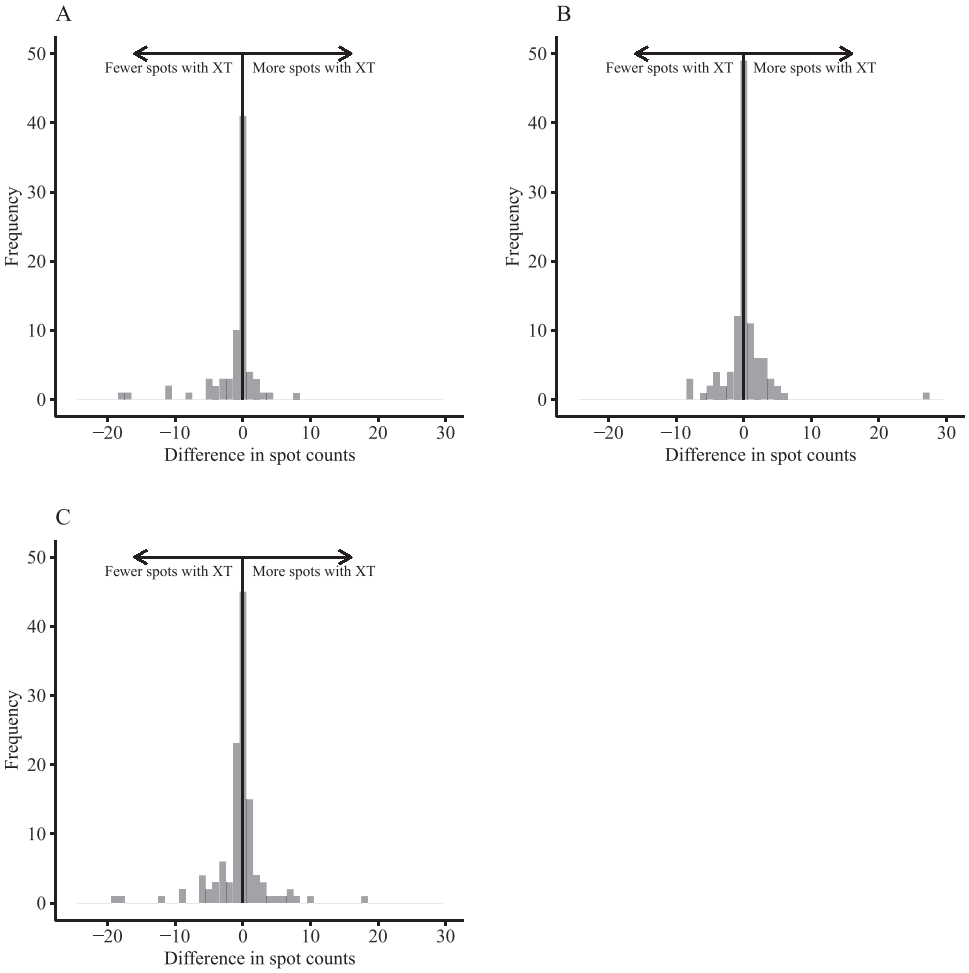

FIG 3 Frequency of spot count differences between samples in each pair, by group. (A) Group 1, ~4.5-h delay without XT versus ~4.5-h delay with XT; (B) group 2, ~24-h delay without XT versus ~24-h delay with XT; (C) group 3, ~4.5-h delay without XT versus ~24-h delay with XT.

(54.5%) were borderline with XT and either positive ($n = 4$) or negative ($n = 2$) without XT. One pair (9.1%) was negative when processed without XT and positive when processed with XT (Table 5). The kappa value of agreement was 0.80 (95% CI, 0.69 to 0.90). Figure 2C plots the spot counts of each pair against one another. About one-third (36.9%) of the pairs had no difference in spot counts between the two samples (Table 3 and Fig. 3C). Of these, 36 (80.0%) had a spot count of 0 on both samples and 1 (2.2%) had a spot count of 50 on both. Seventy-seven pairs had a difference in spot counts; 30 (39.0%) had a greater number of spots and 47 (61.0%) had a lower number of spots

TABLE 4 Frequency of test result combinations between samples with and without T-Cell Xtend for group 2 (~24-h delay without Xtend versus ~24-h delay with Xtend)

	Xtend						
	Positive		Borderline		Negative		
No Xtend	*n*	%[a]	*n*	%[a]	*n*	%[a]	Total
Positive	24	22.4	3	2.8	0	0.0	27
Borderline	2	1.9	1	0.9	3	2.8	6
Negative	1	0.9	5	4.7	68	63.6	74
Total	27		9		71		107

[a]Of grand total.

TABLE 5 Frequency of test result combinations between samples with and without T-Cell Xtend for group 3 (~4.5-h delay without Xtend versus ~24-h delay with Xtend)

	Xtend						
	Positive		Borderline		Negative		
No Xtend	*n*	%[a]	*n*	%[a]	*n*	%[a]	Total
Positive	26	21.3	4	3.3	0	0.0	30
Borderline	2	1.6	2	1.6	2	1.6	6
Negative	1	0.8	2	1.6	83	68.0	86
Total	29		8		85		122

[a]Of grand total.

on the sample processed with XT (Table 3). Similar to the case with groups 1 and 2, the median of the bootstrapped median spot count differences was zero (95% CI, 0 to 0).

DISCUSSION

Addition of XT to blood samples prior to processing with T-SPOT is intended to limit the effect of processing delay—which reduces interferon gamma-producing T cells—on samples processed 8 to 32 h after collection (12, 13). We designed three experiments in adults and adolescents at high risk for LTBI or progression to TB disease to investigate the ability of XT to mitigate the negative impact of processing delay on the T-SPOT assay. In all groups, we expected to observe an equal or greater number of spots in samples processed with XT compared to those without XT; 66%, 74%, and 62% of the pairs in groups 1, 2, and 3, respectively, fit into this pattern. Among participants who had different spot counts between samples, 72%, 48%, and 61% in groups 1, 2, and 3, respectively, had fewer spots in the sample with XT. These findings suggest that the addition of XT does not consistently prevent loss of spots. Additionally, in group 3, we investigated the impact of XT after overnight processing delay compared to early processing (~4.5-h delay) without XT. We found high concordance (91%) in qualitative test results between the two groups. Two-thirds of paired samples had different spot counts. Of those, 61% had fewer spots with XT after an ~24-h processing delay compared with no XT after an ~4.5-h processing delay. Prior studies have shown a significant reduction in spot-forming T cells after as little as a 2-h processing delay compared with immediate processing (10). Thus, it is plausible that processing after ~4.5 h after collection is already too late to use as the reference standard when assessing the impact of XT. Although the XT package insert claims 96.6% (340/352) agreement between the T-SPOT assay with and without the XT, no detail or reference is provided on their study design and very few published studies have investigated the utility of XT in mitigating the effect of processing delay on the T-SPOT assay using paired samples. Lenders and colleagues compared samples from 215 persons being evaluated for TB or treated TB patients that were processed with a 3- to 4-h delay (day 0) without XT to samples processed with an ~24-h delay (day 1) with XT. Similar to group 3 findings in our study, they found high agreement (97.1%) between day 0 without XT and day 1 with XT (14).

This study has certain limitations. First, our study had a small sample size, which limited our ability to detect the significance of small differences between samples in each pair. Second, early processing in this study was defined as ~4.5 h after blood samples had been collected, which previous studies suggest may be too late to use as a reference (10). Thus, immediate processing of blood samples for T-SPOT may show differences that are missed with an ~4.5-h delay. Third, our study population was homogenous because 97% of the participants were born in East Asia. Furthermore, our analysis was a convenience sample of a population at high risk of LTBI or progression to TB disease. Any genetically associated differences in T-cell response that might have affected T-SPOT results would have affected the results of our study and would not be

generalizable to other populations. Fourth, many of the pairs with no difference in spot counts between samples with and without XT had zero spots on both samples, which biased the median spot difference toward zero for all groups.

In conclusion, our study suggests that adding XT to the processing of stored samples may not prevent spot loss after processing delays of ~24 h in persons at high risk of LTBI or progression to TB disease. Further studies with larger sample sizes using immediate processing as the reference standard in low-prevalence settings similar to the United States are needed to confirm our findings.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

ACKNOWLEDGMENTS

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention (CDC). References in the manuscript to any specific commercial products, process, service, manufacturer, or company do not constitute its endorsement or recommendation by the U.S. government or CDC.

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