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Supplementary appendix

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Supplement to: Huang Z, LaCourse S M, W Kay A, et al. CRISPR detection of circulating cell-free *Mycobacterium tuberculosis* DNA in adults and children, including children with HIV: a molecular diagnostics study. *Lancet Microbe* 2022; published online May 31. https://doi.org/10.1016/S2666-5247(22)00087-8.

Supporting information

Contents

Supplementary methods **2**
Key Reagents 2
Preparation of target gene standards 2
cfDNA extraction 2
CRISPR-TB assay 2
Realtime-PCR 2
Supplementary Results **3**
Evaluation of cfDNA extraction method efficiency 3
Optimization of CRISPR reaction steps 4
Optimization of the PCR pre-amplification step 4
Supplementary Figures **5**
Supplementary Figure 1 5
Supplementary Figure 2 6
Supplementary Figure 3 7
Supplementary Figure 4. 8
Supplementary Figure 5 9
Supplementary Figure 6 10
Supplementary Figure 7 11
Supplementary Figure 8 12
Supplementary Figure 9 13
Supplementary Figure 10. 14
Supplementary Figure 11 15
Supplementary Figure 12 16
Supplementary Figure 13 17
Supplementary Figure 14 18
Supplementary Figure 15 19
Supplementary Figure 16 20
Supplementary Tables **21**
Supplementary Table 1 21
Supplementary Table 2 22
Supplementary Table 3 23
Supplementary Table 4 24
Supplementary Table 5 25
Supplementary Table 6 26
Supplementary Table 7 27
Supplementary Table 8 28
Supplementary Table 9 29
Supplementary Table 10 30
Supplementary Table 11 31
Supplementary Table 12 32
Supplementary Table 13 33
References **34**

Supplementary methods

Key Reagents. Quick-cfDNA Serum & Plasma Kit (D4076) was purchased from Zymo Research. Invitrogen AccuPrime Taq DNA Polymerase System (12339024) and nuclease-free water were purchased from ThermoFisher Scientific (4387936). EnGen Lba Cas12a (M0653T) and NEBuffer 2.1 (B7202S) were purchased from New England Biolabs. Primers, gRNAs, and probes (Table S5) were synthesized by Integrated DNA Technologies. *Mycobacterium tuberculosis* strain H37Ra strain was obtained from BEI Resources (NR-122, Lot 3685764), and 5 non-tuberculous mycobacteria list in Table S6 were obtained from ATCC.

Preparation of target gene standards. For stable experimental results, plasmids containing target DNA fragments were prepared as standards. Isolated *Mtb* genomic DNA and specific primers (Table S5) to *Mtb* IS6110, esxB, gyrB target regions were used to amplify target sequences, which were cloned into the TOPO vector and subsequently transformed into chemically competent *E. coli* cells (TOPO TA Cloning Kits, K4500-01SC). After selective culture, plasmid DNA was extracted from *E. coli* clones containing these target fragments using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, D4019), quantified using a Qubit dsDNA HS Assay Kit (Life Technologies), and diluted in nuclease-free water to working concentrations.

cfDNA extraction. Circulating cfDNA was isolated using the Quick-cfDNA Serum & Plasma. A total of 200 µL thawed serum was incubated with 50 µL of S&P 5× Digestion Buffer and 20 µL of Proteinase K at 55 °C for 30 min, then supplemented with 540 µL of S&P DNA Binding Buffer, after which a Zymo-Spin III-S Column was used to adsorb DNA. After washing columns with S&P DNA Prep Buffer and S&P DNA Wash Buffer, cfDNA was eluted in 50 µL nuclease-free water, and stored at -80 °C until use.

CRISPR-TB assay. CRISPR-TB assays require efficient target amplification prior to the CRISPR-mediated signal amplification. In the optimized PCR reactions (see Supplemental Results), 5 µL of isolated cfDNA (supplementary methods) was mixed with 15 µL of PCR mix containing 2 µL of 10X AccuPrime™ PCR Buffer I, 0.2 µL of AccuPrime™ Taq DNA Polymerase, and 11.8 µL of nuclease-free water, 0.5 µL forward primer (10 µM), and 0.5 µL reverse primer (10 µM). PCR reactions were subjected to a standard PCR protocol [denaturation (2 min at 94°C), amplification (38 cycles: 10 s at 98°C, 10 s at 60°C, 15 s at 72°C) and elongation (5 min at 72°C)]. In the optimized CRISPR reaction (see Supplemental Results), 2 µL of the PCR reaction was transferred to a 96-well half-area plate and mixed with 28 µL of the CRISPR reaction reagents (3 µL of 10× NEBuffer 2.1, 3 µL of 300 nM gRNA, 1 µL of 1 µM EnGen Lba Cas12a, 1.5 µL of 10 µM fluorescent probe 1, and 19.5 µL of nuclease-free water), then incubated for at 37°C for 20 minutes in the dark. CRISPR-mediated fluorescence signal was then excited at 485 nm and read at 525 nm using a SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices). The mean plus 3 times standard deviation (SD) of the CRISPR-TB value of the adult healthy control samples was used to set the threshold for positive sample results in serum from individuals with presumed TB cases (1.4×10^6 a.u., Table S8).

Realtime-PCR. Realtime-PCR was performed using TaqPath qPCR Master Mix CG (Thermo Fisher, A16245) and the same primers (Table S5, IS6110 primers) used for CRISPR-mediated target detection. In each reaction, 5 µL of cfDNA sample was mixed with 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 0.5 µL of TaqMan Probe (10 µM), 10 µL of TaqPath™

qPCR Master Mix, and 3.5 µL of nuclease-free water. Reactions were performed using a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) with a UNG incubation (2 min at 50°C) and polymerase activation (20 s at 95°C) step prior to the PCR amplification profile (40 cycles: 15 s at 95°C, 1 min at 60°C).

Lateral flow CRISPR-TB assay. Lateral flow CRISPR-TB assays employed isothermal recombinase polymerase amplification (RPA) assays to amplify its IS6110 target amplicon. Briefly, RPA pellets (TwistDx, TABAS03KIT) were resuspended in 29.5 µL of the supplied Rehydration Buffer, and 11.8 µL of this RPA solution, 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 0.7 µL of nuclease-free water, 2 µL of MgOAC solution (280 mM), and 5 µL of the isolated cfDNA sample were mixed and incubated at 42°C for 20 min. Next, 2 µL of the RPA reaction was mixed with 28 µL of CRISPR reaction reagents (3 µL of 10× NEBuffer 2.1, 3 µL of 300 nM gRNA, 1 µL of 1 µM EnGen Lba Cas12a, 1.5 µL of 10 µM fluorescent probe-3, and 19.5 µL of nuclease-free water) and incubated for at 37°C for 4 minutes. CRISPR reactions were then added to a test strip (HybriDetect 1, Milenia Biotec.) and after a 2 min interval signal development was read by the naked eye or capture on a smartphone and analyzed by an NIH Image J software macro. Samples that produce weak or undetectable T-lines when compared to that of the negative control sample were considered to be *Mtb*-cfDNA positive.

Supplementary Results

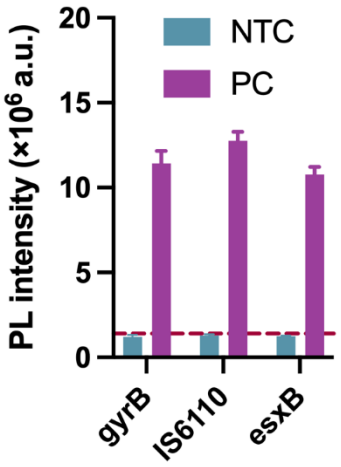
Evaluation of cfDNA extraction method efficiency. Efficient cfDNA target detection requires effective isolation of trace amounts of short DNA targets from blood [0.11 copies/µL (median), 7.20 ± 29.15 copies/µL (mean ± standard deviation)][1]. To address this problem, we evaluated the relative performance of a NaI extraction method that revealed the greatest cfDNA extraction efficiency in a previous studiy[2], and two commercial kits [Kit 1, AllPrep DNA/RNA Mini Kit (80204, QIAGEN); Kit 2, Quick-cfDNA Serum & Plasma Kit], to determine cfDNA yield and size distribution, purification time, and performance in conjunction with the CRISPR-TB assay. For both commercial kits, nucleic acid was extracted at room temperature using the approved protocol (200 µL plasma/serum, 50 µL elution volume). In the NaI isolation method, 200 µL plasma/serum was mixed with 50 µL of proteinase digestion buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 5% SDS) and 20 µL of proteinase K (20 mg/ml, New England Biolabs, P8107S). After incubation at 55°C for 30 min, samples were supplemented with 1 µL of glycogen (20 mg/mL, Thermo Fisher, 10814010), 405 µL of NaI solution (7.6 M, Thermo Fisher, S324-100), and 270 µL of isopropanol, and then incubated at room temperature with shaking for 30 min. Resulting precipitates were collected by centrifugation at 10, 000 g for 5 min at room temperature, washed with 40% isopropanol and 70% ethanol, and finally resuspended in 50 µL TE buffer. Thereafter, precipitates were stored at -80 °C until use. Aliquots of DNA extracted by these three methods were quantified and analyzed by Qubit and microfluidic DNA chip gel electrophoresis (Agilent Technology), respectively.

The Quick-cfDNA Serum & Plasma Kit (Kit 2) exhibited acceptable total DNA yield (23.38 ng/200 µL serum, Figure S2a), the best efficiency in isolating the targeted cfDNA size range (200-500 bp; Figure S2b-d) and produced robust CRISPR-fluorescence signal (Figure S2e). The NaI method had the highest total DNA yield, and comparable target DNA size extraction efficiency and CRISPR fluorescence response, but was considered too complicated and time-consuming to apply in a clinical application (Table S7).

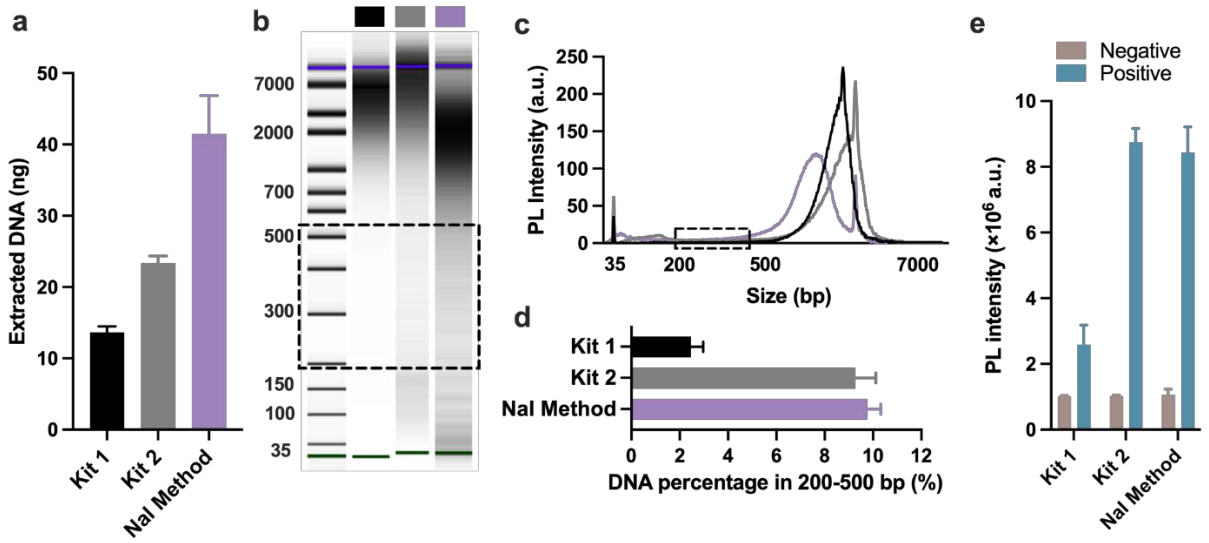
Optimization of CRISPR reaction steps. To develop the most sensitive and robust CRISPR-TB method, we systematically optimized the CRISPR step and the PCR pre-amplification step, starting with the CRISPR reaction as it provides the final assay output. CRISPR reaction performance was optimized by modifying the ratio of gRNA/Cas12a to fluorescent probe, reaction temperature, and incubation time to maximize fluorescence response dynamics in reactions spiked with a constant amount of target gene (Figure S3). For the Cas12a substrate-dependent kinetics study, assays were conducted with molar ratios of Cas12a/gRNA to fluorescent probe set at 1:10, 1:15, 1:20, 1:25, and 1:30. For the temperature-dependent kinetics study, reactions were performed using a 1:20 Cas12a/gRNA to fluorescent probe ratio and incubated at 25 °C, 37 °C, and 41 °C. For the target-dependent kinetics study, the system was conducted with reactions that were performed using a 1:20 Cas12a/gRNA to fluorescent probe ratio and 10^6,10^7, 10^8, and 10^9 copies/µL of the target fragments. CRISPR reactions demonstrated good activity over a wide temperature range (27-42°C), but reducing the probe concentration reduced end-point fluorescence intensity and narrowed quantification range, and increasing the temperature or reducing the gRNA/Cas12a to fluorescent probe ratio reduced the time to achieve maximum signal (Figure S3a-b). Choosing a shorter response time, however, reduced sensitivity, so that CRISPR reactions were set at 37°C for 20 min using gRNA/Cas12a to fluorescent probe ratio of 1:15. CRISPR reaction using the optimized reaction condition had a limit of detection (LoD) of approximately 10^8 copies/µL when directly spiked with template DNA (Figure S3c), indicating that an efficient PCR pre-amplification step is necessary for ultra-sensitivity detection.

Optimization of the PCR pre-amplification step. PCR pre-amplification was optimized by evaluating signal produced when the optimized CRISPR reaction was spiked with a constant volume of a PCR reaction. This analysis first evaluated which polymerase and PCR buffer combination that produced the most CRISPR signal when these conditions were used to amplify a constant amount of a PCR DNA target. This analysis tested three polymerases [Vent DNA Polymerase (New England Biolabs, M0257S) and Taq and AccuPrime Taq DNA Polymerase (Thermofisher, EP0404 and 12339024)] and a normal buffer [NB, 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 15 mM MgCl2, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP]. The two Taq polymerases were also evaluated in NB supplemented with thermostable AccuPrime protein reagent and 10% glycerol. Assay optimization studies determined that AccuPrime Taq polymerase provided the highest amplification efficiency, which could be further improved by the addition of a thermostable protein (TABP) that improved primer-template hybridization efficiency[3] (Figure S4a). Increasing the number of amplification cycles from 32 to 38 also resulted on an increase in the LOD of the CRISPR-TB reaction 40-fold to 0.25 copy/µL (Figure S4b and 5), with a linear quantification range of 0.5 – 1000 copies/µL (Fig. 1f and Figure S5d).

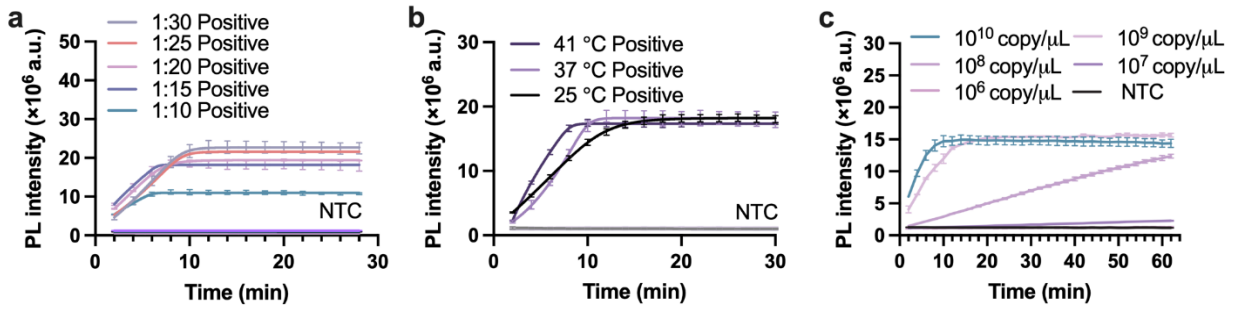
Supplementary Figures



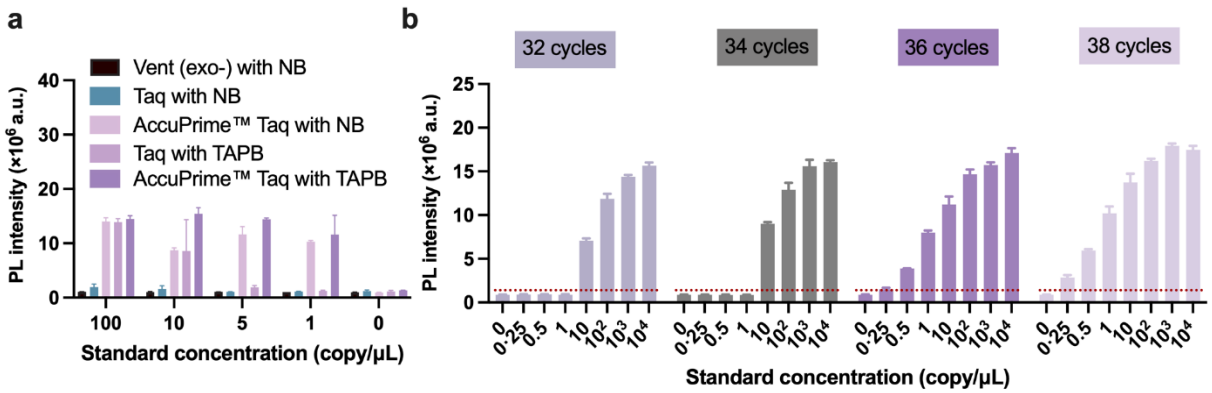
Supplementary Figure 1. CRISPR-assay signal detected for the indicated targets upon CRISPR analysis of PCR reactions spiked with a plasmid standard containing 100 copy/µL of the gyrB, IS6100, and esxB gene plasmids (positive control, PC) or nuclease-free water (non-template control, NTC). DNA targets were PCR-amplified for 35 cycles, and then incubated with CRISPR reagents for 20 min at 37 °C for 20 using a 1:15 ratio of gRNA/Cas12a and reporter oligonucleotide probe. Bar graph data represents the mean ± SD of 3 technical replicates for each sample.



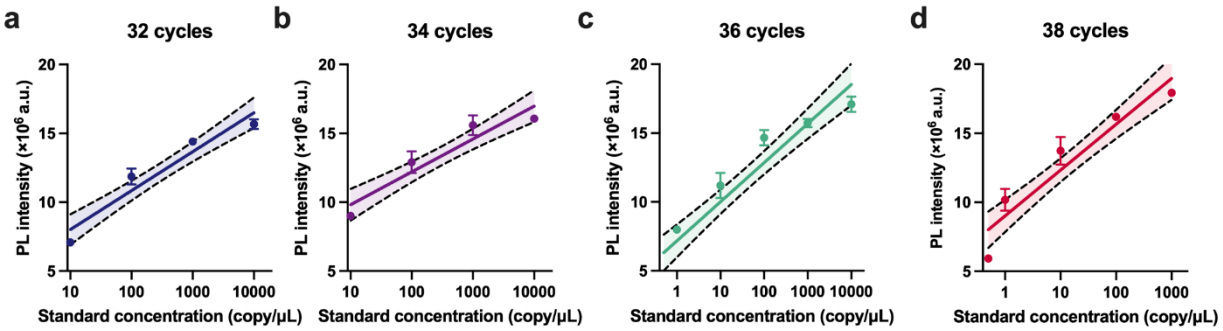
Supplementary Figure 2. Evaluation of three cfDNA extraction methods using 200 µL healthy serum aliquots spiked with 100 copy/µL of the IS6110 gene for cfDNA (**a**) yield, (**b**) size distribution, and (**c**) CRISPR-TB signal. Kit 1: AllPrep DNA/RNA Mini Kit; Kit 2: Quick-cfDNA Serum & Plasma Kit. Bar graph data represent the mean ± SD of 3 technical replicates for each sample.



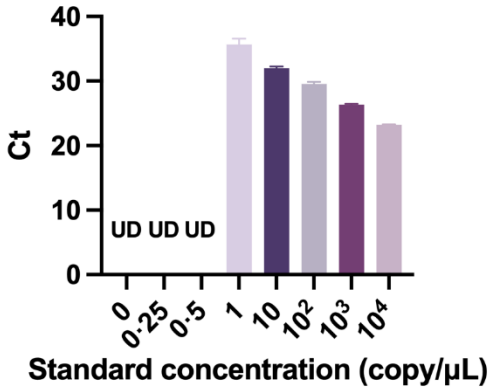
Supplementary Figure 3. Optimization of the gRNA/Cas12a-based CRISPR fluorescence reporting system. (**a**) Substrate-dependent (gRNA/Cas12a to fluorescent probe ratio), (**b**) temperature-dependent, and (**c**) target-dependent effects on PL intensity. An aliquot containing 10^9 target amplicon copies and nuclease-free water were analyzed as positive and non-template control (NTC) samples, respectively. Graphs depict the mean ± SD of 3 technical replicates for each sample Data presented are normalized to the highest signal intensity detected in the corresponding experiment.



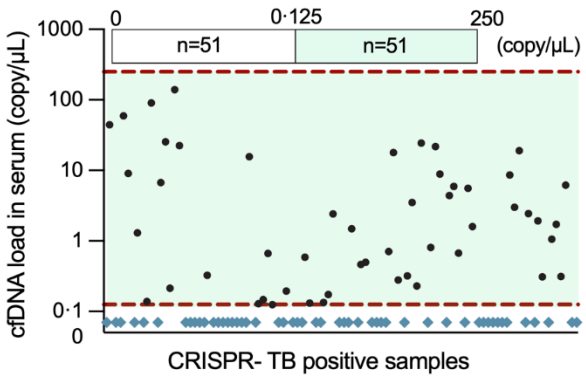
Supplementary Figure 4. Optimization of the PCR pre-amplification step by adjusting the **(a)** DNA polymerase and buffer combination with 36 cycles and (**b**) number of PCR cycles with AccuPrime™ Taq and TAPB. Bar graphs depict the mean ± SD of 3 technical replicates for each sample, and the red dashed line indicates the lowest limit of positive signal cut-off threshold. NB: normal buffer reference; TAPB: normal buffer supplemented with thermostable AccuPrime protein reagent and 10% glycerol.



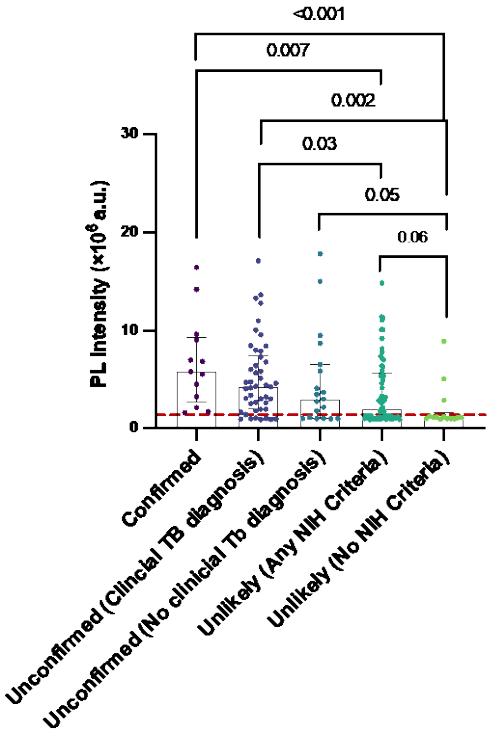
Supplementary Figure 5. Standard curves of CRISPR-TB assays performed with (**a**) 32, (**b**) 34, (**c**) 36, and (**d**) 38 cycle PCR pre-amplification step, using the indicated amount of template DNA. Graphs depict the mean ± SD of 3 technical replicates for each sample. Shading denotes the 95% confidence interval of the fitted line.



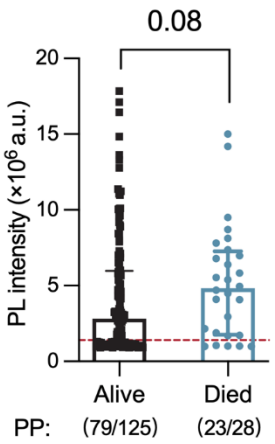
Supplementary Figure 6. Evaluation of the LOD for an optimized real-time PCR reaction for the IS6110 gene target performed using same primers as the CRISPR-TB IS6110 assay. Bar graphs indicate the mean ± SD of 3 technical replicates for each indicated sample concentration. UD, undetermined Ct value.



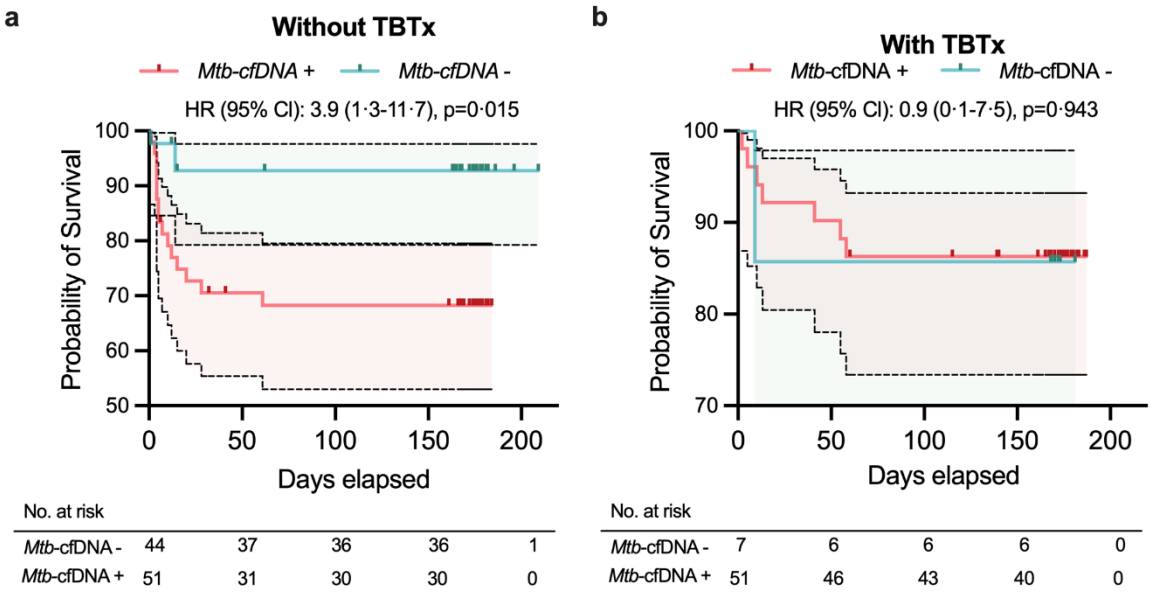
Supplementary Figure 7. *Mtb*-cfDNA levels in CRISPR-TB positive (PL intensity ≥ 1.4×10^6 a.u.) baseline serum samples (n=102) collected from hospitalized ART-naïve PUSH study children living with HIV and at high risk for TB**.** Black dots indicate cfDNA level calculated for samples that fall within the quantitation range of the standard curve (green shaded area; 0.125 -250 copy/µL). Blue dots indicate CRISPR-TB positive samples below the limit of quantification of the standard curve (red dashed line) that were assigned a 0.07 copy/µL value for visualization purposes only. *Mtb*-cfDNA level determined by comparison with the standard curve were divided by four to determine accurate serum *Mtb*-cfDNA levels to account for a four-fold DNA concentration that occurred during the cfDNA isolation procedure.



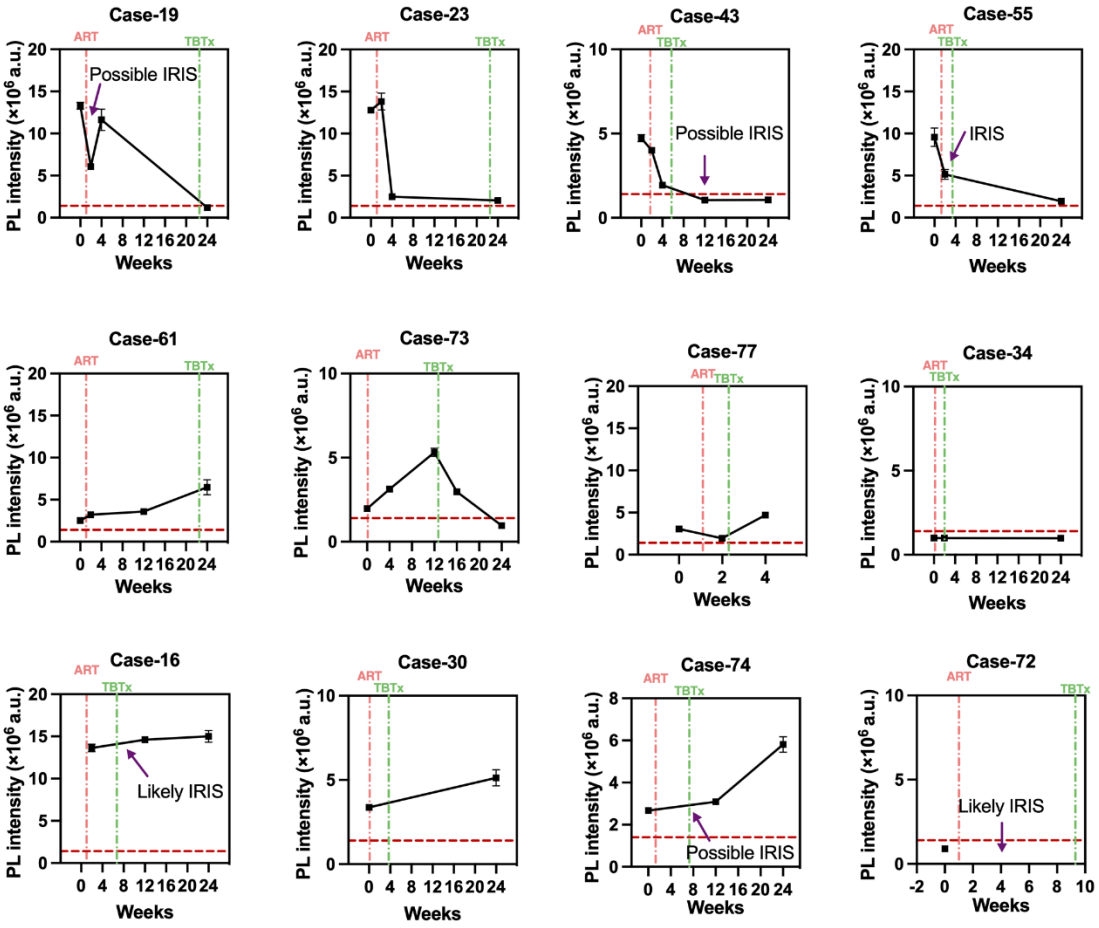
Supplementary Figure 8. Relative *Mtb* cfDNA signal in PUSH cohort subgroups. Clinical TB diagnosis – initiated TB treatment; No clinical TB diagnosis – did not initiate TB treatment. NIH criteria: positive CXR or TB exposure within last two years or TST ≥5mm or NIH TB symptoms: persistent cough (>14 days), fever (>7 days), failure to thrive, or lethargy (>7 days), failure to thrive=wasted (WHZ<-2 or MUAC<12.5) or underweight (WAZ<-2) at enrollment. The graph depicts the median and interquartile range of the indicated groups, p-values for differences were calculated using the Wilcoxon rank sum test, and the red dashed line indicates the lowest limit of positive signal cut-off threshold.



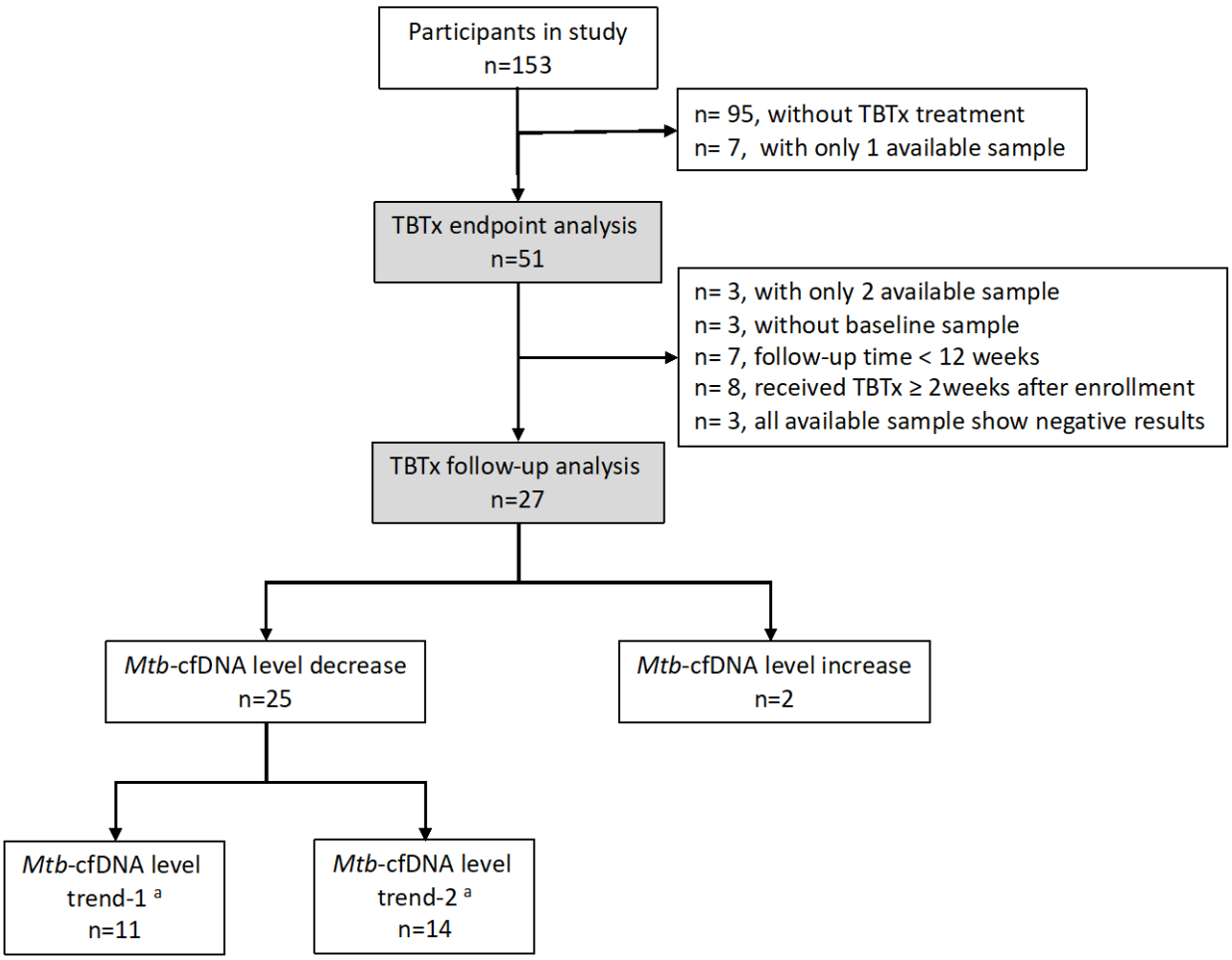
Supplementary Figure 9. Relative baseline *Mtb*-cfDNA signal and positive proportion (PP) children who were alive throughout or died during the study. The graph depicts the median and interquartile range of these two groups p-values for differences were calculated using the Wilcoxon rank sum test, and the red dashed line indicates the lowest limit of positive signal cut-off threshold.



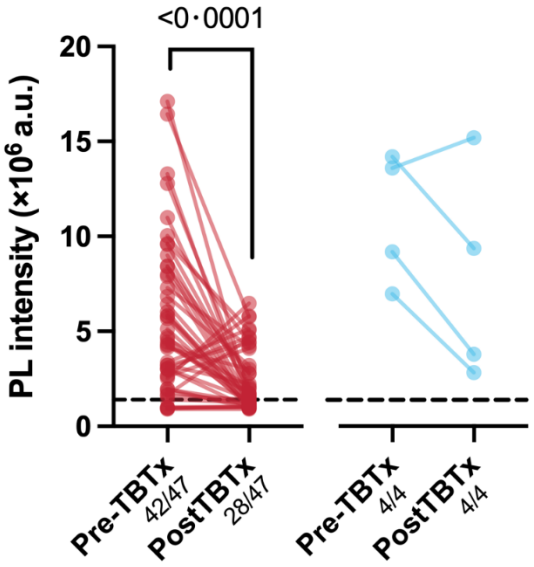
Supplementary Figure 10. Survival curve for children (a) without and (b) with TBTx. Children were stratified by positive and negative CRISPR-TB signals, indicating hazard ratios from the Cox proportional hazards models.



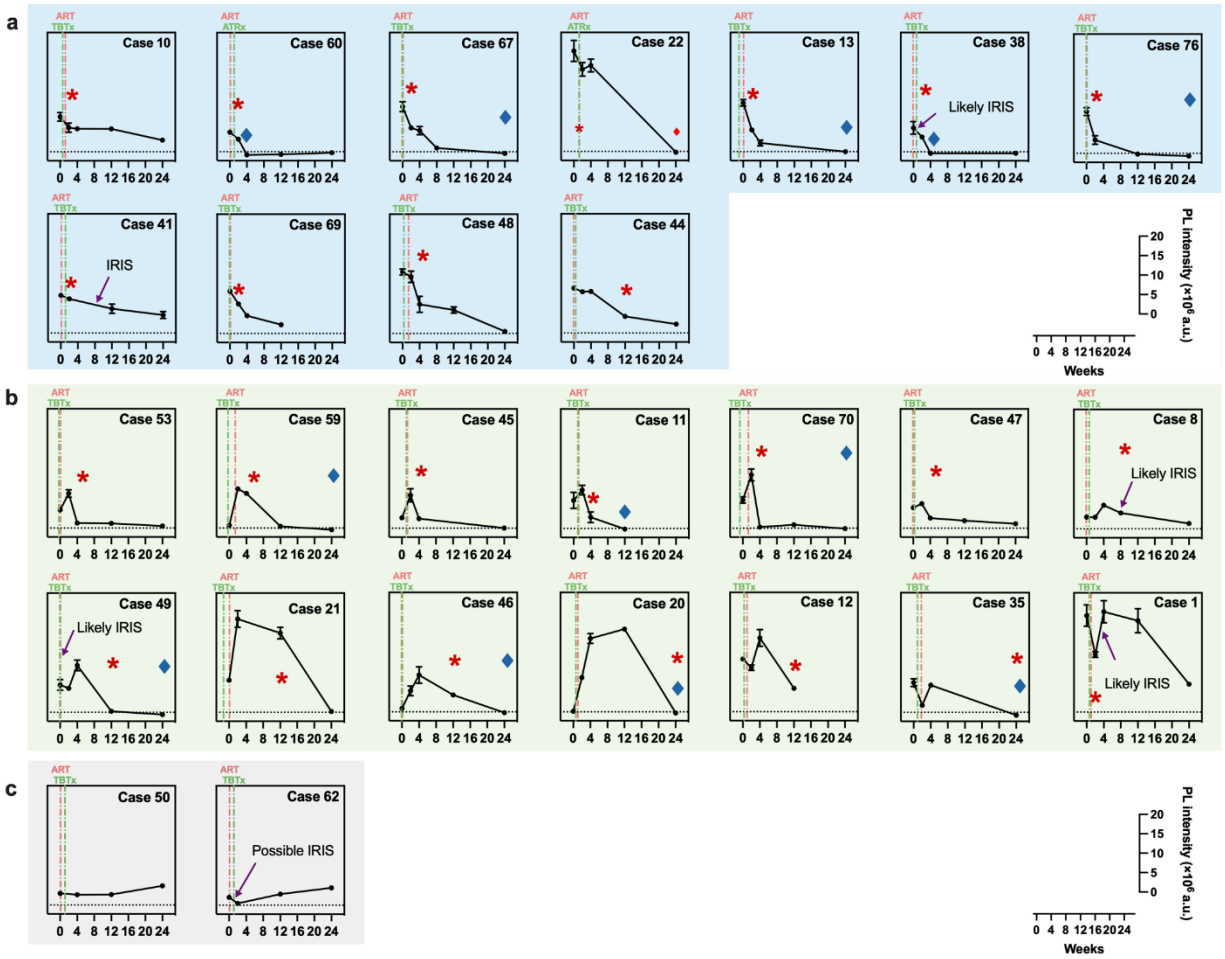
Supplementary Figure 11. *Mtb*-cfDNA dynamics in the 12 children with incident TB. Graphs indicate the mean ± SD CRISPR-TB values from 3 technical replicates for each sample. Vertical dashed lines indicate ART (red) and incident TB diagnosis/TBTx (green) initiation times, and the horizontal red dashed line indicates the cut-off for positive CRSIPR-TB signal. Purple arrows indicate the timing of potential IRIS events, as defined by the panel group using criteria established by the International Network for the study of HIV associated IRIS (INSHI)[4]. Rows 1-2: Children with two more samples available prior to TBTx initiation. Row 3: children with follow-up samples available only after TBTx, or with a single sample (Case #72). Most children demonstrated *Mtb*-cfDNA decreases after ART initiation and prior TBTx initiation, potentially due to immune containment in response to ART, although two children demonstrate *Mtb*-cfDNA increases prior to TBTx initiation.



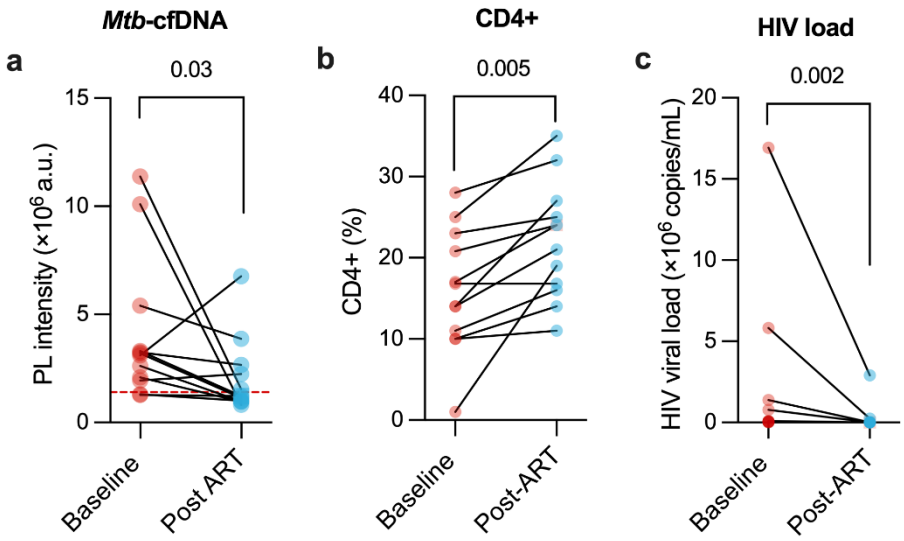
Supplementary Figure 12. Flow chart for inclusion of PUSH cohort children for evaluation *Mtb*-cfDNA response in children receiving TBTx. Fifty-one children who received TBTx and had ≥ 2 available samples were included to evaluate *Mtb*-cfDNA changes before and after TBTx initiation. *Mtb*-cfDNA dynamic changes following TBTx initiation were evaluated among 27 children who own at least one *Mtb*-cfDNA positive sample, received TBTx within 2 weeks after study enrollment, had more than two longitudinal serum samples, including a sample collected pre-TBTx initiation and a sample collected at > 12 weeks post-TBTx initiation. [a] Children with *Mtb*-cfDNA decreases were segregated by the dynamics of this response, a continuous decrease (Trend 1) or an increase versus baseline prior to a decrease (Trend 2).



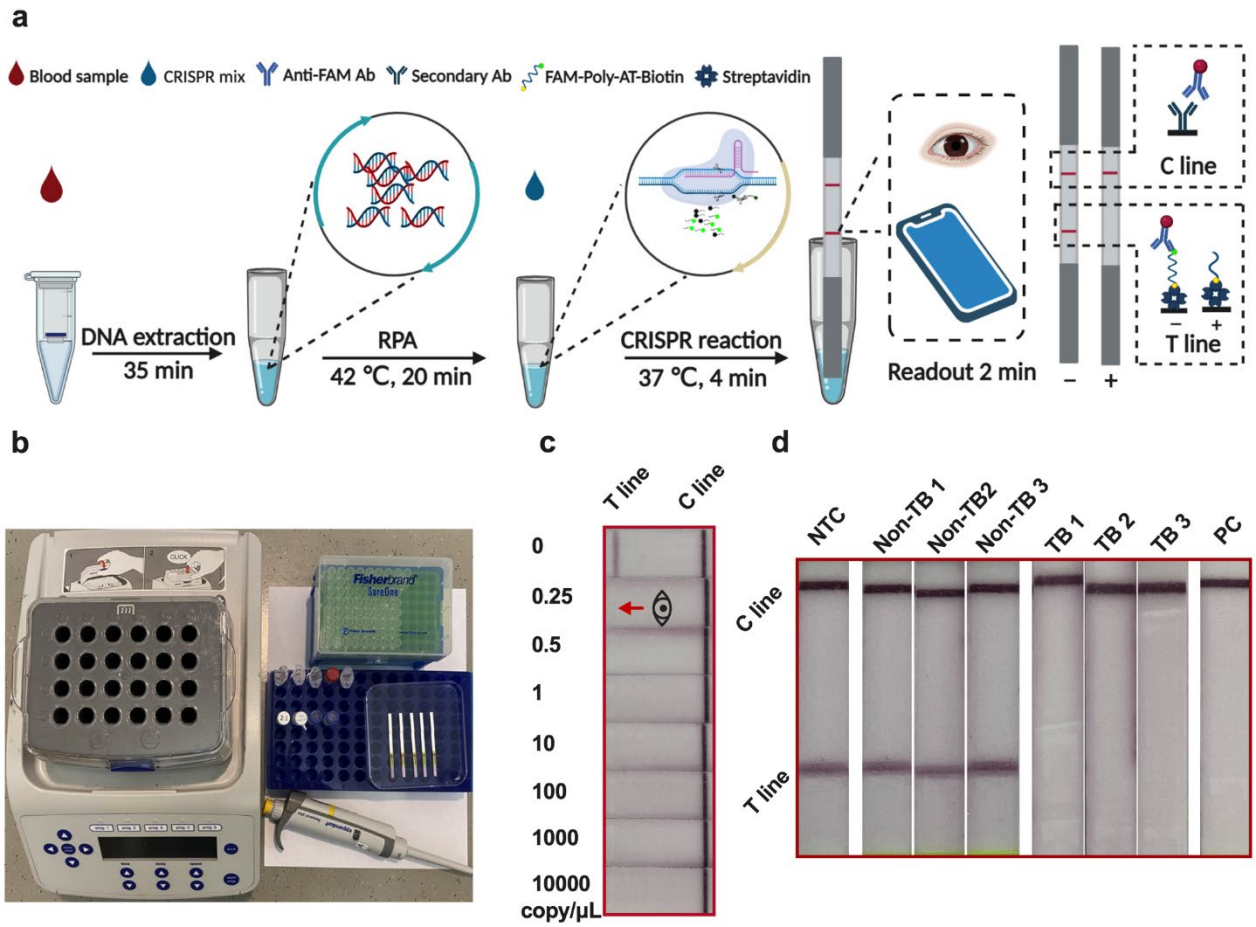
Supplementary Figure 13. Differences in *Mtb*-cfDNA level in and between children with (red, n=47) and without (blue, n=4) clinical responses to TBTx initiation. Pre- versus post-TBTx differences were evaluated by Wilcoxon signed rank test.



Supplementary Figure 14. Evaluation of the *Mtb*-cfDNA dynamic response following TBTx initiation in 27 children (**Fig. S12**) classified as confirmed or unconfirmed TB cases. *Mtb*-cfDNA levels revealed (**a**) a continuous decline for 11 children, (**b**) a peak followed by a decline in 14 children, and (**c**) a gradual increase in 2 children. Graphs indicate the mean ± SD CRISPR-TB values from 3 technical replicates for each sample. Dashed lines indicate ART (red) and TBTx (green) treatment at the implied time points, and the cut-off for positive CRISPR-TB signal (black). A red asterisk (*) indicated the first sample with a CRISPR-TB value lower than the peak value for each child, and a blue diamond (♦) indicates the first observed sample revealing complete *Mtb*-cfDNA clearance. The purple arrows indicate the time points at which IRIS occurred, as defined by the panel group according to International Network for the study of HIV associated IRIS (NSHI). Dashed lines indicate ART (red) and TBTx (green) treatment at the indicated time points, and the cut-off for positive CRISPR-TB results (black).



Supplementary Figure 15. Changes in (**a**) *Mtb*-cfDNA level, (**b**) CD4+ cell percent, and (**c**) HIV viral load for 12 children reclassified from unconfirmed TB to unlikely TB based on symptom improvement (subgroup B in Fig. 2a) before and after ART-initiation (baseline and post-ART), where p-values for differences were calculated using the Wilcoxon signed rank test.



Supplementary Figure 16. A portable, paper strip-based CRISPR detection platform for *Mtb*-cfDNA detection. (**a**) Schematic of the assay, which employs column-based cfDNA extraction (35 min), thermostatic RPA amplification (20 min), sensitive CRISPR signal amplification (4 min) and a lateral flow assay paper strip readout (2 min). In this method, the RPA-amplified target amplicon activates the gRNA/Cas12a complex to cleave a single-stranded oligonucleotide reporter modified with biotin and FITC. Upon addition to the lateral flow assay test strip, the CRISPR reaction mixes with gold nanoparticles (AuNPs) conjugated with anti-FITC antibodies. Intact reporter oligonucleotides are bound by streptavidin conjugated to the strip at the test (T) line. Cleaved reporter and intact reporter that is not bound at the T line is bound at the control (C) line by antibodies that directly recognize the anti-FITC antibody. Both cleaved and intact oligonucleotides are bound by the AuNPs that produce a dark band at their binding site. Thus, CRISPR recognition of its target DNA sequence will produce a loss of signal at the T-line, while not altering signal at the C line. (**b**) Equipment required for the portable test (tubes, reagents, heat block, nuclease-free water, pipettes, pipette tips and lateral flow strips). (**c**) The visually detectable paper strip LOD (0.25 copy/μL) is comparable to that detectable by the standard CRISPR-TB assay, and this assay can (**d**) accurately identify TB and Non-TB cases using cfDNA extracted from their serum. NTC: no template control (nuclease-free water); PC, positive control (IS6110 standard).

Supplementary Tables

Supplementary Table 1. Demographic information of adults and children of Eswatini cohort evaluated by CRISPR-TB in Fig. 2f-g

Characteristics	Adults [a] (N=45)			Children [a] (N=28)		
	Confirmed TB [b] N=26 Median (IQR) or n (%)	Clinical diagnosed TB [b] N=2 Median (IQR) or n (%)	Non-TB control [b] N=17 Median (IQR) or n (%)	Confirmed TB [b] N=4 Median (IQR) or n (%)	Unconfirmed TB [b] N=2 Median (IQR) or n (%)	Non-TB control [b] N=22 Median (IQR) or n (%)
Demographics						
Age (years)	38.1 (23.2-46.3)	29.4 (27.9-31.0)	39.0 (23.0-52.7)	8.5 (2.2-13.8)	1.6 (1.1-2.1)	5.9 (4.3-9.6)
Female sex	13 (50.0%)	0 (0%)	11 (64.7%)	2 (50%)	0 (0%)	15 (68.2%)
HIV positive	13 (50.0%)	1 (50%)	3 (17.6%)	0 (0%)	2 (100%)	1 (4.5%)
Hospitalized	3 (11.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
TB features						
Signs/symptoms of TB	25 (96.2%)	2 (100%)	2 (11.8%)	4 (100%)	2 (100%)	4 (18.2%)
CXR consist with TB	18 (69.2%)	2 (100%)	0 (0%)	3 (75.0%)	2 (100%)	1 (4.5%)
Positive culture	14 (53.8%)	0 (0%)	0 (0%)	3 (75.0%)	0 (0%)	0 (0%)
Positive smear	10 (38.5%)	0 (0%)	0 (0%)	3 (75.0%)	0 (0%)	0 (0%)
Positive Xpert	25 (96.2%)	0 (0%)	0 (0%)	3 (75.0%)	0 (0%)	0 (0%)
Positive T-Spot	2 (7.7%)	0 (0%)	4 (23.5%)	0 (0%)	0 (0%)	4 (18.2%)
Positive QFT	13 (50.0%)	0 (0%)	5 (29.4%)	2 (50.0%)	1 (50%)	11 (50%)
EPTB	3 (11.5%)	2 (100%)	0 (0%)	1 (25.0%)	0 (0%)	0 (0%)

[a] Adult, age ≥ 15 years old; children, age <15 years old.

[b] Adults and children were enrolled as confirmed TB cases if diagnosed by *Mtb* liquid culture and/or Gene Xpert Ultra (Xpert). Clinically diagnosed, presumptive TB patients did not have a positive culture or Xpert result, but were determined to have TB by treating clinicians based on symptoms and radiographic evidence, and were retrospectively classified as "unconfirmed TB" using NIH case definition[5] (children) or "clinically diagnosed TB" using WHO guidelines[6] (adults). Non-TB controls were recruited from household contacts of TB index cases who were asymptomatic or had TB excluded following resolution of mild symptoms or additional tests, and evaluated at two and six months after enrollment to confirm TB exclusion decisions.

Supplementary Table 2. Criteria for TB case definitions*		
Case Definitions:	**Adults (≥15 years)**	**Children (<15 years)**
Confirmed TB	A positive result on a GeneXpert-Ultra or culture collected on a respiratory specimen.	
Clinically diagnosed TB*: Clinically diagnosed TB (adults) Unconfirmed TB (children)	A TB case initiated in accordance with the "WHO Definitions and reporting framework for tuberculosis." A clinically diagnosed TB case is one that does not fulfill the criteria for bacteriological confirmation but has been diagnosed with active TB by a clinician or other medical practitioner who has decided to give the patient a full course of TB treatment. This includes cases diagnosed on the basis of X-ray abnormalities or suggestive histology and extrapulmonary cases without laboratory confirmation. Clinically diagnosed cases later found to be bacteriologically positive (before or after starting treatment) are reclassified as bacteriologically confirmed.	See details in Supplementary Table S3. Criteria for NIH pediatric TB case definitions adapted from Graham 2015.
Non-TB control	Household contacts to enrolled index cases are evaluated for TB symptoms of any duration, including cough, fevers, weight loss or night sweats. If any symptom is present, the participant is followed up longitudinally to assess for resolution of symptoms or evaluated by CXR and Xpert-Ultra to exclude tuberculosis. If the symptoms do not resolve or the evaluation is consistent with tuberculosis, the participant is excluded from the control population. Controls are reassessed at 2 and 6 months to evaluate for sub-clinical disease progression.	
* Clinically diagnosed, presumptive TB patients did not have a positive culture or Xpert result, but were determined to have TB by treating clinicians based on symptoms and radiographic evidence, and were retrospectively classified as "unconfirmed TB" using NIH pediatric TB case definitions[5] (children) or "clinically diagnosed TB" using WHO guidelines[6] (adults).		

290

Supplementary Table 3. Criteria for NIH pediatric TB case definitions (adapted from Graham 2015) *

Confirmed TB

Bacteriologic confirmation from a respiratory (positive *Mtb* culture or Xpert MTB/RIF result) or stool specimen (positive Xpert MTB/RIF result).

Unconfirmed TB

Bacteriologic confirmation NOT obtained and at least 2 of the following:

- Symptoms suggestive of TB
  - Persistent cough (cough>2 weeks, non-remitting cough)
  - Weight loss/Failure to thrive (unexplained weight loss, >5% reduction in weight in last 3 months, deviation from growth trajectory, crossing of percentile lines in prior 3 months, WAZ <=-2 or WHZ <=-2 in absence of prior growth trajectory AND not responding to nutritional rehabilitation or ART if HIV+)
  - Persistent unexplained fever (fever >1 week reported by guardian, or objectively recorded at least once (>38.0)
  - Persistent unexplained lethargy or decreased playfulness reported by caregiver
  - Infants 0-16 days: neonatal pneumonia, unexplained hepatosplenomegaly, or sepsis
- CXR consistent with TB
  - Read by 2 independent, blinded reviewers; Quality of CXR indicated; Standardized forms with predetermined terminology and yes/no options for CXR reader.
  - Example criteria from SATVI:
    - 1) Airway compression or tracheal displacement
    - 2) Soft tissue density suggestive of lymphadenopathy
    - 3) Air space opacification
    - 4) Nodular picture (military or larger, widespread, and bilateral)
    - 5) Pleural effusion
    - 6) Cavities
    - 7) Calcified parenchyma (Ghon focus)
    - 8) Vertebral spondylitis
- Close TB exposure or evidence of *Mtb* infection (TST or IGRA)
- Positive response to TB treatment (clinical features suggestive of TB at baseline have improved and no new clinical feature suggestive of TB.
- Death judged to be possibly or likely TB-related as evaluated by an expert review panel. See **Supplementary Table 4**).

Unlikely TB

Bacteriologic confirmation NOT obtained, criteria for unconfirmed TB NOT met

*Children were classified as having confirmed TB, unconfirmed TB, or unlikely TB, based the 2015 NIH consensus clinical case definitions (ref[5]), which were modified to include stool Xpert data and possible/likely TB-related death as alternate factors for unconfirmed TB classification.

Supplementary Table 4. Proposed criteria to judge TB-related mortality.*	
Classification	**Definition**
Unlikely TB-related	Confirmed or presumed alternate etiology of death, clinical presentation not consistent with intrathoracic TB.
Possible TB-related	Clinical presentation compatible with intrathoracic TB (respiratory symptoms and/or diagnosis of pneumonia) and unable to r/o TB.
Likely TB-related	Confirmed TB without alternate diagnosis preceding death.
* Criteria were proposed by an expert panel, which included four clinician-scientists with expertise in pediatric TB. Three were external to the parent trial study team and one was a co-investigator on the trial. TB classification for all participants was known during the review of whether mortalities were related to TB.	

292

Supplementary Table 5. Oligonucleotide sequences (5'-3') used in this study.			
Oligonucleotide ID	**Sequence**	**Fragment size**	**Target**
IS6110-F IS6110-R gRNA -IS6110	GGTCGGAAGCTCCTATGACAATGCACTAGCC TTGAGCGTAGTAGGCAGCCTCGAGTTCGAC UAAUUUCUACUCUUGUAGAUAUCAGCUCGGUCUUGUAUAG	208	IS6110
esxB-F esxB-R gRNA-esxB	TAAAGAGAGAAAGTAGTCCAGCATGGCAGAG TCTCGTCGAGTTCCTGCTTCTGCTTATTGG UAAUUUCUACUCUUGUAGAUGAGCGGAUCUCCGGCGACCU	236	esxB
gyrB-F gyrB-R gRNA-gyrB	CTGTTGTTGACGTTGTTGTTCCGGTTCATGC CTGAATGCCGTCTTCCTTGTTGATCTTCTTC UAAUUUCUACUCUUGUAGAUGCACAACCGCCGCUGUACAA	119	gyrB
Probe-1	FAM-TTTTTTTTTTTT-BHQ	--	Universal, CRISPR-TB
Probe-2	FAM-AACGGCCTATACAAGACCGAGCTG-BHQ	--	IS6110, qPCR
Probe-3	FAM-TTTTTTTTTTTT-Bio	--	Universal, paper strip-based CRISPR-TB
Underlined sequence indicates the region complementary to the IS6110, esxB, or gyrB target sequence.			

293

Supplementary Table 6. Sources from bacterial analyzed in Fig. 2d.			
No.	**Organism**	**Source**	**Test sample type**
1	*Mycobacterium gordonae*	ATCC 35760	Genomic DNA
2	*Mycobacterium kansasii*	ATCC 12478	Genomic DNA
3	*Mycobacterium avium*	ATCC BAA-968	Genomic DNA
4	*Mycobacterium abscessus*	ATCC 19977	Genomic DNA
5	*Mycobacterium intracellulare*	ATCC 35774	Genomic DNA

294

Supplementary Table 7. Comparison of three cfDNA extraction methods			
	Kit 1 (AllPrep DNA/RNA Mini Kit)	**Kit 2 (Quick-cfDNA Serum & Plasma Kit)**	**NaI Method**
Extraction principle	Affinity column chromatography	Affinity column chromatography	Precipitation
Total DNA yield	Low	Median	High
Extraction efficiency for small DNA	Low	High	High
Running time	< 10 min	< 35 min	> 80 min
Operational complexity	Easy	Easy	Complex

Supplementary Table 8. Lower limit of the positive cut-off threshold for CRISPR-TB signal as defined by CRISPR-TB signal from serum from three adult healthy donors[#].

	Healthy Donor 1	Healthy Donor 2	Healthy Donor 3
Test 1	1.07E+06	1.23E+06	1.03E+06
Test 2	1.26E+06	1.27E+06	1.13E+06
Test 3	1.03E+06	1.14E+06	1.01E+06
Test 4	1.04E+06	1.13E+06	9.83E+05
Test 5	9.31E+05	1.18E+06	1.03E+06
Test 6	8.60E+05	9.32E+05	9.43E+05
Test 7	9.73E+05	9.47E+05	9.67E+05
Test 8	1.22E+06	1.14E+06	1.23E+06
Test 9	1.12E+06	9.68E+05	1.06E+06
Test 10	9.73E+05	9.62E+05	9.41E+05
Average	1.05E+06	1.09E+06	1.03E+06
SD	1.26E+05	1.27E+05	8.98E+04
Cut-off *	1.43E+06	1.47E+06	1.30E+06
Cut-off *	1.40E+06		

Healthy donors are clinically confirmed healthy individuals without TB symptoms and history of TB infection and exposure.

* Lower limit of the positive cut-off threshold for CRISPR-TB defined as Average + 3×SD.

Supplementary Table 9. CRISPR-TB diagnostic performance with TB [a] and non-TB cases in Eswatini cohort.		
Adult	TB (n=28)	Non-TB (n=17)
Mtb-cfDNA +	27	1
Mtb-cfDNA -	1	16
Sensitivity-% (95% CI)	96.4 (81.7-99.9%)	--
Specificity-% (95% CI)	--	94.1% (71.3-99.9%)
Child	TB (n=6)	Non-TB (n=22)
Mtb-cfDNA +	5	1
Mtb-cfDNA -	1	21
Sensitivity-% (95% CI)	83.3% (35.9-99.6%)	--
Specificity-% (95% CI)	--	95.5% (77.2-99.9%)
Pooled	TB (n=34)	Non-TB (n=39)
Mtb-cfDNA +	32	2
Mtb-cfDNA -	2	37
Sensitivity-% (95% CI)	94.1% (80.3-99.3%)	--
Specificity-% (95% CI)	--	94.9% (82.7-99.4%)
[a] TB, including confirmed TB and clinically diagnosed/unconfirmed TB.		

297

Supplementary Table 10. The clinical presentation of five EPTB case in PUSH cohort				
Case ID	**TB Classification**	**EPTB**	**EPTB clinical presentation**	**CRISPR-TB results**
Case 1	Confirmed TB	yes	CXR with military TB - bilateral reticulonodular opacities, pericardial effusion, vertebral spondylitis	Positive
Case 11	Confirmed TB	yes	Lymphadenitis	Positive
Case 15	Unconfirmed TB	yes	CXR with military TB, vertebral spondylitis, airspace opacification	Positive
Case 64	Unconfirmed TB	yes	CXR with military TB + Ghon focus, vertebral spondylitis	Positive
Case 65	Unconfirmed TB	yes	CXR with military TB + Ghon focus, vertebral spondylitis	Positive

298

Supplementary Table 11. CRISPR-TB based early detection of incident TB* in the PUSH cohort.

Case ID	TB classification	Clinical presentation	Days from initial CRISPR-TB analysis to TB diagnosis	Initial CRISPR-TB results
Case 16	Unconfirmed	Symptoms, CXR findings	47	Positive
Case 19	Unconfirmed	Symptoms, CXR findings, TBTx response	160	Positive
Case 23	Unconfirmed	Symptoms, CXR findings, TBTx response	101	Positive
Case 30	Unconfirmed	CXR findings, TB contact, TBTx response	26	Positive
Case 34	Unconfirmed	Symptoms, CXR findings, TBTx response	14	Negative
Case 43	Unconfirmed	Symptoms, CXR findings, TBTx response	40	Positive
Case 55	Unconfirmed	Symptoms, CXR findings, TBTx response	24	Positive
Case 61	Unconfirmed	Symptoms, CXR findings, TBTx response	35	Positive
Case 72*	Unconfirmed	Symptoms, CXR findings	65	Negative
Case 73*	Unconfirmed	Symptoms, CXR findings, TBTx response	89	Positive
Case 74*	Unconfirmed	CXR findings, TBTx response	51	Positive
Case 77	Unconfirmed	Symptoms, TBTx response	16	Positive

* New clinical diagnoses and TB treatment initiation ≥14 days after enrollment.
** Met the criteria for unlikely TB at enrollment but revealed TB-associated symptoms or CXR findings or TBTx response at after enrollment.

Supplementary Table 12. Response and clearance time points of *Mtb*-cfDNA for the 25 cases shown in Fig. S14 a-b

Case ID	Group	Mtb-cfDNA decrease		*Mtb*-cfDNA clearance	
		Visit-point	Days post TBTx initiation	Visit-point	Days post TBTx initiation
Case 10	1	Week-2	16	--	--
Case 60	1	Week-2	8	Week-4	22
Case 67	1	Week-2	15	Week-24	169
Case 22	1	Week-2	13	Week-24	174
Case 13	1	Week-2	21	Week-24	175
Case 38	1	Week-2	10	Week-4	24
Case 76	1	Week-2	15	Week-12	78
Case 41	1	Week-2	7	--	--
Case 69	1	Week-2	13	--	--
Case 48	1	Week-4	33	Week-24	Nearing negative cut-off threshold
Case 44	1	Week-12	83	--	--
Case 53	2	Week-4	24	Week-24	Nearing negative cut-off threshold
Case 59	2	Week-4	30	Week-24	178
Case 45	2	Week-4	30	Week-24	170
Case 11	2	Week-4	20	Week-12	76
Case 70	2	Week-4	33	Week-24	180
Case 47	2	Week-4	26	Week-24	Nearing negative cut-off threshold
Case 8	2	Week-8	49	Week-24	Nearing negative cut-off threshold
case 49	2	Week-12	84	Week-24	172
Case 21	2	Week-12	92	Week-24	Nearing negative cut-off threshold
Case 46	2	Week-12	89	Week-24	167
Case 20	2	Week-24	175	Week-24	175
Case 12	2	Week-12	89	--	--
Case 35	2	Week-24	180	Week-24	180
Case 1	2	Week-24	175	--	--

300

Supplementary Table 13. Cases meeting unlikely TB criteria at initial evaluation demonstrated symptom resolution or weight gain following ART without TBTx. (Group A in Fig. 3a)

Case ID	Initial NIH criteria	Initial CRISPR-TB results
Case 79	Symptoms, CXR+	Positive
Case 80	Symptoms, CXR+	Positive
Case 81	Symptoms, CXR+	Positive
Case 82	Symptoms, CXR+	Positive
Case 83	Symptoms, CXR+	Positive
Case 84	Symptoms, CXR+	Positive
Case 85	Symptoms, CXR+	Positive
Case 86	Symptoms, TB Contact	Positive
Case 87	Symptoms, CXR+	Positive
Case 88	Symptoms, TB Contact	Positive
Case 89	Symptoms, CXR+	Negative
Case 90	Symptoms, CXR+, TB Contact	Negative

References

1. Ushio R, Yamamoto M, Nakashima K, et al. Digital PCR assay detection of circulating Mycobacterium tuberculosis DNA in pulmonary tuberculosis patient plasma. *Tuberculosis (Edinb)* 2016; **99**: 47-53.
2. Fong SL, Zhang JT, Lim CK, Eu KW, Liu Y. Comparison of 7 Methods for Extracting Cell-Free DNA from Serum Samples of Colorectal Cancer Patients. *Clinical Chemistry* 2009; **55**(3): 587-9.
3. Arnheim N, Erlich H. Polymerase chain reaction strategy. *Annual review of biochemistry* 1992; **61**(1): 131-56.
4. Meintjes G, Lawn SD, Scano F, et al. Tuberculosis-associated immune reconstitution inflammatory syndrome: case definitions for use in resource-limited settings. *Lancet Infect Dis* 2008; **8**(8): 516-23.
5. Graham SM, Cuevas LE, Jean-Philippe P, et al. Clinical case definitions for classification of intrathoracic tuberculosis in children: An update. *Clin Infect Dis* 2015; **61Suppl 3**: S179-87.
6. WHO. Definitions and reporting framework for tuberculosis-2013 revision. In: Programme GT, editor.: WHO; 2013.