SUPPLEMENTAL MATERIAL

MOVER approximated CV: a tool for quantifying precision in ratiometric droplet digital PCR assays

Christian Dide-Agossou,[1†] Karen Rossmassler,[2,3] Justin Reid,[2,3] Juhi Purohit,[2,3] Rada M Savic,[4,5,6,7] Payam Nahid,[4,5,6,8] Patrick P J Phillips[8] Camille M Moore,[9] Nicholas D Walter[2,3,4]

† Corresponding author (CDA)

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dMIQE SUPPORTING INFORMATION

1. SPECIMENS

This study used 244 RNA-preserved human sputa collected longitudinally in Study 31, an international, multicenter, randomized, open-label, phase 3, noninferiority trial conducted at sites of the Centers for Disease Control and Prevention Tuberculosis Trials Consortium and the National Institutes of Health AIDS Clinical Trials Group [1]. Sputa were collected in sterile RNAase-free cups containing a guanidine thiocyanate (GTC) solution, the composition of which is described elsewhere [2,3]. Briefly, expectorated sputum was preserved within 5 minutes in the GTC solution. Sputa were needle sheared, centrifuged at 9000×g to separate intact *Mtb* from the eukaryotic cell lysate and other soluble materials. Finally, the pellet containing GTC-stabilized *Mtb* was resuspended in TRIzol (Invitrogen, #15596026) at −80°C until batch shipment on dry ice to the University of Colorado.

2. NUCLEIC ACID EXTRACTION

RNA was extracted using the phenol/chloroform method. Briefly, tubes containing sputum/TRIzol mixture were homogenized prior to RNA extraction. 0.4 ml of 0.1 mm silica beads (Matrix B) (MP Biomedicals, #116911050) were added to each tube. Cells were disrupted by bead beating three times at 30 seconds at 6 M/S on Fastprep24 bead-beater (MP Biomedicals), resting on ice for 1 min in between bead beating cycles. Lysates were centrifuged at 20,800×g for 1 minute and the supernatant was transferred to a new tube and mixed with chloroform. After homogenization, tubes were centrifuged for 10 min at 20,800×g and the aqueous phase was transferred to a new tube with 0.1 volume 5 M ammonium acetate and equal volume of isopropanol. RNA was precipitated overnight at -30°C, followed by 10 minutes of centrifugation at 4°C at 20,800×g. The isopropanol was aspirated and the pellet was washed

twice in 500 µl 70% ethanol, and centrifuged at 20,800×g for 5 minutes after each wash. The pellet was air dried for 10 minutes at room temperature and resuspended in 80 µl nuclease-free water. The RNA was purified using the Maxwell RSC simply RNA tissue kit on the Maxwell RSC instrument (Promega, #AS1340) following the protocol described by Promega (Maxwell RSC simply RNA Tissue Kit, Promega).

For every batch, a negative extraction control was included to test for cross-contamination between samples.

3. NUCLEIC ACID ASSESSMENT AND STORAGE

RNA concentration (ng/µl) was quantified via Quantus (Promega) with the QuantiFluor RNA System kit (Promega, #E3310). RNA samples were stored at -80°C. RNA purity and integrity were not measured.

4. REVERSE TRANSCRIPTION

SuperScript™ III VILO™ Master Mix (Invitrogen, #11755500) was used to generate cDNA from purified RNA in a 10 µl reaction. Each reaction used 8 µl purified RNA mixed with 2 µl reverse transcriptase. The cycling conditions were set as follows:

a. 25°C for 10 minutes

b. 42°C for 120 minutes

c. 85°C for 5 minutes

The resulting cDNA was stored at -80°C until use. To test for residual DNA, we performed a reverse transcription without the addition of reverse transcriptase (RT-) for a random subset of samples. For all samples, the C_T value for the RT- reaction was at least 8 higher than the corresponding RT + reaction, indicating negligible DNA contamination.

5. PCR OLIGONUCLEOTIDES DESIGN AND TARGET INFORMATION

The primer/probe sets targeting the pre-rRNA and 23S rRNA gene are summarized in Table S1. *in silico* verification of the PCR primer/probe sets specificity was performed using command-line BLASTN version 2.12.0+ (Zhang et al., 2000). Verification was performed against downloaded genomes from the Integrated Microbial Genomes & Microbiomes system.

Table S1. PCR primer/probe sequences and information.

Target	Location	Amplicon length	Primer/Probe	Sequence	5' Modification	Internal	3' Modification	Manufacturer
ETS	1471792-1471858	66	Forward	CCGTTTGTTTTGTCAGGATATTTCT				IDT
			Probe	AATACCTTTGGCTCCCTTT	56-FAM		MGBNFQ	Fisher/ABI
			Reverse	CAAAACCCAAACACTCCCTTTG				IDT
23S	1474392-1474461	69	Forward	GCAGCGAAAGCGAGTCTGA				IDT
			Probe	AGGGCGACCCACACGCGC	56-HEX	ZEN	3IABkFQ	IDT
			Reverse	CCAGAACACGCCACTATTCACA				IDT

6. dPCR PROTOCOL

Each sample was partitioned into approximately into ~20,000 droplets using either a manual droplet generator (QX200, Bio-Rad) or an auto droplet generator (AutoQX200, Bio-Rad). Transcript copies were amplified using Bio-Rad C1000 Touch thermal cycler followed by quantification using Bio-Rad QX200 Droplet reader. The primer/probe concentrations, and thermocycling conditions are described in Table S2. Reactions were run in duplex (ETS with 23S) with dPCR SuperMix for Probes (no dUTP) ((Bio-Rad, #1863024).

Table S2. dPCR primer/probe concentrations and thermocycling conditions.

Target	Primer/Probe	dPCR concentration	Template treatment	dPCR thermocycling conditions
ETS	Forward	900 nM	Initial heating denaturation	1. 95C for 5 min (4.8C/sec ramp rate) 2. 95C for 30 sec (4.8C/sec ramp rate) 3. 60C for 30 sec (2.5C/sec ramp rate) 4. go to (2) 39 more times 5. 15C for 30 sec (1.5C/sec ramp rate)
	Probe	250 nM		
	Reverse	900 nM		
23S	Forward	900 nM		
	Probe	250 nM		
	Reverse	900 nM		

7. dPCR DATA ANALYSIS

One positive and at least two negative controls were routinely including in every dPCR experiment. Copies per partition of each target and the ratio between the targets were calculated within each duplexed reaction by the QuantaSoft software (Bio-Rad, AP v1.0). Per Bio-Rad's recommendation, a dPCR assay needs at least 10,000 droplets to be accepted. To separate positive and negative partitions, the threshold was set as the average of the mean amplitude of positive and negative droplets of the positive control. To avoid leading zeros, the ratio provided by QuantaSoft was multiplied by 10,000, resulting in the RS ratio. Figure S1 below show examples of positive and negative experimental results.

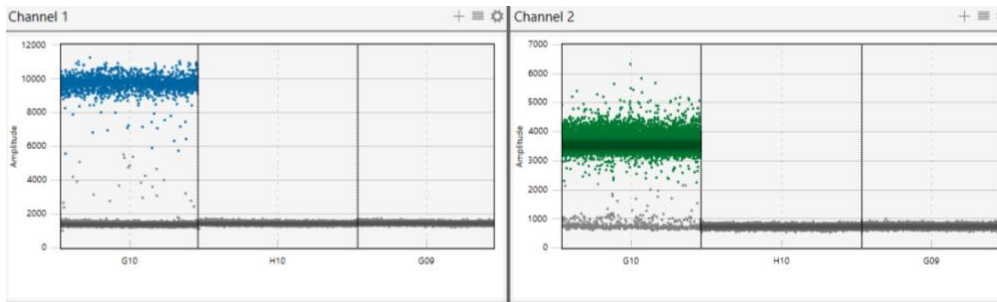

Fig. S1 Example of a dPCR assay showing ETS1 signal (blue) and 23S signal (green) for a positive control (G10) and two negative controls (G09 and H10).

ETHICS STATEMENT AND INSTITUTIONAL REVIEW BOARDS

This manuscript uses specimens from a clinical trial. All participants provided written informed consent for the use of their sputum and clinical information to develop new biomarkers measuring the effectiveness of treatment. The institutional review boards that supervised these studies are listed below.

Cohort	Study Location	Supervising Institutional Review Boards
Sputum Transcriptomic	Uganda	1. Uganda National Council for Science and Technology. Protocol HS 259

Expression Profiling (STEPS-EXPRESS 31): A Substudy of TBTC Study 31/ACTG 5349		2. Makerere University Faculty of Medicine Research and Ethics Committee. Protocol 2006-017 3. Mulago Hospital Institutional Review Board. Protocol 2006-017 4. Colorado Multiple Institutions Review Board. Protocol 10-0290 5. University of California San Francisco Human Research Protection Program Institutional Review Board. Protocol H8660-27882
	Vietnam	1. Vietnam Ministry of Health Ethical Committee in National Biological Medical Research. Protocol 12/QD-BYT 2. University of California San Francisco Human Research Protection Program Institutional Review Board. Protocol H8660-27882-06 3. US Centers for Disease Control and Prevention Institutional Review Board 2. Protocol 6560.0

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