Supplementary Table S1. **Sensitivity pattern of clinical isolates and specimens included in the study.** The table reports RIF and INH molecular pattern for MTBC isolates and MTBC-positive specimens, based on MTBDR*plus* and/or Sanger sequencing. For 4 cases, only Xpert MTB-RIF result was available (*). Samples were made available by the members of the TM-REST, EDCTP TB CHILD Consortia, and from Hospital Universitari de Bellvitge (Spain). Phenotypic DST was also considered when available. One hundred and sixty-three specimens (83%; 47 smear-positive culture positive plus 116 smear-negative culture negative samples) were collected and directly tested on-site at the Nsambya hospital in Kampala, Uganda within the TB CHILD project. The remaining samples were tested in Supranational Reference Laboratory Milan. Ethical approval and patients' consensus were obtained for all clinical samples.

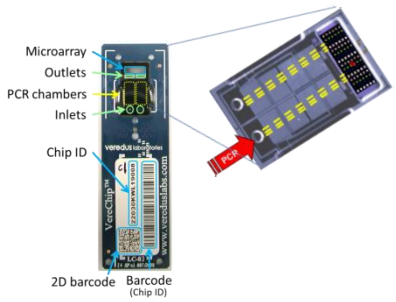
MTB pattern (MTBDRplus/sequencing)											
MTBC clinical isolates (N°91)						MTBC direct specimens (N°80) - AFB positive					
N°	RIF	INH	*rpoB*	*katG*	*inhA*	N°	RIF	INH	*rpoB*	*katG*	*inhA*
15	R	R	S531L	S315T1	WT	9	S	R	WT	WT	C-15T
1	R	R	WT	WT	WT	2	R	R	S531L	S315T1	WT
5	S	R	WT	WT	WT	1	R	R	S531L	WT+S315T1	WT
16	R	R	S531L	WT	C-15T	6	S	R	WT	WT	WT
7	R	R	S531L	WT	WT	2	R	R	D516V	S315T1	WT
2	R	S	S531L	WT	WT	2	R	R	S531L	WT	WT
2	R	R	H526D	S315T1	WT	4	S	R	WT	S315T1	WT
1	R	R	L511P	S315N	WT	1	R	R	S531L	S315T1/T2	WT
1	R	R	H526D	S315R	WT	1	R	R	Q513P	S315T1	WT
1	R	R	H526Y	S315N	WT	1	S	R	WT	S315N	WT
1	R	S	D516V	WT	WT	1	R	S	S531L	WT	WT
2	R	R	S531L	S315T1	T-8A	1	-	-	D516V	S315T1	T-8C
1	R	R	L530M+S531P	S315T1	T-8C	16	-	-	WT	WT	WT
2	R	R	S531L	S315T2	WT	1	R	R	S531L	WT	C-15T
3	R	R	D516V	S315T1	T-8A	15	S	S	WT	WT	WT
1	R	R	D516V	S315T1	T-8C	4*	-	-	WT	-	-
11	S	R	WT	WT	C-15T	11	S	S	-	-	-
5	R	R	D516V	S315T1	WT	1	R	R	Δ 518-525 WT, Δ 530-533 WT	S315T1	WT
5	S	R	WT	S315T1	WT	1	-	-	WT	S315T1	WT
1	R	R	H526D	S315T1	WT						
3	R	R	S531L	S315T1	C-15T						
1	R	R	Q513P	S315T1	WT						
1	S	R	WT	S315N	WT						
2	R	R	H526Y	S315T1	C-15T						
1	S	S	WT	WT	WT						

Supplementary Figure S2. **VerePLEX Biosystem**. (a) The platform includes: a compact Temperature Control System (TCS) consisting of 5 independent modules for amplification and

hybridization phases, and an optical reader (OR) able to excite Cy5-tagged amplicons which allows to automatically analyze the microarray providing an user-friendly diagnostic report within few seconds by the use of a dedicated software compatible with a standard PC and (b) LabOnChip device with detailed PCR and microarray areas. (c) Microarray layout for MDR-TB detection (100 custom hybridization spots and 26 control probes).

(a)

(b)



(c)

	1	2	3	4	5	6	7	8	9	10	11
1	AT683	D516V_m1	S531L_m2	empty	MYC4a	MYC10a	AT683	AT683	D516V_m1	S531L_m2	empty
2	L511_w3a	AT730	S315_w2	InhA - 15C>T_m3	AT809	MYC17a	MYC16a	L511_w3a	AT730	S315_w2	InhA - 15C>T_m3
3	L511P_m3	empty	S315T1_m 2	InhA - 8T>A_m2	MYC5a	AT776	BG1	L511P_m3	empty	S315T1_m 2	InhA - 8T>A_m2
4	D516V_w5	H526D_m2	H526_w14	InhA - 8T>C_m2	MYC6a	MYC19a	rpoB14	D516V_w5	H526D_m2	H526_w14	InhA - 8T>C_m2
5	AT809	H526Y_m5	S315T2_m 1	katG6	rpoB9	MYC31a	BG2	AT809	H526Y_m5	S315T2_m 1	katG6
6	AT683	S531L_w1	InhA_w3	InhA1	MYC8a	MYC15a	AT683	AT683	S531L_w1	InhA_w3	InhA1

	12	13	14	15	16	17	18	19	20	21
1	MYC4a	MYC10a	AT683	AT683	D516V_m1	S531L_m2	empty	MYC4a	MYC10a	AT683
2	AT809	MYC17a	MYC16a	L511_w3a	AT730	S315_w2	InhA - 15C>T_m3	AT809	MYC17a	MYC16a
3	MYC5a	AT776	BG1	L511P_m3	empty	S315T1_m 2	InhA - 8T>A_m2	MYC5a	AT776	BG1
4	MYC6a	MYC19a	rpoB14	D516V_w5	H526D_m2	H526_w14	InhA - 8T>C_m2	MYC6a	MYC19a	rpoB14
5	rpoB9	MYC31a	BG2	AT809	H526Y_m5	S315T2_m 1	katG6	rpoB9	MYC31a	BG2
6	MYC8a	MYC15a	AT683	AT683	S531L_w1	InhA_w3	InhA1	MYC8a	MYC15a	AT683

Supplementary Text S3.

LoC for species identification and MDR-TB assay

PCR primers and probes

Species identification: Primers for species identification were previously designed for the *orfB* of the insertion sequence IS*6110* of *M. tuberculosis* (122 bp fragment) and the 16S rRNA (235 bp in size containing hypervariable region A) genes of most relevant mycobacterial species as described in Lazzeri *et al* (26). Probes specific for the identification of MTBC, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, *M. simiae*, *M. xenopi*, *M. haemophylum*, *M. chelonae*, *M. abscessus* and *M. fortuitum* were designed.

Molecular DST (RIF and INH resistance): Fragments of *rpoB* (222 bp), *katG* (97 bp), and *inhA* (103 bp) genes as the most frequently mutated genes involved in the MDR phenotype in MTBC were amplified designing specific primers to be used on the LoC (Supplementary Table S4).

Wild-type *rpoB* hot-spot (codon 510-513, 515-518, 523-526, 530-533), *inhA* (-21/-7) and *katG* (codon 313-317), were targeted with specific probes, as well as mutations L511P (ctg/ccg), D516V

(gac/gtc) , H526Y (cac/tac), S531L (tcg/ttg) for *rpoB*, c-15t t-8a, t-8c for *inhA*, and S315T (agc/acc, agc/aca) for *katG*.

Amplification locus controls were added for the interpretation of the results (Table 1).

PCR protocol

Two different PCR Master Mixes containing water for molecular biology, PCR buffer, $MgCl_2$, dNTPs, Taq DNA polymerase, primers for multiplex amplification of specific targets (Master Mix 1: primers for species identification, *katG* and *inhA*, Master Mix 2: primers for *rpoB*) and internal PCR controls for the confirmation of valid results were provided by the manufacturer.

Amplification protocol consists of 1 cycle at 95°C for 15 minutes to activate the DNA polymerase, 20 cycles of denaturation at 95°C for 30 s and primer annealing at 62°C for 120 s, 40 cycles of denaturation at 95°C for 10 s, primer annealing at 50°C for 10 s and extension at 72°C for 20 s, followed by a final extension at 72°C for 180 s (total time required for the amplification step including hands-on steps: 105 minutes).

LoC procedure

After preparation of the amplification mix and addition of DNA, 11.5 µL of Master Mix 1 were loaded in one inlet of the chip and 11.5 µL of Master Mix 2 in the other one, the chip was sealed with clamps and then positioned inside one Temperature Control Module (TCM), starting the PCR Program.

After the amplification step, PCR products are driven to the detection area (DA) by the use of a hybridization solution (HYB) containing hybridization controls, Phosphate Buffered Saline (PBS), Tween20 surfactant, Denhardt's solution, Salmon Sperm DNA, Sodium Cloride, formamide, Tetramethylammonium Chloride and betaine provided by the manufacturer. Thus, at the end of the PCR phase, chip was removed from the TCM to load 14.5 µL of HYB per each inlet, making the solution flow from the outlet to the DA. Chip was loaded again inside the TCM for the hybridization phase: amplicons are denaturated at 95°C for 5 minutes and hybridized on the microarray at 50°C for 30 minutes (total time required for the hybridization step including hands-on

steps: 35 minutes). LoC was recovered and transferred in a 50 mL conical tube containing the washing buffer (containing Sodium Dodecyl Sulfate 0.05%, and Saline-Sodium Citrate 0.1X) and centrifuged at 1500 g for 2 minutes and dried in an empty tube using the same settings. At the end of the drying step, the chip was analyzed with the OR, automatically generating a report based on the diagnostic rules fixed for each probe.

Software analysis

E@syCheck software rules for generation of a diagnostic report from the analysis of the microarray data (spot position in the layout, ON/OFF status, spot intensity/size/shape, signal mean, background signal, signal area, signal median: max 65535, background median: 257, signal standard deviation) were fixed after the evaluation of expected WT / expected MUT values from each probe (generally a probe is ON when signal median is at least three times over background median).

Supplementary Table S4. List of the primers used for the MDR-TB LoC. Reverse primers are Cy5-tagged.

Primer	Sequence (5'-3')	Tm (°C)	Target gene	Locus Tag	Genome coordinates
MYC1p	AGTGGCGAACGGGTGAGTAA	61.50	16S rDNA	Rvnr01	1410253
MYC2p_cy5	CGTATCTCAGTCCCAGTGTG	57.40			1410487
MYC13p	GACCACCAGCACCTAACC	57.30	IS*6110*	Acc. X17348	925187
MYC14p_cy5	GACCCGCCAGCCCAGGAT	64.20			925308
rpoB99U20	GGACGTGGAGGCGATCACAC	65.50	*rpoB*	Rv0667	761012
rpoB302L19_cy5	CCGTAGTGCGACGGGTGCA	67.60			761233
katG135U20	TGGGCTTGGGCTGGAAGAGC	66.10	*katG*	Rv1908c	2154519
katG211L21_cy5	CATTTCGTCGGGGTGTTCGTC	62.30			2154615
inhA184U21	CGCTCGTGGACATACCGATTT	61.80	*inhA*	Rv1484	1673391
inhA266L21_cy5	ACGGGATACGAATGGGGGTTT	62.00			1673493

Supplementary Table S5. The limit of detection (LoD) of the assay for MDR-TB identification was evaluated using serial dilutions of Quantitated Bacterial DNA PCR control of MTB H37Rv (Advanced Biotechnologies Inc., Columbia, Maryland, USA) in the range of 400, 200, 120, 100, 40, 4 DNA copies/µL each tested at least in triplicate. The LoD was defined as the minimum DNA concentration needed to obtain positive signals from MTBC identification and WT probes spotted on the array. For each target spotted on the array the percentages of WT probes resulted ON with

standard deviation among replicates and the number of chips tested for each concentration are reported.

	Target	MTB		*rpoB*		*katG*		*inhA*	
Concentrations	**Chip No. tested**	% ON mean	dev st	% ON mean	dev st	% ON mean	dev st	% ON mean	dev st
4 DNA copies/uL	3	100.00	0.00	75.00	36.32	33.33	57.74	100.00	0.00
40 DNA copies/uL	4	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
100 DNA copies/uL	4	100.00	0.00	83.33	9.13	83.33	33.33	100.00	0.00
120 DNA copies/uL	9	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
200 DNA copies/uL	12	100.00	0.00	97.92	5.18	91.67	28.87	100.00	0.00
400 DNA copies/uL	4	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00

DNA hybridization to MTBC identification probes was obtained up to the concentration of 4 DNA copies/µL; for *katG*, *inhA* and *rpoB* targets probes positive signals were always obtained at 40 DNA copies/µL.