How well do routine molecular diagnostics detect rifampicin heteroresistance in *Mycobacterium tuberculosis*?

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Running Head: Routine detection of rifampicin heteroresistance

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ABSTRACT

Rifampicin heteroresistance – where rifampicin-resistant and -susceptible tuberculosis (TB) bacilli co-exist – may result in failed standard TB treatment and potential spread of rifampicin-resistant strains. Detection of rifampicin heteroresistance in routine rapid diagnostic tests (RDTs) allows for patients to receive prompt and effective multidrug-resistant-TB treatment, and may improve rifampicin-resistant TB control.

The limit of detection (LOD) of rifampicin heteroresistance for phenotypic drug susceptibility testing by the proportion method is 1%, yet is insufficiently documented for RDTs. We therefore aimed to determine, for the four RDTs (XpertMTB/RIF, XpertMTB/RIF Ultra, GenoTypeMTBDR*plus*v2.0, and GenoscholarNTM+MDRTBII), the LOD per probe and mutation, validated by colony-forming-unit-counting and targeted deep sequencing (Deeplex-MycTB).

We selected one rifampicin-susceptible and four rifampicin-resistant strains, with mutation D435V, H445D, H445Y, and S450L respectively, mixed them in various proportions in triplicate, tested them with each RDT, and determined the LODs per mutation type. Deeplex-MycTB revealed concordant proportions of the minority resistant variants in the mixtures. The Deeplex-MycTB-validated-LODs ranged from 20-80% for XpertMTB/RIF, 20-70% for Xpert Ultra, 5-10% for GenoTypeMTBDR*plus*v2.0, and 1-10% for GenoscholarNTM+MTBII for the different mutations.

Deeplex-MycTB, GenoTypeMTBDR*plus*v2.0, and GenoscholarNTM+MDRTBII, provide explicit information on rifampicin heteroresistance for the most frequently detected mutations. Classic Xpert and Ultra report rifampicin heteroresistance as rifampicin resistance, while Ultra may denote rifampicin heteroresistance through ’mixed patterns’ of wild-type and mutant melt probe melt peak temperatures.

46 Overall, our findings inform end-users that the threshold for reporting resistance in case of
47 rifampicin heteroresistance is the highest for Classic Xpert and Ultra, to resolve phenotypic
48 and genotypic discordant rifampicin-resistant TB results.

49 **INTRODUCTION**

50 Resistance to rifampicin (RIF) – the most potent core drug in the standard tuberculosis (TB)
51 treatment regimen (1) – is a major barrier to TB control. In 2017, 71% of global RIF resistant-
52 TB (RR-TB) cases were not diagnosed (2). The diagnosis of RR-TB may be complicated by RIF
53 heteroresistance, observed in patient samples where RR and RIF-susceptible (RS) strains co-
54 exist (3, 4), which may be missed and diagnosed as RS because of detection limits. RIF
55 heteroresistance may arise from an existing resistant clonal subpopulation or from a mixed
56 infection of independent strains with RR and RS profiles. Such heteroresistance, also known
57 as 'unfixed' resistance, precedes full blown resistance ('fixed' resistance, 100% RR) as a
58 result of further resistance selection under treatment (4-7). Failure to detect these minority
59 resistant variants can thus result in unsuccessful treatment and potential spread of RR-TB
60 strains (3, 4, 8).

61 The World Health Organization currently endorses use of rapid diagnostic tests (RDTs) for
62 timely detection of RR-TB strains: XpertMTB/RIF (classic Xpert), Xpert Ultra (Ultra), and the
63 Line Probe Assays (LPAs) GenoTypeMTBDR*plus*v2.0 (LPA-Hain) and Genoscholar
64 NTM+MDRTBII (LPA-Nipro) (9-11). Among these RDTs, only the LPAs are currently known to
65 explicitly detect RIF heteroresistance in case of mixtures with mutations covered by the
66 assay, exemplified by both wild-type and mutant bands being present, also known as 'mixed
67 patterns' (4, 12).

68 In this study, we define RIF heteroresistance limit of detection (LOD) as the minimum
69 proportion of mutant bacilli in a total mycobacterial population present in a sample, needed
70 for RIF resistance to be detected (4). It is known that phenotypic drug susceptibility testing
71 by the proportion method determines at least 1% resistant subpopulation in clinical samples
72 (4, 13, 14). In the abovementioned RDTs however, the RIF heteroresistance LOD in

73 association with the specific *rpoB* mutation, is insufficiently documented. In the case of
74 classic Xpert, previous studies report LOD values ranging from 65 to 100% (15, 16), for Ultra,
75 the first validation study conducted by the manufacturer presented LODs only for mutations
76 L430P, H445N (20-40%), and S450L (5-10%) (17), whereas LODs of Genoscholar
77 NTM+MDRTB II have not been reported yet.
78 High coverage depths achieved through pre-selected amplified genes allow targeted deep
79 sequencing to capture and quantify minority resistant variants of *Mycobacterium*
80 *tuberculosis* mutants and detect RIF heteroresistance with high sensitivity (18, 19). As an
81 example of such an approach, Deeplex®-MycTB (Genoscreen, France; Deeplex) employs
82 ultra-deep sequencing of a single, 24-plexed amplicon mix to detect drug resistance-
83 associated mutations in *M. tuberculosis* complex (MTBC) strains, in addition to
84 mycobacterial species identification and MTBC strain genotyping, with a 24-48 hours
85 turnaround time starting from smear positive clinical samples or primary cultures. Among
86 the 18 main gene targets associated with 1st and 2nd– line drug resistance included in
87 Deeplex-MycTB, the *rpoB* gene – associated with RR - is covered by two amplicons of which
88 one comprises the main mutation hotspot region also known as the rifampicin resistance
89 determining region (RRDR) (20).
90 Precise documentation of LODs for most relevant *rpoB* mutations and for the state-of-the-
91 art RDTs is necessary for timely and more accurate identification of RIF heteroresistance and
92 prompt initiation of appropriate treatment. Therefore, we determined the LODs of classic
93 Xpert, Ultra, LPA-Hain, and LPA-Nipro for detecting RIF heteroresistance linked with RR
94 mutations S450L, D435V, H445D, and H445Y, in relation with the different probes used in
95 each RDT. These four mutations are most frequently detected in association with RR-TB in
96 the global MTBC strain population according to large-scale studies (10, 21). We used

97 simulated mixtures of selected, cultured RR and RS-TB strains, at ratios initially based on
98 colony-forming-unit (CFU) counts after McFarland standardization of the bacillary
99 suspensions. Targeted deep sequencing by Deeplex-MycTB was used as reference for
100 quantitative assessment of the RR:RS ratios.
101
102 **MATERIALS AND METHODS**
103 **Selection of strains**
104 We selected one RS- and four RR- *M. tuberculosis* strains from the Belgian Coordinated
105 Collection of Microorganisms hosted in the Institute of Tropical Medicine Antwerp (22), on
106 the basis of the presence of mutations confirmed by Sanger sequencing and captured by
107 mutation probes of LPA-Hain and LPA-Nipro: the Beijing 2.2.1.1 strains TB-TDR-0090 (ITM-
108 041208 with *rpoB* mutation S450L (S531L in *E.coli* numbering)) and TB-TDR-0100 (ITM-
109 041220, D435V (D516V)); the LAM 4.3.4.2 strains TB-TDR-0036 (ITM-000930, H445D
110 (H526D)) and TB-TDR-0131 (ITM-041289, H445Y (H526Y)) (23). The RS strain was the Euro-
111 American lineage 4.9 TDR-0140 (ITM-091634, *rpoB* wildtype).
112 **Bacillary suspensions and baseline colony-forming-unit (CFU) counting**
113 We prepared two batches of McFarland standard 1 (24, 25) suspensions for each RR and the
114 RS strain.. To check if numbers of bacilli were similar among the cultures after McFarland
115 standardization, we performed CFU counting by spread plating of serial dilutions until 10^{-4}-
116 10^{-6} (Figure S1 a). From each dilution, 100µl was plated in triplicate on Dubos agar plates
117 that were sealed with a double layer of parafilm, placed in ziplock bags and incubated at
118 37°C for four weeks, before colony counting. The first batch was used to prepare RR:RS
119 mixtures for testing by classic Xpert, LPA-Hain, and LPA-Nipro, and the second batch was
120 prepared for assessment by Ultra, which was only released after initial testing.

121 **Simulation of RIF heteroresistance**

122 RIF heteroresistance was simulated for each mutation type, by mixing McFarland standard 1
123 suspensions of the RS and respective RR strains in triplicate in the following proportions
124 (R:S): 0:100, 1:99, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, and 100:0
125 (Figure S1 b), and vortexing the mixtures for 20 seconds. Replicate 3 of each RR:RS mixture
126 per batch was tested by targeted deep sequencing (Deeplex-MycTB), the results of which
127 served for cross-validation of variant quantification.

128 **Subjection of RR:RS mixtures to RDTs**

129 We subjected mixtures of RR- and RS-TB bacillary suspensions to classic Xpert and Ultra (10,
130 11), and thermolysates to LPA-Hain and LPA-Nipro following manufacturer's instructions. We
131 recorded the LODs and corresponding RDT probe reaction per mutation type, in comparison
132 with values cross-validated by CFU counts and variant quantification with Deeplex-MycTB.
133 First reading of the LPA strips was done by the person who prepared the mixtures and
134 performed the tests, while second reading was done by a colleague who was blinded to the
135 sample information to ensure objective reading of raw results. Additionally, for LPA-Nipro,
136 the Genoscholar Reader - a mobile application developed by Nipro (Osaka, Japan,
137 https://itunes.apple.com/by/app/genoscholar-reader/id1149733183?mt=8) was utilized.
138 In their standard reporting, Classic Xpert and Ultra (Supplemental File Figure S4 a) report RIF
139 heteroresistance, above or equal its LOD, as RR. Users can indirectly infer RIF
140 *hetero*resistance from Ultra data, as shown by the simultaneous presence of wild-type and
141 mutant (Mut) melt peak temperatures at the 'Melt Peaks' tab (Supplemental File Figure S4
142 b). When generating the results in portable document format, users must tick the 'Melt
143 Peaks' box to include the melt peak temperatures associated with each wild-type and Mut
144 melt probe, which may denote RIF heteroresistance, in the extended report (Supplemental

145 File Figure S4 b). Full resistance is detected by presence of both rpoB4A and rpoB3 Mut melt
146 probes for mutation S450L, whereas RIF heteroresistance is detected only by the rpoB4A
147 Mut melt probe in combination with the corresponding wild-type melt probe (Figure 1).

148 **Quantitative assessment of mutant proportions by Deeplex-MycTB**

149 Per batch, we quantitatively assessed proportions of resistant subpopulations in replicate 3
150 of the prepared RR:RS mixtures by Deeplex-MycTB. These proportions were determined by
151 calculating the mean percentages of minority resistant variants across all mutation positions
152 borne by each of the RR strains in the *rpoB* gene and other gene targets depending on the
153 strain genetic background.

154 Thermolysates of the RR:RS mixtures prepared as previously described (26) were subjected
155 to amplicon deep sequencing, using Deeplex-MycTB Kits for the amplification according to
156 the manufacturer's instructions. Replicates 3 of all first batch mixtures were tested with the
157 classical 18-gene target version, while replicate 3 of the second batch with S450L-WT
158 mixtures was tested with a customized version, including 5 gene targets (*rpoB, katG, inhA,*
159 *fabG1, gyrA*). Amplicons were purified using Agencourt® AMPure® XP magnetic beads
160 (Beckman Coulter, USA) and quantified by fluorescence quantification in 96-well plates on
161 Victor. Paired-end libraries of 150-bp read length were prepared using the Nextera XT DNA
162 Sample Preparation kit (Illumina Inc., San Diego, 160 CA, USA) and sequenced on Illumina
163 MiniSeq using standard procedures. Variant calling was performed using a dedicated,
164 parameterized web application developed by Genoscreen. The nominal threshold for calling
165 minority resistant variants – indicating heteroresistance for drug resistance associated
166 mutations – is set at a minimum of 3% of all reads, after filtering and depending on the
167 coverage depths, to minimize false positive calls due to background technical noise (20, 27,
168 28). Variants present in lower proportions than 3% in the relevant *rpoB* mutation positions

169 were detected separately from the web application,(29) without application of this nominal
170 threshold in the analysis pipeline.
171
172 **RESULTS**
173 After McFarland standardization of the different strain cultures, the mean CFU counts for
174 dilutions 10^{-4}-10^{-6} of the three replicates of both first and second batches of the different RS
175 and RR bacillary suspensions were very similar (Table S1 a and b). The proportions obtained
176 from the Deeplex-MycTB analysis were overall consistent with the expected mixture ratios
177 and the relative variation seen among CFU counts, as relative deviations from expected
178 values were limited to 0-16% (Table 1, Figure S2).
179 We excluded replicate 3 from the first batch of S450L-WT preparation due to substantially
180 high deviation across all mixture ratios, as revealed by Deeplex-MycTB (Supplemental Figure
181 S3). This deviation potentially reflects pipetting variation or bacillary clumping despite
182 similar CFU counts of the respective RR and RS strains. Hence for the S450L-WT mixture,
183 replicate 3 of batch 2 – which was initially only tested by Ultra and Deeplex – was subjected
184 also to classic Xpert and the LPAs, given the good correlation between the two batches and
185 to ensure triplicate testing for all mixtures.
186 In line with these levels of experimental variation, all *rpoB* variants from 5:95 RR:RS mixtures
187 were called by the Deeplex application in proportions ranging from 4.3% (H445Y) to 7.5%
188 (H445D)(Table 1). When the analysis pipeline was used without applying this threshold,
189 expected variants of 1:99 mixtures were also detected in percentages ranging from 1.0%
190 (H445Y) to 1.6% (D435V), which were above background levels of 0.0 to 0.05% detected in
191 the 0:100 mixtures (i.e. wild type strain only) on these specific sequence positions. The
192 average minimum coverage depth observed from the Deeplex analyses was 1595 reads.

193 Among the available classical RDTs, LPAs had a lower LOD to detect RIF heteroresistance
194 compared to classic Xpert and Ultra. The proportion of variants required to be detectable
195 through LPA-Hain was 5% for mutation S450L and 5-10% for mutations D435V, H445D, and
196 H445Y (Table S2). LPA-Nipro performed similarly, with 1-5%, 5%, 5-10% and 10% resistant
197 bacilli detected for mutations S450L, H445D, D435V, and H445Y respectively (Table S2). LPA
198 reading results were consistent between the two readers. Additionally, the 'automated'
199 Genoscholar reader had similar results with manual reading for mutations H445Y and S450L;
200 whereas it had lower sensitivity for mutations D435V (20% Genoscholar reader vs 5%
201 manual reading) and H445D (10% Genoscholar reader vs 5% manual reading) (Table S3,
202 Figure S5 a-d).
203 In contrast, Classic Xpert detected mutation S450L only in mixtures with at least 20-40%
204 resistant bacilli, mutation H445D with at least 40-60% and mutations D435V and H445Y with
205 at least 70-80% mutant bacilli (Table S2). Likewise, Ultra required a minimum of 20-30%
206 resistant bacilli to detect mutation S450L, 40-50% for D435V, 40-60% for H445D and 60-70%
207 for H445Y (Table S2; Figure 1).
208 Notably, in case of S450L at 20% mutant bacilli, only Ultra rpoB4 Mut melt A probe was
209 observed, whereas both rpoB4 Mut melt A and rpoB3 Mut melt probes were present in case
210 of 100% S450L (Figure 1). For mutations D435V, H445D, and H445Y only one Mut melt probe
211 was observed, whether hetero- or fully resistant. The melt peak temperatures did not differ
212 among hetero- or fully resistant populations for all four mutations tested (Figure 1).
213

214 **DISCUSSION**

215 Consistent with previous studies, we found that LPA-Hain and LPA-Nipro detect rifampicin
216 heteroresistance better than classic Xpert and Xpert Ultra in samples with sufficient *M.*

217 *tuberculosis* complex target DNA and subpopulations that carry the most frequently
218 occurring RR-conferring mutations. The consistent CFU counts (Table S1 a and b) and NGS
219 data from targeted deep sequencing (Table 1, Figure S2) did not suggest sub-optimal
220 preparation of the mixtures, supporting the LODs we observed that differed by capturing
221 RDT probe and mutation type. Clearly, Deeplex-MycTB provided useful quantitative
222 information for validating qualitative observations from the RDTs. We also show here that in
223 contrast with classic Xpert, Xpert Ultra may denote rifampicin heteroresistance through
224 'mixed patterns' of wild-type and mutant melt probe melt peak temperatures, which can be
225 leveraged to inform target end-users such as reference laboratory staff and researchers.
226 The different LODs observed can be linked to the inherent detecting mechanisms of the
227 RDTs. Xpert is an automated cartridge-based assay that employs heminested real-time
228 polymerase chain reaction assay and molecular beacon technology in which short
229 overlapping probes bind to the rifampicin resistance-determining region of the wild-type *M.*
230 *tuberculosis rpoB* gene (15, 30). The line probe assays on the other hand rely on multiplex
231 amplification and reverse hybridization involving both wild-type and mutant probes on a
232 membrane strip (31).
233 The LODs of LPA-Hain were consistent with a previous study that employed version 1 of the
234 LPA-Hain kit for mutations H445Y and S450L (4). The initial visual reading of LPA results was
235 consistent with results of second blinded reading for both LPA-Hain and LPA-Nipro, and
236 Genoscholar Reader for LPA-Nipro, although we observed that the mobile application was
237 less sensitive than visual reading for mutations D435V and H445D.
238 Classic Xpert performed relatively poorly in detecting heteroresistant mixtures with
239 mutation S450L, which is by far the globally most prevalent allele in RR-TB (10).
240 Nevertheless, the 20-40% LOD we found for classic Xpert was lower (more sensitive)

241 compared to previous studies which recorded 65-90% LOD (15, 16). Ultra performed
242 similarly to classic Xpert in detecting minority resistant variants of mutations S450L and
243 H445D, but did relatively better in capturing those of mutations D435V and H445Y (Table 2).
244 The 20% Ultra LOD for S450L was slightly less sensitive than the 5-10% LOD reported for the
245 same mutation by Chakravorty and colleagues (17) who tested single mixtures of RR and RS
246 DNA. We observed higher (less sensitive) LODs for the non-S450L mutations, consistent with
247 their findings.
248 Apart from the slight differences in the LODs recorded for classic Xpert and Ultra, we
249 observed that Ultra allows users to infer from raw results on the computer screen – under
250 'Melt Peaks' tab – the phenomenon of heteroresistance (Supplemental File Figure S4 b,
251 Figure 1), which is not possible from classic Xpert data. RIF heteroresistance may be rapidly
252 detected by Ultra through observed melt peak temperatures of both wild-type and Mut melt
253 probes akin to mixed patterns of absent wild-type and developed mutant bands in LPA.
254 Further, in a sample with S450L mutation, observing only the melt peak temperature of
255 rpoB4 Mut melt A probe (vs rpoB4 Mut melt A and rpoB3 Mut melt probes for 100% RR) and
256 corresponding wild-type melt probe may also denote RIF heteroresistance (Figure 1).
257 This information is deemed useful for conducting research on Ultra data and could be
258 practical in the field if export of the melt peak temperatures through the LIS port becomes
259 feasible in future software updates, together with other raw data (e.g. melt peak
260 temperatures of wild-type and Mut melt probes associated with RR mutation). Currently, the
261 raw data of Xpert Ultra are only available directly from the module/computer where it was
262 tested, and cannot yet be automatically extracted and shared through the .gxx files. Further,
263 local staff are not usually trained to interpret melt peak temperatures, as it entails
264 considerable effort for the NTPs to train staff in peripheral settings for such advanced

265 interpretation of raw results. We have formally requested access from the Xpert Ultra
266 manufacturer for automated extraction of melt peak temperatures at central level via non-
267 proprietary connectivity platforms.
268 Automated capture of the melt peak temperatures by connectivity solutions such as
269 DataToCare (Savics, Belgium), GXAlert (SystemOne, USA), or C360 (Cepheid, USA) will avoid
270 tedious and error prone manual transcription of the values from the computer screen. It
271 may also allow the laboratory staff and in-country expert Xpert Ultra users to interpret the
272 melt peak temperatures and their association with specific RR mutations with support of
273 global expert bodies such as the Foundation for Innovative New Diagnostics and Global
274 Laboratory Initiative so that TB reference laboratories can advise peripheral laboratories and
275 clinics optimally. With possible integration into e-Health patient charts beyond tuberculosis
276 diagnostics, this will allow to transform Xpert Ultra data into usable information for the
277 National TB Programs using a combination of unique patient ID and geographical
278 information (10, 32). This may not only greatly benefit the remote resolution of discordant
279 results to improve patient management, but also aid in building more systematic data on the
280 prevalence and impact of heteroresistance on a programmatic level, critical for improving
281 interventions for patients with confirmed heteroresistance.
282 Our study has limitations. We performed LPA testing of thermolysates in order to allow
283 'optimal' reading of results as indirect LPA testing increases the intensity of the bands.
284 Routine LPA is commonly done directly on clinical specimens, where background
285 hybridization is more common and can be harder to distinguish from heteroresistance, as
286 both phenomena may produce faint(er) bands which are often disregarded. Thus, the
287 sensitivity for heretoresistance detection that we determined for the LPAs likely represents
288 the upper bound of values achievable in clinical practice. Inclusion of a culture step however,

289 may lose minority subpopulations (33) and bias the ratio of mutant and wildtype
290 populations especially if there is fitness loss, and cause delay in obtaining results. Moreover,
291 the LPAs will not report heteroresistance for mutations not covered by a mutant probe, and
292 are not recommended for testing paucibacillary smear-negative samples due to their lower
293 sensitivity of detecting *M. tuberculosis* compared with Xpert and culture (34-36).
294 We addressed potential variability resulting from bacillary clumping and pipetting by
295 inclusion of biological replicates, showing differences in LOD of maximum two dilutions
296 amongst replicates with the same mutation.
297 In conclusion, we report distinct abilities of LPA-Hain, LPA-Nipro, classic Xpert, and Ultra to
298 detect minority resistant variants representing the most common RR-conferring mutations
299 against the quantitative results of Deeplex-MycTB. The LPAs have more sensitive LODs than
300 classic Xpert and Ultra in samples with sufficient *M. tuberculosis* complex target DNA,
301 although they report heteroresistance only for the four most common undisputed mutations
302 – D435V, H445D, H445Y, and S450L. For mutations without confirmatory mutant (MUT)
303 band, such as L452P, RIF heteroresistance cannot be detected, and with faint intensity of the
304 WT band (31), it may be difficult to distinguish between WT and (hetero)resistance. Ultra
305 can detect RR and RIF heteroresistance associated with all *rpoB* mutations within the
306 hotspot, albeit requiring a higher proportion of mutant bacilli than LPA. The LPAs and
307 Deeplex-MycTB provide direct information on the occurrence of RIF heteroresistance,
308 whereas Ultra, after informing that RR was detected, may suggest RIF heteroresistance only
309 through additional examination of wild-type and Mut melt probes and corresponding melt
310 peak temperatures in the raw data on the computer screen or in the generated extended
311 report (Supplemental File Figure S4 b).

312 The clinical importance of heteroresistance is likely substantial (3), akin to ’fixed‘, i.e. 100%
313 resistance. The proportion method for phenotypic drug susceptibility testing, which has
314 been around for over half a century, by design, tests for ≥1% resistant subpopulations (4, 13,
315 14), with strong predictive value for poor treatment outcome, at least for the core drugs,
316 e.g. fluoroquinolones (37) and rifampicin (4). Moreover, samples with ≥5% minority *gyrA*
317 resistant variants were found to have the same minimum inhibitory concentration level as
318 that of samples with 100% fluoroquinolone resistance (L. Rigouts, in press, (37)), while for
319 *rpoB*, the Mycobacterium Growth Indicator Tube phenotypic drug susceptibility testing
320 results were similar for samples with ≥5% minority resistant variants and 100% RIF
321 resistance (4). Taken together, this implicates that in samples with resistant subpopulations
322 of at least 5%, classification of heteroresistance as ‘RR’, even when less sensitive than the
323 proportion method, is probably key, notwithstanding the lack of direct evidence on clinical
324 impact. Our findings can thus inform and guide TB reference laboratory staff, healthcare
325 providers, and researchers, that the threshold for reporting resistance in case of rifampicin
326 heteroresistance is the highest for Classic Xpert and Ultra, to resolve phenotypic and
327 genotypic discordant rifampicin-resistant TB results. Prospective large-scale clinical studies
328 using next generation sequencing approaches (3, 8) are necessary to establish the
329 proportion of mutants that predicts poor outcome of treatment with the specific drug.
330

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476

477 **Figure Legend**

478 Figure 1. Presence/absence of Xpert Ultra wild-type and mutant melt probes detected for
479 the rifampicin-resistant(RR):rifampicin-susceptible(RS) mixtures per mutation type at various
480 proportions of the minority resistant variants: at the limit of detection (LOD) reported as
481 (hetero)resistant (shaded in yellow), below LOD reported as RS (shaded in pink), and at full
482 RR reported as resistant (shaded in green).

483 **Tables**

484 Table 1. Proportions of minority resistant variants detected among the rifampicin-
485 resistant:rifampicin-susceptible mixtures by targeted deep sequencing (Deeplex-MycTB).
486 The values represent the percentage of sequence reads bearing the indicated *rpoB*
487 mutation. Gray boxes represent proportions below the nominal threshold of 3% for calling
488 minority resistant variants used in the Deeplex-MycTB application.

RR:RS proportions	Minority resistant variant proportion detected for each specific mutation by Deeplex-MycTB			
	TB-TDR-0036 (H445D/H526D)	TB-TDR-0090 (S450L/S531L)	TB-TDR-0100 (D435V/D516V)	TB-TDR-0131 (H445Y/H526Y)
0:100	0.03	0.05	0.0	0.03
1:99	1.3	1.3	1.6	1.0
5:95	7.5	5.0	6.0	4.3
10:90	14.7	10.0	10.0	8.7
20:80	28.6	21.7	25.2	20.8
30:70	39.9	28.4	35.4	26.0
40:60	55.9	41.6	43.6	37.9
50:50	60.0	50.1	60.6	46.0
60:40	69.4	61.9	67.0	57.5
70:30	77.6	64.9	69.6	66.7
80:20	85.5	69.8	86.2	79.0
100:0	98.3	99.9	98.3	98.9

489

490 Table 2. (Range of) limits of detection for rifampicin heteroresistance among triplicates, tested by different rapid diagnostic tests, per mutation

491 type, with quantification of the respective lowest proportion by targeted deep sequencing (Deeplex-MycTB).

Strain ID	Rifampicin resistance-conferring mutation		LPA-Hain %LOD[1,a]	Deeplex %QP[1,b]	LPA-Nipro %LOD[1,a]	Deeplex %QP[1,b]	Xpert %LOD[1,a]	Deeplex %QP[1,b]	Ultra %LOD[2,a]	Deeplex %QP[2,b]
TB-TDR-0036	H445D*	H526D^	5-10	7.5	5	7.5	40-60	55.9	40-60	
TB-TDR-0090	S450L*	S531L^	5	5	1-5	5	20-40	21.7	20-30	21.7
TB-TDR-0100	D435V*	D516V^	5-10	6	5-10	6	70-80	69.6	40-50	
TB-TDR-0131	H445Y*	H526Y^	5-10	4.3	10	8.7	70-80	66.7	60-70	

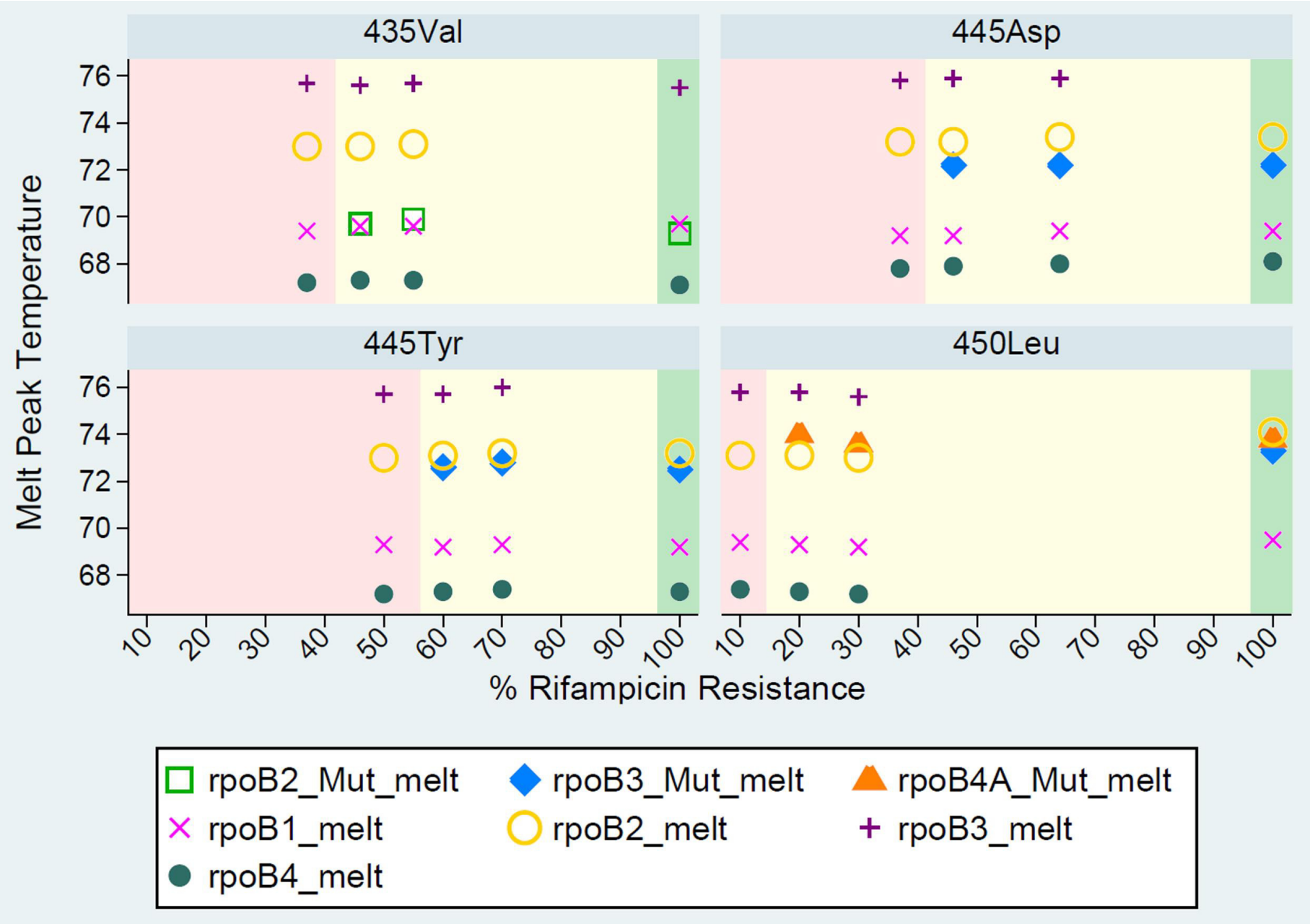
492 *in *M. tuberculosis* numbering; ^in *E.coli* numbering ; Deeplex-MycTB = targeted deep sequencing; RDTs rapid diagnostic tests; LPA-Hain =

493 GenoType MTDBR*plus* v2.0; LPA-Nipro = Genoscholar NTM + MTB II; Xpert = classic Xpert MTB/RIF; Ultra = Xpert MTB/RIF Ultra; [1] = batch 1; [2] =

494 batch 2; [a]Limit of detection (range) among triplicates for the RDTs; [b]quantified proportion of minority resistant variants determined by Deeplex

495 on replicate 3.

496


435Val
445Asp
445Tyr
450Leu
Melt Peak Temperature
76
74
72
70
68
10
20
30
40
50
60
70
80
90
100
% Rifampicin Resistance
rpoB2_Mut_melt
rpoB3_Mut_melt
rpoB4A_Mut_melt
rpoB1_melt
rpoB2_melt
rpoB3_melt
rpoB4_melt