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 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 rifmapicin heteroresistance through additional examination of wild-type and mutant melt probes and corresponding melt peak temperatures.

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

50 **INTRODUCTION**

51 Resistance to rifampicin (RIF) – the most potent anti-TB drug – is a major barrier to
52 tuberculosis (TB) control. In 2017, 71% of global RIF resistant-TB (RR-TB) cases were not
53 diagnosed (1). The diagnosis of RR-TB may be complicated by RIF heteroresistance, observed
54 in patient samples where RR and RIF-susceptible (RS) strains co-exist (2, 3), which may be
55 missed and diagnosed as RS because of detection limits. RIF heteroresistance may arise from
56 an existing resistant clonal subpopulation or from a mixed infection of independent strains
57 with RR and RS profiles. Such heteroresistance, also known as 'unfixed' resistance, precedes
58 full blown resistance ('fixed' resistance, 100% RR) as a result of further resistance selection
59 under treatment (3-6). Failure to detect these minority resistant variants can thus result in
60 unsuccessful treatment and potential spread of RR-TB strains (2, 3, 7).

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) (8-10). 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' (3, 11).

68 In this study, we define RIF heteroresistance limit of detection (LOD) as the minimum
69 proportion of mutant bacilli in the sample needed for RIF resistance to be detected. It is
70 known that phenotypic drug susceptibility testing by the proportion method determines at
71 least 1% resistant subpopulation in clinical samples (3, 12, 13). In the abovementioned RDTs
72 however, the RIF heteroresistance LOD in association with the specific *rpoB* mutation, is
73 insufficiently documented. In the case of classic Xpert, previous studies report LOD values

74 ranging from 65 to 100% (14, 15), for Ultra, the first validation study conducted by the
75 manufacturer presented LODs only for mutations L430P, H445N (20-40%), and S450L (5-
76 10%) (16), whereas LODs of Genoscholar NTM+MDRTB II have not been reported yet.
77 High coverage depths achieved through pre-selected amplified genes allow targeted deep
78 sequencing to capture and quantify minority resistant variants of *Mycobacterium*
79 *tuberculosis* mutants and detect RIF heteroresistance with high sensitivity (17, 18). As an
80 example of such an approach, Deeplex®-MycTB (Genoscreen, France; Deeplex) employs
81 ultra-deep sequencing of a single, 24-plexed amplicon mix to detect drug resistance-
82 associated mutations in *M. tuberculosis* complex (MTBC) strains, in addition to
83 mycobacterial species identification and MTBC strain genotyping, with a 24-48 turnaround
84 time starting from smear positive clinical samples or primary cultures. Among the 18 main
85 gene targets associated with 1st and 2nd– line drug resistance included in Deeplex-MycTB, the
86 *rpoB* gene – associated with RR - is covered by two amplicons of which one comprises the
87 main mutation hotspot region also known as the rifampicin resistance determining region
88 (RRDR) (19).
89 Precise documentation of LODs for most relevant *rpoB* mutations and for the state-of-the-
90 art RDTs is necessary for timely and more accurate identification of RIF heteroresistance and
91 prompt initiation of appropriate treatment. Therefore, we determined the LODs of classic
92 Xpert, Ultra, LPA-Hain, and LPA-Nipro for detecting RIF heteroresistance linked with RR
93 mutations S450L, D435V, H445D, and H445Y, in relation with the different probes used in
94 each RDT. These four mutations are most frequently detected in association with RR-TB in
95 the global MTBC strain population according to large-scale studies (20, 21). We used
96 simulated mixtures of selected, cultured RR and RS-TB strains, at ratios initially based on
97 colony-forming-unit (CFU) counts after McFarland standardization of the bacillary

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

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

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

128 We subjected mixtures of the RR-TB bacillary suspensions to classic Xpert and Ultra (9, 10),
129 and thermolysates to LPA-Hain and LPA-Nipro following manufacturer's instructions. We
130 recorded the LODs and corresponding RDT probe reaction per mutation type, in comparison
131 with values cross-validated by CFU counts and variant quantification with Deeplex-MycTB.
132 We excluded replicate 3 from the first batch of S450L-WT preparation due to substantially
133 high deviation across all mixture ratios, as revealed by Deeplex-MycTB (Supplemental Figure
134 S2). This deviation potentially reflects pipetting variation or bacillary clumping despite
135 similar CFU counts of the respective RR and RS strains. Hence for the S450L-WT mixture,
136 replicate 3 of batch 2 – which was initially only tested by Ultra and Deeplex – was subjected
137 also to classic Xpert and the LPAs, given the good correlation between the two batches and
138 to ensure triplicate testing for all mixtures.
139 First reading of the LPA strips was done by the person who prepared the mixtures and
140 performed the tests, while second reading was done by a colleague who was blinded to the
141 sample information to ensure objective reading of raw results. Additionally, for LPA-Nipro,
142 the Genoscholar Reader - a mobile application developed by Nipro (Osaka, Japan,
143 https://itunes.apple.com/by/app/genoscholar-reader/id1149733183?mt=8) was utilized.

144 In their standard reporting, Classic Xpert and Ultra (Supplemental File Figure S3 a) report RIF
145 heteroresistance, above or equal its LOD, as RR. Users can indirectly infer RIF
146 *hetero*resistance from Ultra data, as shown by the simultaneous presence of wild-type and
147 mutant (Mut) melt peak temperatures at the 'Melt Peaks' tab (Supplemental File Figure S3
148 b-d). When generating the results in portable document format, users must tick the 'Melt
149 Peaks' box to include the melt peak temperatures associated with each wild-type and Mut
150 melt probe, which may denote RIF heteroresistance, in the extended report (Supplemental
151 File Figure S3 f). Full resistance is detected by presence of both rpoB4A and rpoB3 Mut melt
152 probes for mutation S450L, whereas RIF heteroresistance is detected only by the rpoB4A
153 Mut melt probe in combination with the corresponding wild-type melt probe (Supplemental
154 File Figure S3 e and g).

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

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

161 Thermolysates of the RR:RS mixtures prepared as previously described (24) were subjected
162 to amplicon deep sequencing, using Deeplex-MycTB Kits for the amplification according to
163 the manufacturer's instructions. Replicates 3 of all first batch mixtures were tested with the
164 classical 18-gene target version, while replicate 3 of the second batch with S450L-WT
165 mixtures was tested with a customized version, including 5 gene targets (*rpoB, katG, inhA,*
166 *fabG1, gyrA*). Amplicons were purified using Agencourt® AMPure® XP magnetic beads
167 (Beckman Coulter, USA) and quantified by fluorescence quantification in 96-well plates on

168 Victor. Paired-end libraries of 150-bp read length were prepared using the Nextera XT DNA
169 Sample Preparation kit (Illumina Inc., San Diego, 160 CA, USA) and sequenced on Illumina
170 MiniSeq using standard procedures. Variant calling was performed using a dedicated,
171 parameterized web application developed by Genoscreen. The nominal threshold for calling
172 minority resistant variants – indicating heteroresistance for drug resistance associated
173 mutations – is set at a minimum of 3% of all reads, after filtering and depending on the
174 coverage depths, to minimize false positive calls due to background technical noise (19, 25,
175 26). Variants present in lower proportions than 3% in the relevant *rpoB* mutation positions
176 were detected separately from the web application,(27) without application of this nominal
177 threshold in the analysis pipeline.
178
179 **RESULTS**
180 After McFarland standardization of the different strain cultures, the mean CFU counts for
181 dilutions 10^{-4}-10^{-6} of the three replicates of both first and second batches of the different RS
182 and RR bacillary suspensions were very similar (Table S1 a and b). The proportions obtained
183 from the Deeplex-MycTB analysis were overall consistent with the expected mixture ratios
184 and the relative variation seen among CFU counts, as relative deviations from expected
185 values were limited to 0-16% (Figure 1).
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 S3). 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 S6,
202 Figure S4 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 S4). Likewise, Ultra required a minimum of 20-30%
206 resistant bacilli to detect mutation S450L, 40-50% for D435V, 60% for H445D and 60-70% for
207 H445Y (Table S5; Supplementary File Figure S3 b-e).
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 (Supplemental File Figure S3 e and g). For mutations D435V, H445D, and
211 H445Y only one Mut melt probe was observed, whether hetero- or fully resistant. The melt
212 peak temperatures did not differ among hetero- or fully resistant populations for all four
213 mutations tested (Supplemental File Figure S3 b-d; h-j).
214

215 **DISCUSSION**

216 Focusing on the most commonly detected *rpoB* mutations in the MTBC strain population -
217 D435V, H445D, H445Y and S450L (20, 21) - we determined the RIF heteroresistance LOD of
218 the WHO-endorsed RDTs - classic Xpert, Ultra, LPA-Hain and LPA-Nipro - against CFU
219 counting and Deeplex-MycTB (19, 26) as reference. As seen from analysis of different
220 heteroresistant mixtures defined based on bacterial colony counts, Deeplex-MycTB provided
221 useful information for validating qualitative observations from the RDTs.

222 The LPA-Hain LODs ranging between 5 and 10% was consistent with a previous study that
223 employed version 1 of the LPA-Hain kit (3). Further, the initial visual reading of LPA results
224 was consistent with results of second blinded reading and Genoscholar Reader, although we
225 observed that the mobile application was less sensitive than visual reading for mutations
226 D435V and H445D.

227 We performed LPA testing of thermolysates in order to allow 'optimal' reading of results.
228 Routine LPA is commonly done directly on clinical specimens, where background
229 hybridization is more common and can be harder to distinguish from heteroresistance, as
230 both phenomena may produce faint(er) bands which are often disregarded. Thus, the
231 sensitivity for heretoresistance detection that we determined for the LPAs likely represent
232 the upper bound of values achievable in clinical practice. Moreover, LPA will not detect
233 heteroresistance for mutations not covered by a mutant probe.

234 The LODs recorded for classic Xpert and Ultra were higher (less sensitive) than for the LPAs.
235 As a foremost example, classic Xpert performed relatively poorly in detecting heteroresistant
236 mixtures with mutation S450L, which is by far the globally most prevalent allele in RR-TB (9,
237 21). Nevertheless, the 20-40 % LOD we found for classic Xpert was lower (more sensitive)
238 compared to previous studies which recorded 65-90% LOD (14, 15). Ultra performed

239 similarly to classic Xpert in detecting minority resistant variants of mutations S450L (20-30%
240 vs 20-40%) and H445D (60% vs 40-60%), but did relatively better in capturing those of
241 mutations D435V (40-50% vs 70-80%) and H445Y (60-70% vs 70-80%). The 20% Ultra LOD for
242 S450L was slightly less sensitive than the 5-10% LOD reported for the same mutation by
243 Chakravorty and colleagues (16), who tested single mixtures of RR and RS DNA. We observed
244 higher (less sensitive) LODs for the non-S450L mutations, consistent with findings of
245 Chakravorty et al. (16).
246 Apart from the slight differences in the LODs recorded for classic Xpert and Ultra, we
247 observed that Ultra allows users to infer from raw results on the computer screen – under
248 'Melt Peaks' tab – the phenomenon of heteroresistance (Supplemental File Figure S3 b-e),
249 which is not possible from classic Xpert data. RIF heteroresistance may be rapidly detected
250 by Ultra through observed melt peak temperatures of both wild-type and Mut melt probes
251 akin to mixed patterns of absent wild-type and developed mutant bands in LPA. Further, in a
252 sample with S450L mutation, observing only the melt peak temperature of rpoB4 Mut melt
253 A probe (vs rpoB4 Mut melt A and rpoB3 Mut melt probes for 100% RR) and corresponding
254 wild-type melt probe may also denote RIF heteroresistance (Supplemental File Figure S3 e
255 and g).
256 This information is deemed useful for conducting research on Ultra data and could be
257 practical in the field if export of the melt peak temperatures through the LIS port becomes
258 feasible in future software updates, together with other raw data (e.g.wild-type and Mut
259 melt probes associated with RR mutation). Export to connectivity solutions such as
260 DataToCare (Savics, Belgium), GXAlert (SystemOne, USA), or C360 (Cepheid, USA), with
261 possible integration into e-Health patient charts beyond tuberculosis diagnostics, allows to
262 transform this data into usable information for the National TB Programs using a

263 combination of unique patient ID and geographical information (9, 28). This may not only
264 greatly benefit the remote resolution of discordant results to improve patient management,
265 but also aids to build more systematic data on the prevalence and impact of
266 heteroresistance on a programmatic level.
267 We addressed potential variability resulting from bacillary clumping and pipetting by
268 inclusion of biological replicates, showing differences in LOD of maximum two dilutions
269 amongst replicates with the same mutation.
270 In conclusion, we report distinct abilities of LPA-Hain, LPA-Nipro, classic Xpert, and Ultra to
271 detect the most common RR-conferring mutations in varying mutant proportions against
272 quantitative results of Deeplex-MycTB. We found that the LPAs have more sensitive LODs
273 than Ultra, although they detect heteroresistance only for the four most common
274 undisputed mutations – D435V, H445D, H445Y, and S450L. For mutations without
275 confirmatory mutant (MUT) band, such as L452P, RIF heteroresistance cannot be detected,
276 and with faint intensity of the WT band (29), it may be difficult to distinguish between WT
277 and (hetero)resistance. Ultra on the other hand, can detect RR and RIF heteroresistance
278 associated with all *rpoB* mutations within the hotspot, albeit requiring a higher proportion of
279 mutant bacilli than LPA. The LPAs and Deeplex-MycTB provide direct information on the
280 occurrence of RIF heteroresistance, whereas Ultra, after informing that RR was detected,
281 may suggest RIF heteroresistance only through additional examination of wild-type and Mut
282 melt probes and corresponding melt peak temperatures in the raw data on the computer
283 screen or in the generated extended report (Supplemental File Figure S3 b-e, f).
284 The clinical importance of heteroresistance is likely substantial (2), akin to ’fixed‘, i.e. 100%
285 resistance. The proportion method for phenotypic drug susceptibility testing, which has
286 been around for over half a century, by design, tests for ≥1% resistant subpopulations (3, 12,

287 13), with strong predictive value for poor treatment outcome, at least for the core drugs,
288 e.g. fluoroquinolones (30) and rifampicin (3). Moreover, samples with ≥5% minority *gyrA*
289 resistant variants were found to have the same minimum inhibitory concentration level as
290 that of samples with 100% fluoroquinolone resistance (L. Rigouts, submitted for publication,
291 (30)), while for *rpoB*, the Mycobacterium Growth Indicator Tube phenotypic drug
292 susceptibility testing results were similar for samples with ≥5% minority resistant variants
293 and 100% RIF resistance (3). Taken together, this implicates that in samples with resistant
294 subpopulations of at least 5%, classification of heteroresistance as 'RR', even when less
295 sensitive than the proportion method, is probably key, notwithstanding the lack of direct
296 evidence on clinical impact. Our findings can thus inform and guide TB reference laboratory
297 staff, healthcare providers, and researchers, that the threshold for reporting resistance in
298 case of rifampicin heteroresistance is the highest for Classic Xpert and Ultra, to resolve
299 phenotypic and genotypic discordant rifampicin-resistant TB results. Prospective large-scale
300 clinical studies using next generation sequencing approaches (2, 7) are necessary to establish
301 the proportion of mutants that predicts poor outcome of treatment with the specific drug.

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314

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425

426 **Tables**

427 Table 1. Proportions of minority resistant variants detected among the rifampicin-
428 resistant:rifampicin-susceptible mixtures by targeted deep sequencing (Deeplex-MycTB).
429 The values represent the percentage of sequence reads bearing the indicated *rpoB*
430 mutation. Gray boxes represent proportions below the nominal threshold of 3% for calling
431 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

432

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

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

Strain number	**Rifampicin resistance-conferring mutation**		**LPA-Hain[1,a]**	**Deeplex[1,b]**	**LPA-Nipro[1,a]**	**Deeplex[1,b]**	**Xpert[1,a]**	**Deeplex[1,b]**	**Ultra[2,a]**	**Deeplex[2,b]**
TB-TDR-0036	H445D*	H526D^	5-10	7.5	5	7.5	40-60	55.9	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	

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

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

437 batch 1; [2] = batch 2; [a]Limit of detection (range) among triplicates for the RDTs versus Deeplex quantification on the lowest proportion of

438 replicate 3; [b]minority resistant variant proportion as determined by Deeplex on replicate 3.

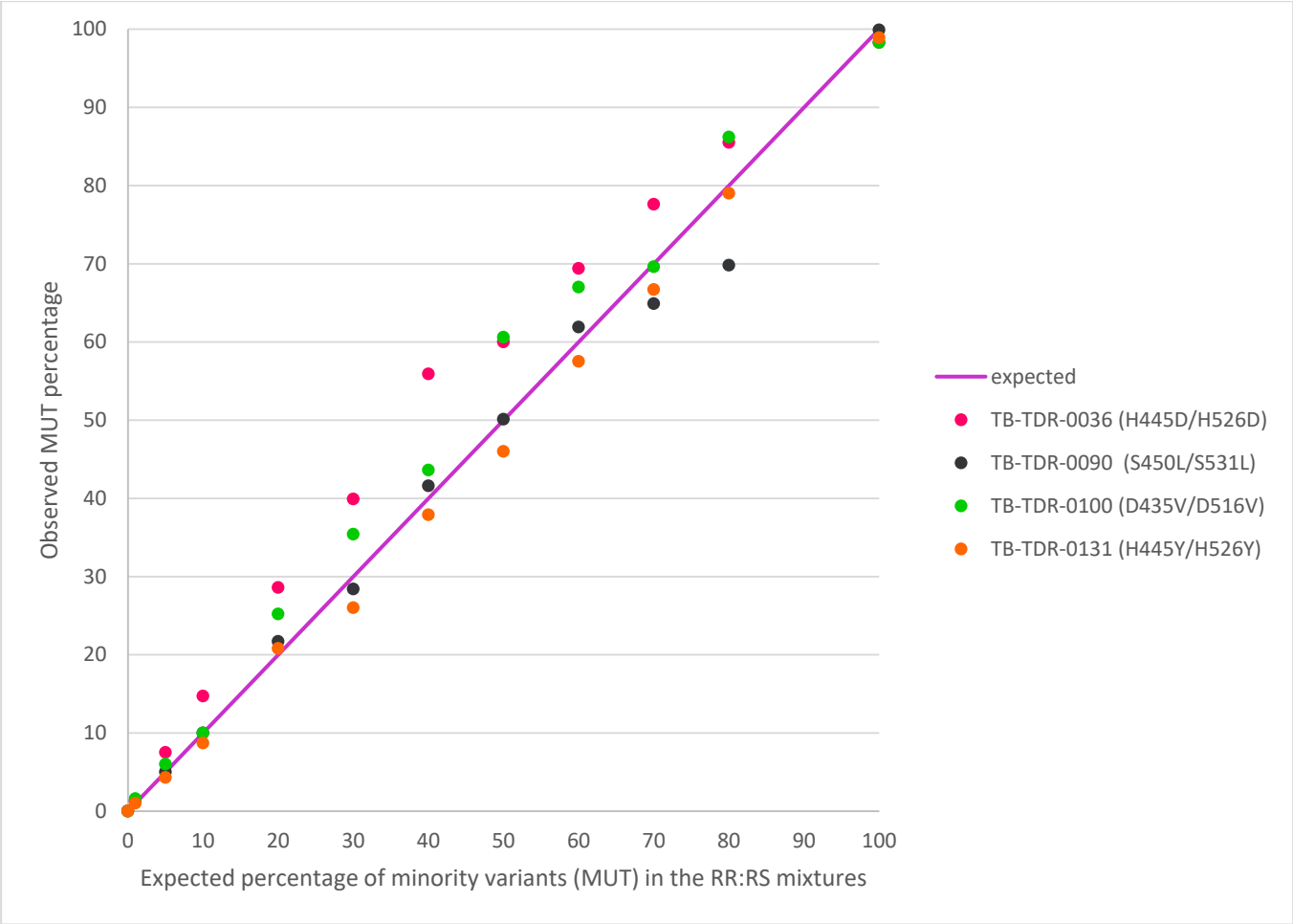

Figure 1. Minority resistant variant proportions detected across all mutation positions by targeted deep sequencing (Deeplex-MycTB) among the rifampicin-resistant:rifampicin-susceptible mixtures. The legend shows the RR-TB strain numbers and the corresponding RR-conferring mutations in *Mycobacterium tuberculosis* / *Escherichia coli* numbering system.