



Line probe assays for drug-resistant tuberculosis detection

Interpretation and reporting guide for laboratory staff and clinicians



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Abbreviations

Am	amikacin
CB	clinical breakpoint
CC	critical concentration
Cm	capreomycin
DST	drug-susceptibility testing
Eto	ethionamide
FL-LPA	first line – line probe assay
FQ	fluoroquinolone
H	isoniazid
Km	kanamycin
Lfx	levofloxacin
LPA	line probe assay
NTM	non-tuberculous mycobacteria
MIC	minimum inhibitory concentration
MDR-TB	multidrug-resistant tuberculosis
Mfx	moxifloxacin
MTBC	Mycobacterium tuberculosis complex
MUT	mutation probe
Pto	prothionamide
QRDR	quinolone-resistance determining region
R	resistant
RRDR	Rifampicin- resistance determining region
Rif	rifampicin
S	susceptible
SLI	second-line injectable (drug) (i.e. kanamycin, amikacin, capreomycin)
SL-LPA	second line – line probe assay
TB	tuberculosis
WT	wild-type

Glossary

Critical concentration (CC): The lowest concentration of an anti-TB agent in vitro that will inhibit the growth of 99% of phenotypically wild type strains of *Mycobacterium tuberculosis* complex (MTBC).

Clinical breakpoint (CB): concentration/s of an antimicrobial agent which defines a minimum inhibitory concentration (MIC) above the critical concentration that separates strains that will likely respond to treatment from those which will likely not respond to treatment. This concentration is determined by multiple variables, including *in vivo* ones, such as the correlation with available clinical outcome data and PK/PD data including drug dose, as well as MIC distributions and genetic markers. A dose increase can be used to overcome resistance observed at lower dosing, up until the maximum tolerated dose, and therefore a higher clinical breakpoint above which the particular drug is not recommended for use. The clinical breakpoint is used to guide individual clinical decisions in patient treatment.

Minimum inhibitory concentration (MIC): The lowest concentration of an antimicrobial agent that prevents growth of more than 99% of a microorganism in a solid medium or broth dilution susceptibility test.

Typically, when MICs that are tested using a standardised method are aggregated for one species, a single Gaussian-shaped MIC distribution is formed, which corresponds to the phenotypically wild-type (pWT) distribution for that species (i.e. the distribution for organisms that lack phenotypically detectable resistance mechanisms). Additional distributions with higher overall MICs are sometimes identified that correspond to intrinsically or naturally resistant organisms (i.e. phenotypically non wild-type distribution).

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About this guide

This document has been developed to provide practical guidance for the interpretation of the most commonly used first line (FL) and second line (SL) line probe assays (LPA) (i.e. GenoType MTBDR*plus* V2.0 and GenoType MTBDR*s*/ V2.0 assay; Hain Lifescience, Germany). In addition to the interpretation of the mutations identified by the two assays, this guide includes practical recommendations on additional follow up diagnostic actions that should be performed in the presence of specific mutations and the clinical implications that these mutations have on the selection of appropriate tuberculosis (TB) treatment regimens.

This guide is intended to provide information for both laboratory staff and clinicians on:

- the association of the specific mutations detected by the test with phenotypic drug resistance;
- the instances when specific resistance conferring mutations are not identified and resistance can only be inferred;
- the clinical implications of the specific mutations detected by the assays.

In addition, the guide provides specific support for staff at national and regional TB reference laboratories to better understand and manage possible discrepancies between phenotypic and genotypic drug susceptibility testing (DST).

This guide outlines the mutations identified by both FL- and SL-LPA test strips, including information related to their association with phenotypic drug resistance based on the work published by Miotto *et al.* (1) and with the minimum inhibitory concentration (MIC) for first and second line drugs as recently reported by WHO (2, 3). Test interpretation, the follow-up diagnostic testing, and clinical implications related to the presence of specific mutations as well of the clinical implications for instances when resistance is inferred are also reported.

Lastly, the guide illustrates individual case studies to demonstrate how information should be reported to clinicians, examples of LPA results, and a recommended reporting template.

Introduction

In the past two decades, improved understanding of the molecular basis of drug resistance has led to the development of a plethora of genotypic-based methods for the rapid determination of susceptibility and resistance to anti-TB drugs. Besides the rapidity of diagnosis, molecular testing has additional advantages such as the possibility to be applied directly to clinical specimens (without the need for isolating the strain first on solid or liquid culture) and to specimens containing non-viable bacteria (e.g. bacteria killed by heat or chemical inactivation), the potential for high throughput and the decrease of laboratory biosafety requirements.

In 2008, the World Health Organization (WHO) endorsed the use of the first line (FL) line probe assay (LPA), the GenoType MTBDR*plus* (referred to as GenoType MTBDR*plus* V1), for the rapid detection of multidrug-resistant TB (MDR-TB) (4). Subsequently, newer versions of the LPA technology have been developed since 2011, including (i) the GenoType MTBDR*plus* version 2 (referred to as GenoType MTBDR*plus* V2), and (ii) the Nipro NTM+MDRTB detection kit 2 (referred to as “Nipro”, Tokyo, Japan). These newer LPAs aim to improve the sensitivity of (*Mycobacterium tuberculosis* complex) MTBC detection and to simultaneously detect resistance to rifampicin (Rif) and isoniazid (H).

In 2015, FIND (the Foundation for Innovative New Diagnostics) evaluated the Nipro and the GenoType MTBDR*plus* V2 LPAs and compared them with the GenoType MTBDR*plus* V1. The study demonstrated equivalence between these three commercially available LPAs for detecting TB and resistance to Rif and H (5).

The first commercial LPA for detection of resistance to second-line TB drugs was the GenoType MTBDR*s/* version 1.0, developed by Hain Lifescience a decade ago (referred to as GenoType MTBDR*s/* V1). An updated version of this assay (GenoType MTBDR*s/* V2) which detects the mutations associated with fluoroquinolone and second-line injectable (SLI) drug resistance detected by version 1.0, as well as additional mutations (described in the section below), became available in 2015.

The following year, WHO released recommendations for the use of the commercially available FL-LPAs (i.e. GenoType MTBDR*plus* V1, GenoType MTBDR*plus* V2, and Nipro) as the initial test instead of phenotypic drug-susceptibility testing (DST) to detect resistance to Rif and H (6). As well, recommendations for the use of GenoType MTBDR*s/* (V1 and V2) to detect resistance to fluoroquinolones and SLI drugs in patients with rifampicin-resistant/MDR-TB and to guide the initiation of appropriate MDR-TB treatment regimen were also issued by WHO in 2016 (7).

For a more detailed description of the placement of FL- and SL-LPA within the TB diagnostic algorithms, refer to the Global Laboratory Initiative (GLI) document *GLI model TB diagnostic algorithms* (updated in June 2018) (8).

The purpose of this document, which focuses on the two most widely used LPAs (i.e. GenoType MTBDR*plus* V2 and GenoType MTBDR*s/* V2), is to provide guidance to

laboratory staff and clinicians on the interpretation of the results for both, FL- and SL-LPA, and to better understand and manage possible discrepancies between phenotypic and genotypic DST.

Principle of Line Probe Assay

Line probe assays are a family of DNA strip-based tests that determine the drug resistance profile of a MTBC strain through the pattern of binding of amplicons (DNA amplification products) to probes targeting the most common resistance associated mutations to first- and second-line agents and to probes targeting the corresponding wild-type (WT) DNA sequence.

LPAs are WHO-approved tests for rapid detection of drug resistance to first- and second-line agents. They can be used for testing of culture isolates (indirect testing), as well as direct testing of acid fast bacilli (AFB) smear microscopy positive specimens (FL-LPA), and both smear positive and smear negative sputum specimens (SL-LPA) (6, 7).

Mutations are detected by: (i) the binding of amplicons to probes targeting the most commonly occurring mutations (MUT probes) or (ii) inferred by the lack of hybridization (i.e. lack of binding) of the amplicons to the corresponding WT probes.

The post-hybridization reaction leads to the development of coloured bands on the test strip detecting probe binding.

It is important to remember that similarly to other rapid tests currently endorsed by WHO, LPAs have some limitations:

- Although LPA can detect the mutations that are most frequently identified in resistant strains, some mutations that confer resistance are outside the regions covered by the test and therefore resistance cannot be completely excluded even in the presence of all WT probes. Thus, in some cases additional phenotypic DST may be necessary to provide full assessment.
- Some mutations are identified specifically by MUT probes, whereas others are only inferred by the absence of binding of the amplicons to WT probes. The lack of binding of a WT probe without simultaneous binding of a mutant probe is likely caused by the presence of a resistance mutation. However, systematic errors are possible due to synonymous and non-synonymous mutations (e.g. phylogenetic mutations). Globally this is rare (<1% of isolates), but these isolates can be frequent locally (9).
- LPA is less efficient than conventional culture-based DST in finding resistance in samples harboring both drug-susceptible and -resistant bacteria (i.e. hetero-resistance). More specifically, with LPA, it is possible to detect resistant bacteria with mutations detected by the MUT probes if resistant bacteria represent at least 5% of the total population. However, resistant bacteria with mutations inferred by the absence of WT probes would probably be missed if the resistant population is less than 95% of the total bacterial population (10, 11).

Overall, sensitivities and specificities for the different drugs targeted by LPAs are reported in detail elsewhere (12, 13). Briefly, FL-LPA showed a sensitivity and specificity for the detection of Rif resistance of 96.7% and 98.8%, respectively, and for the

detection of H resistance, a sensitivity and specificity of 90.2% and 99.2%, respectively (12). SL-LPA (GenoType MTBDRsI V1) showed a pooled sensitivity and specificity for the detection of fluoroquinolone resistance by direct testing of 86.2% and 98.6%, respectively, and a pooled sensitivity and specificity for the detection of second-line injectables drugs resistance of 87.0% and 99.5%, respectively (13).

GenoType MTBDRplus Version 2

The GenoType MTBDRplus (Figure 1a) targets specific mutations in the Rif-resistance determining region (RRDR) of the *rpoB* gene (from codon 505 to 533) (Figure 2) to detect Rif resistance and mutations in the *inhA* promoter (from -16 to -8 nucleotides upstream) and the *katG* (codon 315) regions to identify H resistance. The specific nucleotide changes detected by the test are reported in the Annex Section.

Figure 1. Configuration of GenoType MTBDRplus V2 (a) and GenoType MTBDRsI V2 (b) strips

a (14)

Line	
1	Conjugate Control
2	Amplification Control
3	<i>M. tuberculosis</i> complex TUB
4	<i>rpoB</i> Locus Control <i>rpoB</i>
5	<i>rpoB</i> wild type probe 1 <i>rpoB</i> WT1
6	<i>rpoB</i> wild type probe 2 <i>rpoB</i> WT2
7	<i>rpoB</i> wild type probe 3 <i>rpoB</i> WT3
8	<i>rpoB</i> wild type probe 4 <i>rpoB</i> WT4
9	<i>rpoB</i> wild type probe 5 <i>rpoB</i> WT5
10	<i>rpoB</i> wild type probe 6 <i>rpoB</i> WT6
11	<i>rpoB</i> wild type probe 7 <i>rpoB</i> WT7
12	<i>rpoB</i> wild type probe 8 <i>rpoB</i> WT8
13	<i>rpoB</i> mutation probe 1 <i>rpoB</i> MUT1
14	<i>rpoB</i> mutation probe 2A <i>rpoB</i> MUT2A
15	<i>rpoB</i> mutation probe 2B <i>rpoB</i> MUT2B
16	<i>rpoB</i> mutation probe 3 <i>rpoB</i> MUT3
17	<i>katG</i> Locus Control <i>katG</i>
18	<i>katG</i> wild type probe <i>katG</i> WT
19	<i>katG</i> mutation probe 1 <i>katG</i> MUT1
20	<i>katG</i> mutation probe 2 <i>katG</i> MUT2
21	<i>inhA</i> Locus Control <i>inhA</i>
22	<i>inhA</i> wild type probe 1 <i>inhA</i> WT1
23	<i>inhA</i> wild type probe 2 <i>inhA</i> WT2
24	<i>inhA</i> mutation probe 1 <i>inhA</i> MUT1
25	<i>inhA</i> mutation probe 2 <i>inhA</i> MUT2
26	<i>inhA</i> mutation probe 3A <i>inhA</i> MUT3A
27	<i>inhA</i> mutation probe 3B <i>inhA</i> MUT3B
	Colored marker

b (15)

Line	
1	Conjugate Control
2	Amplification Control
3	<i>M. tuberculosis</i> complex TUB
4	<i>gyrA</i> Locus Control <i>gyrA</i>
5	<i>gyrA</i> wild type probe 1 <i>gyrA</i> WT1
6	<i>gyrA</i> wild type probe 2 <i>gyrA</i> WT2
7	<i>gyrA</i> wild type probe 3 <i>gyrA</i> WT3
8	<i>gyrA</i> mutation probe 1 <i>gyrA</i> MUT1
9	<i>gyrA</i> mutation probe 2 <i>gyrA</i> MUT2
10	<i>gyrA</i> mutation probe 3A <i>gyrA</i> MUT3A
11	<i>gyrA</i> mutation probe 3B <i>gyrA</i> MUT3B
12	<i>gyrA</i> mutation probe 3C <i>gyrA</i> MUT3C
13	<i>gyrA</i> mutation probe 3D <i>gyrA</i> MUT3D
14	<i>gyrB</i> Locus Control <i>gyrB</i>
15	<i>gyrB</i> wild type probe <i>gyrB</i> WT
16	<i>gyrB</i> mutation probe 1 <i>gyrB</i> MUT1
17	<i>gyrB</i> mutation probe 2 <i>gyrB</i> MUT2
18	<i>rrs</i> Locus Control <i>rrs</i>
19	<i>rrs</i> wild type probe 1 <i>rrs</i> WT1
20	<i>rrs</i> wild type probe 2 <i>rrs</i> WT2
21	<i>rrs</i> mutation probe 1 <i>rrs</i> MUT1
22	<i>rrs</i> mutation probe 2 <i>rrs</i> MUT2
23	<i>eis</i> Locus Control <i>eis</i>
24	<i>eis</i> wild type probe 1 <i>eis</i> WT1
25	<i>eis</i> wild type probe 2 <i>eis</i> WT2
26	<i>eis</i> wild type probe 3 <i>eis</i> WT3
27	<i>eis</i> mutation probe 1 <i>eis</i> MUT1
	Colored marker

GenoType MTBDRs/ Version 2

The second version of GenoType MTBDRs/ (Figure 1b) includes the quinolone-resistance determining region (QRDR) of *gyrA* (from codon 85 to 96) (Figure 3) and of *gyrB* (from codon 536 to 541) (16) genes for detection of resistance to fluoroquinolones and the *rrs* (nucleic acid position 1401, 1402 and 1484) and the *eis* promoter region (from -37 to -2 nucleotides upstream) for detection of resistance to SLI drugs. It is worth noting that the precise regions covered by all MUT probes have not been disclosed and that only some of the regions covered by WT probes are known (see Figures 2 and 3). The specific nucleotide changes detected by the MUT probes are reported in Annex Section.

Line Probe Assay interpretation and reporting

The LPA has two internal controls on the strip: the **Conjugate Control** (line 1), and the **Amplification Control** (line 2) (Figure 1). The Conjugate Control line should always be visible to document the efficiency of conjugate binding and substrate reaction. The Amplification Control serves as reference for the interpretation of WT and MUT probes: only those bands whose intensities are about as strong as or stronger than that of the Amplification Control band are to be considered. In case of a positive test result, the signal of the Amplification Control zone can be weak or even vanish totally. This can occur more frequently upon indirect testing, whereas it is rare upon direct testing. The absence of Amplification Control might be due to competition of the single reactions during amplification. In this case the test has been carried out correctly and can be interpreted. In case of a negative test result, both Conjugate Control and Amplification Control bands should always be visible (i.e. valid negative result). The absence of Amplification Control in a negative test indicates mistakes during setup and/or performance of the amplification reaction, or presence of amplification inhibitors. In this case, the test result is **invalid** and must be repeated.

The **TUB reaction band** (line 3) is only present if the DNA amplified is from members of the MTBC. The presence of non-tuberculous mycobacteria (NTM) in the specimen can result in random banding patterns, with several species testing positive at some *rpoB* WT bands due to the gene similarities among the species. Therefore, in the presence of NTM rather than MTBC bacteria, the TUB band will always be absent, and the result reported as MTBC not detected.

The gene **locus control** bands for the different target regions analyzed on the DNA strip are located just before their respective WT and MUT bands. These locus control bands must always be present for the assay to be considered valid for the corresponding target. However, when only one gene locus control band is missing, the results for the other genes for which the gene locus control band is present can be interpreted.

The LPA is defined as **indeterminate** for a specific drug or group of drugs if the corresponding locus control for that specific drug or group of drugs is missing while the test is valid (i.e. Conjugate Control and TUB bands are visible with or without the Amplification Control). In this case, the assay should be repeated before reporting the results. If the same result is obtained upon re-testing, report the results for the interpretable loci following the guideline, while report as indeterminate the result for the drugs or group of drugs for which the locus control is missing. Systematic reasons

for these indeterminate results could be mutations or deletions in the locus control region, as well as the complete or partial deletion of a target gene. In this cases, sequencing should be requested to identify the specific mutation.

The **WT reactions zones** comprise regions of the genome with known resistance mutations. The **MUT reaction zones** correspond to probes that identify the most common resistance mutations of the gene interrogated.

Resistance is detected when MUT probes are developed, whereas in the absence of WT probes, resistance can only be inferred (see below for details).

The concomitant detection of all WT probes and one of the MUT probes in the corresponding target region, indicates the presence of heteroresistance (i.e. coexistence of susceptible and resistant bacteria in the same sample). In this case the result should be reported as resistant.

Revisions to manufacturer interpretations (14, 15)

Use of the term "Resistance not detected" instead of "Susceptible" to define the bacteria resistance profile

Given the limitations of LPA and in particular the fact that resistance cannot be completely excluded even in the presence of all WT probes (i.e. not all mutations that confer resistance are covered by these tests or mutations that are covered may be below the limit of detection), it is more appropriate to report the result as "Resistance detected" or "Resistance not detected".

Differentiation of resistance into "Resistance inferred" and "Resistance detected"

The term "Resistance inferred" is used whenever one or more WT probes in regions of the gene known to confer resistance to the drug are not developed, and none of the MUT probes in the corresponding region is developed. In this case the precise mutation cannot be reported, only the region where the mutation lies is identified.

The term "Resistance detected" is used whenever one or more MUT probes identifying specific mutations conferring resistance to the drugs are developed (regardless of whether WT probes are developed or not).

Stratification of resistance mutations for Isoniazid (H) and Moxifloxacin (Mfx) into mutations associated with a "low-level increase in MIC" and "high-level increase in MIC"

Mutations conferring resistance to H and Mfx are stratified into mutations associated with a low-level increase in MIC and high-level increase in MIC depending on their associated MICs distribution. This stratification has important implications for the inclusion of H and Mfx in the treatment regimen since resistance due to mutations associated with low-level increases in MICs for H or Mfx may be overcome by increasing the drug dose.

For H, *in vitro* evidence suggests that when specific *inhA* promoter mutations, which are generally associated with low-level increase in MIC, are detected (and in the absence of any *katG* mutation), increasing the drug dose may be effective; thus, using H at a maximum dose of 15mg/kg per day could be considered. In the case of *katG* mutations, which are more commonly associated with high-level increase in MIC, the use of isoniazid even at higher dose is less likely to be effective. The presence

of combined mutations in the *inhA* promoter and *katG* gene results in substantial increases in the MIC, unlikely to be compensated for by increased dosing (17).

For Mfx, if mutations associated with MIC increases above the critical concentration (CC) but below the clinical breakpoint (CB), which are defined as mutations associated with a low-level increase in MIC, high dose Mfx (up to 800 mg daily to adults), is likely to be effective (18–21). When resistance to Mfx is inferred (i.e. the specific mutation is unknown), the presence of mutations associated with at least low-level increase in MIC is inferred and therefore a high drug dose may still be effective. However, in this case, it is recommended to perform DST for Mfx at the CB, and if available, sequencing to determine the specific mutation. If the MTBC strain is resistant to Mfx at the CB due to the presence of mutations associated with high-level increase in MIC, the drug cannot be considered as an effective medicine.

Whenever there is more than one probe per drug that provides information (e.g. concomitant detection of resistance mutations associated with different increases in MIC), the criteria used for the interpretation is that the mutations associated with high-level increase in MIC overrule mutations associated with low-level increase in MIC.

In summary, the results should be reported according the following hierarchy (where the ">" sign means overrule):

- For *H*: Mutation associated with high-level increase in MIC detected > Mutation associated with high-level increase in MIC inferred > Mutation associated with at least low-level increase in MIC detected > Mutation associated with at least low-level increase in MIC inferred > Resistance not detected.
- For *Mfx*: Mutation associated with high-level increase in MIC detected > Mutation associated with at least low-level increase in MIC detected > Mutation associated with at least low-level increase in MIC inferred > Resistance not detected.
- For *Rif*, *levofloxacin (Lfx)*, *amikacin (Am)*, *kanamycin (Km)* and *capreomycin (Cm)*: Resistance detected > Resistance inferred > Resistance not detected.

In Summary

Case	WT reaction zones	MUT reaction zones	Interpretation
1	All WT probes are developed	All MUT probes are not developed	Resistance not detected.
2	One or more WT probes are not developed	One or more MUT probes in the corresponding region are developed	Depending on the specific drug: – Resistance detected (Rif, SLI drugs); – Mutations associated with high-level increase in MIC detected (H and Mfx); – Mutations associated with at least low-level increase in MIC detected (H and Mfx).
3	One or more WT probes are not developed	No MUT probes developed	Depending on the specific drug: – Resistance inferred (Rif, SLI drugs); – Mutations associated with high-level increase in MIC inferred (H and Mfx); – Mutations associated with at least low-level increase in MIC inferred (H and Mfx).
4	All WT probes are developed	One MUT probe developed	Resistance detected (due to heteroresistance); Interpret according to case 2.

Exclusion of eis WT3 probe

To date there is no clear evidence that the mutation **c-2a** in the *eis* promoter region represents, on its own, a valid marker of resistance (1). Therefore, if *eis* WT3 probe is not developed, the test interpretation for Km has been revised into “Resistance not detected”.

Interpretation of resistance profile for ethionamide and prothionamide

Mutations leading to an overexpression of *inhA* gene, such those detected by FL-LPA, are associated with cross-resistance to ethionamide (Eto) and prothionamide (Pto) (1, 22, 23). Therefore, if these mutations are detected by the test, resistance to Eto and Pto should be reported and the medicines not used in the treatment regimen. However, it is important to note that even in the absence of mutations in the *inhA* promoter region, resistance to Eto/Pto cannot be excluded. Mutations conferring resistance to Eto/Pto may in fact be present in genomic regions not targeted by LPA (e.g. *ethA*, *ethR*).

Reporting of results for kanamycin and capreomycin

WHO has recently released a rapid communication on key changes to treatment of MDR and Rif-resistant TB (24) anticipating key aspects of the new WHO MDR-TB treatment guidelines that will be released in late 2018. These changes are based on the results of a meta-analysis aimed to estimate the association of treatment success and death with the use of individual drugs, and the optimal number and duration of treatment with those drugs in patients with MDR-TB (25).

On the use of SLI drugs, the results of this meta-analysis show that compared with regimens without any injectable drug, amikacin provided modest benefits, while kanamycin and capreomycin were associated with no benefits or worse outcomes. Therefore, WHO is no longer recommending the use of kanamycin and capreomycin, due to the increased risk of treatment failure and relapse associated with their use

in longer MDR-TB regimens. In addition, programmes using the standardized shorter MDR-TB regimen should replace kanamycin with amikacin, without waiting for existing stocks of kanamycin to be used up (24).

Acknowledging the fact that it would not be immediately possible to achieve the new WHO standards of care in every individual MDR-TB patient, and that kanamycin and capreomycin will likely be used during the transition phase, the interpretation of SL-LPA for kanamycin and capreomycin has been included in this document with the purpose to provide interim guidance.

Additional follow-up diagnostic actions to guide appropriate TB treatment

Depending on the specific region interrogated by FL- and SL-LPA, one or more follow-up diagnostic actions are either recommended or suggested as optional to better guide the choice of the treatment regimen. The decision to perform the optional follow-up diagnostic actions should be guided by considerations on the individual patient's risk for resistance and by the prevalence of resistance in the specific geographical setting, as these factors affect the positive predictive value of the test.

Different follow-up diagnostic actions are recommended or suggested as optional, depending on the specific drug considered. These actions are briefly summarized below:

Rifampicin (Rif):

- If resistance is inferred by the absence of binding of the amplicons to WT probes (i.e. one or more WT probes not developed), sequencing of *rpoB* gene is suggested as optional to identify the specific mutation. For the interpretation of *rpoB* mutations refer to the WHO Technical guide on the use of next-generation sequencing technology (3). It is important to note that phenotypic DST performed in MGIT should not be considered as the ideal confirmatory test given that it misses well-established Rif resistance associated mutations (i.e. "disputed" mutations) (26–29).

Isoniazid (H):

- If resistance is inferred by the absence of binding of the amplicons to WT probes in the *katG* region (i.e. one or more WT probes not developed), sequencing of *katG* gene is suggested as optional to identify the specific mutation. For the interpretation of *katG* mutations refer to the WHO Technical guide on the use of next-generation sequencing technology (3).
- If mutations associated with low-level increase in MIC are detected (i.e. MUT probes developed in the *inhA* promoter region in the absence of mutations in the *katG* target region), sequencing of *inhA* coding region and *katG* gene is suggested as optional. This is due to the fact that the concomitant presence of additional mutations in the *inhA* coding region or in positions other than 315 in the *katG* gene (mutations not detected by GenoType MTBDRplus) (30, 31), which are globally rare but could be more frequent in specific geographical settings, may cause substantial MIC increases, too high to be compensated for by increased drug dosing.
- If mutations associated with low-level increase in MIC are inferred by the absence of binding of the amplicons to WT probes in the *inhA* promoter region (and no mutations

in the *katG* target region are detected), it is recommended to repeat the testing to confirm the result. Optional follow-up diagnostic actions include sequencing of *inhA* promoter to identify the specific mutation or perform phenotypic DST for H.

Moxifloxacin (Mfx):

- If mutations associated with low-level increase in MIC are detected (i.e. MUT1, MUT2, MUT3A probes developed in *gyrA* and/or MUT1, MUT2 probes developed in *gyrB* regions), phenotypic DST for Mfx is recommended to exclude resistance at CB.
- If mutations associated with low-level increase in MIC are inferred by the absence of binding of the amplicons to WT probes in the *gyrA* or *gyrB* regions (i.e. WT probes not developed), phenotypic DST for Mfx is recommended to exclude resistance at CB. Optional follow-up actions include sequencing of *gyrA* and/or *gyrB* QRDR to identify the specific mutation and/or to perform phenotypic DST for Mfx (and/or Lfx) at CC (depending on laboratory capacity).

Amikacin (Am), kanamycin (Km),¹ capreomycin (Cm):¹

- If resistance is inferred by the absence of binding of the amplicons to WT probes in the *rrs* region (i.e. one or more WT probes not developed), it is recommended to repeat the test to confirm the result. If the result is confirmed, phenotypic DST for Am, Km, Cm should be performed to confirm resistance. Sequencing of *rrs* gene is suggested as optional to identify the specific mutation.

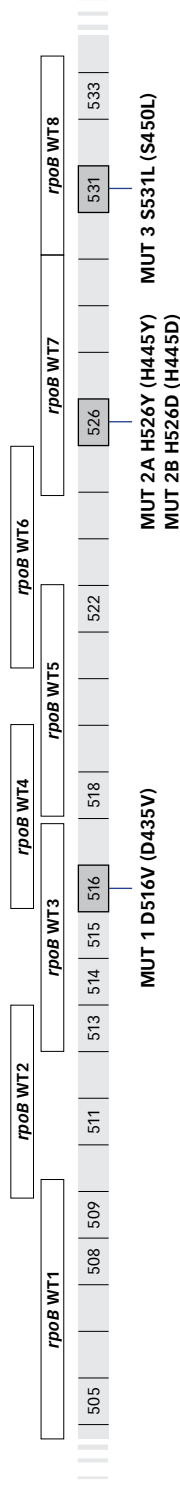
¹ The use of Km and Cm in the treatment of rifampicin-resistant/MDR-TB is no longer recommended (24). Laboratories should continue to report results for Cm and Km until the recommendations are fully implemented.

First Line (FL) – Line Probe Assay (LPA) interpretation

Rifampicin

Figure 2. Rifampicin resistance determining region interrogated by GenoType MTBDRplus

Rifampicin-resistance determining region (RRDR) of *rpoB* gene, codons covered by the WT probes and the specific mutations recognized by the MUT probes in GenoType MTBDRplus Ver 2.0- for *E. coli* vs. MTB codon numbering and amino acid nomenclature (14). Overall, the specificity of GenoType MTBDRplus Ver 2.0 for Rif resistance is very good, yet when in doubt on the validity of a Rif resistance result, request *rpoB* sequencing as gold standard.



Target region	MTBDRplus Probe	Mutation or region interrogated	Result interpretation	Additional diagnostic action ^a	Clinical implications
<i>rpoB</i> WT1	<i>rpoB</i> WT1 not developed	Mutation(s) at codons 505–509 (424–428) ^b	Resistance to rifampicin (Rif) inferred	Optional: Perform sequencing of <i>rpoB</i> to identify the specific mutation.	Rifampicin is not effective
<i>rpoB</i> WT2	<i>rpoB</i> WT2 not developed	Mutation(s) at codons 510–513 (429–432) ^b	Resistance to Rif inferred	Optional: Perform sequencing of <i>rpoB</i> to identify the specific mutation.	Rifampicin is not effective
<i>rpoB</i> WT2/3	<i>rpoB</i> WT2 and WT3 not developed	Mutation(s) at codons 510–517 (429–436) ^b	Resistance to Rif inferred	Optional: Perform sequencing of <i>rpoB</i> to identify the specific mutation.	Rifampicin is not effective

FIRST LINE (FL) – LINE PROBE ASSAY (LPA) INTERPRETATION

Target region	MTBDRp/lus Probe	Mutation or region interrogated	Result interpretation	Additional diagnostic action ^a	Clinical implications
<i>rpoB</i> WT3/4	<i>rpoB</i> MUT1 developed	D516V (D435V) ^b	Resistance to Rif detected	No additional diagnostic action required.	Rifampicin is not effective
	<i>rpoB</i> WT3, WT4 and MUT1 not developed	Mutation(s) at codons 513–519 (432–438) ^b	Resistance to Rif inferred	Optional: Perform sequencing of <i>rpoB</i> to identify the specific mutation.	Rifampicin is not effective
<i>rpoB</i> WT4/5	<i>rpoB</i> WT4 and WT5 not developed	Mutation(s) at codons 516–522 (435–441) ^b	Resistance to Rif inferred	Optional: Perform sequencing of <i>rpoB</i> to identify the specific mutation.	Rifampicin is not effective
	<i>rpoB</i> WT5/6	Mutation(s) at codons 518–525 (437–444) ^b	Resistance to Rif inferred	Optional: Perform sequencing of <i>rpoB</i> to identify the specific mutation.	Rifampicin is not effective
<i>rpoB</i> WT7	<i>rpoB</i> MUT2A developed	H526Y (H445Y) ^b	Resistance to Rif detected	No additional diagnostic action required	Rifampicin is not effective
	<i>rpoB</i> MUT2B developed	H526D (H445D) ^b	Resistance to Rif detected	No additional diagnostic action required	Rifampicin is not effective
<i>rpoB</i> WT8	<i>rpoB</i> WT7, MUT2A and MUT2B not developed	Mutation(s) at codons 526–529 (445–448) ^b	Resistance to Rif inferred	Optional: Perform sequencing of <i>rpoB</i> to identify the specific mutation.	Rifampicin is not effective
	<i>rpoB</i> MUT3 developed	S531L (S450L) ^b	Resistance to Rif detected	No additional diagnostic action required	Rifampicin is not effective
	<i>rpoB</i> WT8 and MUT3 not developed	Mutation(s) at codon 530–533 (449–452) ^b	Resistance to Rif inferred	Optional: Perform sequencing of <i>rpoB</i> to identify the specific mutation.	Rifampicin is not effective

^a The decision to perform the optional diagnostic actions should be guided by considerations on the individual patient's risk group for resistance and by the prevalence of resistance in the specific geographical setting, as these factors affect the positive predictive value of the test. Silent mutations may be of greater concern in low rifampicin-resistant prevalence settings.

^b MTB codon numbering according to Andre *et al* (32) is reported in parenthesis.

Isoniazid

Target region	MTBDRplus Probe	Mutation or region interrogated	Result interpretation	Additional diagnostic action ^a	Clinical implications
katG WT	katG MUT1 or MUT2 developed	S315T1 /S315T2	Mutation associated with high-level increase in MIC detected.	No additional diagnostic action required	Isoniazid is unlikely to be effective even at high dose (17).
	katG WT, MUT1 and MUT2 not developed ^b	Mutation(s) at codon 315	Mutation associated with high-level increase in MIC inferred.	Optional: Perform sequencing of <i>katG</i> to identify the specific mutation.	Isoniazid is unlikely to be effective even at high dose (17).
inhA WT1	inhA MUT1 developed	c-15t	Mutation associated with at least low-level increase in MIC detected. Resistance to Eto/Pto detected.	Optional: Perform sequencing of <i>inhA</i> coding region and <i>katG</i> gene. No additional diagnostic action for Eto/Pto.	Isoniazid at high dose is likely effective (17). Ethionamide/prothionamide are not effective.
	inhA MUT2 developed	a-16g ^d	Mutation likely associated with at least low-level increase in MIC detected. Resistance to Eto/Pto likely detected.	Optional: Perform sequencing of <i>inhA</i> coding region and <i>katG</i> gene. No additional diagnostic action for Eto/Pto.	Isoniazid at high dose is likely effective (23). Ethionamide/prothionamide are likely not effective.
	inhA WT1, MUT1 and MUT2 not developed	Mutation(s) in the -15 region ^d	Mutation likely associated with at least low-level increase in MIC inferred. Resistance to Eto/Pto likely inferred.	Recommended: Repeat SL-LPA to confirm the result. Optional: – Perform sequencing to identify specific mutation. – Perform phenotypic DST for H, Eto/Pto.	Isoniazid at high dose is likely effective (23). Ethionamide/prothionamide are likely not effective.

FIRST LINE (FL) – LINE PROBE ASSAY (LPA) INTERPRETATION

Target region	MTBDR _{plus} Probe	Mutation or region interrogated	Result interpretation	Additional diagnostic action ^a	Clinical implications
<i>inhA</i> WT2	<i>InhA</i> MUT3A developed	t-8c ^d	Mutation likely associated with at least low-level increase in MIC detected. Resistance to Eto/Pto likely detected.	Optional^c: Perform sequencing of <i>inhA</i> coding region and <i>katG</i> gene. No additional diagnostic action for Eto/Pto.	Isoniazid at high dose is likely effective (23). Ethionamide/prothionamide are likely not effective.
	<i>InhA</i> MUT3B developed	t-8a ^d	Mutation likely associated with at least low-level increase in MIC detected. Resistance to Eto/Pto likely detected.	Optional^c: Perform sequencing of <i>inhA</i> coding region and <i>katG</i> gene. No additional diagnostic action for Eto/Pto.	Isoniazid at high dose is likely effective (23). Ethionamide/prothionamide are likely not effective.
	<i>inhA</i> WT2, MUT3A and MUT3B not developed	Mutation in the -8 region ^d	Mutation likely associated with at least low-level increase in MIC inferred. Resistance to Eto/Pto likely inferred.	Recommended: Repeat FL-LPA to confirm the result. Optional: – Perform sequencing to identify specific mutation. – Perform phenotypic DST for H, Eto/Pto.	Isoniazid at high dose is likely effective (23). Ethionamide/prothionamide are likely not effective.

^a The decision to perform the optional follow-up diagnostic actions should be guided by considerations on the individual patient's risk group for resistance and by the prevalence of resistance in the specific geographical setting, as these factors affect the positive predictive value of the test.

^b The partial or whole deletion of the *katG* gene, which is associated with high-level increase in MIC, results in the complete absence of *katG* locus bands (i.e. *katG* locus control, WT and MUT probes are not developed).

^c The concomitant presence of additional mutations in the *inhA* coding region or in positions other than 315 in the *katG* gene (mutations not detected by genotype MTBDR_{plus}) (30, 37), which are globally rare but could be more frequent in specific geographical settings, may cause substantial MIC increases, too high to be compensated for by increased drug dosing.

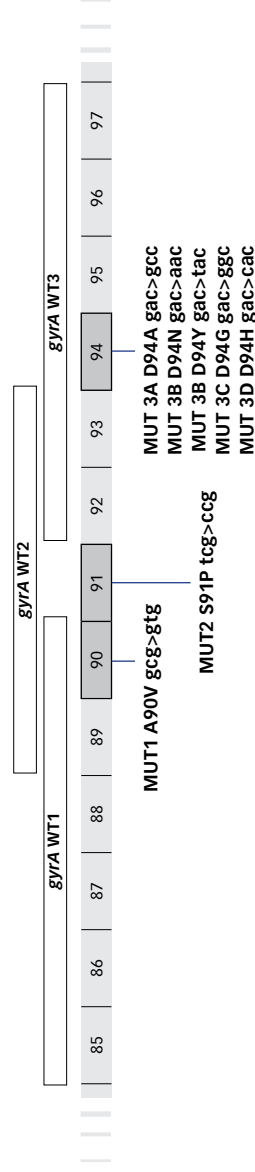
^d Additional data correlating these mutations with phenotypic DST for Isoniazid is needed to increase the confidence in the association of these mutations with drug resistance.

Second Line (SL) – Line Probe Assay (LPA) interpretation

Fluoroquinolones

Figure 3. Quinolone resistance determining region of *gyrA* gene interrogated by GenoType MTBDRsl.

Quinolone-resistance determining region (QRDR) of *gyrA* gene, the codons covered by the WT probes and the specific mutations (both amino acid and nucleotide changes) recognized by the MUT probes in GenoType MTBDRsl Ver 2.0 (15).



Target region	MTBDRs/ Probe	Mutation or region interrogated	Result interpretation	Additional diagnostic action ^a	Clinical implications
<i>gyrA</i> WT 1	<i>gyrA</i> WT1 not developed	Codon(s) 85–89	Resistance to Lfx inferred. Mutation associated with at least low-level increase in MIC for Mfx inferred.	<p>Recommended: Perform phenotypic DST for Mfx at CB to exclude resistance.</p> <p>Optional:</p> <ul style="list-style-type: none"> – Perform sequencing of <i>gyrA</i> QRDR to identify specific mutation; – Perform phenotypic DST for Lfx and Mfx at CC. 	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB. <p>Note. These recommendations do not apply if sequencing, available before treatment initiation, identifies mutations not associated with resistance to FQ, or if phenotypic DST shows susceptibility at CC.</p>

SECOND LINE (SL) – LINE PROBE ASSAY (LPA) INTERPRETATION

Target region	MTBDRs/ Probe	Mutation or region interrogated	Result interpretation	Additional diagnostic action ^a	Clinical implications
gyrA WT2	gyrA MUT1 developed	A90V	Resistance to Lfx detected. Mutation associated with at least low-level increase in MIC for Mfx detected.	Recommended: Perform phenotypic DST for Mfx at CB to exclude resistance.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB.
	gyrA MUT2 developed	S91P	Resistance to Lfx detected. Mutation associated with at least low-level increase in MIC for Mfx detected.	Recommended: Perform phenotypic DST for Mfx at CB to exclude resistance.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB.
gyrA WT3	gyrA WT2, MUT1 and MUT2 not developed	Codon(s) 89–93	Resistance to Lfx inferred. Mutation associated with at least low-level increase in MIC for Mfx inferred.	Recommended: Perform phenotypic DST for Mfx at CB to exclude resistance. Optional: – Perform sequencing of <i>gyrA</i> QRDR to identify specific mutation, – Perform phenotypic DST for Lfx and Mfx at CC.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB. Note. These recommendations do not apply if sequencing, available before treatment initiation, identifies mutations not associated with resistance to FQ, or if phenotypic DST shows susceptibility at CC.
	gyrA MUT3A developed	D94A	Resistance to Lfx detected. Mutation associated with at least low-level increase in MIC for Mfx detected.	Recommended: Perform phenotypic DST for Mfx at CB to exclude resistance.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB.
	gyrA MUT3B developed	D94N or D94Y	Resistance to Lfx detected. Mutation associated with high-level increase in MIC for Mfx detected.	No additional diagnostic action required.	Levofloxacin is not effective. Moxifloxacin is not effective.

Target region	MTDRs/ Probe	Mutation or region interrogated	Result interpretation	Additional diagnostic action ^a	Clinical implications
gyrA WT3	gyrA MUT3C developed	D94G	Resistance to Lfx detected. Mutation associated with high-level increase in MIC for Mfx detected.	No additional diagnostic action required.	Levofloxacin is not effective. Moxifloxacin is not effective.
	gyrA MUT3D developed	D94H	Resistance to Lfx detected. Mutation associated with high-level increase in MIC for Mfx detected.	No additional diagnostic action required.	Levofloxacin is not effective. Moxifloxacin is not effective.
	gyrA WT3, MUT3A, MUT3B, MUT3C, MUT3D not developed	Codon(s) 92–96	Resistance to Lfx inferred. Mutation associated with at least low-level increase in MIC for Mfx inferred.	<p>Recommended: Perform phenotypic DST for Mfx at CB to exclude resistance.</p> <p>Optional:</p> <ul style="list-style-type: none"> – Perform sequencing of <i>gyrA</i> QRDR to identify specific mutation; – Perform phenotypic DST for Lfx and Mfx at CC. 	<p>Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB.</p> <p>Note. These recommendations do not apply if sequencing, available before treatment initiation, identifies mutations not associated with resistance to FQ, or if phenotypic DST shows susceptibility at CC.</p>

^a The decision to perform the optional follow-up diagnostic actions should be guided by considerations on the individual patient's risk group for resistance and by the prevalence of resistance in the specific geographical setting, as these factors affect the positive predictive value of the test.

SECOND LINE (SL) – LINE PROBE ASSAY (LPA) INTERPRETATION

Target region	MTBDRs/ Probe	Mutation or region interrogated	Result interpretation	Additional diagnostic action ^a	Clinical implications
gyrB WT	gyrB MUT1 developed	N538D (codon 499) ^b	Resistance to Lfx detected. Mutation associated with at least low-level increase in MIC for Mfx detected.	Recommended: Perform phenotypic DST for Mfx to exclude resistance at CB.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB.
	gyrB MUT2 developed	E540V (codon 501) ^b	Resistance to Lfx detected. Mutation associated with at least low-level increase in MIC for Mfx detected.	Recommended: Perform phenotypic DST for Mfx to exclude resistance at CB.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB.
	gyrB WT, MUT1 and MUT2 not developed	Codon(s) 536–541 (codon 497–502) ^b	Resistance to Lfx inferred. Mutation associated with at least low-level increase in MIC for Mfx inferred.	Recommended: Perform phenotypic DST for Mfx to exclude resistance at CB. Optional: – Perform sequencing of <i>gyrA</i> QRDR to identify specific mutation, – Perform phenotypic DST for Lfx and Mfx at CC.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB. Note: These recommendations do not apply if sequencing, available before treatment initiation, identifies mutations not associated with resistance to FQ, or if phenotypic DST shows susceptibility at CC.

^a The decision to perform the optional follow up diagnostic actions should be guided by considerations on the individual patient's risk group for resistance and by the prevalence of resistance in the specific geographical setting, as these factors affect the positive predictive value of the test.

^b Codon numbering system according to Carnus *et al* (33).

Second line injectables^a

Target region	MTBDRs/ Probe	Mutation or region interrogated	Result interpretation for Kanamycin (Km)	Result interpretation for Amikacin (Am)	Result interpretation for Capreomycin (Cm)	Additional diagnostic action ^b	Clinical implications
rrs WT1	rrs MUT1 developed	a1401g	Resistance to Km detected.	Resistance to Am detected.	Resistance to Cm detected.	No additional diagnostic action required.	Amikacin, kanamycin and capreomycin are not effective.
	rrs WT1 and MUT1 not developed	Mutation(s) in the 1400 region	Resistance to Km inferred.	Resistance to Am inferred ^c	Resistance to Cm inferred.	Recommended: Repeat the SL-LPA test and if the result is confirmed, perform phenotypic DST for Am, Km, Cm. Optional: Perform sequencing to identify specific mutation.	Kanamycin and capreomycin are likely not effective. Phenotypic DST result should guide the choice to use Amikacin in the treatment regimen.
rrs WT2	rrs MUT2 developed	g1484t	Resistance to Km detected.	Resistance to Am detected.	Resistance to Cm detected.	No additional diagnostic action required.	Amikacin, kanamycin and capreomycin are not effective.
	rrs WT2 and MUT2 not developed	Mutation in the 1484 region	Resistance to Km inferred.	Resistance to Am inferred.	Resistance to Cm inferred.	Recommended: Repeat the SL-LPA test and if the result is confirmed, perform phenotypic DST for Am, Km and Cm. Optional: Perform sequencing to identify specific mutation.	Amikacin, kanamycin and capreomycin are likely not effective.

SECOND LINE (SL) – LINE PROBE ASSAY (LPA) INTERPRETATION

Target region	MTBDRs/ Probe	Mutation or region interrogated	Result interpretation for Kanamycin (Km)	Result interpretation for Amikacin (Am)	Result interpretation for Capreomycin (Cm)	Additional diagnostic action ^b	Clinical implications
<i>eis</i> WT1	<i>eis</i> WT1 not developed	Mutation(s) in the -37 region	Resistance to Km inferred.	Resistance to Am not detected.	Resistance to Cm not detected.	Optional: Perform sequencing to identify specific mutation	Amikacin is likely effective. Capreomycin is likely effective. Kanamycin is not effective.
<i>eis</i> WT2	<i>eis</i> MUT1 developed	c-14t	Resistance to Km detected.	Resistance to Am not detected.	Resistance to Cm not detected.	Optional: Perform sequencing to identify specific mutation	Amikacin is likely effective. Capreomycin is likely effective. Kanamycin is not effective.
	<i>eis</i> WT2 and MUT1 not developed	Mutation(s) in the -10 to -15 region	Resistance to Km inferred.	Resistance to Am not detected.	Resistance to Cm not detected.	Optional: Perform sequencing to identify specific mutation	Amikacin is likely effective. Capreomycin is likely effective. Kanamycin is likely not effective.
<i>eis</i> WT3	<i>eis</i> WT3 not developed	Mutation(s) in the -2 region Note. No evidence that mutations in this region are associated with resistance (7)	Resistance to Km not detected.	Resistance to Am not detected.	Resistance to Cm not detected.	Optional: Perform sequencing to identify specific mutation	Amikacin is likely effective. Capreomycin is likely effective. Kanamycin is likely effective.

^a WHO is no longer recommending the use of kanamycin and capreomycin due to the increased risk of treatment failure and relapse associated with their use in longer MDR-TB regimens (23). The interpretation of SL-LPA for kanamycin and capreomycin has been included in this document with the purpose to provide interim guidance during the transition phase.

^b The decision to perform additional diagnostic actions indicated as optional should be guided by considerations on the individual patient's risk group for resistance and by the prevalence of resistance in the specific geographical setting, as these factors affect the positive predictive value of the test.

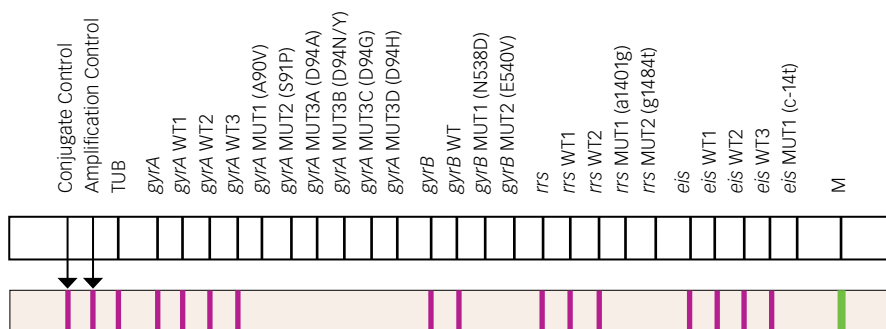
^c As no MUT probe in the *r*/S 1400 region develop, the precise mutation cannot be identified. Since this banding profile may be caused by a mutation likely not associated with Am resistance (e.g. c1402t), resistance to the drug is inferred until phenotypic DST is performed and the result used to re-evaluate the treatment regimen.

Drug-resistant TB cases assessment guide based on SL-LPA

CASE 1

No resistance mutations detected or inferred in any of the genomic regions included in SL-LPA.

All WT bands developed and no MUT bands developed in SL-LPA.



Genotypic report:

Resistance not detected

Additional Diagnostic Action:

Optional:

- Perform phenotypic DST for Lfx at CC (e.g. CC: 1.0 mg/L in MGIT and 7H10) and/or for Mfx at CC and CB (e.g. CC: 0.25 mg/L in MGIT and 0.5 mg/L on 7H10; CB: 1.0 mg/L in MGIT and 2.0 mg/L on 7H10).
- Perform phenotypic DST for SLI drug of interest.

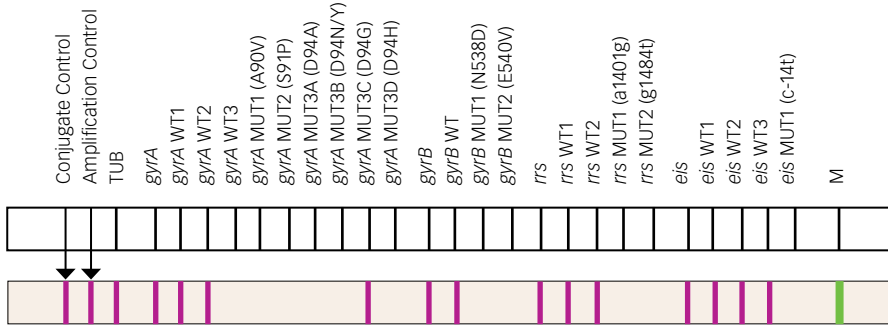
The decision to perform these optional follow-up actions should be guided by considerations on the individual patient's risk group for resistance (e.g. prior exposure to SL medicines, suspect treatment failure), and by the prevalence of resistance in the specific geographical setting, as these factors affect the predictive values of the test.

Clinical implications:

Start MDR-TB treatment. Review treatment regimen based on phenotypic DST results.

CASE 2

Detection of resistance mutations associated with high-level increase in MIC for Mfx



If one of the following MUT probe is developed:

- *gyrA* MUT3C (i.e. *gyrA* D94G) (see picture above as example)
- *gyrA* MUT3D (i.e. *gyrA* D94H)
- *gyrA* MUT3B (i.e. *gyrA* D94N/Y)

Genotypic report:

Levofloxacin: Resistance detected

Moxifloxacin: Mutation associated with high-level increase in MIC for Mfx detected

Additional Diagnostic Action:

Optional: Perform phenotypic DST for SLI drug of interest.

Clinical implications:

Mfx even at a high dose cannot be considered as an effective medicine.

CASE 3

Detection of mutations associated with at least low-level increase in MIC for Mfx



If one of the following MUT probe is developed:

- *gyrA* MUT1 (i.e. *gyrA* A90V) (see picture above as example)
- *gyrA* MUT2 (i.e. *gyrA* S91P)
- *gyrA* MUT3A (i.e. *gyrA* D94A)
- *gyrB* MUT1 (i.e. *gyrB* N538D)
- *gyrB* MUT2 (i.e. *gyrB* E540D)

Genotypic report:

Levofloxacin: Resistance detected.

Moxifloxacin: Mutation associated with at least low-level increase in MIC for Mfx detected.

Additional Diagnostic Action:

Recommended: Perform phenotypic DST for Mfx at CB according to Case 1.

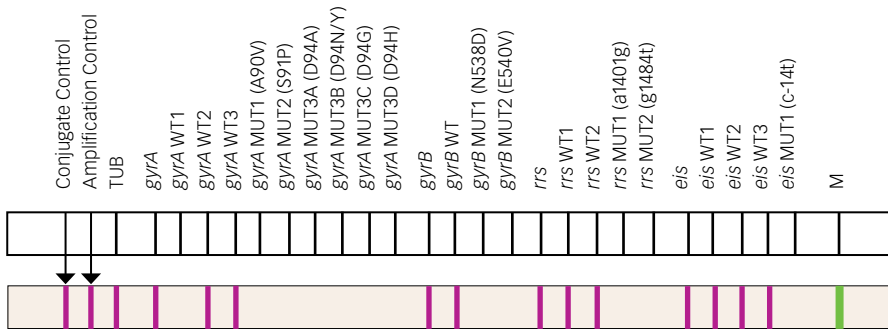
Optional: Perform phenotypic DST for SLI drug of interest.

Clinical implications:

Mfx could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB.

CASE 4

*Precise mutation unknown, only inferred in the fluoroquinolones (FQ) regions (i.e. *gyrA* and *gyrB*)*



If one of the following WT bands is not developed:

- *gyrA* WT1 (i.e. *gyrA* WT1 probe missing) (see picture above as example),
- *gyrA* WT2 (i.e. *gyrA* WT2 probe missing),
- *gyrA* WT3 (i.e. *gyrA* A WT3 probe missing),
- *gyrB* WT (i.e. *gyrB* WT probe missing),

and none of the MUT probe is developed in the *gyrA* and *gyrB* regions.

Genotypic report:

Levofloxacin: Resistance inferred.

Moxifloxacin: Mutation associated with at least low-level increase in MIC for Mfx inferred.

Additional Diagnostic Action:

Recommended: Perform phenotypic DST for Mfx at CB according to Case 1.

Optional but recommended in some settings:¹ *gyrA* and *gyrB* QRDR sequencing to identify resistance mutation and exclude synonymous mutations or non-synonymous mutations not causing resistance (systematic false positive results) (interpret based on Cases 2–4 and follow respective recommendations for phenotypic DST).

If sequencing is not available, perform phenotypic DST at CC for Lfx and/or Mfx according to case 1.

Optional: Perform phenotypic DST for SLI drug of interest.

Clinical implications:

Levofloxacin is not effective.

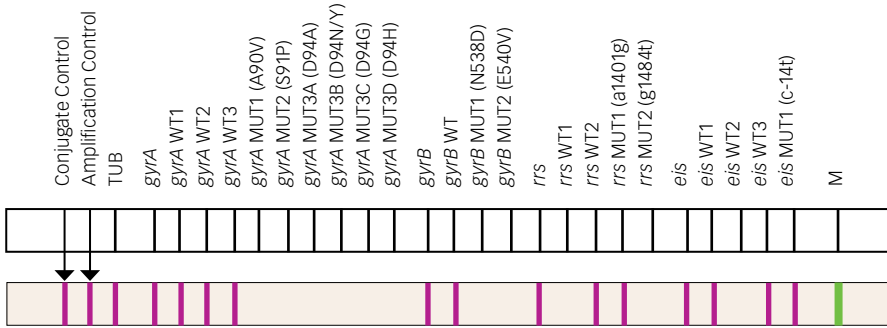
Mfx could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB.

Note. These recommendations do not apply if sequencing, available before treatment initiation, identifies mutations not associated with resistance to FQ, or if phenotypic DST shows susceptibility at CC.

¹ The lack of binding of a WT probe without simultaneous binding of a mutant probe is caused by the presence of a resistance mutation (e.g. *gyrA* G88A). However, systematic errors are possible due to synonymous or non-synonymous mutations. Globally this is rare (<1% of isolates), but these isolates can be frequent locally. Unfortunately, it cannot be predicted in which settings these cases are frequent, which means that each laboratory has to decide whether sequencing of the QRDR region is required based on the local epidemiology. For example, the *gyrA* A90G mutation, which prevents binding of *gyrA* WT2, is frequent in the Republic of the Congo and the Democratic Republic of the Congo and a synonymous mutation codon at 96 of *gyrA*, which prevents binding of *gyrA* WT3, is frequent in Medellín (Colombia) (9). In both of these settings, sequencing would therefore be recommended.

CASE 5

Detection of mutations that cause resistance to SLI.



If one of the following MUT bands is developed:

- *rrs* MUT1 (i.e. *rrs* a1401g) (see picture above as example)
- *rrs* MUT2 (i.e. *rrs* g1484t)
- *eis* MUT1 (i.e. *eis* c-14t)

Genotypic report:

In case of *rrs* mutations only or mutations in both *rrs* and *eis*:

- Amikacin: Resistance detected
- Kanamycin: Resistance detected
- Capreomycin: Resistance detected

In case of *eis* mutation c-14t only:

- Amikacin: Resistance not detected
- Kanamycin: Resistance detected
- Capreomycin: Resistance not detected

Additional Diagnostic Action:

Optional: Perform phenotypic DST for FQs according to Case 1.

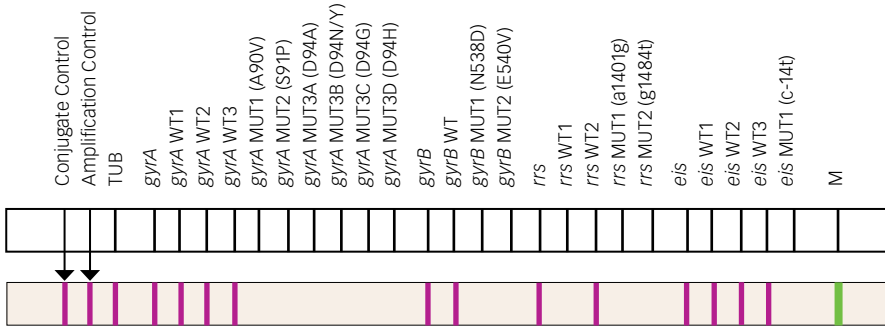
Clinical implications:

In case of *rrs* mutations only or mutations in both *rrs* and *eis*, resistance to SLI drugs is predicted.

In case of *eis* mutation c-14t only: amikacin is effective.

CASE 6

Precise mutation unknown, only inferred, in the rrs region



If one of the following WT bands is not developed:

- *rrs* WT1 (*rrs* probe WT1 not developed) (see picture above as example),
- *rrs* WT2 (*rrs* probe WT2 not developed),

and none of the MUT probes is developed in the *rrs* region.

Genotypic report:

In case of mutation in the *rrs* 1400 region:

- Amikacin: Resistance inferred¹
- Kanamycin: Resistance inferred
- Capreomycin: Resistance inferred

In case of mutation in the *rrs* 1484 region:

- Amikacin: Resistance inferred
- Kanamycin: Resistance inferred
- Capreomycin: Resistance inferred

Additional Diagnostic Action:

Recommended: Repeat the assay and if the result is confirmed, perform phenotypic DST for Am.

Optional: Perform sequencing to identify precise mutation.
Perform phenotypic DST for FQs according to Case 1.

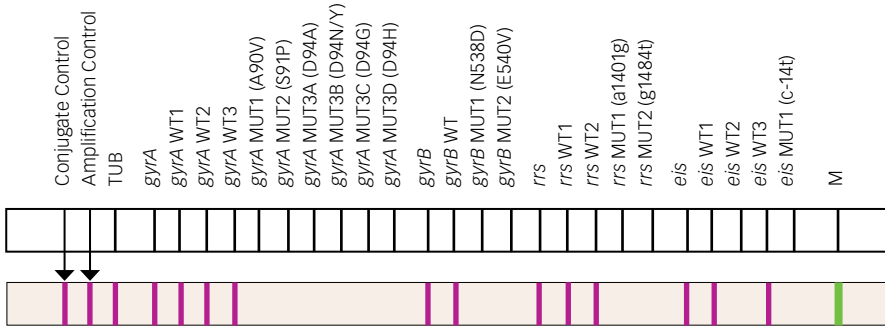
Clinical implications:

Kanamycin and capreomycin are likely not effective.

¹ As no MUT probe in the *rrs* 1400 region develop, the precise mutation cannot be identified. Since this banding profile may be caused by a mutation likely not associated with Am resistance (e.g. c1402t), resistance to the drug is inferred until phenotypic DST is performed and the result used to re-evaluate the treatment regimen.

CASE 7

Precise mutation unknown, only inferred, in the eis region



If one of the following WT bands is not developed:

- *eis* WT1 (*eis* probe WT1 not developed) (e.g. *eis* g-37t),
- *eis* WT2 (*eis* probe WT2 not developed) (e.g. *eis* c-12t or g-10a) (see picture above as example),

and none of the MUT probes is developed in the *eis* region.

Genotypic report:

In case mutations in *eis* are inferred (and no additional mutations in the *rrs* region):

- Amikacin: Resistance not detected
- Kanamycin: Resistance inferred
- Capreomycin: Resistance not detected

Additional Diagnostic Action:

Optional: Perform phenotypic DST for FQs according to case 1 and for Am and Cm.

Clinical implications:

Amikacin is likely effective.

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Annex 1.

FL-LPA Reporting format – Practical examples

Please note that the “Conclusion” section has been included for convenience but should not be part of the laboratory report.

Example 1

Drug	Gene	Mutation	Interpretation	Conclusion
Rif ^a	<i>rpoB</i>	H526Y	Resistance to Rif detected.	Rifampicin is not effective.
H ^a	<i>katG</i>	Mutation(s) in codon 315 region	Mutation associated with high-level increase in MIC inferred.	Isoniazid is unlikely to be effective even at high dose.
	<i>inhA</i>	t-8a		
Eto/Pto	<i>inhA</i>	t-8a	Resistance to Eto/Pto likely detected.	Ethionamide and prothionamide are likely not effective.

Example 2

Drug	Gene	Mutation	Interpretation	Conclusion
Rif	<i>rpoB</i>	No mutation detected	Resistance to Rif not detected.	Rifampicin is effective.
H ^a	<i>katG</i>	S315T	Mutation associated with high-level increase in MIC detected.	Isoniazid is not effective even at high dose.
	<i>inhA</i>	c-15t		
Eto/Pto	<i>inhA</i>	c-15t	Resistance to Eto/Pto detected.	Ethionamide and prothionamide are not effective.

Example 3

Drug	Gene	Mutation	Interpretation	Conclusion
Rif	<i>rpoB</i>	Mutation(s) in codons 516-522 (435-441)	Resistance to Rif inferred.	Rifampicin is not effective.
H	<i>katG</i>	No mutation detected	Mutation likely associated with at least low-level increase in MIC detected.	Isoniazid at high dose is likely effective.
	<i>inhA</i>	t-8c		
Eto/Pto	<i>inhA</i>	t-8c	Resistance to Eto/Pto likely detected.	Ethionamide and prothionamide are likely not effective.

^a If more than one probe per drug that provides information the results should be reported according the following hierarchy (where the “>” sign means overrule):
 For H: Mutation associated with high-level increase in MIC detected > Mutation associated with high-level increase in MIC inferred > Mutation associated with at least low-level increase in MIC detected > Mutation associated with at least low-level increase in MIC inferred > Resistance not detected.
 For Rif: Resistance detected > Resistance inferred > Resistance not detected.

Annex 2.

SL-LPA Reporting format – Practical examples

Please note that the “Conclusion” section has been included for convenience but should not be part of the laboratory report.

Example 1

Drug	Gene	Mutation	Interpretation	Conclusion
Lfx ^a	<i>gyrA</i>	D94A	Resistance to Lfx detected.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB. Kanamycin is not effective. Amikacin is not effective. Capreomycin is not effective.
	<i>gyrB</i>	No mutation		
Mfx ^a	<i>gyrA</i>	D94A	Mutation associated with at least low-level increase in MIC for Mfx detected.	
	<i>gyrB</i>	No mutation		
Km ^a	<i>rrs</i>	a1401g	Resistance to Km detected.	
	<i>eis</i> promoter	Mutation(s) in the -10 to -15 region		
Am	<i>rrs</i>	a1401g	Resistance to Am detected.	
Cm	<i>rrs</i>	a1401g	Resistance to Cm detected.	

Example 2

Drug	Gene	Mutation	Interpretation	Conclusion
Lfx	<i>gyrA</i>	A90V	Resistance to Lfx detected.	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB. Kanamycin is not effective. Amikacin is effective. Capreomycin is effective.
	<i>gyrB</i>	No mutation		
Mfx	<i>gyrA</i>	A90V	Mutation associated with at least low-level increase in MIC for Mfx detected.	
	<i>gyrB</i>	No mutation		
Km	<i>rrs</i>	No mutation	Resistance to Km inferred.	
	<i>eis</i> promoter	Mutation(s) in the -37 region		
Am	<i>rrs</i>	No mutation	Resistance to Am not detected.	
Cm	<i>rrs</i>	No mutation	Resistance to Cm not detected.	

Example 3

Drug	Gene	Mutation	Interpretation	Conclusion
Lfx	<i>gyrA</i>	Mutation(s) at codon 89–93	Resistance to Lfx inferred	Levofloxacin is not effective. Moxifloxacin could be used at higher dose. The regimen should be re-evaluated based on phenotypic DST results at CB. Note. These recommendations do not apply if sequencing, available before treatment initiation, identifies mutations not associated with resistance to FQ, or if phenotypic DST shows susceptibility at CC.
	<i>gyrB</i>	No mutation		
Mfx	<i>gyrA</i>	Mutation(s) at codon 89-93	Mutation associated with at least low-level increase in MIC for Mfx inferred.	
	<i>gyrB</i>	No mutation		
Km	<i>rrs</i>	No mutation	Resistance to Km not detected	
	<i>eis</i> promoter	Mutation(s) in the -2 region		
Am	<i>rrs</i>	No mutation	Resistance to Am not detected	
Cm	<i>rrs</i>	No mutation	Resistance to Cm not detected	

^a If more than one probe per drug that provides information the results should be reported according the following hierarchy (where the ">" sign means overrule):

For *Lfx*, *Am*, *Km*, *Cm*: Resistance detected > Resistance inferred > Resistance not detected.

For *Mfx*: Mutation associated with high-level increase in MIC detected > Mutation associated with at least low-level increase in MIC detected > Mutation associated with at least low-level increase in MIC inferred > Resistance not detected.

Annex 3.

Specific nucleotide changes detected by MUT probes

Please note that some of the amino acid (AA) changes identified by FL- and SL-LPA are caused by nucleotide changes that are not specifically recognized by the MUT probes. For instance, the *gyrA* mutation A90V is caused by two possible nucleotide changes, namely (i) gcg>gtg or (ii) gcg>gtc. However, only the former nucleotide change (gcg>gtg) will be recognized by the *gyrA* MUT1 probe, while the latter (gcg>gtc) will be detected only by the absence of *gyrA* WT2 (i.e. *gyrA* WT2 not detected).

	MUT probe	AA change	Nucleotide change
rpoB MUT probes	MUT1	D516V (D435V)	gac>gtc
	MUT2A	H526Y (H445Y)	cac> tac
	MUT2B	H526D (H445D)	cac> gac
	MUT3	S531L (S450L)	tcg>ttg

	MUT probe	AA change	Nucleotide change
katG MUT probes	MUT1	S315T	agc>acc
	MUT2	S315T	agc>aca

	MUT probe	AA change	Nucleotide change
gyrA MUT probes	MUT1	A90V	gcg>gtg
	MUT2	S91P	tcg>ccg
	MUT3A	D94A	gac>gcc
	MUT3B	D94N	gac>aac
	MUT3B	D94Y	gac>tac
	MUT3C	D94G	gac>ggc
	MUT3D	D94H	gac>cac

	MUT probe	AA change	Nucleotide change
gyrB MUT probes	MUT1	N538D (N499D)	aac>gac
	MUT2	E540V (E501V)	gaa>gta



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