World Health Organization

Line probe assays for detection of drug-resistant tuberculosis

Interpretation and reporting manual for laboratory staff and clinicians

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Acronyms and abbreviations

7H10	Middlebrook 7H10 medium
Am	amikacin
CB	clinical breakpoint
CC	critical concentration
DST	drug-susceptibility testing
Eto	ethionamide
FQ	fluoroquinolone
H	isoniazid
Lfx	levofloxacin
LPA	line probe assay
MDR-TB	multidrug-resistant tuberculosis
MGIT	BACTEC™ Mycobacterial Growth Indicator Tube™ 960
Mfx	moxifloxacin
MIC	minimum inhibitory concentration
MTBC	*Mycobacterium tuberculosis* complex
MUT probe	mutation probe
Pto	prothionamide
PZA	pyrazinamide
QRDR	quinolone-resistance determining region
R	resistant
Rif	rifampicin
S	susceptible
SL-LPA	second-line line probe assay
TB	tuberculosis
WT	wild type

Glossary

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

Clinical breakpoint (CB): concentration(s) of an antimicrobial agent that defines a minimum inhibitory concentration (MIC) above the critical concentration that separates strains that are likely to respond to treatment from those that will probbably not respond. This concentration is determined by correlation with clinical outcome data, the distribution of MICs, genetic markers and data on pharmacokinetics and pharmacodynamics, including drug dose. An increased dose can be used to overcome resistance observed at lower doses, up to the maximum tolerated dose, i.e., the CB above which the drug is not recommended for use. The CB is used to guide clinical decisions in the treatment of individual patients. The CB is not applicable for surveillance of drug resistance.

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 tested in a standardized method are aggregated for one species, a single Gaussian-shaped MIC distribution is observed, which corresponds to the phenotypically wild-type (WT) distribution of that species (i.e., the distribution of organisms that lack phenotypically detectable resistance mechanisms). Additional distributions with higher overall MICs may be identified that correspond to intrinsically or naturally resistant organisms (i.e., phenotypically non-wild type distribution).

Preface

This document was developed to provide practical guidance on interpretation of the most commonly used first- and second-line line probe assays (LPAs) (i.e., GenoType MTBDR*plus* V2.0 and GenoType MTBDRsl V2.0 assays; Bruker-Hain). In this updated manual, the interpretation of mutations identified by the two assays has been revised to align them with the most recent WHO catalogue of mutations in *Mycobacterium tuberculosis* complex (MTBC) and their association with drug resistance *(1)* and to present the latest changes in instructions for use of the assays *(2)*.

The manual is intended for both laboratory staff and clinicians. It provides information on:

— the association of specific mutations detected by the most commonly used line probe assays with phenotypic drug resistance;
— instances in which specific resistance-conferring mutations are not identified and resistance can only be inferred;
— actions to be performed when certain mutations are detected in the assays; and
— the clinical implications of specific LPA mutations for selection of appropriate tuberculosis (TB) treatment regimens.

In addition, this document provides support for staff at national and regional TB reference laboratories in understanding and managing any discrepancies between phenotypic and genotypic drug susceptibility testing (DST).

The manual outlines the mutations identified with both first- and second-line LPA test strips, including information on their association with phenotypic drug resistance based on the WHO catalogue of mutations in MTBC and their association with drug resistance *(1)* and the MICs for first- and second-line drugs reported by WHO *(3, 4)*. Test interpretation, follow-up diagnostic testing and the clinical implications of the presence of specific mutations and inferred resistance are also described.

The guide also presents case studies of examples of LPA test results and describes how results should be reported to clinicians, with recommended, customizable reporting templates (Annexes 1 and 2).

Introduction

In the past two decades, better understanding of the molecular bases of resistance to tuberculosis (TB) drugs has resulted in the development of various genotypic assays for rapid determination of resistance to anti-TB agents. Molecular testing has several advantages besides the rapidity of diagnosis, including: direct use of clinical specimens (without time-consuming solid or liquid culture to isolate *Mycobacterium tuberculosis* complex (MTBC) from a patient sample) and of specimens containing non-viable bacteria (e.g., bacteria killed by heat or chemical inactivation), greater potential for high-throughput testing and fewer laboratory biosafety requirements for testing procedures (i.e., low complexity-nucleic acid amplification testing).

In 2008, WHO endorsed use of the first line probe assay (LPA), the GenoType MTBDR*plus* version 1 (referred to as GenoType MTBDR*plus* V1), for rapid detection of multidrug-resistant TB (MDR-TB) *(5)*. In 2011, newer versions of the LPA technology became available, including the GenoType MTBDR*plus* version 2 (referred to as GenoType MTBDR*plus* V2) and the Nipro (Tokyo, Japan) non-tuberculous mycobacteria +MTBDR detection kit 2 (referred to as "Nipro"). The aim of these newer LPAs was to improve the sensitivity of MTBC detection and simultaneously detect resistance to rifampicin (Rif) and isoniazid (H). In 2015, the Foundation for Innovative New Diagnostics compared the Nipro and the GenoType MTBDR*plus* V2 LPAs with GenoType MTBDR*plus* V1 and found equivalence among the three commercially available LPAs for detecting MTBC and resistance to Rif and H *(6)*.

The first commercial LPA for detection of resistance to second-line TB drugs was the GenoType MTBDRsl version 1.0 (referred to as GenoType MTBDRsl V1), developed by Hain Lifescience more than a decade ago. An updated version of this assay (GenoType MTBDRsl V2) for detecting both the mutation associated with resistance to fluoroquinolone (FQ) and second-line injectable drugs detected with version 1.0 as well as other mutations (described below) became available in 2015.

The following year, the WHO recommended use of the commercially available first-line LPAs (i.e., GenoType MTBDR*plus* V1, GenoType MTBDR*plus* V2 and Nipro) for initial testing instead of phenotypic drug-susceptibility testing (DST) to detect resistance to Rif and H *(7)*. WHO also recommended use of GenoType MTBDRsl (V1 and V2) to detect resistance to FQs and amikacin (Am) in patients with Rif-resistant/MDR-TB and to guide initiation of an appropriate MDR-TB treatment regimen *(8)*. Most recently, in 2021, the WHO recommended use of the LPA Genoscholar PZA-TB II (Nipro) for detection of resistance to pyrazinamide (PZA) in isolates from patients with bacteriologically confirmed pulmonary TB *(9)*. For a more detailed description of the place of first- and second-line LPA within TB diagnostic algorithms, refer to module 3 of the 2021 WHO operational handbook on tuberculosis *(10)*.

This document focuses on the two currently most widely used LPAs (GenoType

MTBDR*plus* V2 and GenoType MTBDRsl V2). It provides guidance to laboratory staff and clinicians in interpreting and reporting the results of both first- and second-line LPAs for better understanding and management of possible discrepancies between phenotypic and genotypic DST and the impact of LPA results on decisions about further TB testing and treatment.

Principle of the line probe assay

LPAs are a family of DNA strip-based tests that allow users to determine the drug resistance profile of an MTBC strain by interpreting a pattern of bands that represent lines of immobilized probes that are bound (or hybridized) to MTBC amplicons (DNA amplification products). LPA probes are designed to target the most common mutations associated with resistance to first- and second-line anti-TB agents and specific MTBC wild-type (WT) DNA sequences.

LPAs are approved by WHO for rapid detection of drug resistance to first- and second-line anti-TB agents, including PZA. They can be used to test culture isolates (indirect testing, e.g., Genoscholar PZA-TB II) and for direct testing of acid-fast bacilli smear microscopy-positive specimens (first-line LPA) and both smear-positive and smear-negative sputum specimens (second-line LPA) (7, 8).

Mutations are detected by binding of amplicons to probes that target the most common mutations (MUT probes) (with, e.g., first- and second-line LPAs) or by lack of amplicon binding (i.e., lack of hybridization) to the corresponding WT probes (e.g., PZA-LPA), defined as "inferred resistance". The post-hybridization reaction leads to development of coloured bands on the strip at the site of probe binding.

It is important to note that, like other molecular tests currently endorsed by WHO, LPAs have some limitations:

- Although LPAs can detect the mutations most frequently identified in resistant strains, some mutations that confer resistance are outside the regions covered by the test and resistance cannot be completely excluded, even in the presence of all WT probes. Thus, in some cases additional phenotypic DST may be necessary for a full assessment of the presence of a resistant strain.
- Some mutations are identified specifically by MUT probes, whereas others are inferred only by the absence of binding of the amplicons to WT probes. The lack of binding of a WT probe without simultaneous binding of an MUT probe is probably due to the presence of a resistance mutation. Systematic errors are possible if there are synonymous and non-synonymous mutations (e.g., phylogenetic mutations) *(11)*. This is rare (< 1% of isolates), although the frequency of these isolates may increase in in certain settings *(12)*.
- LPA is less efficient than conventional culture-based DST in detecting resistance in samples that harbour both drug-susceptible and -resistant bacteria (i.e., heteroresistance). Specifically, LPA can be used to identify 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 represents less than 95% of the total bacterial population *(13, 14)*.

The overall sensitivity and specificity of LPAs for different drugs are reported in detail elsewhere *(9)*. Briefly, first-line LPA showed a sensitivity of 95.8% and a specificity of 98.4% for detection of Rif resistance by direct testing and a sensitivity of 94.5% and a specificity of 99.3% for detection of H resistance. Second-line LPAs (GenoType MTBDRsl) had a pooled sensitivity of 86.2% and a specificity of 98.6% for the detection of FQ resistance by direct testing and a pooled sensitivity of 87.0% and a specificity of 99.5% for detection of resistance to second-line injectable drugs. PZA-LPA (Genoscholar PZA-TB II) showed a pooled sensitivity of 81.2% and a specificity of 97.8% for detection of resistance to PZA in MTBC isolates *(9)*. Additional information on the PZA-LPA can be found in the information sheet in the annex to the 2021 WHO operational handbook on tuberculosis: Module 3 *(10)*.

GenoType MTBDR*plus* Version 2

GenoType MTBDR*plus* (Fig. 1a) targets specific mutations in the Rif resistance-determining region of the *rpoB* gene (from codon 505 to 533) (Fig. 2) to detect Rif resistance and mutations in the *inhA* promoter (from –16 to –8 nucleotides upstream)

Fig. 1. Configuration of GenoType MTBDR*plus* V2 a and GenoType MTBDRsl V2 b strips

a *(2)*

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

b *(15)*

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

and *katG* (codon 315) genomic regions to identify resistance to H *(2)*. The specific nucleotide changes detected by the test are reported in Annex 3.

GenoType MTBDR*sl* Version 2

The second version of GenoType MTBDRsl (Fig. 1b) includes the quinolone-resistance determining region (QRDR) of *gyrA* (from codon 85 to 96) (Fig. 3) and of *gyrB* (from codon 536 to 541) genes for detection of resistance to FQs and the *rrs* (nucleic acid positions 1401, 1402 and 1484) and the *eis* promoter regions (from –37 to –2 nucleotides upstream) for detection of resistance to Am *(15)*. The precise regions covered by all MUT probes have not been disclosed, and only some of the regions covered by WT probes are known (see Fig. 2 and 3). The specific nucleotide changes detected by the MUT probes are reported in Annex 3.

Interpretation and reporting

The LPA has two internal controls on the strip: "conjugate control" (line 1) and "amplification control" (line 2) (Fig. 1). The conjugate control line should always be visible in order to document the efficiency of conjugate binding and substrate reaction. The amplification control line serves as reference for interpretation of WT and MUT probes: only those bands of which the intensity is about a strong as or stronger than that of the amplification control line should be considered. In the case of a positive test result (i.e., a positive *M. tuberculosis* control band), the signal of the amplification control zone may be weak or even vanish. This may occur more frequently in indirect testing. The absence of amplification control may be due to competition among single reactions during amplification. This indicates that the test has been conducted correctly and need not be repeated with the same sample.

In first-line LPA, when there are strong signals of WT bands but weak or no staining of the amplification control band, a single WT band that is significantly fainter than the remaining WT bands of the same locus (or the Locus control for *katG*) should be considered negative *(2)*. In the case of a negative test result, both the conjugate and the amplification control bands should always be visible to ensure a valid negative result. The absence of an amplification control band in a negative test indicates mistakes during setup and/or performance of the amplification reaction or the presence of amplification inhibitors. In this case, the test result is invalid and must be repeated.

The ***M. tuberculosis* control band** (line 3) is present only if the DNA amplified is from members of the MTBC. In rare cases, the *M. tuberculosis* control band band is missing because of competition among the single amplification reactions during the polymerase chain reaction. When an evaluable resistance pattern develops, however, the presence of an MTBC strain should be suspected, and the test should be repeated. In rare cases in direct testing, only the conjugate and amplification control bands and the *M. tuberculosis* control band may be visible, in the absence of an evaluable resistance pattern. This may indicate the presence of an MTBC strain at very low concentration, below the limit of detection. In such instances, the test should be repeated on the corresponding culture isolate (i.e., indirect testing). The presence of non-tuberculous mycobacteria in the specimen can result in random banding patterns, with several species testing positive at some *rpoB* WT bands because of gene similarity among the species. In the presence of non-tuberculous rather that

MTBC bacteria, the *M. tuberculosis* control band will always be absent, and the result should be reported as "MTBC not detected".

The gene **locus control** bands for the different target regions analysed on the DNA strip are located just before their respective WT and MUT probe bands. The locus control bands must always be present in order for the assay to be considered valid for the corresponding target. In rare cases, all bands of a gene locus (including the locus control band) may be missing. In direct testing, such a banding pattern cannot be evaluated, and the test must be repeated. In indirect testing, however, the complete absence of the *katG* locus indicates resistance to H of the strain tested due to mutations or deletions in the locus control region or to complete or partial deletion of the gene *(2)*. The **WT reaction zones** comprise regions of the genome with known resistance mutations. The **MUT probe reaction zones** correspond to probes that identify the most common resistance mutations of the gene being examined.

Resistance is detected when MUT probes are developed, whereas, in the absence of WT probes, resistance can only be inferred (see below for details). Concomitant detection of all WT probes and any of the MUT probes in the corresponding target region indicates the presence of heteroresistance (i.e., susceptible and resistant bacteria in the same sample). In this case, the result should be reported as "resistant".

Revisions to manufacturers' interpretations *(2, 15)*

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

Given the limitations of LPA and in particular the fact that resistance cannot be totally excluded even in the presence of all WT probes (as not all mutations that confer resistance are covered by these tests, and mutations that are covered may occur below the limit of detection), it is most 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 when 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, only the region in which the mutation is located and not the precise mutation can be reported.

The term "Resistance detected" is used when one or more MUT probes that identify specific mutations conferring resistance to the drugs are developed (regardless of whether WT probes are developed).

Stratification of resistance mutations for H and moxifloxacin (Mfx) into mutations associated with "low-level resistance" and "high-level resistance"

Mutations that confer resistance to H and Mfx are stratified into those associated with low- and high-level resistance and, depending on the distribution of the associated MICs, with a low and a high increase in MIC, respectively. This stratification has important implications for the inclusion of H and Mfx in a treatment regimen, as resistance due to mutations associated with low-level resistance 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 resistance, are detected (in the absence of any *katG* mutation), increasing the drug dose might be effective; thus, administration of H at a maximum dose of 15 mg/kg per day could be considered. In the case of *katG* mutations, which are more commonly associated with high-level resistance, administration of H at an even higher dose is less likely to be effective. The presence of combined mutations in the *inhA* promoter and the *katG* gene results in substantial increases in the MIC (i.e., high-level resistance), which is unlikely to be compensated for by increasing the dose *(17)*.

For Mfx, if mutations associated with MIC increase above the CC but below the CB, which are defined as mutations associated with low-level resistance, high-dose Mfx (up to 800 mg daily for adults) might be effective. When resistance to Mfx is inferred (i.e., the specific mutation is unknown), the presence of mutations associated with at least low-level resistance is inferred, and therefore a high dose of Mfx might still be effective. In this case, however, it is recommended that DST be performed for Mfx at the CB, and, if available, sequencing be conducted to determine the specific mutation. If the MTBC strain is resistant to Mfx at the CB because of the presence of mutations associated with high-level resistance, the drug cannot be considered effective.

When more than one probe per drug provides information (e.g., concomitant detection of mutations associated with different resistance levels), the criterion for interpretation is that the mutations associated with high-level resistance overrule mutations associated with low-level resistance. Similarly, mutations detected by MUT probes overrule mutations that are only inferred by the absence of WT probes.

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

- *For H:* Mutation associated with high-level resistance detected > Mutation associated with high-level resistance inferred > Mutation associated with at least low-level resistance detected > Mutation associated with at least low-level resistance inferred > Resistance not detected
- *For Mfx:* Mutation associated with high-level resistance detected > Mutation associated with at least low-level resistance detected > Mutation associated with at least low-level resistance inferred > Resistance not detected
- *For Rif, levofloxacin (Lfx), Am, kanamycin and capreomycin:* Resistance detected > Resistance inferred > Resistance not detected

In summary, according to whether WT and MUT probes are present, the following four cases may occur:

Case	WT reaction zones	MUT probe 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 drug: • Resistance detected (Rif, Am) • Mutations associated with high-level resistance detected (H and Mfx) • Mutations associated with at least low-level resistance detected (H and Mfx)
3	One or more WT probes are not developed.	No MUT probes are developed.	Depending on the drug: • Resistance inferred (Rif, Am) • Mutations associated with high-level resistance inferred (H and Mfx) • Mutations associated with at least low-level resistance inferred (H and Mfx)
4	All WT probes are developed.	One MUT probe is developed.	Resistance detected (due to heteroresistance); interpret according to case 2.

Interpretation of resistance profile for amikacin

The WHO Catalogue of mutations in MTBC *(1)* defines two additional markers for Am resistance: (i) the *eis* c-14t mutation, identified by the *eis* MUT1 probe, is classified as a definitive marker for resistance (group 1); and (ii) the *rrs* c1402t, inferred by the absence of the *rrs* WT1, is recognized as a group 2 mutation (i.e., a mutation associated with resistance-interim). Therefore, the interpretation of second-line-LPA for Am has been revised accordingly.

Exclusion of the eis WT3 probe

To date, there is no clear evidence that the mutation c-2a in the *eis* promoter region is on its own a valid marker of resistance *(16)*. Therefore, if the *eis* WT3 probe is not developed, the test interpretation for kanamycin has been revised to "Resistance not detected".

Interpretation of resistance profiles for ethionamide and prothionamide

Mutations leading to an overexpression of *inhA* gene, such those detected by first-line LPA, are associated with resistance to ethionamide (Eto) *(1)* and prothionamide (Pto). Therefore, if these mutations are detected, resistance to the two drugs should be reported, and they should be excluded from the treatment regimen. Even in the absence of mutations in the *inhA* promoter region, however, resistance to Eto and Pto cannot be excluded. Mutations conferring resistance to these drugs may in fact be present in genomic regions not targeted by LPA (e.g., *ethA*, *ethR*) *(1)*.

Reporting of results for kanamycin and capreomycin

WHO currently recommends that injectable medicines be phased out as a priority in all treatment regimens and be replaced by bedaquiline, which makes rapid DST for second-line injectables unnecessary *(17)*. In addition, since 2018, WHO no longer recommends use of kanamycin or capreomycin because of the increased risks of

treatment failure and relapse associated with their use in longer MDR-TB regimens *(18)*. Am is the only second-line injectable agent still recommended for use in MDR-TB regimens when options for composition of the treatment regimen are limited *(17)*. Therefore, interpretation of second-line LPA for kanamycin and capreomycin is not considered in this manual.

Definition of additional follow-up diagnosis to guide initiation of appropriate treatment

Depending on the region interrogated by first- and second-line LPA, one or more follow-up diagnostic actions are either recommended or suggested to guide treatment regimens. A decision to conduct the optional follow-up diagnostic actions should be guided by considerations of the risk group of the patient for resistance and by the prevalence of resistance in the setting, as these factors affect the positive predictive value of the test.

The follow-up diagnostic actions that are recommended or suggested depend on the drug. The actions are summarized briefly below.

Rifampicin (Rif):

- If resistance is inferred from the absence of binding of the amplicons to WT probes (i.e., one or more WT probes not developed), sequencing of the *rpoB* gene is suggested to identify the specific mutation. For interpretation of *rpoB* mutations, see the WHO catalogue *(1)*.

Isoniazid (H):

- If resistance is inferred from 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 the *katG* gene is suggested to identify the specific mutation. For interpretation of *katG* mutations, see the WHO catalogue *(1)*.
- If mutations associated with low-level resistance are detected (i.e., MUT probes developed in the *inhA* promoter region in the absence of mutations in the *katG* target region), sequencing of the *inhA* coding region and the *katG* gene is suggested, because 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*) *(19, 20)*, which are globally rare but could be more frequent in some settings, may substantially increase the MIC to a level too high to be compensated for by increasing the dose of the drug.
- If mutations associated with low-level resistance are inferred from the absence of binding of the amplicons to WT probes in the *inhA* promoter region (and no mutations are detected in the *katG* target region), it is recommended that the test be repeated to confirm the result. Optional follow-up diagnostic actions include sequencing of the *inhA* promoter to identify the specific mutation or performing phenotypic DST for H.

Moxifloxacin (Mfx):

- If mutations associated with low-level resistance 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 resistance are inferred from the absence of binding of the amplicons to WT probes in the *gyrA* or *gyrB* region (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 phenotypic DST for Mfx (and/or Lfx) at CC (depending on laboratory capacity).

Amikacin (Am):

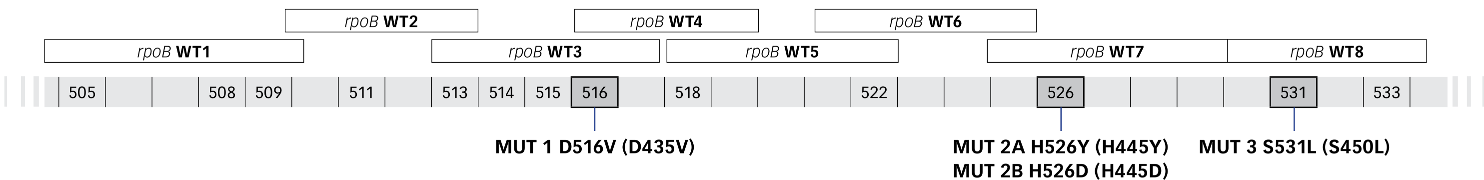
- If resistance is inferred from the absence of binding of the amplicons to WT probes in the *rrs* region (i.e., one or more WT probes are not developed), it is recommended that testing be repeated to confirm the result. Sequencing of *rrs* gene is suggested to identify the specific mutation.
- If resistance is inferred from the absence of binding of the amplicons to the WT2 probe in the *eis* region and no MUT1 probe is developed, it is recommended that testing be repeated to confirm the result. Sequencing of the *eis* gene, including the promoter region, is suggested to identify the specific mutation.

Interpretation of first-line line probe assay results

Rifampicin

The Rif resistance-determining region of the *rpoB* gene, codons covered by the WT probes and the specific mutations recognized by the MUT probes in MTBDR*plus* Ver 2.0 *(2)* - for E. coli vs. MTB codon numbering and amino acid nomenclature are shown in Fig. 2. Overall, the specificity of MTBDR*plus* Ver 2.0 for Rif resistance is very good. If the validity of a Rif resistance result is doubtful, request *rpoB* sequencing as the gold standard.

Fig. 2. Rifampicin resistance-determining region interrogated by GenoType MTBDR*plus*



Target region	MTBDR*plus* probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[a]	Clinical implications
rpoB WT1	*rpoB* WT1 not developed	Mutation(s) in codons 505–509 (424–428)[b]	Resistance to rifampicin (Rif) inferred	**Optional:** Perform sequencing of *rpoB* to identify the specific mutation.	Rif is not effective[c]
rpoB WT2	*rpoB* WT2 not developed	Mutation(s) in codons 510-513 (429–432)[b]	Resistance to Rif inferred	**Optional:** Perform sequencing of *rpoB* to identify the specific mutation.	Rif is not effective[c]
rpoB WT2/3	*rpoB* WT2 and WT3 **not** developed	Mutation(s) in codons 510–517 (429–436)[b]	Resistance to Rif inferred	**Optional:** Perform sequencing of *rpoB* to identify the specific mutation.	Rif is not effective[c]
rpoB WT3/4	*rpoB* MUT1 developed	D516V (D435V)[b]	Resistance to Rif detected	No additional diagnostic action required.	Rif is not effective

Target region	MTBDR*plus* probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[a]	Clinical implications
	rpoB WT3, WT4 and MUT1 not developed	Mutation(s) in codons 513–519 (432–438)[b]	Resistance to Rif inferred	**Optional:** Perform sequencing of *rpoB* to identify the specific mutation.	Rif is not effective[c]
rpoB WT4/5	*rpoB* WT4 and WT5 **not** developed	Mutation(s) in codons 516–522 (435–441)[b]	Resistance to Rif inferred	**Optional:** Perform sequencing of *rpoB* to identify the specific mutation.	Rif is not effective[c].
rpoB WT5/6	*rpoB* WT5 and WT6 not developed	Mutation(s) in codons 518–525 (437–444)[b]	Resistance to Rif inferred	**Optional:**Perform sequencing of *rpoB* to identify the specific mutation.	Rif is not effective[c]
rpoB WT7	*rpoB* MUT2A developed	H526Y (H445Y)[b]	Resistance to Rif detected	No additional diagnostic action required	Rif is not effective
	rpoB MUT2B developed	H526D (H445D)[b]	Resistance to Rif detected	No additional diagnostic action required	Rif is not effective
	rpoB WT7, MUT2A and MUT2B not developed	Mutation(s) in codons 526–529 (445–448)[b]	Resistance to Rif inferred	**Optional:** Perform sequencing of *rpoB* to identify the specific mutation.	Rif is not effective[c]
rpoB WT8	*rpoB* MUT3 developed	S531L (S450L)[b]	Resistance to Rif detected	No additional diagnostic action required	Rif is not effective
	rpoB WT8 and MUT3 **not** developed	Mutation(s) in codons 530–533 (449–452)[b]	Resistance to Rif inferred	**Optional:** Perform sequencing of *rpoB* to identify the specific mutation.	Rif is not effective[c]

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

[b] MTB codon numbering according to Andre et al. *(21)* is reported in parentheses.

[c] This recommendation does not apply if sequencing identifies a silent mutation.

Isoniazid

Target region	MTBDR*plus* probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[a]	Clinical implications
katG WT	*katG* MUT1 or MUT2 developed	S315T1 /S315T2	Mutation associated with high-level resistance detected.	No additional diagnostic action required	H is unlikely to be effective even at a high dose *(17)*.
	katG WT, MUT1 and MUT2 **not** developed[b]	Mutation(s)in codon 315 region	Mutation associated with high-level resistance inferred.	**Optional:** Perform sequencing of *katG* to identify the specific mutation.	H is unlikely to be effective even at a high dose *(17)*.
inhA WT1	*inhA* MUT1 developed	c-15t	Mutation associated with at least low-level resistance detected. Resistance to Eto and Pto detected.	**Optional:**[c] Perform sequencing of *inhA* coding region and *katG* gene. No additional diagnostic action for Eto and Pto	H at high dose is likely to be effective *(17)*. Eto and Pto are not effective.
	inhA MUT2 developed	a-16g[d]	Mutation likely associated with at least low-level resistance detected. Resistance to Eto and Pto detected.	**Optional:**[c] Perform sequencing of *inhA* coding region and *katG* gene. No additional diagnostic action for Eto and Pto	H at a high dose is likely to be effective. Eto and Pto are not effective.
	InhA WT1, MUT1 and MUT2 not developed	Mutation(s) in the –15 region[d]	Mutation is likely to be associated with at least low-level resistance (inferred). Resistance to Eto and Pto inferred	**Recommended:** Repeat second-line-LPA to confirm the result. **Optional:** Perform sequencing to identify specific mutation.	H at high dose is likely to be effective *(17)*. Eto and Pto are unlikely to be effective.
inhA WT2	*InhA* MUT3A developed	t-8c[d]	Mutation associated with at least low-level resistance detected. Resistance to Eto and Pto detected.	**Optional:**[c] Perform sequencing of *inhA* coding region and *katG* gene. No additional diagnostic action for Eto and Pto	H at a high dose is likely to be effective *(17)* . Eto and Pto are not effective.

Target region	MTBDR*plus* probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[a]	Clinical implications
	InhA MUT3B developed	t-8a[d]	Mutation associated with at least low-level resistance detected Resistance to Eto and Pto detected	**Optional:**[c] Perform sequencing of *inhA* coding region and *katG* gene. No additional diagnostic action for Eto and Pto	H at a high dose is likely to be effective *(17)* . Eto and Pto are not effective.
	InhA WT2, MUT3A and MUT3B **not** developed	Mutation(s) in the –8 region[d]	Mutation associated with at least low-level resistance inferred Resistance to Eto and Pto inferred	**Recommended:** Repeat first-line LPA to confirm the result. **Optional:** Perform sequencing to identify specific mutation.	H at a high dose is likely to be effective *(17)* . Eto and Pto are unlikely to be effective.

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

[b] Partial or whole deletion of the *katG* gene, which is associated with high-level resistance, results in 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*) *(19, 20)*, which are globally rare but could be more frequent in some settings, may cause substantial increases in the MIC, too high to be compensated for by increased the dose of the drug.

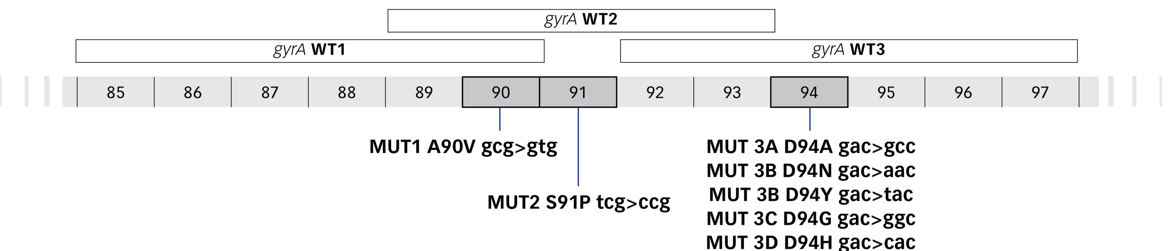
[d] Additional data correlating these mutations with phenotypic DST for isoniazid is needed to increase the confidence in the association of these mutations with drug resistance.

Interpretation of second-line line probe assay results

Fluoroquinolones

The QRDR of the *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 are shown in Fig. 3.

Fig. 3. Quinolone resistance-determining region of *gyrA* gene interrogated with GenoType MTBDRsl



Target region	MTBDRs*l* probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[a]	Clinical implications
gyrA WT 1	*gyrA* WT1 **not** developed	Mutation(s) in codons 85–89	Resistance to Lfx inferred Mutation associated with at least low-level resistance for Mfx inferred	**Recommended:** Perform phenotypic DST for Mfx at CB to exclude resistance. **Optional:** • Perform sequencing of *gyrA* QRDR to identify specific mutation. • Perform phenotypic DST for Lfx and Mfx at CC.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to 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.

Target region	MTBDR*sl* probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[a]	Clinical implications
gyrA WT2	*gyrA* MUT1 developed	A90V	Resistance to Lfx detected. Mutation associated with at least low-level resistance to Mfx detected.	**Recommended:** Perform phenotypic DST for Mfx at CB to exclude resistance.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to phenotypic DST results at CB.
	gyrA MUT2 developed	S91P	Resistance to Lfx detected. Mutation associated with at least low-resistance for Mfx detected.	**Recommended:** Perform phenotypic DST for Mfx at CB to exclude resistance.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to phenotypic DST results at CB.
	gyrA WT2, MUT1 and MUT2 **not** developed	Mutation(s) in codons 89–93	Resistance to Lfx inferred. Mutation associated with at least low-level resistance for Mfx inferred.	**Recommended:** Perform phenotypic DST for Mfx at CB to exclude resistance. **Optional:** • Perform sequencing of *gyrA* QRDR to identify specific mutation • Perform phenotypic DST for Lfx and Mfx at CC.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to 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 WT3	*gyrA* MUT3A developed	D94A	Resistance to Lfx detected. Mutation associated with at least low-level resistance for Mfx detected.	**Recommended:** Perform phenotypic DST for Mfx at CB to exclude resistance.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to phenotypic DST results at CB.
	gyrA MUT3B developed	D94N or D94Y	Resistance to Lfx detected. Mutation associated with high-level resistance for Mfx detected.	No additional diagnostic action required.	Lefx is not effective. Mfx is not effective.

Target region	MTBDRsl probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[a]	Clinical implications
gyrA WT3	*gyrA* MUT3C developed	D94G	Resistance to Lfx detected. Mutation associated with high-level resistance for Mfx detected.	No additional diagnostic action required.	Lfx is not effective. Mfx is not effective.
	gyrA MUT3D developed	D94H	Resistance to Lfx detected. Mutation associated with high-level resistance for Mfx detected.	No additional diagnostic action required.	Lfx is not effective. Mfx is not effective.
	gyrA WT3, MUT3A, MUT3B, MUT3C and MUT3D **not** developed	Mutation(s) in codons 92–96	Resistance to Lfx inferred. Mutation associated with at least low-level resistance for Mfx inferred.	**Recommended:** Perform phenotypic DST for Mfx at CB to exclude resistance. **Optional:** • Perform sequencing of *gyrA* QRDR to identify specific mutation • Perform phenotypic DST for Lfx and Mfx at CC.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to 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.
gyrB WT	*gyrB* MUT1 developed	N538D (N499D)[b]	Resistance to Lfx detected. Mutation associated with at least low-level resistance for Mfx detected.	**Recommended:**Perform phenotypic DST for Mfx to exclude resistance at CB.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to phenotypic DST results at CB.
	gyrB MUT2 developed	E540V (E501V)[b]	Resistance to Lfx detected. Mutation associated with at least low-level resistance for Mfx detected.	**Recommended:** Perform phenotypic DST for Mfx to exclude resistance at CB.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to phenotypic DST results at CB.

Target region	MTBDRsl probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[a]	Clinical implications
	gyrB WT, MUT1 and MUT2 **not** developed	Mutation(s) in codons 536–541 (497–502)[b]	Resistance to Lfx inferred. Mutation associated with at least low-level resistance for Mfx inferred.	**Recommended:** Perform phenotypic DST for Mfx to exclude resistance at CB. **Optional:** • Perform sequencing of *gyrA* QRDR to identify specific mutation; • Perform phenotypic DST for Lfx and Mfx at CC.	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to 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 consideration of the individual patient's risk group for resistance and by the prevalence of resistance in the setting, as these factors affect the positive predictive value of the test.
[b] Codon numbering system according to Camus et al. *(22)* in parentheses

Amikacin[a]

Target region	MTBDR*sl* probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[b]	Clinical implications
rrs WT1	*rrs* MUT1 developed	a1401g	Resistance to Am detected	No additional diagnostic action required	Am is not effective.
	rrs WT1 and MUT1 not developed	Mutation(s) in the 1400 region	Resistance to Am inferred	**Optional:** Perform sequencing to identify specific mutation.	Am is unlikely to be effective.
rrs WT2	*rrs* MUT2 developed	g1484t	Resistance to Am detected	No additional diagnostic action required	Am is not effective.
	rrs WT2 and MUT2 **not** developed	Mutation in the 1484 region	Resistance to Am inferred	**Recommended:** Repeat second-line LPA to confirm the result. **Optional:** Perform sequencing to identify specific mutation.	Am is unlikely to be effective.

Target region	MTBDR*sl* probe	Mutation or region interrogated	Interpretation	Additional diagnostic action[b]	Clinical implications
eis WT1	*eis* WT1 not developed	Mutation(s) in the –37 region	Resistance to Am not detected	No additional diagnostic action required	Am is likely to be effective.
eis WT2	*eis* MUT1 developed	c-14t	Resistance to Am detected	No additional diagnostic action required	Am is not effective.
	eis WT2 and MUT1 **not** developed	Mutation(s) in the –10 to –14 region	Resistance to Am not detected	**Recommended:** Repeat the second-line LPA to confirm the result. **Optional:** Perform sequencing to identify specific mutation.[c]	Am is likely to be effective.
eis WT3	*eis* WT3 not developed	Mutation(s) in the –2 region **Note.** No evidence that mutations in this region are associated with resistance[d]	Resistance to Am not detected	No additional diagnostic action required.	Am is likely to be effective.

[a] WHO no longer recommends use of kanamycin or capreomycin because of the increased risks of treatment failure and relapse associated with their use in longer MDR-TB regimens *(18)*. Interpretation of second-line LPA for kanamycin and capreomycin is therefore not included in this document.

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

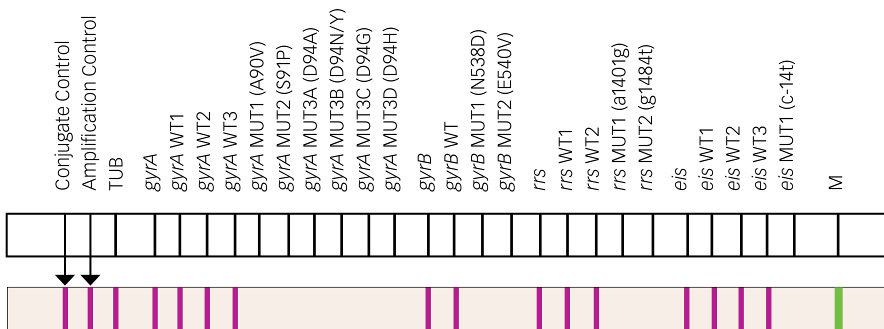
[c] If sequencing reveals the presence of the *eis* mutation c-14t, which for some reason was not detected by the *eis* MUT1 probe, Am is not effective.

[d] Miotto P, et al. A standardised method for interpreting the association between mutations and phenotypic drug resistance in *Mycobacterium tuberculosis*. Eur Respir J. 2017;50(6):1701354.

Assessment of drug-resistant TB cases based on second-line line probe assay results

Case 1. No resistance mutations detected or inferred in any of the genomic regions included in second-line line probe assay

All WT bands developed, and no MUT probe bands developed in second-line LPA



Genotypic report:

Resistance not detected

Additional diagnostic action:

Optional:

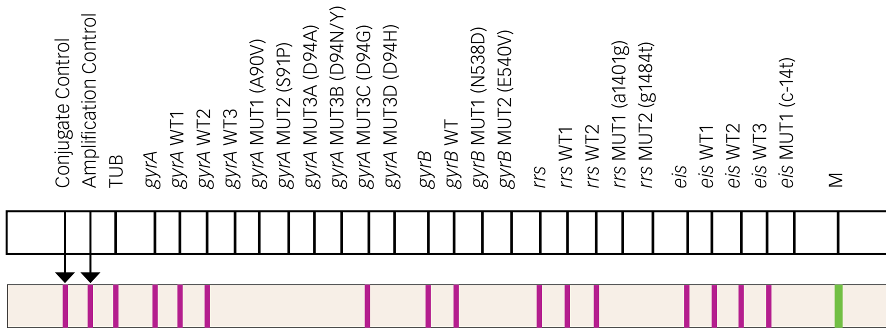
- Perform phenotypic DST for Lfx at CC (e.g., CC: 1.0 mg/L in MGIT and 7H10) and for Mfx at CC and CB (e.g., CC: 0.25 mg/L in MGIT and 0.5 mg/L on 7H10; CB: 1.0 mg/L in MGIT and 2.0 mg/L on 7H10).
- Perform phenotypic DST for Am if indicated.

The decision to perform these optional follow-up actions should be guided by consideration of the individual patient's risk group for resistance (e.g., prior exposure to second-line medicines, suspected treatment failure) and by the prevalence of resistance in the setting, as these factors affect the predictive value of the test.

Clinical implications:

Start MDR-TB treatment. Review treatment regimen according to phenotypic DST results.

Case 2. Detection of resistance mutations associated with high-level resistance to moxiifloxacin



If one of the following MUT probes 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:

Lfx: Resistance detected
Mfx: Mutation associated with high-level resistance for Mfx detected

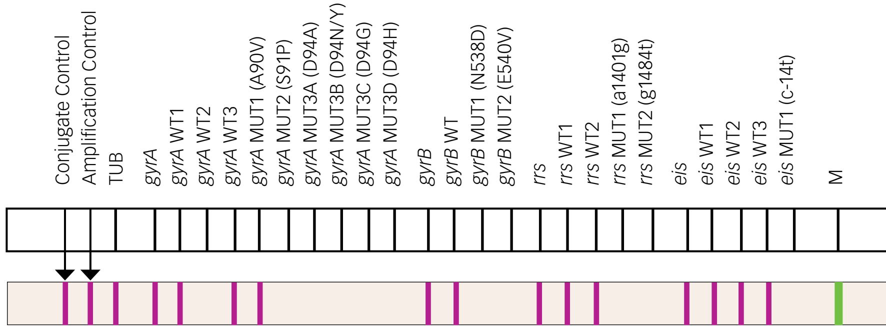
Additional diagnostic action:

Perform phenotypic DST for Am if indicated.

Clinical implications:

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

Case 3. Detection of mutations associated with at least low-level resistance to moxifloxacin



If one of the following MUT probes 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:

Lfx: Resistance detected
Mfx: Mutation associated with at least low-level resistance for Mfx detected

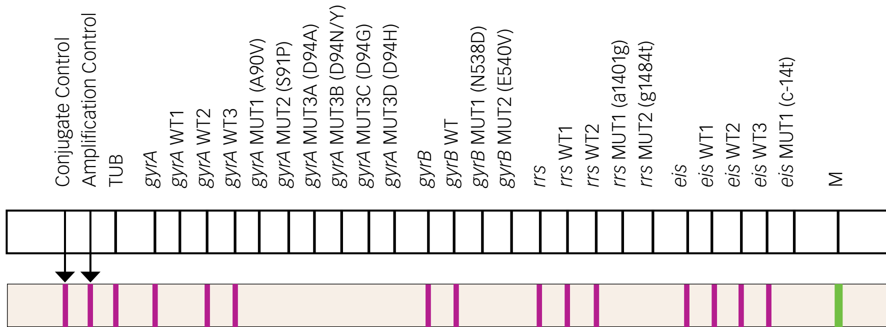
Additional diagnostic action:

Recommended: Perform phenotypic DST for Mfx at CB according to case 1.
Perform phenotypic DST for Am if indicated.

Clinical implications:

Mfx could be used at a higher dose. The regimen should be re-evaluated according to phenotypic DST results at CB.

Case 4. Precise mutation unknown, only inferred for fluoroquinolones (i.e., *gyrA* and *gyrB*)



If one of the following WT bands is not developed:

- *gyrA* WT1 (i.e. *gyrA* WT1 probe missing) (see example above),
- *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 probes is developed in the *gyrA* and *gyrB* regions.

Genotypic report:

Lfx: Resistance inferred
Mfx: Mutation associated with at least low-level resistance 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 that do not cause resistance (systematic false-positive results) (interpret according to cases 2–4, and follow the respective recommendations for phenotypic DST). If sequencing is unavailable, perform phenotypic DST at CC for Lfx and/or Mfx as for case 1.

Optional: Perform phenotypic DST for Am if indicated.

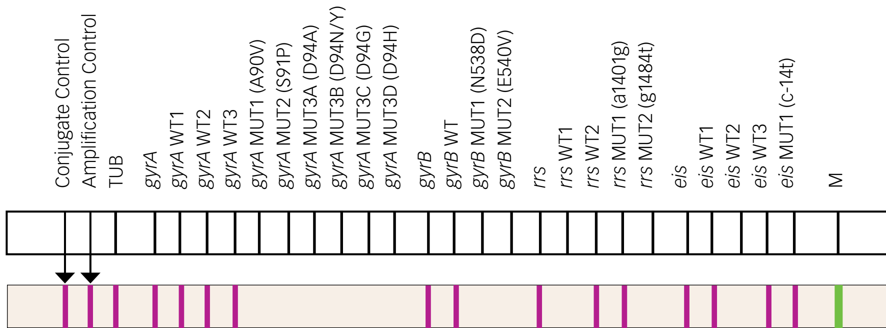
[1] Lack of binding of a WT probe without simultaneous binding of a mutant probe is due to the presence of a resistance mutation (e.g., *gyrA* G88A). Systematic errors are possible due to synonymous or non-synonymous mutations; however, this is rare (< 1% of isolates), but these isolates may be frequent locally. Unfortunately, the settings in which these cases are frequent cannot be predicted. Thus, each laboratory must decide on the basis of local epidemiology whether sequencing of the QRDR region is necessary. For example, the *gyrA* A90G mutation, which prevents binding of *gyrA* WT2, is frequent in 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) *(12)*. In both of these settings therefore, sequencing would be recommended.

Clinical implications:

Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to the 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.

Case 5. Detection of mutations that cause resistance to amikacin



If one of the following MUT probe bands is developed:

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

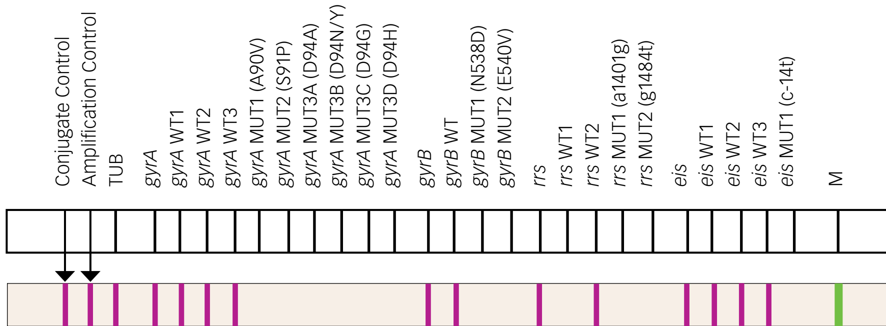
Genotypic report:

Resistance to Am detected.

Additional diagnostic action:

Perform phenotypic DST for FQs according to case 1.
Clinical implications: Am is not 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 example above),
- *rrs* WT2 (*rrs* probe WT2 not developed)

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

Genotypic report:

Resistance to Am inferred

Additional diagnostic action:

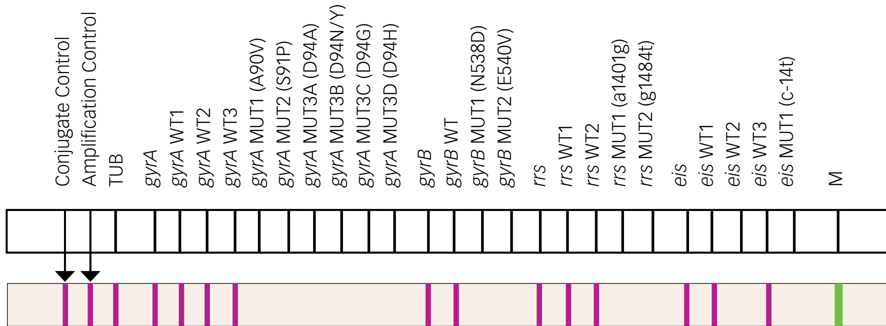
Recommended: If only the *rrs* WT2 probe or both *rrs* WT1 and WT 2 probes are not detected (and no MUT probes are detected), repeat the assay to confirm the result.

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

Clinical implications:

Am is unlikely to be effective.

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 example above),

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

Genotypic report:

Resistance to Am not detected (if no additional mutations in the *rrs* region are present)

Additional diagnostic action:

Recommended: Repeat the test to confirm the result.

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

Clinical implications:

Am is likely to be effective.

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Annex 1.

Reporting format for first-line line probe assay results and practical examples

The "Conclusion" column has been included for convenience but should not be part of the laboratory report.

Example 1

Drug	Gene	Mutation	Interpretation	Conclusion
Rif[a]	*rpoB*	H526Y	Resistance to Rif detected	Rif is not effective.
H[a]	*katG*	Mutation(s) in codon 315 region	Mutation associated with high-level resistance to H inferred	H is unlikely to be effective even at a high dose.
	inhA	t-8a		
Eto and Pto	*inhA*	t-8a	Resistance to Eto and Pto likely detected	Et0 and Pto are likely not effective.

Example 2

Drug	Gene	Mutation	Interpretation	Conclusion
Rif	*rpoB*	No mutation detected	Resistance to Rif not detected	Rif is effective.
H[a]	*katG*	S315T	Mutation associated with high-level resistance to H detected	H is not effective even at a high dose.
	inhA	c-15t		
Eto and Pto	*inhA*	c-15t	Resistance to Eto and Pto detected	Eto and Pto are not effective.

Example 3

Drug	Gene	Mutation	Interpretation	Conclusion
Rif	*rpoB*	Mutation(s) in codons 516–522 (435–441)	Resistance to Rif inferred	Rif is not effective.
H	*katG*	No mutation detected	Mutation likely to be associated with at least low-level resistance to H detected	H at a high dose is likely to be effective.
	inhA	t-8c		
Eto and Pto	*inhA*	t-8c	Resistance to Eto and Pto likely detected	Eto and Pto are likely not effective.

[a] If more than one probe per drug provides information, the results should be reported according the following hierarchy (where ">"means overrule):
For H: Mutation associated with high-level resistance detected > Mutation associated with high-level resistance inferred > Mutation associated with at least low-level resistance detected > Mutation associated with at least low-level resistance inferred > Resistance not detected
For Rif: Resistance detected > Resistance inferred > Resistance not detected

Annex 2.
Reporting format for second-line line probe assay results and practical examples

The "Conclusion" column has been included for convenience but should not be part of the laboratory report.

Example 1

Drug	Gene	Mutation	Interpretation	Conclusion
Lfx[a]	*gyrA*	D94A	Resistance to Lfx detected	Lfx is not effective. Mfx could be used at higher dose. The regimen should be re-evaluated according to phenotypic DST results at CB.
	gyrB	No mutation detected		
Mfx[a]	*gyrA*	D94A	Mutation associated with at least low-level resistance to Mfx detected	
	gyrB	No mutation detected		
Am[a]	rrs	a1401g	Resistance to Am detected	Am is not effective.
	eis promoter	No mutation detected		

Example 2

Drug	Gene	Mutation	Interpretation	Conclusion
Lfx	*gyrA*	A90V	Resistance to Lfx detected	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to phenotypic DST results at CB.
	gyrB	No mutation detected		
Mfx	*gyrA*	A90V	Mutation associated with at least low-level resistance to Mfx detected	
	gyrB	No mutation detected		
Am	rrs	No mutation detected	Resistance to Am detected	Am is not effective.
	eis promoter	c-14t		

Example 3

Drug	Gene	Mutation	Interpretation	Conclusion
Lfx	*gyrA*	Mutation(s) in codons 89-93	Resistance to Lfx inferred	Lfx is not effective. Mfx could be used at a higher dose. The regimen should be re-evaluated according to 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.
	gyrB	No mutation detected		
Mfx	*gyrA*	Mutation(s) in codons 89-93	Mutation(s) associated with at least low-level resistance to Mfx inferred	
	gyrB	No mutation detected		
Am	rrs	No mutation detected	Resistance to Am not detected	Am is effective.
	eis promoter	Mutation(s) in the –37 region		

[a] If more than one probe per drug provides information, the results should be reported according to the following hierarchy (where ">"means overrule):
For Lfx and Am: Resistance detected > Resistance inferred > Resistance not detected
For Mfx: Mutation associated with high-level resistance detected > Mutation associated with at least low-level resistance detected > Mutation associated with at least low-level resistance inferred > Resistance not detected

Annex 3.

Specific nucleotide changes detected with mutation probes

Some of the amino acid (AA) changes identified with first- and second-line LPAs are due to nucleotide changes that are not specifically recognized by the MUT probes. For instance, the *gyrA* mutation A90V is due to two possible nucleotide changes: gcg > gtg or gcg>gtc. However, only the first, gcg > gtg, will be recognized by the *gyrA* MUT1 probe, while the second, 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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