Standard Operating Procedure (SOP)

For

GenoType **MTBDR*plus*** & **Geno**Type **MTBDR*sl***

Version 1.0

USAID
FROM THE AMERICAN PEOPLE

IDDS

ACKNOWLEDGEMENTS

The development of SOPs for MTB Culture and DST is an expression of the commitment by the NTP, NTRL, Regional TB Reference Laboratory, and its development partners for a strengthened TB, HIV and DR-TB response in Bangladesh. The Ministry of Health would like to acknowledge the following experts for their contribution and commitment in the development of SOPs.

SOPs for MTB Culture & DST Development

Name	Designation/Organization
Dr. Khurshid Alam	Line Director, TB Lep & ASP, DGHS
Dr. Shakil Ahmed	Director, MBDC, DGHS
Dr. Afzalur Rahman	Assistant Director, MBDC and PM TB
Dr. Pronab Kumar Modak	Deputy Program Manager (Training), NTP, DGHS
Dr. Md. Ahmadul Hasan Khan	Monitoring & Evaluation Expert, NTP, DGHS
Umme Tasnim Maliha	Microbiologist, NTP, Dhaka
Dr. Iffat Ara Ifa	Microbiologist, NTRL-Dhaka, NTP
Tanvir Huda Rusel	Microbiologist, NTRL-Dhaka, NTP
Md. Mamun Hasan	Microbiologist, RTRL-Khulna, NTP
Piplu Barua	Microbiologist, RTRL-Chattogram, NTP
Sohana Asma	Microbiologist, RTRL-Chattogram, NTP
Tanzida Begum Rumi	Microbiologist, RTRL-Sylhet, NTP
Dr. Nusrat Siddiqua Nira	Microbiologist, RTRL Shyamoli, Dhaka
Dr.Md. Abdul Hamid Salim	MDR TB and Global Fund Advisor, USAID
Dr. Adneen Moureen	TB New Technologies and Diagnostics Advisor, USAID
Dr. Nazis Arefin Saki	National Program Officer, WHO
Sarder Tanzir Hossain	Senior TB Diagnostic Specialist, IDDS, Dhaka
Tafsina Haque Aurin	Program Coordinator-GeneXpert, IDDS, Dhaka
Sharmin Islam	TB Diagnostic Specialist, IDDS, Dhaka
Prianka Dey	TB Diagnostic Specialist, IDDS, Dhaka
Dr. Aung Kya Jai Maug	Country Director, Damien Foundation
Dr. Md. Ashraful Islam	Specialist, Lab Management, CDP-BRAC
Mostafizur Rahman	Program Manager, Coordination, Icddr,b
Dr. Mahfuzur Rahman	Project Manager, IRD

Finally, we would like to acknowledge the support of USAID funded IDDS project for updating and printing the SOPs.

Dr. Md. Khurshid Alam
Line Director TB-L & ASP
National Tuberculosis Control Programme
Directorate General of Health Services
Mohakhali, Dhaka-1212

This publication was produced with the support of the United States Agency for International Development (USAID), Global Health under the terms of the Infectious Disease Detection and Surveillance contract GS00Q14OADU119. Views expressed are not necessarily those of USAID or the United States government.

Contents

GenoType MTBDR*plus* VER 2.0

Molecular Genetic Assay for Identification of the *M. tuberculosis* Complex and its Resistance to Rifampicin and Isoniazid from Clinical Specimens and Cultivated Samples

Please read the instructions on hand completely and carefully before using the kit. Strictly adhere to the established procedure to obtain correct test results.

Intended Use

The GenoType MTBDR*plus* VER 2.0 is a qualitative in vitro test for the identification of the Mycobacterium tuberculosis complex and its resistance to rifampicin (RMP) and/or isoniazid (INH) from pulmonary smear-positive or -negative clinical specimens and cultivated samples. The following species are included in the tuberculosis (TB)-causing *M. tuberculosis* complex: *M. tuberculosis, M. africanum, M. bovis subsp. bovis, M. bovis subsp. caprae, M. bovis BCG, M. microti, M. canettii, and M. pinnipedii*. The identification of RMP resistance is enabled by the detection of the most significant associated mutations of the *rpoB* gene (coding for the -subunit of the RNA polymerase). For detection of INH resistance, the *katG* gene (coding for the catalase peroxidase) and the promoter region of the *inhA* gene (coding for the NADH-enoyl-ACP reductase) are examined. The test is indicated as an aid for diagnosis and intended for use in medical laboratories.

Summary and Explanation

Tuberculosis (TB) is a bacterial infectious disease passed on by droplet infection. In 2017, there were an estimated 10.0 million incident cases of TB globally, and an estimated 1.3 million TB deaths [1]. TB treatment requires a therapy over several months. Emergence and spread of multidrug-resistant tuberculosis (MDR-TB) is a major medical and public problem threatening global health. MDR-TB is defined as TB that is resistant at least to RMP and INH, the two most important first-line anti-TB drugs [2]. MDR-TB is a challenge to TB control due to its complex diagnosis and obstacles in treatment. In 2013, there were an estimated 480,000 cases of MDR-TB among the world's 11 million prevalent cases of TB [1]. As long as MDR-TB is not verified, use of inadequate and hence ineffective antibiotics may lead to further spread of resistant bacteria and amplification of resistance. Therefore, rapid diagnosis and identification of MDR-TB is a prerequisite for appropriate treatment.

Principles of the Procedure

The **GenoType MTBDR*plus*** VER 2.0 is based on the **DNA**•STRIP technology. The whole procedure is divided into three steps: (i) DNA extraction from clinical specimens (pulmonary, decontaminated) or cultured material (solid/liquid medium) – the necessary reagents are not included in the kit, (ii) a multiplex amplification with biotinylated primers, and (iii) a reverse hybridization.

All reagents needed for amplification, such as polymerase and primers, are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single-stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. Thus, the probes reliably discriminate several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. A template ensures the easy and fast interpretation of the banding pattern obtained.

Materials required but not included in the kit

- 0.5 ml Screw cap tubes or 1.5 ml screw cap tubes for aliquots (Sarstedt, Nümbrecht, Germany, see chapter Ordering Information)
- Absorbent paper
- Adjustable pipettes for 10, 20, 200, and 1000 µl
- Disposable gloves
- Disposable sterile pipette tips with filter
- DNA extraction kit (GenoLyse® or GXT DNA/RNA Extraction Kit, see chapter Ordering Information) as well as necessary equipment
- Graduated cylinder
- PCR tubes, DNase and RNase free
- Reagents for cultivation of mycobacteria as well as necessary equipment (when cultivated samples are to be used)
- Sample decontamination reagents as well as necessary equipment
- Shaking water bath + shaking platform or TwinCubator (instrument for manual hybridization) or automated hybridization instrument
- Thermal cycler
- Timer
- Tweezers
- Water (distilled)
- Water (molecular biology grade, for negative controls)

Quality Control

In order to control the correct performance of the test and the proper functioning of kit constituents, each strip includes 5 control zones:

- a Conjugate Control zone (CC) to check the binding of the conjugate on the strip and a correct chromogenic reaction
- an Amplification Control zone (AC) to check for a successful amplification reaction
- three Locus Control zones (*rpoB*, *katG*, and *inhA*) checking the optimal sensitivity of the reaction for each of the tested gene loci

Observe the usual precautions for amplification setup. It is essential that all materials (such as pipette tips) coming in contact with the reagents are free from DNases.
Do not interchange or pool Amplification Mixes or membrane strips from different kits unless the lots are identical. You can find the kit lot and the corresponding lots of the kit constituents on the lot labels included in the kit.
A negative control sample for detection of possible contamination events containing water (molecular biology grade) instead of DNA should be part of each test run; the respective test strip should show the bands CC and AC only.

Specimen Requirements

Decontaminated pulmonary smear-positive or -negative patient specimens such as sputum (induction or expectoration), bronchial material (e.g. bronchoalveolar lavages), or aspirates (e.g. pleural aspirate) as well as cultivated samples (solid/liquid medium) can be used as starting material for DNA extraction. Until the present edition of the instructions on hand, the performance of the test has not been validated with other sample materials than those mentioned above.

Precautions for handling specimens

Patient specimens and cultures made from patient specimens must always be considered as infectious and must be handled accordingly (e.g. see [3] or [4]). Always wear suitable protective clothing and gloves. Samples from patients at risk (infected by pathogenic microorganisms or viruses including Hepatitis B and Human Immunodeficiency Virus (HIV)) and cultures made from those samples must always be labeled and handled under suitable safety conditions according to institutional guidelines.

All specimens that may contain mycobacteria should be handled applying Biosafety Level 2 practices or, when indicated, Biosafety Level 3 practices (e.g. see [3]). Observe all federal, state, and local safety regulations.

Discard used pipette tips immediately after use in a container for biohazardous waste. After finishing the assay, discard all used disposables in a container for biohazardous waste.

Storage and transport

All specimens should be collected and transported as recommended in the CDC publication "Public Health Mycobacteriology: A Guide for the Level III Laboratory" [5], the "Clinical Microbiology Procedures Handbook" [6], or your laboratory procedure manual.

It must be ensured that until decontamination, specimens are kept in sterile plastic containers at a temperature of 2°C to 8°C. The transport of specimens at room temperature has to be carried out as soon as possible and should be done within 1-2 days [7,8]. Specimens used for decontamination must not be older than 4 days.

After decontamination and subsequent resuspension of the bacteria pellet with phosphate buffer, samples can be stored at –20°C or –80°C for a maximum of 5 days until performing DNA extraction.

Preparation

Clinical specimens must be processed using the NALC-NaOH method according to the CDC publication "Public Health Mycobacteriology: A Guide for the Level III Laboratory" [5]. After decontamination, the cell pellet should be resuspended in a maximum of 1 to 1.5 ml of phosphate buffer. When testing patient specimens, higher volumes might hamper the sensitivity of the test. Due to the potential inhomogeneity of the specimen, the decontaminated sample must be mixed before removing the aliquot to be analyzed; otherwise, the sensitivity of the test might be influenced.

When the sample is to be cultivated, cultivation can be performed either on solid medium (e.g. Loewenstein-Jensen, Middlebrook) or in liquid medium (e.g. MGIT (BD Diagnostics, Franklin Lakes, USA)). Handling of potentially infectious specimens must be carried out in a class II safety cabinet.

DNA Extraction

Decontaminated patient samples as well as bacteria grown on solid medium (e.g. Loewenstein-Jensen, Middlebrook) or in liquid medium (e.g. MGIT (BD Diagnostics, Franklin Lakes, USA)) may be used as starting material for DNA extraction. For DNA extraction from NALC-NaOH-decontaminated clinical specimens or cultured material the **GenoLyse**® kit (see chapter Ordering Information) is used. For automated DNA extraction from patient specimens, also the **GenoXtract®** in combination with the GXT DNA/RNA Extraction Kit (see chapter Ordering Information) can be used. For handling instructions, please refer to the respective instructions for use.

The methods mentioned here were used for performance evaluation of the **GenoType MTBDR*plus*** VER 2.0. Until the present edition of the instructions on hand, the performance of the test has not been validated with other DNA extraction methods.

Amplification

All reagents needed for amplification, such as polymerase and primers, are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. Thaw AM-A and AM-B shortly before preparing the master mix, spin down briefly, and mix carefully by pipetting up and down.
Pipette AM-A and AM-B only in a room free from contaminating DNA. To avoid contamination, the DNA solution must be added in a separate working area.

Prepare for each sample

After DNA extraction with **Geno**Lyse®	After DNA extraction with **GXT** DNA/RNA Extraction Kit
- 10 µl AM-A (see Kit Component 2)	– 10 µl AM-A (see Kit Component 2)
- 35 µl AM-B (see Kit Component 2)	– 35 µl AM-B (see Kit Component 2)
- 5 µl DNA solution	– 10 µl DNA solution
Final volume: 50 µl	Final volume: 55 µl

Determine the total number of samples (number of samples to be analyzed plus control samples). Prepare the number of tubes needed. Prepare a master mix containing AM-A and AM-B and mix carefully but thoroughly (do not vortex). Alternatively, the content of an AM-A reaction tube may completely be transferred to an AM-B reaction tube. This will lead to master mix sufficient for 12 amplification reactions (12 tests kit) or for 4x 24 amplification reactions (96 tests kit). Please note that the master mix needs to be prepared freshly each time and needs to be processed quickly. Aliquot 45 µl into each of the prepared PCR tubes and add 5 or 10 µl water (molecular biology grade) to one aliquot (negative control sample). In a separate working area, add 5 or 10 µl DNA solution to each aliquot (except for negative control).

Amplification profile

When using a thermal cycler from Hain Lifescience with the respective preinstallation, select protocol "MDR DIR" for clinical specimens or protocol "MDR CUL" for cultivated samples.

	Clinical specimens	**Cultivated samples**
15 min 95°C	1 cycle	1 cycle
30 sec 95°C 2 min 65°C	20 cycles	10 cycles
25 sec 95°C 40 sec 50°C 40 sec 70°C	30 cycles	20 cycles
8 min 70°C	1 cycle	1 cycle
Heating rate	≤2.2°C/sec	≤2.2°C/sec

The heated lid must be switched on during the entire program.

Amplification products can be stored at –20°C to +8°C.

Hybridization

When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on **Error! Hyperlink reference not valid.**for the name of the hybridization protocol to be used.
The following protocol describes the manual hybridization using a water bath or a TwinCubator.

Preparation

Prewarm shaking water bath to 45°C (the maximum tolerated deviation from the target temperature is +/–1°C) or switch on TwinCubator. Prewarm solutions HYB and STR to 37°C to 45°C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary. Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10 µl concentrate to 1 ml of the respective buffe r. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

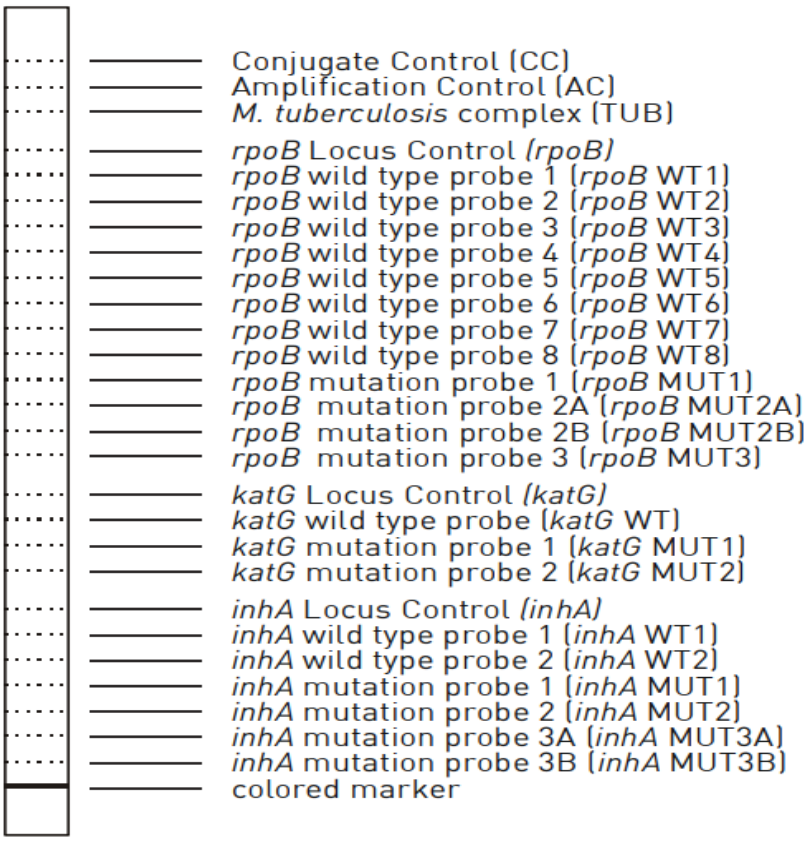
1. **Dispense 20 µl of Denaturation Solution (DEN, blue) in a corner of each of the wells used.**
2. **Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate at room temperature for 5 minutes.**
   Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. Always wear gloves when handling strips.
3. **Carefully add to each well 1 ml of prewarmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous color.**
   Take care not to spill solution into the neighboring wells.
4. **Place a strip in each well.**
   The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers, turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps.
5. **Place tray in shaking water bath/TwinCubator and incubate for 30 minutes at 45°C.**
   Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least 1/3 of its height.
6. **Completely aspirate Hybridization Buffer.**
   For example, use a Pasteur pipette connected to a vacuum pump.
7. **Add 1 ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45°C in shaking water bath/TwinCubator.**
8. **Work at room temperature from this step forward. Completely remove Stringent Wash Solution.**
   Pour out Wash Solution in a waste container and remove all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper. This also applies to all other wash steps.
9. **Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator (pour out RIN after incubation).**
10. Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on shaking platform/TwinCubator. Remove solution and wash each strip twice for 1 minute with 1 ml of

Rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water (e.g. use wash bottle) on shaking platform/TwinCubator (pour out solution each time).
Make sure to remove any trace of water after the last wash.

11. **Add 1 ml of diluted substrate (see above) to each strip and incubate protected from light without shaking.**
    Depending on the test conditions (e.g. room temperature), the substrate incubation time, i.e. the time until the bands are clearly visible, can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.
12. **Stop reaction as soon as bands are clearly visible by briefly rinsing twice with distilled water.**
13. **Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.**

Evaluation and Interpretation of Results

Paste strips and store protected from light. An evaluation sheet is included in the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and AC with the respective lines on the sheet. For technical reasons the distances between single probes on the strips may vary slightly. For an accurate evaluation therefore please use the provided template and align it – separately for each locus – with the respective Locus Control band. Determine the resistance status and note down in the respective column. As a help for interpretation, evaluation examples are given in the subsequent chapter. Each strip has a total of 27 reaction zones (see figure).



Note: The strip is not displayed in original size.

Conjugate Control (CC)
A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.

Amplification Control (AC)
When the test is performed correctly, a control amplicon will bind to the Amplification Control zone. Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone (AC) 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 might t be due to competition of the single reactions during amplification. In this case, the test was performed correctly and does not have to be repeated.

When only the CC and AC bands are developed, this represents a valid negative result. A missing AC band in case of a negative test result indicates mistakes during setup and/or performance of the amplification reaction, or presence of amplification inhibitors. In this case, the test result is not valid and the test has to be repeated with the respective sample. In case of overall strong signal intensities but only weak staining or absence of the Amplification Control band, a single wild type band showing significantly weaker staining than the other wild type bands of the respective locus (or Locus Control band for *katG*) is to be considered negative.

M. tuberculosis complex (TUB)
This zone hybridizes with amplicons generated from all members of the *Mycobacterium tuberculosis* complex. If the TUB zone is negative while no evaluable resistance pattern is developed, the tested specimen does not contain bacteria belonging to the M. tuberculosis complex and cannot be evaluated by this test system. In rare cases, the TUB zone may be negative while an evaluable resistance pattern is developed. If so, the presence of a strain belonging to the
M. tuberculosis complex must be suspected, and the test should be repeated (see below, "special case" no. 3).

Locus Controls *(rpoB, katG, and inhA)*
The Locus Control zones detect a gene region specific for the respective locus. In case of a positive test result (evaluable wild type and mutation banding pattern), the signals of the Locus Control bands may be weak.

Wild type probes
The wild type probes comprise the most important resistance regions of the respective genes (see figure 1, as well as tables 1, 2, and 3). When all wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. This indicates that the strain tested is sensitive to the respective antibiotic. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes indicates a resistance of the tested strain to the respective antibiotic.
Each pattern deviating from the wild ~~type~~type of pattern indicates a resistance of the tested strain. The banding pattern obtained with the *rpoB* probes allows drawing a conclusion about an RMP resistance of the strain tested, the *katG* and the *inhA* banding pattern about an INH resistance.

Mutation probes
The mutation probes detect some of the most common resistance-mediating mutations (see tables 1, 2, and 3). Compared to the other probes, positive signals of the mutation probes *rpoB* MUT2A and

MUT2B may show a lower signal strength. In rare cases, when the *rpoB* MUT3 band is positive, weak staining may be detected at the *rpoB* WT8 band which is to be considered negative.
Each pattern deviating from the wild type pattern indicates a resistance of the tested strain. The banding pattern obtained with the *rpoB* probes allows drawing a conclusion about an RMP resistance of the strain tested, the *katG* and the *inhA* banding pattern about an INH resistance.

Please note:
Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone (AC) are to be considered. Not all bands of a strip have to show the same signal strength.

Note the following special cases

1. There is a possibility that the specimen tested contains a hetero resistant strain. In case of a hetero resistance, a mutated as well as a wild type sequence can be detected in the respective strain; hence, one of the mutation probes as well as the corresponding wild type probe may stain positive on the respective strip. Whether the respective resistance becomes phenotypically evident depends on the ratio of mutated and nonmutated sequences at investigation.
2. There is a possibility that the tested specimen contains more than one *M. tuberculosis* complex strain (due to mixed culture or contamination). If at least one of these strains harbors a mutation, one of the mutation probes as well as the corresponding wild type probe may stain positive. Whether the respective resistance becomes phenotypically evident, depends on the ratio of resistant and sensitive strain at investigation.
3. There is a possibility that due to a mixed infection the tested specimen contains both an *M. tuberculosis* complex strain and a nontuberculous mycobacterium. In rare cases, the TUB band may be missing due to competition of the single amplification reactions during PCR. However, when an evaluable resistance pattern is developed, the presence of a strain belonging to the *M. tuberculosis* complex must be suspected and the test should be repeated.
4. In rare cases, all bands of a gene locus (including the Locus Control band) may be missing completely on a test strip. If this result is generated from a clinical specimen, possible reasons could be, but are not limited to, a DNA concentration in the sample below the limit of detection or the presence of interfering substances in the sample. Such a banding pattern cannot be evaluated, and the test must be repeated. If a cultivated sample generates a result with the complete *katG* locus missing, this indicates an INH resistance of the strain tested.

Resistance regions and common resistance-mediating mutations

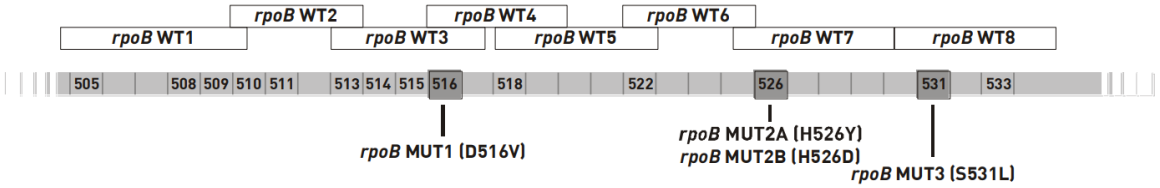


Figure 1: RMP resistance region of the *rproB* gene

rpoB WT1-8: *rpoB* wild type probes; *rpoB* MUT1-3: *rpoB* mutation probes. The numbers specify the positions of the amino acids (codons) for all mutations listed in the table. The codons for which mutation probes were designed are highlighted.

Table 1: Mutations in the *rpoB* gene and the corresponding wild type and mutation bands (according to [9])

Failing wild type bands (S)	Codons analyzed	Developing mutation band	Mutation
rpoB WT1	505-509		F505L
			T508A
			S509T
rpoB WT2	510-513		Q510H
			L511P*
rpoB WT2/WT3	510-517		Q513L*
			Q513P
			del514-516
rpoB WT3/WT4	513-519	*rpoB* MUT1	D516V
			D516Y
			del515
rpoB WT4/WT5	516-522		del518*
			N518I
rpoB WT5/WT6	518-525		S522L
			S522Q
rpoB WT7	526-529	*rpoB* MUT2A	H526Y
		rpoB MUT2B	H526D
			H526R
			H526P*
			H526Q*
			H526N
			H526L
			H526S
			H526C
rpoB WT8	530-533	*rpoB* MUT3	S531L
			S531Q*
			S531W
			L533P

*These rare mutations have only been detected theoretically (in sillico).

Table 2: Mutations in the *katG* gene and the corresponding wild type and mutation bands

Failing wild type bands (S)	Codons analyzed	Developing mutation band	Mutation
katG WT	315	*katG* MUT1	S315T1
		katG MUT2	S315T1

Table 3: Mutations in the *inhA* promoter region and the corresponding wild type and mutation bands

Failing wild type bands (S)	Codons analyzed	Developing mutation band	Mutation
inhA WT1	-15	*inhA* MUT1	C–15T
	-16	*inhA* MUT2	A–16G
inhA WT2	-8	*inhA* MUT3A	T–8C
		inhA MUT3B	T–8A

Evaluation Examples

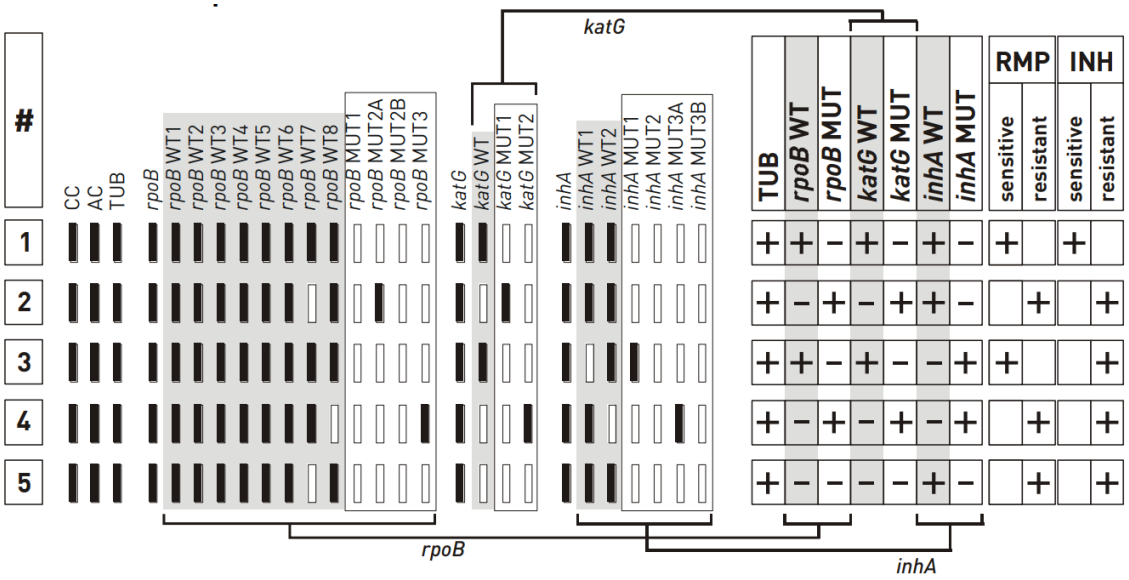


Figure 2: Examples for banding patterns and their evaluations with respect to RMP and/or INH resistance

If all wild type bands display a signal, this is classified as positive and marked in the WT column of the respective gene as "+". If at least one of the wild type bands is absent, this is classified as negative and marked in the WT column as "–". Negative entries are only made to the mutation columns when none of the mutation bands displays a coloration. If at least one of the mutation bands displays a coloration, this is classified as positive and the MUT column of the respective gene is marked with a "+".

Below, two of the examples shown above are explicated:

Example 1 shows the wild type banding pattern. All wild type probes, but none of the mutation probes display a signal; hence, the evaluation chart shows "+" in the three wild type columns and "–" in the three mutation columns. Accordingly, the boxes "RMP sensitive" and "INH sensitive" are marked with a "+".
In **example 5**, one of the *rpoB* and the *katG* wild type probes are missing; hence, the boxes for "*rpoB* WT" and "*katG* WT" are marked with a "–". As none of the mutation probes are developed, these boxes are also marked with a "–". The *inhA* promoter region does not deviate from the wild type pattern. The strain is evaluated as resistant to RMP and INH.

Limitations

Strictly adhere to the established protocols and procedures in order to obtain correct test results and to avoid contaminations.
Use of this assay is limited to qualified personnel well trained in the test procedure and familiar with molecular biological methods.
The test reflects the current state of knowledge of Hain Lifescience.

As any DNA-based assay, this test only screens the nucleic acid sequence and not the amino acid sequence. Therefore, it is possible that mutations in the probe region that do not cause an amino acid exchange (silent mutations) will still produce the absence of one of the wild type bands. A silent mutation in codon 514 of the *rpoB* gene leading to the absence of the *rpoB* WT3 band was observed in rare cases [10]. Hence, if an RMP resistance is detected solely by a missing *rpoB* WT3 band, results of phenotypic drug susceptibility testing should be considered.
Additional mutations within the tested *rpoB* gene region causing RMP resistance have been published [11]. As these mutations are very rare, they were not accessible for validation purposes of this test system but were only detected in silico.

The **GenoType MTBDR*plus*** VER 2.0 only detects those resistances that have their origins in the *rpoB, katG*, and *inhA* regions examined here. Resistances originating from mutations of other genes or gene regions as well as other RMP and INH resistance mechanisms will not be detected by this test.

Theoretically, a resistance can exist in spite of a wild type of pattern. If the sample contains a strain that has developed a heteroresistance and the resistance is caused by a mutation not covered by the mutation probes, the wild type of pattern will appear. Similarly, if the sample contains more than one *M. tuberculosis* complex strain (due to mixed culture or contamination) and one of these harbors a mutation not covered by the mutation probes, the wild type of pattern will also appear.

As any DNA detection method, the test system on hand detects DNA from viable and nonviable bacteria. Therefore, this test may not be used for monitoring the progression or success of treatment of patients with antimicrobial therapy.

The **GenoType MTBDR*plus*** VER 2.0 generates qualitative results. The intensities of the bands on a strip do not give information about the number of cells in a positive sample.
The presence of multiple bacterial species in the sample to be analyzed might hamper the interpretation of the test.

The members of the MTB complex cannot be differentiated.
The test only works within the limits of the genomic regions the primers and probes were chosen from.

As any detection system based on hybridization, the test system on hand bears the possibility that sequence variations in the genomic regions the primers and probes were chosen from but the detection of which the test was not designed for may lead to false results. Due to the high variability of bacterial genomes, it is possible that certain subtypes might not be detected.

Performance evaluation of this assay was carried out using the GenoLyse® kit for DNA extraction from decontaminated pulmonary smear-positive and smear-negative clinical specimens as well as from cultivated samples and using the GXT DNA/RNA Extraction Kit for automated DNA extraction from decontaminated clinical specimens. Until the present edition of the instructions on hand, the performance of the test has not been validated with other DNA extraction methods or sample materials.

The results of this test may only be interpreted in combination with additional laboratory and clinical data available to the responsible physician. In addition, results of phenotypic drug susceptibility testing have to be considered in certain cases.

The user must have or acquire information about the local mutation distribution pattern of the genes investigated with this test. Confirmation of the results by phenotypic drug susceptibility testing may be necessary.

Troubleshooting

Overall weak or no signals (including Conjugate Control zone)
- Room temperature too low or reagents not equilibrated to room temperature.
- No or too little amount of CON-C and/or SUB-C used.
  Repeat reverse hybridization.

Weak or no signals except for Conjugate Control zone
- Quality of extracted DNA does not allow an efficient amplification. Repeat extraction.
- Amplification Mixes (AM-A and AM-B) were not mixed properly, interchanged, or added in wrong amounts. Prepare a new master mix and repeat amplification.
- Incubation temperature too high. Repeat reverse hybridization.

No homogeneous staining
- Strips were not completely immersed during incubation steps.
- Tray was not shaken properly.
  Repeat reverse hybridization.

High background color
- CON-C and/or SUB-C used too concentrated.
- Washing steps were not performed with the necessary care.
- Wash solutions too cold.
  Repeat reverse hybridization.

Unexpected result
- Wrong incubation temperature.
- Hybridization Buffer and/or Stringent Wash Solution were not properly prewarmed or mixed.
- Contamination of neighboring wells by spillage during addition of Hybridization Buffer.
  Repeat reverse hybridization.
- Contamination of extracted DNA with previously extracted or amplified DNA. Repeat extraction.
- Contamination of amplification reagents. In this case, a negative control sample shows additional bands besides CC and AC. Repeat amplification using fresh reagents.
- Depending on the amount of amplified DNA used and on the specific reaction conditions, a strong and fast color development may occur. In such cases, discontinue the substrate incubation as soon as the signals are clearly visible in order to prevent the development of cross-hybridizing bands.
- No pure culture as starting material. Re-culture in order to exclude contamination.
- Improper sampling, storage, transport, or preparation of specimen. Request a new specimen and repeat test.
- Error during DNA extraction. Repeat extraction.

Ordering information

Hain LifeScience	Order no.
GenoType **MTBDR*plus*** VER 2.0 (kit for analysis of 12 samples)	304A
GenoType **MTBDR*plus*** VER 2.0 (kit for analysis of 96 samples)	30496A
GenoLyse® (kit for manual DNA extraction of 12 samples)	51612
GenoLyse® (kit for manual DNA extraction of 96 samples)	51610
GXT DNA/RNA Extraction Kit (kit for automated DNA/RNA extraction of 96 samples using the **Geno**Xtract®)	12.01.02
GenoXtract® (instrument for nucleic acid extraction of up to 12 samples)	8.31.01

Sarstedt, Nümbrecht, Germany	**Order no.**
0.5 ml Screw cap tubes	72.730.105
1.5 ml Screw cap tubes	72.692.005

GenoType **MTBDR*sl*** VER 2.0

Molecular Genetic Assay for Identification of the *M. tuberculosis* Complex and its Resistance to Fluoroquinolones and Aminoglycosides/Cyclic Peptides from Sputum Specimens or Cultivated Samples
Please read the instructions on hand completely and carefully before using the kit. Strictly adhere to the established procedure to obtain correct test results.

Intended Use

The **Geno**Type **MTBDR*sl*** VER 2.0 is a qualitative in vitro test for the identification of the Mycobacterium tuberculosis complex and its resistance to fluoroquinolones (FLQ; e.g. ofloxacin and moxifloxacin) and aminoglycosides/cyclic peptides (AG/CP; injectable antibiotics such as kanamycin, amikacin, capreomycin, and viomycin) from smear-positive or -negative sputum specimens and cultivated samples. The following species are included in the tuberculosis (TB)-causing M. tuberculosis complex: *M. tuberculosis*, *M. africanum*, *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, *M. bovis* BCG, *M. microti*, *M. canettii*, and *M. pinnipedii*. The detection of FLQ resistance is enabled by the detection of the most significant resistance-associated mutations of the *gyrA* and *gyrB* genes (coding for the A-subunit and the B-subunit of the DNA gyrase, respectively). For detection of AG/CP resistance, the 16S rRNA gene (*rrs*) is examined, for detection of low-level kanamycin resistance, the promoter region of the *eis* gene (coding for the acetyltransferase Eis) is examined.

The test is indicated as an aid for diagnosis and intended for use in medical laboratories.

Summary and Explanation

Tuberculosis (TB) is a bacterial infectious disease passed on by droplet infection. In 2014, there were an estimated 9.6 million incident cases of TB globally, and an estimated 1.5 million TB deaths [1]. TB treatment requires a therapy over several months. Emergence and spread of drug-resistant tuberculosis is a major medical and public problem threatening global health. Multidrug-resistant (MDR-)TB is defined as TB that is resistant at least to the first-line drugs rifampicin and isoniazid. The other anti-TB drugs referred to as first-line drugs are pyrazinamide, ethambutol, and streptomycin. All other anti-TB drugs are generally referred to as second-line drugs. Extensively drug-resistant (XDR-)TB is defined as TB that is resistant to rifampicin and isoniazid and additionally to at least one of the fluoroquinolones and an injectable second-line antibiotic (such as kanamycin and amikacin (both AG), or capreomycin and viomycin (both CP)) [2]. Due to its complex diagnosis and obstacles in treatment, XDR-TB is a major challenge to TB control.

As long as XDR-TB is not verified, use of inadequate and hence ineffective antibiotics may lead to further spread of resistant bacteria and amplification of resistance. Therefore, rapid diagnosis and identification of XDR-TB is a prerequisite for appropriate treatment. Each DNA extracted from sputum or a cultivated sample using the GenoLyse® kit can be used for amplification with the **Geno**Type **MTBDR*sl*** VER 2.0 kit (e.g. subsequent to the **Geno**Type **MTBDR*plus*** VER 2.0).

Principles of the Procedure

The GenoType **MTBDRsl** test is based on the **DNA**•STRIP technology. The whole procedure is divided into three steps: (i) DNA extraction from NALC-NaOH- decontaminated sputum specimens or cultured material (solid/liquid medium) – the necessary reagents are not included in the kit, (ii) a multiplex amplification with biotinylated primers, and (iii) a reverse hybridization.
All reagents needed for amplification, such as polymerase and primers, are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single- stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands is ensured by stringent conditions

which result from the combination of buffer composition and a certain temperature. Thus, the probes reliably discriminate several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. A template ensures the easy and fast interpretation of the banding pattern obtained.

Material required but not included in the kit

- Absorbent paper
- Adjustable pipettes for 10, 20, 200, and 1000 µl
- Class II safety cabinet
- Disposable gloves
- Disposable sterile pipette tips with filter
- DNA extraction kit (GenoLyse®, see chapter Ordering Information) as well as necessary equipment
- Graduated cylinder
- PCR tubes, DNase- and RNase-free
- Reagents for cultivation of mycobacteria as well as necessary equipment (when cultivated samples are to be used)
- Sample decontamination reagents as well as necessary equipment
- Shaking water bath + shaking platform *or* TwinCubator (instrument for manual hybridization) *or* automated hybridization instrument
- Thermal cycler
- Timer
- Tweezers
- Water (distilled)
- Water (molecular biology grade; for negative controls)

Quality Control

In order to control the correct performance of the test and the proper functioning of kit constituents, each strip includes 6 control zones:

- a Conjugate Control zone (CC) to check the binding of the conjugate on the strip and a correct chromogenic reaction
- an Amplification Control zone (AC) to check for a successful amplification reaction
- four Locus Control zones (*gyrA*, *gyrB*, *rrs*, and *eis*) checking the optimal sensitivity of the reaction for each of the tested gene loci

Observe the usual precautions for amplification setup. It is essential that all materials (such as pipette tips) coming in contact with the reagents are free from DNases. Do not interchange or pool Amplification Mixes or membrane strips from different kits unless the lots are identical.
A negative control sample for detection of possible contamination events containing water (molecular biology grade) instead of DNA should be part of each test run; the respective test strip should show the bands CC and AC only.

Specimen Requirements

NALC-NaOH-decontaminated smear-positive or -negative sputum samples as well as cultivated samples (solid/liquid medium) can be used as starting material for DNA extraction. Until the present edition of the instructions on hand, the performance of the test has not been validated with other sample materials.

Precautions for handling specimens

Patient specimens must always be considered as potentially infectious and must be handled accordingly (e.g. see [3] or [4]). Always wear suitable protective clothing and gloves. Samples from patients at risk (infected by pathogenic microorganisms including Hepatitis B and Human Immunodeficiency Virus (HIV)) must always be

labeled and handled under suitable safety conditions according to institutional guidelines. Patient specimens must be centrifuged in a class II safety cabinet or in an aerosol-tight rotor. Open aerosol-tight rotor in safety cabinet only. For inactivated samples, a standard rotor can be used for centrifugation outside the safety cabinet.
Positive culture samples are infectious and must always be handled in a class II safety cabinet and according to the CDC publication "Public Health Mycobacteriology: A Guide for the Level III Laboratory" [5].
Discard used pipette tips immediately after use in a container for biohazardous waste. After finishing the assay, discard all used disposables in a container for biohazardous waste.

Storage and transport
All specimens should be collected and transported as recommended in the CDC publication "Public Health Mycobacteriology: A Guide for the Level III Laboratory" [5], the "Clinical Microbiology Procedures Handbook" [6], or your laboratory procedure manual.
It must be ensured that until decontamination takes place, specimens are kept in sterile plastic containers at a temperature of 2-8°C. The transport of specimens at room temperature has to be carried out as soon as possible and should be done within 1-2 days [7,8]. The specimens used for decontamination must not be older than 4 days.
After decontamination and subsequent resuspension of the bacteria pellet with phosphate buffer, samples can be stored at –20°C or –80°C for a maximum of 5 days until performing DNA extraction.

Preparation
Clinical specimens must be processed using the NALC/NaOH method according to the CDC publication "Public Health Mycobacteriology: A Guide for the Level III Laboratory" [5].
After decontamination, the cell pellet should be resuspended in a maximum of 1 to 1.5 ml of phosphate buffer. When testing patient specimens, higher volumes might hamper the sensitivity of the test. Due to the potential inhomogeneity of the specimen, the decontaminated samp le must be mixed before removing the aliquot to be analyzed; otherwise, the sensitivity of the test might be influenced.
When the sample is to be cultivated, cultivation can be performed either on solid medium (e.g. Loewenstein-Jensen, Middlebrook) or in liquid medium (e.g. MGIT (BD Diagnostics, Franklin Lakes, USA)).

DNA Extraction

NALC-NaOH-decontaminated smear-positive or -negative sputum samples as well as mycobacteria grown on solid medium (e.g. Loewenstein-Jensen, Middlebrook) or in liquid medium (e.g. MGIT (BD Diagnostics, Franklin Lakes, USA)) can be used as starting material for DNA extraction. The working area must be free from contaminating DNA.

For DNA extraction from NALC-NaOH-decontaminated clinical specimens or cultured material, the **GenoLyse**® kit (see chapter Ordering Information) is used according to protocol A.
The method described above was used for performance evaluation of the **GenoType MTBDRsl** test. Until the present edition of the instructions on hand, the performance of the test has not been validated with other DNA extraction methods or sample materials.
Each DNA extracted from sputum or a cultivated sample using the **GenoLyse**® kit can be used for amplification with the **GenoType MTBDRsl** VER 2.0 kit (e.g. subsequent to the GenoType MTBDRplus VER 2.0).

Amplification
All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. After thawing, spin down AM-A and AM-B briefly and mix carefully by pipetting up and down. Pipette AM-A and AM-B only in a room free from contaminating DNA. The DNA solution should be added in a separate working area.

Prepare for each sample:

- 10 µl AM-A (see Kit Component 2)
- 35 µl AM-B (see Kit Component 2)
- 5 µl DNA solution

Final volume: 50µl

Determine the number of samples (number of samples to be analyzed plus control samples). Prepare the number of tubes needed. Prepare a master mix containing AM-A and AM-B and mix carefully but thoroughly (do not vortex). Alternatively, the content of an AM-A reaction tube may completely be transferred to an AM-B reaction tube. This will lead to 0.68 ml master mix for 12 amplification reactions (12 tests kit) or, respectively, 4x 1.35 ml for 4x 24 amplification reactions (96 tests kit). Please note that the master mix needs to be prepared freshly each time. Aliquot 45 µl into each of the prepared PCR tubes and add 5 µl water (molecular biology grade) to one aliquot (negative control). In a separate working area, add 5 µl DNA solution to each aliquot (except for negative control).

Amplification profile

When using a thermal cycler from Hain Lifescience with the respective preinstallation, select protocol "MDR DIR" for clinical specimens or protocol "MDR CUL" for cultivated samples.

	Clinical specimens	**Cultivated samples**
15 min 95°C	1 cycle	1 cycle
30 sec 95°C 2 min 65°C	20 cycles	10 cycles
25 sec 95°C 40 sec 50°C 40 sec 70°C	30 cycles	20 cycles
8 min 70°C	1 cycle	1 cycle
Heating rate	≤2.2°C/sec	≤2.2°C/sec

Amplification products can be stored at +8 to –20°C.

Hybridization

When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the hybridization protocol to be used.
The following protocol describes the manual hybridization using a water bath or a **Twin**Cubator.

Preparation

Prewarm shaking water bath to **45°**C (the maximum tolerated deviation from the target temperature is +/–1°C) or switch on **Twin**Cubator. Prewarm solutions HYB and STR to 37-45°C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary. Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (**CON-C with CON-D, SUB-C with SUB-D**) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10 µl concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

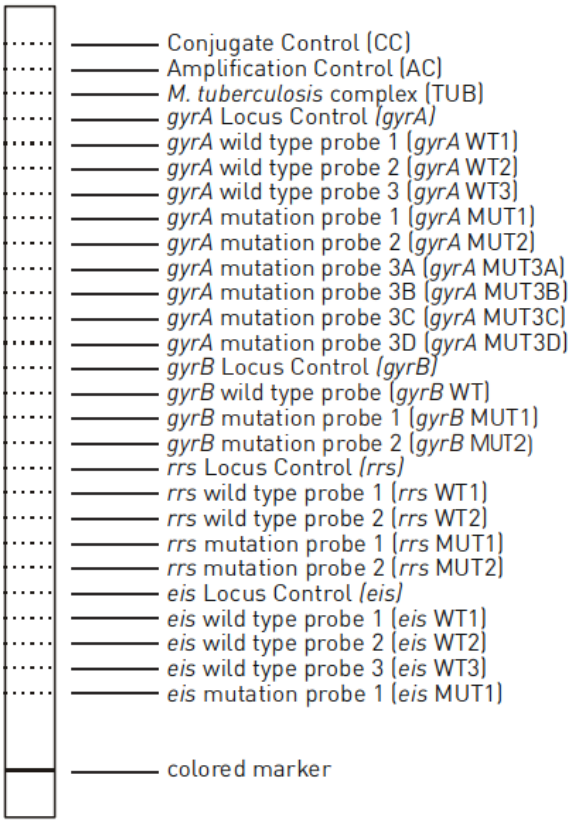
1. **Dispense 20 µl of Denaturation Solution (DEN, blue) in a corner of each of the wells used.**
2. **Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate**

at room temperature for 5 minutes.
Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. Always wear gloves when handling strips.

3. **Carefully add to each well 1 ml of prewarmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous color.**
   Take care not to spill solution into the neighboring wells.
4. **Place a strip in each well.**
   The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers, turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps.
5. **Place tray in Shaking water bath/TwinCubator and incubate for 30 minutes at 45°C.**
   Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least 1/3 of its height.
6. **Completely aspirate Hybridization Buffer.**
   For example, use a Pasteur pipette connected to a vacuum pump.
7. **Add 1 ml of Stringent wash Solution (STR, red) to each strip and incubate for 15 minutes at 45°C in shaking water bath/TwinCubator.**
8. **Work at room temperature from this step forward. Completely remove Stringent wash Solution.**
   Pour out Wash Solution in a waste container and remove all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper. This also applies to all other wash steps.
9. **Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator (pour out RIN after incubation).**
10. **Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on shaking platform/Twin**Cubator**.**
11. **Remove solution and wash each strip twice for 1 minute with 1 ml of rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water (e.g. use wash bottle) on shaking platform/Twin**Cubator **(pour out solution each time).**
    Make sure to remove any trace of water after the last wash.
12. **Add 1 ml of diluted substrate (see above) to each strip and incubate protected from light without shaking.**
    Depending on the test conditions (e.g. room temperature), the substrate incubation time, i.e. the time until the bands are clearly visible, can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.
13. **Stop reaction as soon as bands are clearly visible by briefly rinsing twice with distrilled water.**
14. **Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.**

Evaluation and Interpretation of Results

Paste strips and store protected from light. An evaluation sheet is included in the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and AC with the respective lines on the sheet. For technical reasons the distances between single probes on the strips may vary slightly. **For an accurate evaluation therefore please use the provided template and align it- separately for each locus– with the respective Locus Control band.** Determine the resistance status and note down in the respective column. As a help for interpretation, evaluation examples are given below. Each strip has a total of 27 reaction zones (see figure).



Note: The strip is not displayed in original size.

Conjugate Control (CC)

A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.

Amplification Control (AC)

When the test is performed correctly, a control amplicon will bind to the Amplification Control zone. In case of a positive test result, the signal of the Amplification Control zone can be weak or even vanish totally. This might be due to competition of the single reactions during amplification. In this case the test was performed correctly and does not have to be repeated.

When only the CC and AC bands are developed, this represents a valid negative result. A missing AC band in case of a negative test result indicates mistakes during setup and/or performance of the amplification reaction, or presence of amplification inhibitors. In this case, the test result is not valid, and the test has to be repeated with the respective sample.

M. tuberculosis complex (TUB)

This zone hybridizes with amplicons generated from all members of the *Mycobacterium tuberculosis* complex. If the TUB zone is negative while no evaluable resistance pattern is developed, the tested specimen does not contain bacteria belonging to the *M. tuberculosis* complex and cannot be evaluated by this test system.

LOCUS CONTROLS *(gyrA, gyrB, rrs, eis)*

The Locus Control zones detect gene regions specific for the respective locus. In case of a positive test result (evaluable wild type and mutation banding pattern), the signals of the Locus Control bands may be weak.

gyrA

Both the *gyrA* and *gyrB* genes are examined for detection of resistance to FLQ (e.g., ofloxacin or moxifloxacin). The wild type probes comprise the most important resistance regions of the *gyrA* gene (see table 1). When all wild type probes stain positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe resulting in the absence of the wild type probe signal. The mutation probes detect some of the most common resistance-mediating mutations (See table 1)

Each pattern deviating from the wild type of pattern (see evaluation example 1) indicates resistance to FLQ of the tested strain.

Table 1: Mutations in the *gyrA* gene and the corresponding wild type and mutation bands (according to [9,10,11,12])

Failing wild type band	Developing mutation band	Mutation	Phenotypic resistance
gyrA WT1	-	G88A	FLQ
		G88C	
gyrA WT2	*gyrA* MUT1	A90V	
	gyrA MUT2	S91P	
gyrA WT3	*gyrA* MUT3A	D94A	
	gyrA MUT3B	D94N	
		D94Y	
	gyrA MUT3C	D94G	
	gyrA MUT3D	D94H[1)]	

[1)] This rare mutation has only been detected theoretically (in silico).

gyrB

Both the *gyrA* and *gyrB* genes are examined for detection of resistance to FLQ (e.g., ofloxacin or moxifloxacin).
The wild type probe comprises the most important resistance region of the *gyrB* gene (see table 2). When the wild type probe stains positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the wild type probe resulting in the absence of the wild type probe signal.
The mutation probes detect the most common resistance-mediating mutations (see table 2). Additional mutations within the examined *gyrB* gene region that cause a failing wild type band but are not detected by the mutation probes may also lead to FLQ resistance [13].

Table 2: Mutations in the *gyrB* gene and the corresponding wild type and mutation bands (according to [13])

Failing wild type band	Developing mutation band	Mutation[1)]	Phenotypic resistance
gyrB WT	*gyrB* MUT1	N538D	FLQ
	gyrB MUT2	E540V	

[1)] Amino acid positions are numbered according to [14].

rrs

The *rrs* gene is examined for detection of cross-resistance to AG/CP antibiotics such as kanamycin (KAN) and amikacin (AMK), both AG, or capreomycin (CAP) and viomycin (VIO), both CP.

The wild type probes comprise the most important resistance regions of the *rrs* gene (see table 3). When both wild type probes stain positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe resulting in the absence of the wild type probe signal. The mutation probes detect some of the most common resistance-mediating mutations (See table 3)

Each pattern deviating from the wild type pattern (see evaluation example 1) indicates an AG/CP resistance of the tested strain. The detectable cross- resistances are shown in the table below.

Table 3: Mutations in the *rrs* gene, the corresponding wild type and mutation bands, and the resulting cross-resistances (according to [15,16])

Failing wild type band	Analyzed nucleic acid position	Developing mutation band	Mutation	Phenotypic resistance				See figure 1
rrs WT1	1401	*rrs* MUT1	A1401G	KAN	AMK	CAP		example 2 and 6
	1402	-	C1402T	KAN		CAP	VIO	example 3
rrs WT2	1484	*rrs* MUT2	G1484T	KAN	AMK	CAP	VIO	example 4

KAN, kanamycin; AMK, amikacin; CAP, capreomycin; VIO, viomycin

eis

The *eis* gene is examined for detection of a low-level KAN resistance.

The wild type probes comprise the most important resistance regions of the *eis* gene (see table 4). When all wild type probes stain positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe resulting in the absence of the wild type probe signal.

The mutation probe detects the most common resistance-mediating mutation (see table 4). More mutations within the examined *eis* gene region than those listed in table 4 are known [19]. These mutations that may be causing a failing wild type band but are not detected by the mutation probe may also cause low- level KAN resistance.

Table 4: Mutations in the *eis* promoter region and the corresponding wild type and mutation bands (according to [17,18,19,20])

Failing wild type band	Developing mutation band	Mutation	Phenotypic resistance
eis WT1	-	G-37T	low-level KAN
eis WT2	*eis* MUT1	C-14T	
	-	C-12T	
	-	G-10A	
eis WT3	-	C-2A	

Please note:

Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone (AC) are to be considered. Not all bands of a strip have to show the same signal strength.

When both a mutation probe and the corresponding wild type probe of a strip are developed, this represents a valid result. Possible reasons could be:

- The tested specimen contains a heteroresistant strain.
- The tested specimen contains more than one *M. tuberculosis* complex strain (e.g. due to mixed infection of the patient).

Theoretically, a resistance can exist in spite of a wild type pattern. Possible reasons could be:

- The tested specimen contains a strain that has developed a heteroresistance and the resistance is caused by a mutation not covered by the mutation probes.
- The tested specimen contains a wild type and a resistant strain (e.g. due to mixed infection of the patient) and the resistance is caused by a mutation not covered by the mutation probes.

When a complete gene locus (all bands including the Locus Control band) is missing, this is an invalid result. If this result is generated from a clinical specimen, a possible reason could be, amongst others, that the DNA concentration in the sample is below the limit of detection.

Evaluation Examples

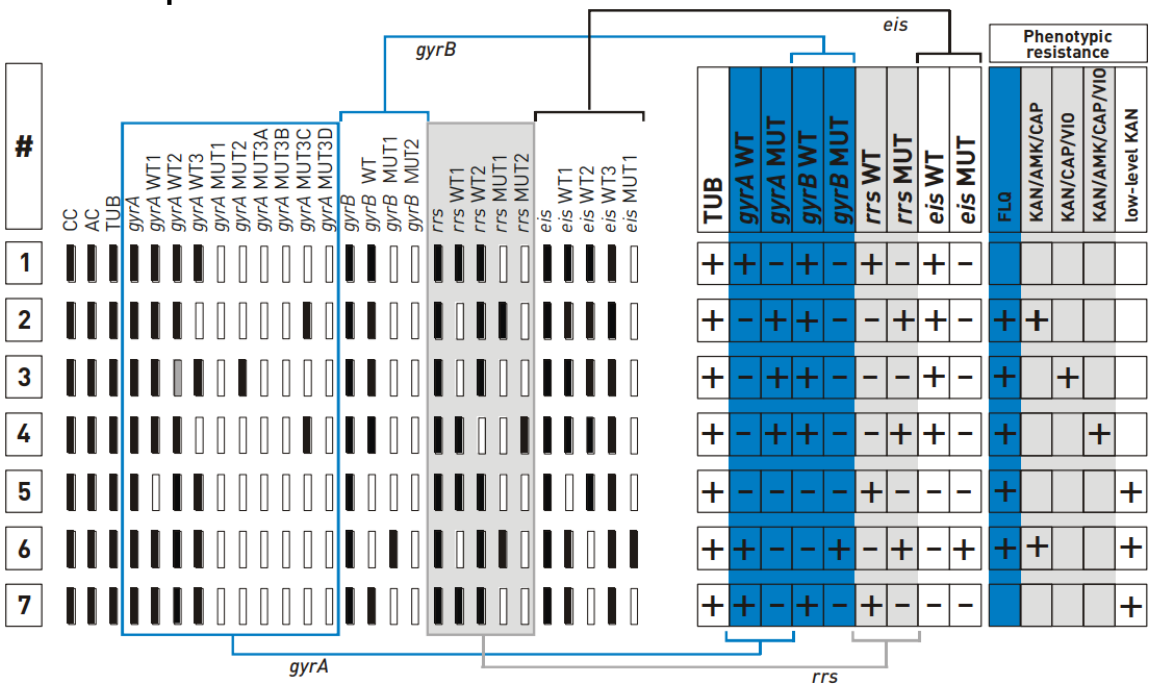


Figure 1: Examples for banding patterns and their evaluation with respect to resistances to fluoroquinolones (FLQ) and/or aminoglycosides/cyclic peptides (AG/CP)

If all wild type bands of a gene display a signal, this is classified as positive and marked in the WT column of the respective gene as "+". If at least one of the wild type bands is absent, this is classified as negative and marked in the WT column as "–". In the MUT columns negative entries are only made if none of the mutation bands of the respective gene displays a coloration. If at least one of the mutation bands displays a coloration, this is classified as positive and the MUT column of the respective gene is marked with a "+". To the resistance columns a "+" is depicted only if at least one entry in the WT and MUT columns deviates from the wild type pattern of the respective gene in example 1.

Below, the examples shown above are explicated:

Example 1 shows the wild type banding pattern. All wild type probes but none of the mutation probes display a signal; hence, the evaluation chart shows "+" in the four wild type columns and "–" in the four mutation columns. Accordingly, no entry is made in the fields of the resistance columns.

Example 2: One of the *gyrA* wild type bands is missing and one of the *gyrA* mutation bands is developed. Hence, the evaluation chart shows a "-" in the "*gyrA* WT" column and a "+" in the "*gyrA* MUT" column. The *gyrB* locus displays the wild type banding pattern resulting in a wild type entry as in example 1. Due to the *gyrA* banding pattern, the strain is evaluated as FLQ-resistant. The *rrs* wild type band "*rrs* WT1" is missing, and the mutation band "*rrs* MUT1" is developed; hence, the field in the "*rrs* WT" column is marked with a "–", the field in the "*rrs* MUT" column is marked with a "+", and the strain is evaluated as cross-resistant to KAN, AMK, and CAP (see table 3 above). Finally, the probes of the *eis* locus display the wild type banding pattern; hence, the columns "*eis* WT" and "*eis* MUT" are marked according to example 1 and no low-level KAN resistance is detected.

Example 3: The "*gyrA* WT2" band is missing (signal intensity is lower than that of the AC) and the "*gyrA* MUT2" band is developed. Accordingly, the field in the "*gyrA* WT" column is marked with a "–" and the field in the "*gyrA* MUT" column is marked with a "+". The *gyrB* locus displays the wild type banding pattern which is depicted accordingly. Due to the *gyrA* result, FLQ resistance is assigned to the tested strain. The *rrs* wild type band "*rrs* WT1" is missing, but none of the *rrs* mutation bands is developed; thus, the fields in the "*rrs* WT" and "*rrs* MUT" columns are marked with a "–" and cross-resistance to KAN, CAP, and VIO is identified (see table 3 above). The *eis* locus displays the wild type banding pattern which is depicted accordingly.

Example 4: One of the *gyrA* wild type bands is missing and one of the *gyrA* mutation bands is developed. In the evaluation chart, a "–" is depicted in the field of the "*gyrA* WT" column and the field of the "*gyrA* MUT" column is marked with a "+". The *gyrB* locus displays the wild type banding pattern which is depicted accordingly. Due to the *gyrA* result, FLQ resistance is assigned to the tested strain. The *rrs* wild type band "*rrs* WT2" is missing and the mutation band "*rrs* MUT2" is developed; thus, the field in the "*rrs* WT" column is marked with a "–", the field in the "*rrs* MUT" column is marked with a "+", and the tested strain is evaluated as cross-resistant to KAN, AMK, CAP, and VIO (see table 3 above). The *eis* locus displays the wild type banding pattern which is depicted accordingly.

Example 5: From both the *gyrA* and the *gyrB* locus one wild type bands is missing and none of the *gyrA* and *gyrB* mutation bands are developed. Therefore, all *gyrA* and *gyrB* columns are marked with a "–" and FLQ resistance is assigned to the tested strain. The *rrs* locus shows the wild type banding pattern which is depicted accordingly. Finally, one of the *eis* wild type bands is missing; hence, both the fields in the "*eis* WT" and "*eis* MUT" columns are marked with a "–" and a low-level KAN resistance is detected.

Example 6 shows the wild type banding pattern for the *gyrA* locus which is depicted accordingly. The *gyrB* wild type band is missing and one of the *gyrB* mutation bands is developed. Hence, in the evaluation chart, a "–" is depicted in the field of the "*gyrB* WT" column and a "+" in the field of the "*gyrB* MUT" column. Due to the *gyrB* result, FLQ resistance is assigned to the tested strain. The *rrs* wild type band "*rrs* WT1" is missing and the mutation band "*rrs* MUT1" is developed; hence, the field in the "*rrs* WT" column is marked with a "–", the field in the "*rrs* MUT" column is marked with a "+", and the strain is evaluated as cross-resistant to KAN, AMK, and CAP (see table 3 above). One of the *eis* wild type bands is missing and the *eis* mutation band is developed. Hence, in the eis WT column, a "–" is depicted, the eis MUT column is marked with a "+", and a low-level KAN resistance is assigned to the tested strain.

Example 7: Both the *gyrA* locus and the *gyrB* locus show the wild type pattern which is depicted accordingly with respect to FLQ resistance. The *rrs* locus shows the wild type pattern which is depicted accordingly. One of the *eis* wild type bands is missing. Hence , a "–" is depicted in both the "*eis* WT" and the "*eis* MUT" column of the evaluation chart, and a low-level KAN resistance is assigned to the tested strain.

Limitations

Strictly adhere to the established protocols and procedures in order to obtain correct test results and to avoid contaminations. Use of this assay is limited to qualified personnel well trained in the test procedure and familiar with molecular biological methods. The test reflects the current state of knowledge of Hain Lifescience.

This result of this test may only be interpreted in combination with additional laboratory and clinical data available to the responsible physician. In addition, result of phenotypic drug susceptibility testing have to be considered in certain cases.

The user must have or acquire information about the local mutation distribution pattern of the genes investigated with this test. Conformation of the test results by phenotypic drug susceptibility testing may be necessary.

As with any DNA-based assay, this test only screens the nucleic acid sequence and not the amino acid sequence. Therefore, it is possible that mutations in the probe region that do not cause an amino acid exchange (silent mutations) will still produce the absence of one of the wild type bands.

The **GenoType MTBDRsl** test only detects those resistances that have their origins in the gyrA, gyrB, rrs, and eis gene regions examined here. Resistances originating from mutations of other genes or gene regions as well as other FLQ or AG/CP resistance mechanisms will not be detected by this test.

The members of the M. tuberculosis complex cannot be differentiated. The presence of multiple bacterial species in the sample to be analyzed might hamper the interpretation of the test. As any DNA detection method the test system on hand detects DNA from viable and nonviable bacteria. Therefore, the **GenoType MTBDRsl** may not be used for monitoring the progression or success of treatment of patients with antimicrobial therapy.

The **GenoType MTBDRsl** generates qualitative results. The intensities of the bands on a strip do not give information about the number of cells in a positive sample.

Performance evaluation of this assay was carried out using the **GenoLyse®** kit for DNA extraction from NALC-NaOH-decontaminated smear-positive and smear-negative sputum samples as well as from cultivated samples. Until the present edition of the instructions on hand, the performance of the test has not been validated with other DNA extraction methods or sample materials.

Troubleshooting

Overall weak or no signals (including Conjugate Control zone)

- Room temperature too low or reagents not equilibrated to room temperature.
- No or too little amount of CON-C and/or SUB-C used.
  Repeat reverse hybridization.

Weak or no signals except for Conjugate Control zone

- Quality of extracted DNA does not allow an efficient amplification. Repeat extraction.
- Amplification Mixes (AM-A and AM-B) were not mixed properly, interchanged, or added in wrong amounts.
  Prepare a new master mix and repeat amplification.
- Incubation temperature too high. Repeat reverse hybridization.

No homogeneous staining

- Strips were not completely immersed during incubation steps.
- Tray was not shaken properly.
  Repeat reverse hybridization.

High background color

- CON-C and/or SUB-C used too concentrated.
- Washing steps were not performed with the necessary care.
- Wash solutions too cold.
  Repeat reverse hybridization.

Unexpected result

- Wrong incubation temperature.
- Hybridization Buffer and/or Stringent Wash Solution were not properly prewarmed or mixed.
- Contamination of neighboring wells by spillage during addition of Hybridization Buffer.
  Repeat reverse hybridization.
- Contamination of extracted DNA with previously extracted or amplified DNA. Repeat extraction.
- Contamination of amplification reagents. In this case, a negative control sample shows additional bands besides CC and AC. Repeat amplification using fresh reagents.
- Depending on the amount of amplified DNA used and on the specific reaction conditions, a strong and fast color development may occur. In such cases, discontinue the substrate incubation as soon as the signals are clearly visible in order to prevent the development of cross-hybridizing bands. If necessary, the amount of amplicon used for reverse hybridization may be reduced down to 5 µl.
- No pure culture as starting material. Re-culture in order to exclude contamination.
- Improper sampling, storage, transport, or preparation of specimen. Request new specimen and repeat test.

- Error during DNA extraction. Repeat extraction.

Ordering Information

	Order no.
GenoType MTBDR*sl* VER 2.0 (kit for analysis of 12 samples)	317A
GenoType MTBDR*sl* VER 2.0 (kit for analysis of 96 samples)	31796A
GenoLyse® (kit for manual DNA extraction of 12 samples)	51612
GenoLyse® (kit for manual DNA extraction of 96 samples)	51610

References

1. World Health Organization. Global tuberculosis report 2018. WHO/CDS/TB/2018.20. World Health Organization, Geneva, Switzerland 2018.
2. Zhang Y, Yew WW. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 2009; 13: 1320–1330.
3. Biosafety in microbiological and biomedical laboratories, 5th edition. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, USA 2009.
4. Protection of laboratory workers from occupationally acquired infections. Approved guideline. Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards), USA, Document M29 (please refer to the latest version).
5. Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, USA 1985.
6. Isenberg HD. Clinical microbiology procedures handbook. American Society for Microbiology, Washington, D.C., USA 1992.
7. Richter E, Beer J, Diel R, Hillemann D, Hoffmann H, Klotz M, Mauch H, Rüsch-Gerdes S. MiQ 5, Tuberkulose, Mykobakteriose. In: Podbielski A, Herrmann M, Kniehl E, Mauch H, Rüssmann H (eds): Mikrobiologisch-infektiologische Qualitätsstandards. Elsevier, Munich, Germany 2010.
8. DIN, Deutsches Institut für Normung e.V. (ed). DIN 58943-4:2009-02: Medical microbiology - Diagnosis of tuberculosis - Part 4: Primary samples for the diagnosis of tuberculosis and mycobacteria – Qualitative and quantitative requirements, extraction, transport and storage. Beuth, Berlin, Germany 2009.
9. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. Detection of rifampicin-resistance mutations in
10. *Mycobacterium tuberculosis*. *Lancet* 1993, 341: 647-650.
11. Alonso M, Palacios JJ, Herranz M, Penedo A, Menéndez A, Bouza E, García de Viedma D. Isolation of *Mycobacterium tuberculosis* strains with a silent mutation in rpoB leading to potential misassignment of resistance category. *J Clin Microbiol* 2011; 49: 2688-2690.
12. Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clin Microbiol Rev* 1995; 8: 496-514.
13. Crudu V, Stratan E, Romancenco E, Allerheiligen V, Hillemann A, Moraru N. First evaluation of an improved assay for molecular genetic detection of tuberculosis as well as RMP and INH resistances. *J Clin Microbiol* 2012; Epub ahead of print, doi:10.1128/JCM.05903-11.
14. World Health Organization. Global tuberculosis report 2015. WHO/HTM/TB/2015.22. World Health Organization, Geneva, Switzerland 2015.
15. Cheng AF, Yew WW, Chan EW, Chin ML, Hui MM, Chan RC. Multiplex PCR amplimer conformation analysis for rapid detection of gyrA mutations in fluoroquinolone-resistant Mycobacterium tuberculosis clinical isolates. Antimicrob Agents Chemother

2004; 48: 596-601.

16. Aubry A, Veziris N, Cambau E, Truffot-Pernot C, Jarlier V, Fisher LM. Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of Mycobacterium tuberculosis: functional analysis of mutant enzymes. Antimicrob Agents Chemother 2006; 50: 104-112.
17. Matrat S, Veziris N, Mayer C, Jarlier V, Truffot-Pernot C, Camuset J, Bouvet E, Cambau E, Aubry A. Functional analysis of DNA gyrase mutant enzymes carrying mutations at position 88 in the A subunit found in clinical strains of Mycobacterium tuberculosis resistant to fluoroquinolones. Antimicrob Agents Chemother 2006; 50: 4170-4173.
18. Kocagöz T, Hackbarth CJ, Unsal I, Rosenberg EY, Nikaido H, Chambers HF. Gyrase mutations in laboratory-selected, fluoroquinolone-resistant mutants of Mycobacterium tuberculosis H37Ra. Antimicrob Agents Chemother 1996; 40: 1768-1774.
19. Malik S, Willby M, Sikes D, Tsodikov OV, Posey JE. New insights into fluoroquinolone resistance in Mycobacterium tuberculosis: functional genetic analysis of gyrA and gyrB mutations. PLoS One 2012; 7: e39754. doi: 10.1371/journal.pone.0039754.
20. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998; 393: 537-44. Erratum in: Nature 1998; 396: 190.
21. Maus CE, Plikaytis BB, Shinnick TM. Mutation of tlyA confers capreomycin resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 2005; 49: 571-577.
22. Maus CE, Plikaytis BB, Shinnick TM. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in Mycobacterium tuberculosis. Antimicrob Agents Chemother 2005; 49: 3192-3197.
23. Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC. Evaluation of genetic mutations associated with Mycobacterium tuberculosis resistance to amikacin, kanamycin and capreomycin: a systematic review. PLoS One 2012; 7: e33275. doi: 10.1371/journal.pone.0033275.
24. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, Hooks DP, Cowan LS, Plikaytis BB, Posey JE. Molecular detection of mutations

associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrob
Agents Chemother 2011; 55: 2032-2041.

25. Chakravorty S, Lee JS, Cho EJ, Roh SS, Smith LE, Lee J, Kim CT, Via LE, Cho SN, Barry CE 3rd, Alland D. Genotypic susceptibility testing of Mycobacterium
tuberculosis isolates for amikacin and kanamycin resistance by use of a rapid sloppy molecular beacon-based assay identifies more cases of low-level
drug resistance than phenotypic Lowenstein-Jensen testing. J Clin Microbiol 2015; 53: 43-51.

26. Zaunbrecher MA, Sikes RD Jr, Metchock B, Shinnick TM, Posey JE. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis
confers kanamycin resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 2009; 106: 20004-20009.

27. Tagliani E, Cabibbe AM, Miotto P, Borroni E, Toro JC, Mansjö M, Hoffner S, Hillemann D, Zalutskaya A, Skrahina A, Cirillo DM. Diagnostic performance of
the new version (v2.0) of GenoType MTBDRsl assay for detection of resistance to fluoroquinolones and second-line injectable drugs: a multicenter study.
J Clin Microbiol 2015; 53: 2961-2969.