GenoType MTBDR*sl*

VER 2.0

Instructions for Use

IFU-317A-01

CE

IVD for in vitro diagnostic use only

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HAIN LIFESCIENCE

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 **GenoType 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 is a bacterial infectious disease spread by droplet infection. In 2013, there were an estimated 9 million incident cases of TB globally, and an estimated 1.5 million deaths occurred [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 **GenoType MTBDR*sl*** VER 2.0 kit (e.g. subsequent to the **GenoType MTBDR*plus*** VER 2.0).

Principles of the Procedure

The **GenoType MTBDR*sl*** test is based on the **DNA•STRIP** technology. The whole procedure is divided into three steps: (i) DNA extraction from 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.

Storage and Disposal of Kit Constituents

1/2 Kit Component 1 of 2

2/2 Kit Component 2 of 2

Store all constituents from Kit Component 1 at 2-8°C. Store all constituents from Kit Component 2 at –20°C and keep strictly separated from contaminating DNA. Avoid repeated freezing and thawing of AM-A and AM-B; when processing only small sample numbers per run, aliquot AM-A and AM-B. Do not use the reagents beyond their expiry date. Dispose of unused reagents and waste in accordance with federal, state, and local regulations.

Precautions for Handling Kit Constituents

Observe all federal, state, and local safety and environmental regulations. Always wear suitable protective clothing and gloves.
When handling kit reagents, the following special safety measures must be applied:
The **Denaturation Solution** (DEN) contains <2% NaOH and is irritating to eyes and skin (R36/38 and S26-37/39-45).
The **Substrate Concentrate** (SUB-C) contains dimethyl sulfoxide and is irritating (R36/37/38, S23-26-36).
For additional information, please refer to the safety data sheets which can be downloaded from: www.hain-lifescience.com/products/msds.html

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 CC and AC bands only.

Specimen Requirements

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 and cultures made from 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)) and cultures made from those samples must always be labeled and handled under suitable safety conditions according to institutional guidelines.

Handling of potentially infectious specimens must be carried out in a class II safety cabinet. Potentially infectious samples 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.

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 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 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 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 MTBDR*sl*** 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 MTBDR*sl*** VER 2.0 kit (e.g. subsequent to the **GenoType MTBDR*plus*** 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: 50 µl

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 2 min	95°C 65°C	20 cycles	10 cycles
25 sec 40 sec 40 sec	95°C 50°C 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

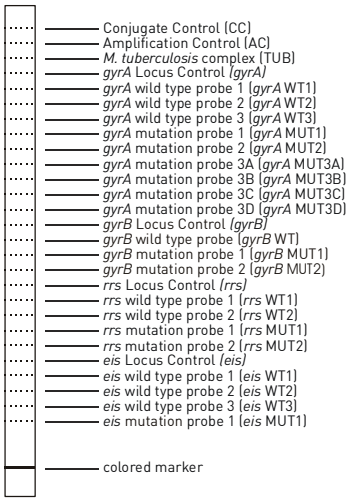
When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the hybridization protocol to be used.
The following protocol describes the manual hybridization using a water bath or a **TwinCubator**.

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 37-45°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/TwinCubator.**
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/TwinCubator (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 distilled 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 CC and AC bands 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 amplification setup, or presence of amplification inhibitors. In this case, the test result is not valid and the respective sample has to be repeated.

***M. tuberculosis* complex (TUB)**
This zone hybridizes, as far as is known, 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 pattern (see evaluation example 1) indicates, as far as is known, 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 the most common resistance-mediating mutations (see table 3).

Each pattern deviating from the wild type pattern (see evaluation example 1) indicates, as far as is known, 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, possible reasons could be, but are not limited to, a DNA concentration in the sample below the limit of detection.

Evaluation examples

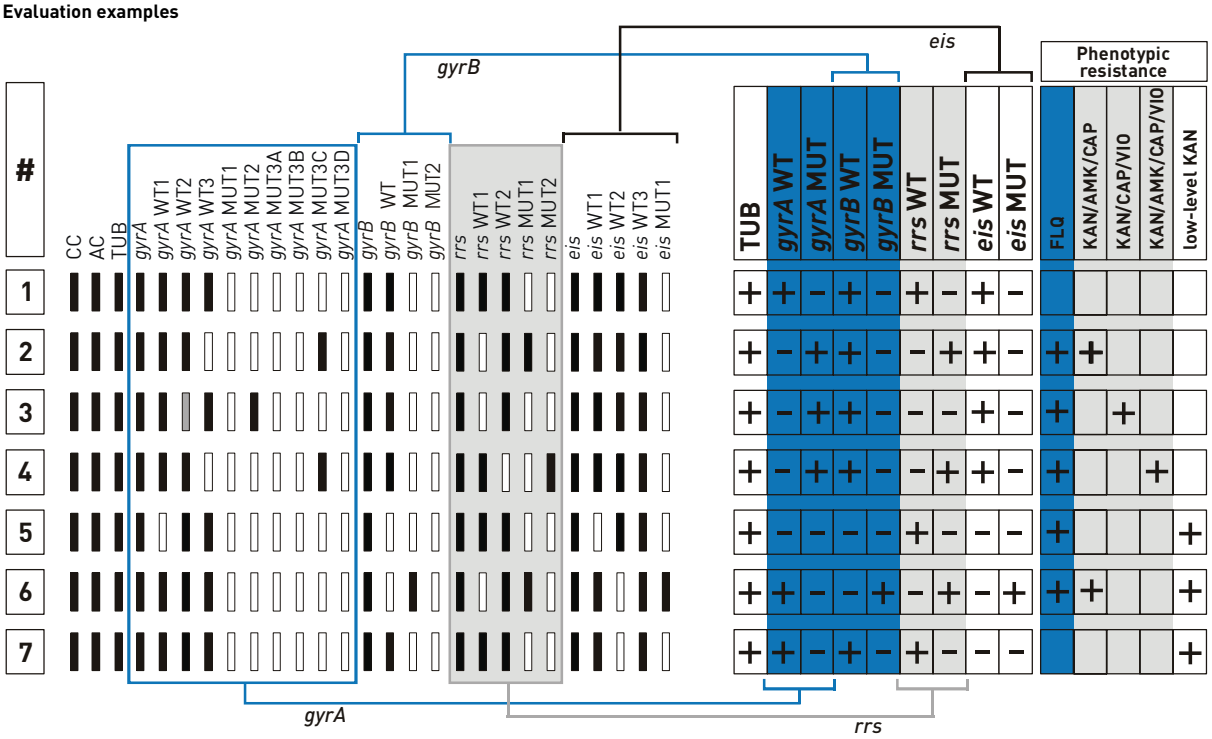


Figure 1: Examples for banding patterns and their evaluation with respect to resistances to FLQ and/or AG/CP

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.

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 MTBDR*sl*** 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 data given in table 3 regarding the cross-resistances between KAN, AMK, CAP, and VIO reflect the current state of knowledge of Hain Lifescience.

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 MTBDR*sl*** may not be used for monitoring the progression or success of treatment of patients with antimicrobial therapy.
The **GenoType MTBDR*sl*** 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 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.

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 test 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) 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 new specimen and repeat test.
- Error during DNA extraction. Repeat extraction.

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
- 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)

Kit Contents

Order no.	**317A**	**31796A**
Tests	**12**	**96**
Kit Component 1 of 2 (store at 2-8°C)		
Membrane strips coated with specific probes (MTBDRsl VER 2.0 STRIPS)	12	2x 48
Denaturation Solution (DEN) contains <2% NaOH, dye	0.3 ml	2x 1.2 ml
Hybridization Buffer (HYB) contains 8-10% anionic tenside, dye	20 ml	120 ml
Stringent Wash Solution (STR) contains >25% of a quaternary ammonium compound, <1% anionic tenside, dye	20 ml	120 ml
Rinse Solution (RIN) contains buffer, <1% NaCl, <1% anionic tenside	50 ml	3x 120 ml
Conjugate Concentrate (CON-C) contains streptavidin-conjugated alkaline phosphatase, dye	0.2 ml	1.2 ml
Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, <1% NaCl	20 ml	120 ml
Substrate Concentrate (SUB-C) contains dimethyl sulfoxide, substrate solution	0.2 ml	1.2 ml
Substrate Buffer (SUB-D) contains buffer, <1% $MgCl_2$, <1% NaCl	20 ml	120 ml
Tray, evaluation sheet	1 of each	4 of each
Instructions for use, template	1 of each	1 of each
Kit Component 2 of 2 (store at –20°C)		
Amplification Mix A (AM-A GT MTBDRsl VER 2.0) contains buffer, specific primers, nucleotides, Taq polymerase	0.15 ml	4x 0.3 ml
Amplification Mix B (AM-B GT MTBDRsl VER 2.0) contains buffer, salts, dye	0.53 ml	4x 1.05 ml

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

Performance Characteristics

Diagnostic performance

The summary of the diagnostic performance characteristics of the **GenoType MTBDR*sl*** VER 2.0 is currently in process.

Analytical performance

Analytical specificity

The specificity of the **GenoType MTBDR*sl*** VER 2.0 is ensured by the accurate design of specific primers and probes which considers, among others, homology comparisons of the sequences published in gene databases, and by stringent reaction conditions.

The analytical specificity was determined with eight *M. tuberculosis* complex strains: *M. tuberculosis*, *M. africanum*, *M. bovis* BCG, *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, *M. canettii, M. microti*, and *M. pinnipedii* (all FLQ- and AG/CP-sensitive). The following 40 strains not detectable with the test system were also analyzed: *Bordetella pertussis, Corynebacterium* spec.*, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Mycobacterium abscessus, M. alvei, M. asiaticum, M. avium, M. celatum, M. chelonae, M. fortuitum, M. gastri, M. genavense, M. goodii, M. gordonae, M. haemophilum, M. immunogenum, M. interjectum, M. intermedium, M. intracellulare, M. kansasii, M. lentiflavum, M. mageritense, M. malmoense, M. marinum, M. mucogenicum, M. peregrinum, M. scrofulaceum, M. shimoidei, M. simiae, M. smegmatis, M. szulgai, M. triplex, M. ulcerans, M. xenopi, Nocardia* spec.*, Pseudomonas aeruginosa, Staphylococcus aureus,* and *S. pneumoniae.*

The eight *M. tuberculosis* complex isolates were correctly identified as FLQ- and AG/CP-sensitive MTBC strains. All other 40 isolates displayed invalid band patterns. Hence, an analytical specificity of 100% was achieved.

Analytical sensitivity

For determination of analytical sensitivity of the **GenoType MTBDR*sl*** for clinical samples, three BCG culture dilutions (FLQ- and AG/CP-sensitive, 1500, 150, and 15 bacteria/ml) were prepared in triplicate. Including a negative control, DNA was extracted using the **GenoLyse®** kit and analyzed with the **GenoType MTBDR*sl*** applying the "MDR DIR" PCR protocol. A limit of detection of 150 bacteria/ml was determined.

For determination of analytical sensitivity of the **GenoType MTBDR*sl*** for culture samples, three BCG culture dilutions (FLQ- and AG/CP-sensitive, 1.65×10^6, 1.65×10^5, and 1.65×10^4 bacteria/ml) were set up in triplicate. Including a negative control, DNA was extracted using the **GenoLyse®** kit and analyzed with the **GenoType MTBDR*sl*** applying the "MDR CUL" PCR protocol. A limit of detection of 1.65×10^5 bacteria/ml was determined.

Reproducibility

Intra-assay precision

In order to determine the intra-assay precision of the **GenoType MTBDR*sl***, three BCG culture dilutions (FLQ- and AG/CP-sensitive, one above, one at, and one below cutoff concentration) and one negative control were set up in quadruplicate and tested under identical conditions applying the "MDR DIR" PCR protocol. DNA extraction was performed using the **GenoLyse®** DNA extraction kit. All parallels showed identical and correct banding patterns and comparable signal strengths. Additionally, signal strengths between different sample dilutions were comparable. Hence, an intra-assay precision of 100% was achieved.

Inter-assay precision

In order to determine the inter-assay precision of the **GenoType MTBDR*sl***, three BCG culture dilutions (one above, one at, and one below cutoff concentration) and a negative control were tested at three different points of time. DNA extraction was performed using the **GenoLyse®** DNA extraction kit and the "MDR DIR" protocol was applied for PCR. Apart from the varied parameter, all other testing conditions were identical. No deviations were detected between parallel samples, that is between runs banding patterns were identical and correct, and signal strengths were comparable. Hence, the inter-assay precision was 100%.

Interfering substances

There are substances that may inhibit PCR reactions. Such inhibitors may, for example, originate from the culture medium. In order to assess if the medium influences the **GenoType MTBDR*sl***, 6 different *M. tuberculosis* complex samples (3x FLQ- and AG/CP-sensitive, 2x FLQ-sensitive and AG/CP-resistant, 1x FLQ- and AG/CP-resistant) were cultured in 4 different media (solid media: Loewenstein-Jensen, Stonebrink, and Middlebrook-7H10; liquid medium: MGIT (BD Diagnostics, Franklin Lakes, USA)). Subsequently, the culture samples were tested with the **GenoType MTBDR*sl*** applying the "MDR CUL" PCR protocol.

All *M. tuberculosis* complex samples showed the same correct results. Hence, it can be excluded that the tested media import inhibitors into the **GenoType MTBDR*sl***.

Interfering substances may also be carried over from the sample material. Hence, the substances indicated in table 1 were tested in order to assess a potential interference with the **GenoType MTBDR*sl***. Defined BCG culture dilutions above, at, and below the detection limit of clinical samples were spiked with various amounts of the potential inhibitors. From all samples, DNA extraction was performed using the **GenoLyse®** DNA extraction. Then the culture dilutions were tested with the **GenoType MTBDR*sl*** applying the "MDR DIR" protocol for PCR.

Table 1: Tested potential interferents of the **GenoType MTBDR*sl***

Substance/class	Description/active ingredient	Substance concentrations
Blood	Whole blood	2.5% v/v to 90% v/v
Blood	Hemoglobin	0.05% v/v to 13.5% v/v
Pus		0.5% v/v to 90% v/v

Interference of the **GenoType MTBDR*sl*** VER 2.0 (invalid test result) was observed in samples containing concentrations greater than 10% whole blood, 1% hemoglobin, and 2.5% pus.

Stability

Shelf life of the **GenoType MTBDR*sl*** test kit when stored as recommended: see box label.

Stability is determined according to DIN EN ISO 23640.

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Hain Lifescience GmbH
Hardwiesenstraße 1, 72147 Nehren, Germany
www.hain-lifescience.de, +49 (0) 74 73- 94 51- 0