GenoType MTBDR*plus*

VER 2.0

Instructions for Use

IFU-304A-02

CE

IVD for in vitro diagnostic use only

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HAIN LIFESCIENCE

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 β-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 facilities and medical laboratories.

Summary and Explanation

Tuberculosis is a bacterial infectious disease passed on by droplet infection. In 2010, there were an estimated 8.8 million incident cases of TB globally, and an estimated 1.1 million deaths occurred [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 2010, there were an estimated 650,000 cases of MDR-TB among the world's 12 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*** test 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 provided, (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. 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 material safety data sheets which can be downloaded from: www.hain-lifescience.com/products/msds.html

Quality Control

In order to validate 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 set-up. It is essential that all materials (such as pipette tips) coming in contact with the reagents are free from DNases.
A negative control sample for detection of possible contamination events containing water instead of DNA should be part of each test run; the respective test strip should show the bands CC and AC only.

Specimen Requirements

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 potentially infectious and must be handled accordingly (e.g. see [3] or [4]). Always wear suitable protective clothing and gloves. Samples from risk patients (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 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. 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. 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.

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. BACTEC, MB-Check) may be used. 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. For automated DNA extraction from patient specimens, also the **GenoXtract** instrument 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 described above were used for performance evaluation of the **GenoType MTBDR*plus*** 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.

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, stir AM-A and AM-B carefully. Pipette AM-A and AM-B only in a room free from contaminating DNA. The DNA sample should be added in a separate area.

Prepare for each sample:

After DNA extraction with **GenoLyse®**
- 10 µl AM-A (see Kit Component 2)
- 35 µl AM-B (see Kit Component 2)
- 5 µl DNA solution
  Final volume: 50 µl

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

Determine the number of samples to be amplified (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). Aliquot 45 µl in each of the prepared PCR tubes and add 5 or 10 µl water (see above) to one aliquot (negative control). Add 5 or 10 µl DNA in a separate area to each aliquot (except for negative control). Please note that the master mix needs to be prepared freshly each time.

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

		cultivated samples	clinical specimens
15 min	95°C	1 cycle	1 cycle
30 sec	95°C	10 cycles	20 cycles
2 min	65°C		
25 sec	95°C	20 cycles	30 cycles
40 sec	50°C		
40 sec	70°C		
8 min	70°C	1 cycle	1 cycle

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

Hybridization

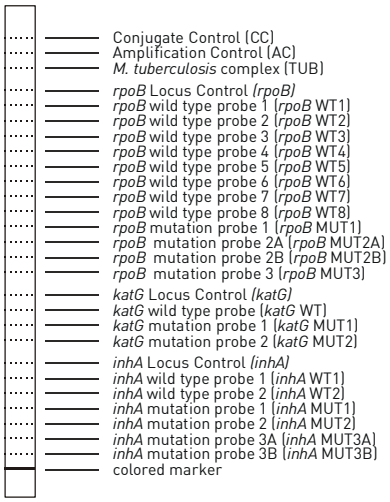
When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the preinstalled hybridization protocol to be used.
The following protocol describes the manual hybridization using a water bath 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 provided with 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. Not all bands of a strip have to show the same signal strength. 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. If this band is developed, the carry-over of amplification inhibitors and mistakes during setup and performance of the amplification reaction can be excluded.
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, however, the amplification reaction was performed correctly and the test 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 set-up, or carry-over 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 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 bacterium does not belong 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 signal of the Locus Control bands may be weak or even vanish totally. In rare cases, the *katG* locus may miss completely (including the Locus Control band). This indicates a resistance to INH of the strain tested. However, if the *rpoB* and/or the *inhA* loci are not developed, the test cannot be evaluated.

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 for 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 hence indicates a resistance of the tested strain to the respective antibiotic.
Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone (AC) are to be considered. The *rpoB* WT probe, however, represents an exception to this evaluation scheme: if the corresponding mutation band *rpoB* MUT3 is not developed, a signal at the *rpoB* WT8 band weaker than that of the AC is to be considered positive.
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.

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.
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 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.

Note the following special cases:
1. There is a possibility that the specimen tested contains a heteroresistant strain. In case of a heteroresistance, 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.

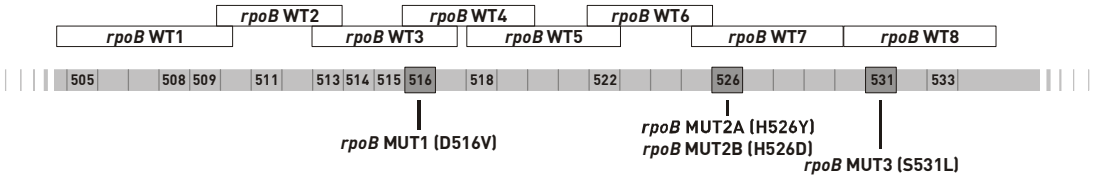


Figure 1: RMP resistance region of the *rpoB* gene
rpoB WT1-8: *rpoB* wild type probes; *rpoB* MUT1-3: *rpoB* mutation probes. The numbers specify the positions of the amino acids (codons) for all mutations listed in the table. The codons for which mutation probes were designed are highlighted.

Table 1: Mutations in the *rpoB* gene and the corresponding wild type and mutation bands (according to [7])

failing wild type band(s)	codons analyzed	developing mutation band	mutation
rpoB WT1	505-509		F505L
			T508A
			S509T
rpoB WT2	510-513		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

* This rare mutation has only been detected theoretically (in silico) yet. It is therefore possible that it cannot be detected in vitro.

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

failing wild type band	codon analyzed	developing mutation band	mutation
katG WT	315	*katG* MUT1	S315T1
		katG MUT2	S315T2

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

failing wild type band	analyzed nucleic acid position	developing mutation band	mutation
inhA WT1	–15	*inhA* MUT1	C15T
	–16	*inhA* MUT2	A16G
inhA WT2	–8	*inhA* MUT3A	T8C
		inhA MUT3B	T8A

Evaluation Examples

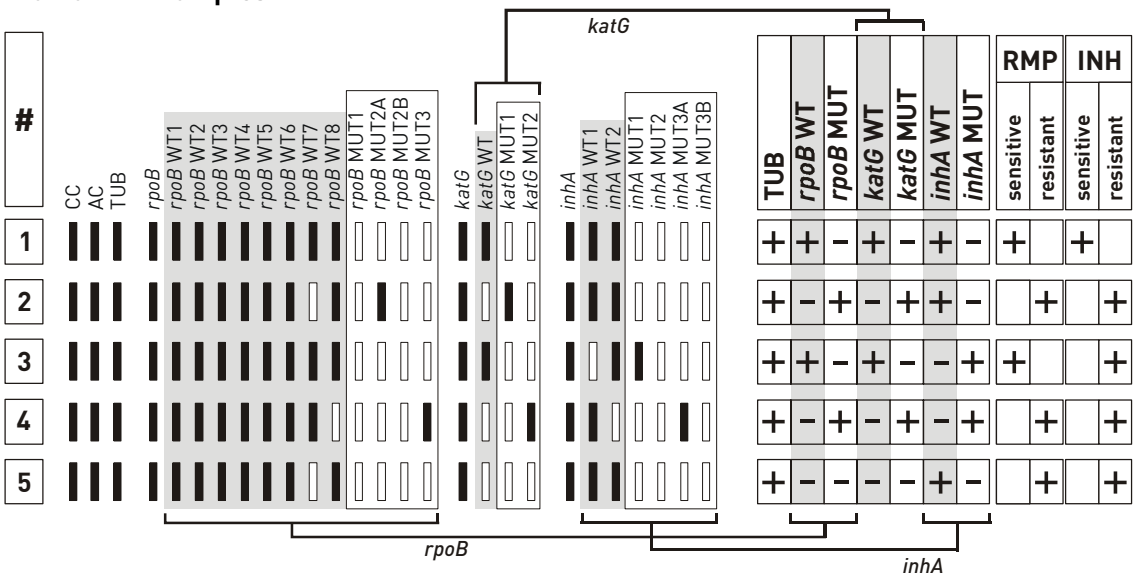


Figure 2: Examples for banding patterns and their evaluation with respect to RMP and/or INH resistance

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 display a coloration. If at least one of the mutation bands display 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 "+".
One of the *rpoB* and the *katG* wild type probes are missing in example 5; 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.

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. A silent mutation in codon 514 of the *rpoB* gene leading to the absence of the *rpoB* WT3 band was observed in rare cases [8]. Hence, if an RMP resistance is detected solely by a missing *rpoB* WT3 band, results of the phenotypical resistance determination should be considered.
In spite of an L533P mutation, the respective strain may still be RMP-sensitive. Hence, if the band WT8 is absent and the *rpoB* MUT3 band does not develop, results of the phenotypical resistance determination should be considered.

Additional mutations within the tested *rpoB* gene region causing RMP resistance have been published [9]. As these mutations are very rare, they were not accessible for validation purposes of this test system but were only detected in silico. An in silico detection, however, does not exclude the possibility that some of these mutations cannot be detected in vitro.
The **GenoType MTBDR*plus*** 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 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 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 pattern will also appear.

As any DNA detection method the test system on hand detects DNA from viable and nonviable bacteria. Therefore, the **GenoType MTBDR*plus*** may not be used for monitoring the progression or success of treatment of patients with antimicrobial therapy.
The **GenoType MTBDR*plus*** 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 *M. tuberculosis* complex cannot be differentiated.

The test only works within the limits of the genomic regions the primers and probes were chosen from.
As with 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 sub-types might not be detected. The test reflects the state of knowledge of Hain Lifescience.
Use of this assay is limited to qualified personnel well trained in the test procedure and familiar with molecular biological methods.

Performance evaluation of this assay was carried out using the **GenoLyse®** kit for DNA extraction from pulmonary smear-positive and smear-negative clinical specimens as well as cultivated samples and using the **GXT DNA/RNA Extraction Kit** for automated DNA extraction from 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 phenotypical resistance determination have to be considered in certain cases (see above).
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 phenotypical resistance determination 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 und AM-B) were mixed up, added in wrong amounts, or not mixed properly. Prepare a new master mix and repeat test.
- 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 and/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.
- Silent mutation in probe region (see chapter Limitations). Use alternative identification method.

Material Required but not Provided

- Absorbent paper
- Adjustable pipettes for 10, 20, 200, and 1000 µl
- Class II safety cabinet
- Disposable gloves
- DNA extraction kit (**GenoLyse®** or **GXT DNA/RNA Extraction Kit**, see chapter Ordering Information) as well as necessary equipment
- Disposable sterile pipette tips with filter
- 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 (heating rate: 3°C/sec, cooling rate: 2°C/sec, precision: +/–0.2°C)
- Timer
- Tweezers
- Water (molecular biology grade, for negative controls)

Kit Contents

Order no.	**304A**	**30496A**
Tests	**12**	**96**
Kit Component 1 of 2 (store at 2-8°C)		
Membrane strips coated with specific probes (MTBDRplus VER 2.0 STRIPS)	12	2x 48
Denaturation Solution (DEN) ***ready to use*** contains <2% NaOH, dye	0.3 ml	2x 1.2 ml
Hybridization Buffer (HYB) ***ready to use*** contains 8-10% anionic tenside, dye	20 ml	120 ml
Stringent Wash Solution (STR) ***ready to use*** contains >25% of a quaternary ammonium compound, <1% anionic tenside, dye	20 ml	120 ml
Rinse Solution (RIN) ***ready to use*** contains buffer, <1% NaCl, <1% anionic tenside	50 ml	3x 120 ml
Conjugate Concentrate (CON-C) ***concentrate*** 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) ***concentrate*** 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 MTBDRplus VER 2.0) contains buffer, nucleotides, Taq polymerase	0.15 ml	4x 0.3 ml
Amplification Mix B (AM-B GT MTBDRplus VER 2.0) contains salts, specific primers, dye	0.53 ml	4x 1.05 ml

Ordering Information

	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
GenoType MTBDR*plus* VER 2.0 and **GenoLyse®** (kit for analysis of 12 samples and kit for manual DNA extraction of 12 samples)	304AM
GenoType MTBDR*plus* VER 2.0 and **GenoLyse®** (kit for analysis of 96 samples and kit for manual DNA extraction of 96 samples)	30496AM
GenoType MTBDR*plus* VER 2.0 and **GXT DNA/RNA Extraction Kit** (kit for analysis of 96 samples and kit for automated DNA extraction of 96 samples using the **GenoXtract**)	30496AA
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 extraction of 96 samples using the **GenoXtract**)	12.01.02
GenoXtract (instrument for DNA extraction of up to 12 samples)	8.31.01

Performance Characteristics

Diagnostic performance

Pulmonary clinical specimens

Diagnostic performance characteristics of the **GenoType MTBDR*plus*** VER 2.0 were determined in a study [10] with 338 specimens (including sputum, bronchoalveolar lavages, and pleural aspirates) compared to culture (successful cultivation on Loewenstein-Jensen solid medium or in BACTEC MGIT 960 liquid medium and subsequent speciation using the **GenoType Mycobacterium CM** test) and phenotypic drug susceptibility testing (DST). Additionally, the samples were examined by microscopy. Clinical data of the patients were included in the evaluation.

The study site was located in a high MDR-TB burden country. Microscopy and cultivation methods were conducted on site. Aliquots of the NALC-decontaminated sputum specimens were shipped to a second laboratory to perform DNA extraction and the **GenoType MTBDR*plus***. Manual DNA extraction was performed with the **GenoLyse®** kit (162 of the 338 sputum samples), automated DNA extraction was carried out on the **GenoXtract** instrument using the **GXT DNA/RNA Extraction Kit** (176 of the 338 sputum samples) according to the respective instructions for use.

A congruent **GenoType MTBDR*plus*** positive, clinical positive result was defined either by positivity of culture and **GenoType MTBDR*plus*** or when only **GenoType MTBDR*plus*** was positive and culture negative, but TB was indicated by previous culture-based findings of the respective patient. A discrepant result (**GenoType MTBDR*plus*** positive and culture negative) does not exclude in all cases a TB infection of the patient as for some patients' histories of a probable TB infection were not available.

Table 1: Performance characteristics of the **GenoType MTBDR*plus*** for detection of MTBC from pulmonary clinical specimens compared to culture/**GenoType Mycobacterium CM** (GT Myco CM) and clinical findings

		smear-positive			smear-negative		
		culture/GT Myco CM and clinic		Sens: 100%	culture/GT Myco CM and clinic		Sens: 80.3%
		positive	negative	Spec: /*	positive	negative	Spec: 98.4%
GenoLyse®	**GenoType MTBDR*plus*** positive	39	0	PPV: 100%	49	1	PPV: 98.0%
	GenoType MTBDR*plus* negative	0	1	NPV: /*	12	60	NPV: 83.3%
		culture/GT Myco CM and clinic		Sens: 100%	culture/GT Myco CM and clinic		Sens: 78.3%
		positive	negative	Spec: /*	positive	negative	Spec: 96.0%
GXT	**GenoType MTBDR*plus*** positive	39	1	PPV: 97.5%	47	3	PPV: 94.0%
	GenoType MTBDR*plus* negative	1	0	NPV: /*	13	72	NPV: 84.7%

Sens, diagnostic sensitivity; Spec, diagnostic specificity; PPV, positive predictive value; NPV, negative predictive value

* no value due to low sample number

From 162 **GenoLyse®** and 176 **GXT** isolates, 78 were positive for MTBC detection with both culture and **GenoType MTBDR*plus***. Hence, these 78 MTBC-positive samples were used for evaluation of resistance detection.

Table 2: Performance characteristics of the **GenoType MTBDR*plus*** for detection of RMP resistance from pulmonary clinical specimens compared to culture/DST

		smear-positive			smear-negative		
		culture/DST		Sens: 100%	culture/DST		Sens: 96.0%
		RMP-R	RMP-S	Spec: 92.3%	RMP-R	RMP-S	Spec: 93.3%
GenoLyse®	**GenoType MTBDR*plus*** RMP-R	25	1	PPV: 96.2%	24	1	PPV: 96.0%
	GenoType MTBDR*plus* RMP-S	0	12	NPV: 100%	1	14	NPV: 93.3%
		culture/DST		Sens: 96.3%	culture/DST		Sens: 86.2%
		RMP-R	RMP-S	Spec: 100%	RMP-R	RMP-S	Spec: 100%
GXT	**GenoType MTBDR*plus*** RMP-R	26	0	PPV: 100%	25	0	PPV: 100%
	GenoType MTBDR*plus* RMP-S	1	12	NPV: 92.3%	4	10	NPV: 71.4%

Sens, diagnostic sensitivity; Spec, diagnostic specificity; PPV, positive predictive value; NPV, negative predictive value; RMP-R, resistant to rifampicin; RMP-S, sensitive to rifampicin

Table 3: Performance characteristics of the **GenoType MTBDR*plus*** for detection of INH resistance from pulmonary clinical specimens compared to culture/DST

		smear-positive			smear-negative		
		culture/DST		Sens: 96.7%	culture/DST		Sens: 96.7%
		INH-R	INH-S	Spec: 87.5%	INH-R	INH-S	Spec: 90.0%
GenoLyse®	**GenoType MTBDR*plus*** INH-R	29	1	PPV: 96.7%	29	1	PPV: 96.6%
	GenoType MTBDR*plus* INH-S	1	7	NPV: 87.5%	1	9	NPV: 90.0%
		culture/DST		Sens: 100%	culture/DST		Sens: 90.6%
		INH-R	INH-S	Spec: 100%	INH-R	INH-S	Spec: 71.4%
GXT	**GenoType MTBDR*plus*** INH-R	28	0	PPV: 100%	29	2	PPV: 93.5%
	GenoType MTBDR*plus* INH-S	0	11	NPV: 100%	3	5	NPV: 62.5%

Sens, diagnostic sensitivity; Spec, diagnostic specificity; PPV, positive predictive value; NPV, negative predictive value; INH-R, resistant to isoniazid; INH-S, sensitive to isoniazid

Cultured material

The diagnostic performance characteristics of the **GenoType MTBDR*plus*** were determined in a study with 74 cultured samples compared to **GenoType Mycobacterium CM** and phenotypic drug susceptibility testing (DST). The study site was located in a low MDR-TB burden country. Manual DNA extraction was performed using the **GenoLyse®** kit according to the instructions for use. From 74 cultures, 49 were positive for *M. tuberculosis* complex (MTBC) and 25 cultures showed growth of nontuberculous mycobacteria. Hence, for resistance detection in cultured material, 49 isolates were available.

Table 4: Performance characteristics of the **GenoType MTBDR*plus*** for detection of MTBC from cultured material compared to culture/**GenoType Mycobacterium CM** (GT Myco CM)

		culture/GT Myco CM		Sens: 100%
		positive	negative	Spec: 100%
GenoType MTBDR*plus*	positive	49	0	PPV: 100%
	negative	0	25	NPV: 100%

Sens, diagnostic sensitivity; Spec, diagnostic specificity; PPV, positive predictive value; NPV, negative predictive value

Table 5: Performance characteristics of the **GenoType MTBDR*plus*** for detection of RMP resistance from cultured material compared to culture/DST

		culture/DST		Sens: /*
		RMP-R	RMP-S	Spec: 100%
GenoType MTBDR*plus*	RMP-R	0	0	PPV: /*
	RMP-S	0	49	NPV: 100%

Sens, diagnostic sensitivity; Spec, diagnostic specificity; PPV, positive predictive value; NPV, negative predictive value; RMP-R, resistant to rifampicin; RMP-S, sensitive to rifampicin

* no value due to low sample number

Table 6: Performance characteristics of the **GenoType MTBDR*plus*** for detection of INH resistance from cultured material compared to culture/DST

		culture/DST		Sens: /*
		INH-R	INH-S	Spez: 100%
GenoType MTBDR*plus*	INH-R	3	0	PPV: /*
	INH-S	0	46	NPV: 100%

Sens, diagnostic sensitivity; Spec, diagnostic specificity; PPV, positive predictive value; NPV, negative predictive value; INH-R, resistant to isoniazid; INH-S, sensitive to isoniazid

* no value due to low sample number

Analytical performance

Analytical specificity

The specificity of the **GenoType MTBDR*plus*** test 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 60 DNA isolates including the following MTBC strains: *M. tuberculosis*, *M. africanum*, *M. bovis, M. canettii,* and *M. microti* (RMP- and INH-sensitive). The following strains not detectable with the test system were analyzed: *Actinomyces naeslundii, Aggregatibacter actinomycetemcomitans, Bacillus cereus, Corynebacterium ammoniagenes, C. bovis, C. durum, Escherichia coli, Gordona rubropertinctus, Klebsiella oxytoca, Mycobacterium abscessus, M. alvei, M. asiaticum, M. avium, M. celatum, M. chelonae, M. chimaera, M. fortuitum* (2 sequevars), *M. frederiksbergense, M. gastri, M. genavense, M. goodii, M. gordonae, M. heckeshornense, M. immunogenum, M. interjectum, M. intermedium, M. intracellulare, M. lentiflavum, M. marinum, M. mucogenicum, M. palustre, M. peregrinum, M. scrofulaceum, M. shimoidei, M. simiae, M. smegmatis, M. szulgai, M. triplex, M. ulcerans, M. xenopi,* MRSA, *Nocardia abscessus, N. africana, N. amarae, N. asteroides, N. farcinica, Porphyromonas gingivalis, Prevotella intermedia, Rhodococcus erythropolis, Saccharomonospora glauca, Tannerella forsythia, Treponema denticola, Tsukamurella inchonensis, T. pulmonis.*

The five MTBC isolates were correctly identified as RMP- and INH-sensitive MTBC strains. All other 55 isolates displayed no TUB band and no evaluable band pattern for RMP and INH resistances. Hence, an analytical specificity of 100% was achieved.

Analytical sensitivity

For determination of analytical sensitivity of the **GenoType MTBDR*plus***, ten replicates of MTBC-negative sputum samples were spiked with four BCG culture dilutions (1000, 500, 200, 160, and 100 bacteria/ml, respectively), extracted once using the **GenoLyse®** kit and once using the **GXT DNA/RNA Extraction Kit**, and analyzed with the **GenoType MTBDR*plus***. A limit of detection of 160 bacteria/ml was determined with both extraction methods.

Reproducibility

Intra-assay precision

In order to determine the intra-assay precision of the **GenoType MTBDR*plus***, two BCG cultures (RMP- and INH-sensitive, 1,500 and 150 bacteria/ml, respectively) and an *M. avium* culture (10,000 bacteria/ml) were set up in triplicate and, including a contamination control, tested under identical conditions. DNA extraction was performed once using the **GenoLyse®** DNA extraction kit, and once using the **GXT DNA/RNA Extraction Kit**. All parallels showed identical banding patterns and comparable signal strengths. Additionally, signal strengths between the two DNA extraction methods and between different dilutions of the same samples 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*plus***, two BCG cultures (RMP- and INH-sensitive, 1,500 and 150 bacteria/ml, respectively) and an *M. avium* culture (10,000 bacteria/ml) were set up in triplicate and, including a contamination control, tested in nine runs: on three different days, using three different sets of instruments, and conducted by three different operators. DNA extraction was performed once using the **GenoLyse®** DNA extraction kit and once using the **GXT DNA/RNA Extraction Kit**. 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 signal strengths were comparable. Moreover, signal strengths were comparable between different DNA extraction methods and different bacterial concentrations. 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*plus***, 6 different *M. tuberculosis* complex samples (4 RMP- and INH-resistant, 2 RMP- and INH-sensitive) were cultured in 4 different media (solid media: Loewenstein-Jensen, Stonebrink, and Middlebrook-7H10, liquid medium: MGIT (Becton Dickinson)). Then the culture samples were tested with the **GenoType MTBDR*plus***.
All *M. tuberculosis* complex samples showed the same results. Hence, it can be excluded that the tested media import inhibitors into the **GenoType MTBDR*plus*** test.

Stability
Stability is determined according to DIN EN 13640.
Shelf life of the **GenoType MTBDR*plus*** test kit when stored as recommended: see box label.

References

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Important Changes in IFU-304A-02

Chapter	Change
Performance Characteristics	The summary of diagnostic performance characteristics of the **GenoType MTBDR*plus*** VER 2.0 for patient specimens has been updated.

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