GenoType NTM-DR
VER 1.0

Instructions for Use
IFU-297-01

for in vitro diagnostic use only

09/2015
**GenoType NTM-DR**  
**Molecular Genetic Assay for Detection of Resistance to Macrolides and Aminoglycosides in various Nontuberculous Mycobacterial Species (NTM) from Cultured Material**

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 NTM-DR is a qualitative in vitro test for detection of antibiotic resistance in members of the *Mycobacterium avium* complex, the *Mycobacterium abscessus* complex, and *Mycobacterium chelonae*. The *M. avium* complex includes the species *M. avium*, *M. intracellulare*, and *M. chimaera*. The *M. abscessus* complex includes the subspecies *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *ballanti*, and *M. abscessus* subsp. *massiliense*.  

Antibiotic resistances detectable by this test are resistances to macrolides (clarithromycin, azithromycin) and aminoglycosides (kanamycin, amikacin, gentamicin). Macrolide resistance is identified (i) by the characterization of a resistance-associated part of the *erm*(41) gene (coding for an erythromycin ribosome methyltransferase only present in members of the *M. abscessus* complex) [1], and (ii) by examining the most common resistance-associated mutations of the *rrl* gene (coding for the 23S rRNA) [2]. For detection of aminoglycoside resistance, the most significant resistance-associated mutations of the *rrs* gene (coding for the 16S rRNA) [3-5] are examined. The test is indicated as an aid for diagnosis and intended for use in medical laboratories.

**Summary and Explanation**

Mycobacterioses are infectious diseases caused by bacteria of the genus *Mycobacterium*. The most significant is tuberculosis (TB) caused by the members of the *Mycobacterium tuberculosis* complex. The genus *Mycobacterium* comprises numerous species which are divided into three groups: (i) the *Mycobacterium tuberculosis* complex, (ii) *M. leprae* causing leprosy, and (iii) atypical or nontuberculous mycobacteria (NTM). In view of the varying pathogenicity and apathogenicity of some species, a fast and certain identification of the *M. tuberculosis* complex and hence its differentiation from the NTMs is most essential. NTM can cause chronic mycobacterioses. Infectiousness and symptoms vary in a broad range and depend both on the pathogen as well as on the immunocompetence of the person affected [6]. The most common mycobacterioses of patients suffering from cystic fibrosis or chronic pulmonary diseases are caused by *M. avium* and members of the *M. abscessus* complex [7] and are often poorly curable due to various resistances. Macrolides are important components of the therapy; however, resistances may limit treatment options. One potential resistance mechanism is mediated by mutations within the *rrl* gene, particularly through substitutions of single nucleotides at positions 2058 and 2059. A mechanism of resistance that solely affects members of the *M. abscessus* complex is mediated by the gene *erm*(41). Thus, macrolide resistance arises either due to a resistance-mediating mutation in the *rrl* gene or when a functional *erm*(41) gene harbors a T at position 28. Macrolide sensitivity arises if gene *erm*(41) shows a deletion or harbors a C at position 28, provided there is no resistance-mediating mutation in the *rrl* gene. Moreover, the subspecies of *M. abscessus* complex are differentiated on the basis of the *erm*(41) gene variants described [1].  

Aminoglycosides are another important element of therapy. Aminoglycoside resistance of mycobacteria is primarily caused by a modification of the ribosomal 305 subunit. For example, in *M. abscessus* subsp. *abscessus* mutations at position 1408 of the *rrs* gene lead to amikacin and kanamycin resistance.  

As long as no resistance is verified, use of inadequate and hence ineffective antibiotics may lead to further spread of resistant bacteria and amplification of resistance. Therefore, rapid diagnosis and determination of the resistance status is a prerequisite for appropriate treatment.

**Principles of the Procedure**

The GenoType NTM-DR test is based on the DNA-STRIP technology. The whole procedure is divided into three steps: (i) DNA extraction from 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:

Hybridization Buffer [HYB] and Substrate Concentrate [SUB-C] are not classified as hazardous. Due to their ingredients, however, hazard statement EUH210 applies: Safety data sheet available on request.

- Denaturation Solution [DEN] contains >2% sodium hydroxide.
- Warning!
  - H315: Causes skin irritation.
  - H319: Causes serious eye irritation.
  - P280: Wear protective gloves/protective clothing/eye protection.
  - P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
  - P313: Get medical advice/attention.

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 5 control zones:

- a Conjugate Control zone (CC) to check the binding of the conjugate on the strip and a correct chromogenic reaction
- a Universal Control zone (UC) which detects, as far as is known, all mycobacteria and members of the group of gram-positive bacteria with a high G+C content
- three Locus Control zones (erm[41], rrl, rrs) 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 band only.

**Specimen Requirements**

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. 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 [8] or [9]). 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” [10], the “Clinical Microbiology Procedures Handbook” [11], 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 [12,13]. 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” [10]. After decontamination, the cell pellet should be resuspended in a maximum of 1 to 1.5 ml of phosphate buffer. 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

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. The working area must be free from contaminating DNA.

For DNA extraction, the GenoLyse® kit (see chapter Ordering Information) is used according to protocol A. For handling instructions, please refer to the respective instructions for use.

The method described above was used for performance evaluation of the GenoType NTM-DR 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, 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 Cul".

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>95</td>
</tr>
<tr>
<td>22</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>46</td>
<td>95</td>
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<tr>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td>8</td>
<td>70</td>
</tr>
</tbody>
</table>

Heating rate: ≤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/Twin Cubator 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/Twin Cubator.

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 bands CC and UC with the respective lines on the sheet. The supplied template also serves as an aid for evaluation and must be aligned with the bands CC and UC, as well as – separately for each present locus – with the respective Locus Control band. Determine the species with the help of the interpretation chart and the name of the identified species in the respective column. 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 24 reaction zones (see figure).

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Conjugate Control (CC)
Universal Control (UC)
SP1
SP2
SP3
SP4
SP5
SP6
SP7
SP8
SP9
SP10
erm (41) Locus Control (erm41)
erm (41) T28
rrl Locus Control (rrl)
rrl wild type probe (rrl WT)
rrl mutation probe 1 (rrl MUT1)
rrl mutation probe 2 (rrl MUT2)
rrl mutation probe 3 (rrl MUT3)
rrl mutation probe 4 (rrl MUT4)
rrs Locus Control (rrs)
rrs wild type probe (rrs WT)
rrs mutation probe 1 (rrs MUT1)
colored marker

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.

Universal Control (UC)
This zone detects, as far as is known, all mycobacteria and members of the group of gram-positive bacteria with a high G+C content. If this zone and the Conjugate Control zone stain positive but the remaining banding pattern cannot be assigned to a specific mycobacterium, additional methods have to be applied to identify the respective bacterial species.

Species-specific probes (SP1-SP10)
Specific probes, for evaluation see interpretation chart.

Locus Controls (erm(41), rrl, and rrs)
The Locus Control zones detect a gene region specific for the respective locus. Since the erm(41) locus is only present in members of the M. abscessus complex, the erm(41) probe will only stain positive for members of this complex. In case of a positive test result (evaluable wild type or mutation banding pattern), the signals of the Locus Control bands may be weak.

erm(41)
The erm(41) gene is examined for detection of resistance to macrolides (clarithromycin or azithromycin) and is only present in members of the M. abscessus complex.

The erm(41) C28 probe detects a genotype that carries a C at position 28 of the erm(41) gene. When the erm(41) C28 probe stains positive, this indicates that the tested strain is sensitive to macrolides (except for strains with an additional rrl mutation) [1].

The erm(41) T28 probe detects a genotype that carries a T instead of a C at position 28 of the erm(41) gene. When the erm(41) T28 probe stains positive, this indicates that the tested strain is resistant to macrolides [1].

The probes erm(41) C28 and erm(41) T28 are only relevant for M. abscessus subsp. abscessus and M. abscessus subsp. bolletii, but not for M. abscessus subsp. massiliense. Due to deletions in the erm(41) gene of M. abscessus subsp. massiliense the gene is nonfunctional, leading to macrolide sensitivity in spite of a developed erm(41) T28 band (except for strains with an additional rrl mutation) [1].

rrl
The rrl gene is examined for detection of resistance to macrolides (clarithromycin or azithromycin).

The wild type probe comprises the most important resistance region of the rrl gene (see table 1). 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 1).

Each pattern deviating from the wild type pattern (see evaluation example 1) indicates, as far as is known, a macrolide resistance of the tested strain.
Table 1: Mutations in the \textit{rrl} gene and the corresponding wild type and mutation bands [2]

<table>
<thead>
<tr>
<th>Failing wild type band</th>
<th>Codons analyzed</th>
<th>Developing mutation band</th>
<th>Mutation</th>
<th>Phenotypic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{rrl} WT</td>
<td>2058-2059</td>
<td>\textit{rrl} MUT1</td>
<td>A2058C</td>
<td>Macrolides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{rrl} MUT2</td>
<td>A2058G</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>A2058T</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{rrl} MUT3</td>
<td>A2059C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{rrl} MUT4</td>
<td>A2059G</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>A2059T</td>
<td></td>
</tr>
</tbody>
</table>

\textit{rrs}

The \textit{rrs} gene is examined for detection of resistance to aminoglycosides (kanamycin, amikacin, gentamicin). The wild type probe comprises the most important resistance region of the \textit{rrs} 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 probe detects the most common resistance-mediating mutation (see table 2). Each pattern deviating from the wild type pattern (see evaluation example 1) indicates, as far as is known, an aminoglycoside resistance of the tested strain.

Table 2: Mutations in the \textit{rrs} gene and the corresponding wild type and mutation bands [3-5]

<table>
<thead>
<tr>
<th>Failing wild type band</th>
<th>Codons analyzed</th>
<th>Developing mutation band</th>
<th>Mutation</th>
<th>Phenotypic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{rrs} WT</td>
<td>1406-1409</td>
<td>\textit{rrs} MUT1</td>
<td>A1408G</td>
<td>Aminoglycosides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>T1406A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>C1409T</td>
<td></td>
</tr>
</tbody>
</table>

Please note:

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

Identification of macrolide resistance mediated by the \textit{erm}(41) gene is only possible for members of the \textit{M. abscessus} complex. If the strain investigated does not belong to the \textit{M. abscessus} complex, the entire \textit{erm}(41) gene locus (all bands including the Locus Control band) is absent.

In case the entire \textit{rrl} and/or \textit{rrs} gene locus (all bands including the Locus Control band) is missing while at least one species-specific band (SP1-SP10) is developed, the test result is invalid.

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 mycobacterial 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.

Additional mycobacteria species can be identified with the \textit{GenoType Mycobacterium CM} and the \textit{GenoType Mycobacterium AS}. 

Additional comments and considerations:

- The use of universal control zones (UC) is essential for consistency and reliability.
- The presence of more than one band in a wild type pattern can indicate the presence of heteroresistant strains.
- The absence of a band in a mutation pattern can indicate the presence of a wild type strain.
- The combination of wild type and mutation bands provides a comprehensive view of the resistance profile.
- The \textit{rrs} gene and \textit{erm}(41) gene are key targets for identifying aminoglycoside resistance in mycobacteria.
- The identification of specific bands (SP1-SP10) is crucial for accurate species identification.
- The test results should be interpreted with caution, considering the potential for false positives and false negatives.
- The use of standardized procedures and quality control measures is essential for maintaining the accuracy and reliability of the test results.
### Interpretation Chart

<table>
<thead>
<tr>
<th>M. avium</th>
<th>M. intracellulare</th>
<th>M. chimaera</th>
<th>M. abscessus subsp. abscessus</th>
<th>M. abscessus subsp. massiliense</th>
<th>M. abscessus subsp. bovis</th>
<th>other mycobacterial species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band No. 1 (CC): Conjugate Control</td>
<td>Band No. 2 (UC): Universal Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Band Staining:**
- **Staining:** Black
- **Facultative Staining:** Gray
- **No Staining:** White

**Notes:**
1. Please note the special criteria for resistance detection in *M. abscessus* subsp. *massiliense* (see evaluation examples 4 and 5).
2. Species may be identified with the GenoType Mycobacterium CM and the GenoType Mycobacterium AS.

### Evaluation Examples

**Figure 1:** Examples for banding patterns and their evaluation with respect to macrolide (MA) and/or aminoglycoside (AG) resistance.
If the wild type band displays a signal, this is classified as positive and marked in the WT column of the respective gene as “+”. If the wild type band 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 “+”. In the resistance columns a “+” is assigned only if at least one entry in the WT and MUT columns or the “erm(41) C28” and “erm(41) T28” columns deviates from the wild type pattern depicted in example 1.

Below, the examples shown above are explicated:

Example 1 shows the wild type banding pattern of M. abscessus subsp. abscessus. All wild type probes and “erm(41) C28” but none of the mutation probes display a signal; hence, the evaluation chart shows a “−” in the “erm(41) C28” column and in the two wild type columns and a “−” in the “erm(41) T28” column and in the two mutation columns. Accordingly, no entry is made in the resistance columns.

Example 2 shows a possible banding pattern of M. abscessus subsp. bolletii. The “erm(41) C28” band is missing and the “erm(41) T28” band is developed. Hence, the evaluation chart shows a “−” in the “erm(41) C28” column and a “+” in the “erm(41) T28” column. The rrl wild type band is missing, and the mutation band “rrl MUT2” is developed; hence, the field in the “rrl WT” column is marked with a “−”, the field in the “rrl MUT” column is marked with a “+”, and the strain is evaluated as resistant to macrolides. Finally, the rrs wild type band 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 “+”; hence, an aminoglycoside resistance is detected.

Example 3 shows the wild type banding pattern of M. avium. The “erm(41)” locus is missing as is correct; hence, no entries are made in the respective columns. The rrl locus and the rrs locus display the wild type banding pattern resulting in a wild type entry as in example 1. The strain is evaluated as sensitive to macrolides and aminoglycosides.

Example 4 shows a possible banding pattern of M. abscessus subsp. massiliense. The “erm(41) C28” band is missing and the “erm(41) T28” band is developed. Hence, the evaluation chart shows a “−” in the “erm(41) C28” column and a “+” in the “erm(41) T28” column. Since the probes erm(41) C28 and erm(41) T28 are, however, not relevant for the evaluation of a phenotypic resistance to macrolides in M. abscessus subsp. massiliense [see chapter Evaluation and Interpretation of Results], there must be an additional mutation in the rrl gene to evaluate the strain as resistant to macrolides. Here, the rrl wild type band is present, and no mutation band is developed; hence, the field in the “rrl WT” column is marked with a “+”; the field in the “rrl MUT” column is marked with a “−”, and the strain is evaluated as sensitive to macrolides. The rrs locus displays the wild type banding pattern which is depicted accordingly.

Example 5 shows a possible banding pattern of M. abscessus subsp. massiliense. The “erm(41) C28” band is missing and the “erm(41) T28” band is developed. Hence, the evaluation chart shows a “−” in the “erm(41) C28” column and a “+” in the “erm(41) T28” column. Since the probes erm(41) C28 and erm(41) T28 are, however, not relevant for the evaluation of a phenotypic resistance to macrolides in M. abscessus subsp. massiliense [see chapter Evaluation and Interpretation of Results], there must be an additional mutation of the rrl gene to evaluate the strain as resistant to macrolides. Here, the rrl wild type band is actually missing, and the mutation band “rrl MUT3” is developed; hence, the field in the “rrl WT” column is marked with a “−”, the field in the “rrl MUT” column is marked with a “+”, and the strain is evaluated as resistant to macrolides. The rrs locus displays the wild type banding pattern which is depicted accordingly.

Example 6 shows a possible banding pattern of M. chelonae. The “erm(41)” locus is missing as is correct; hence, no entries are made to the respective columns. The rrl wild type band is missing and the mutation band “rrl MUT1” is developed; hence, the field in the “rrl WT” column is marked with a “−”, the field in the “rrl MUT” column is marked with a “−”, and the tested strain is evaluated as resistant to macrolides. Finally, the rrs wild type band 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 an aminoglycoside resistance is detected.

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 NTM-Dx only detects those resistances that have their origins in the rrl, rrs and rrl regions examined here. Resistances originating from mutations of other genes or gene regions as well as other macrolide and aminoglycoside resistance mechanisms will not be detected by this test.

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 NTM-Dx may not be used for monitoring the progression or success of treatment of patients with antimicrobial therapy.

The GenoType NTM-Dx 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 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 subtypes might not be detected.

Performance evaluation of this assay was carried out using the Genolyse® kit for DNA extraction 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
- DNA extraction kit [GenaLyse®, see chapter Ordering Information] as well as necessary equipment
- Disposable sterile pipette tips with filter
- Graduated cylinder
- PCR tubes, DNase- and RNase- free
- Reagents for cultivation of mycobacteria as well as necessary equipment
- Reagents for sample decontamination 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

<table>
<thead>
<tr>
<th>Kit Component 1 of 2</th>
<th>Order no.</th>
<th>Tests</th>
<th>29712</th>
<th>29796</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane strips coated with specific probes (NTM-DR STRIPS)</td>
<td></td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation Solution (DEN) contains &lt;2% NaOH, dye</td>
<td></td>
<td></td>
<td>0.3 ml</td>
<td>2x 1.2 ml</td>
</tr>
<tr>
<td>Hybridization Buffer (HYB) contains &lt;10% anionic tenside, dye</td>
<td></td>
<td></td>
<td>20 ml</td>
<td>120 ml</td>
</tr>
<tr>
<td>Stringent Wash Solution (STR) contains &gt;25% of a quaternary ammonium compound, &lt;1% anionic tenside, dye</td>
<td></td>
<td></td>
<td>20 ml</td>
<td>120 ml</td>
</tr>
<tr>
<td>Rinse Solution (RIN) contains buffer, &lt;1% NaCl, &lt;1% anionic tenside</td>
<td></td>
<td></td>
<td>50 ml</td>
<td>3x 120 ml</td>
</tr>
<tr>
<td>Conjugate Concentrate (CDN-C) contains streptavidin-conjugated alkaline phosphatase, dye</td>
<td></td>
<td></td>
<td>0.2 ml</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, &lt;1% NaCl</td>
<td></td>
<td></td>
<td>20 ml</td>
<td>120 ml</td>
</tr>
<tr>
<td>Substrate Concentrate (SUB-C) contains &lt;70% dimethyl sulfoxide, &lt;10% 4-nitro blue tetrazolium chloride, &lt;10% 5-bromo-4-chloro-3-indolyl phosphate</td>
<td></td>
<td></td>
<td>0.2 ml</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>Substrate Buffer (SUB-D) contains buffer, &lt;1% MgCl₂, &lt;1% NaCl</td>
<td></td>
<td></td>
<td>20 ml</td>
<td>120 ml</td>
</tr>
<tr>
<td>Tray, evaluation sheet</td>
<td></td>
<td></td>
<td>1 of each</td>
<td>4 of each</td>
</tr>
<tr>
<td>Instructions for use, template</td>
<td></td>
<td></td>
<td>1 of each</td>
<td>1 of each</td>
</tr>
</tbody>
</table>

### Kit Component 2 of 2

<table>
<thead>
<tr>
<th>Kit Component 2 of 2</th>
<th>Order no.</th>
<th>Tests</th>
<th>29712</th>
<th>29796</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification Mix A (AM-A GT NTM-DR) contains buffer, nucleotides, Taq polymerase</td>
<td></td>
<td></td>
<td>0.15 ml</td>
<td>4x 0.3 ml</td>
</tr>
<tr>
<td>Amplification Mix B (AM-B GT NTM-DR) contains salts, specific primers, dye</td>
<td></td>
<td></td>
<td>0.53 ml</td>
<td>4x 1.05 ml</td>
</tr>
</tbody>
</table>

### Ordering Information

| GenoType NTM-DR (kit for analysis of 12 samples) | Order no. | 29712 |
| GenoType NTM-DR (kit for analysis of 96 samples) | Order no. | 29796 |
| GenoLyse® (kit for manual DNA extraction of 12 samples) | Order no. | 51612 |
| GenoLyse® (kit for manual DNA extraction of 96 samples) | Order no. | 51610 |
Performance Characteristics
For the performance evaluation of the GenoType NTM-DR the test was carried out as described in the instructions for use.

Diagnostic performance
Diagnostic performance characteristics of the GenoType NTM-DR were determined in a study with 96 samples. The test panel consisted of 88 strains identifiable with the GenoType NTM-DR test kit (covering all species identifiable with the test system), and 8 strains not identifiable with the test kit. DNA extraction from cultured material was performed with the GenoLyse® kit according to the respective instructions for use. For evaluation of species identification, all samples were characterized by the GenoType NTM-DR, by sequencing, and by the GenoType Mycobacterium CM VER 1.0. One sample contained a mixed culture of two identifiable strains. This sample was excluded from evaluation. The other 95 isolates showed correct results. For evaluation of resistance detection, the GenoType NTM-DR was compared to phenotypic drug susceptibility testing (DST). All 87 isolates identifiable with the kit showed correct results.

Table 1: Performance characteristics of the GenoType NTM-DR for species identification from cultured material compared to sequencing/GenoType Mycobacterium CM VER 1.0 (GT Myco CM)

<table>
<thead>
<tr>
<th>GenoType NTM-DR</th>
<th>Sequencing/GenoType Mycobacterium CM VER 1.0</th>
<th>Diagnostic sensitivity: 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>diagnostic specificitv: 100%</td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>87 0</td>
<td>Positive predictive value: 100%</td>
</tr>
<tr>
<td>negative</td>
<td>0 8</td>
<td>Negative predictive value: 100%</td>
</tr>
</tbody>
</table>

Table 2: Performance characteristics of the GenoType NTM-DR for detection of clarithromycin resistance from cultured material compared to DST

<table>
<thead>
<tr>
<th>GenoType NTM-DR</th>
<th>DST</th>
<th>Diagnostic sensitivity: 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>resistant</td>
<td>20 0</td>
<td>Diagnostic specificity: 100%</td>
</tr>
<tr>
<td>sensitive</td>
<td>0 67</td>
<td>Positive predictive value: 100%</td>
</tr>
</tbody>
</table>

Table 3: Performance characteristics of the GenoType NTM-DR for detection of amikacin/tobramycin resistance from cultured material compared to DST

<table>
<thead>
<tr>
<th>GenoType NTM-DR</th>
<th>DST</th>
<th>Diagnostic sensitivity: 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>resistant</td>
<td>5 0</td>
<td>Diagnostic specificity: 100%</td>
</tr>
<tr>
<td>sensitive</td>
<td>0 82</td>
<td>Positive predictive value: 100%</td>
</tr>
</tbody>
</table>

Analytical performance
Analytical specificity
The specificity of the GenoType NTM-DR 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 of the GenoType NTM-DR was determined with DNA isolates of the following Mycobacterium species identifiable by this test: M. abscessus subsp. abscessus, M. abscessus subsp. bovis, M. chelonae, M. chimaera, M. intracellularare, M. abscessus subsp. massiliense. Furthermore, the following strains not detectable with the test system were analyzed: Actinomyces naeslundii, Aggregatibacter actinomycetemcomitans, Bacillus cereus, Bacteroides pertussis, Corynebacterium amycolatum, C. jeikeium, C. minutissimum, C. spec., C. ulcerans, Escherichia coli, Gordonia rubra, Pseudomonas aeruginosa, Klebsiella oxytoca, K. pneumoniae, Mycobacterium africanum, M. alvei, M. asiaticum, M. bovis, M. tuberculosis, M. fortuitum, M. frederiksenii, M. gai, M. genavense, M. goodie, M. gordonae, M. haemophilum, M. heckehornense, M. immunogenum, M. interjectum, M. kansasi, M. lentiflavum, M. mageritense, M. malmoense, M. marinum, M. mucogenicum, M. palustre, M. peregrinum, M. pinnipedii, M. scrofulaceum, M. shimoidei, M. smegmatis, M. szulgai, M. triple, M. tuberculosis, M. ulcerans, M. xenopi, MRS, Nocardia asteroides, N. brasiliensis, N. farcinica, N. spec., Porphyromonas gingivalis, Prevotella intermedia, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Tannerella forsythia, Treponema denticola, Tsukamurella inklonis, T. pulmonis.
All species identifiable with the test were correctly identified. All other isolates not detectable with the test system displayed no specific banding pattern for species identification and no evaluable banding pattern for macrolide and aminoglycoside resistances. Hence, an analytical specificity of 100% was achieved.

Analytical sensitivity [Limit of Detection, LoD]
Analytical sensitivity of the GenoType NTM-DR was determined using culture dilutions of M. abscessus subsp. abscessus. Each culture dilution was prepared in 20 parallels. DNA was extracted using the GenoLyse® kit and analyzed with the GenoType NTM-DR applying the "MDR CUL" PCR protocol. A limit of detection (LoD) lowest bacterial concentration that generates a positive test result with a confidence of 95% of 3.3x10^9 bacteria/ml was determined.

Reproducibility
Intra-assay precision
In order to determine the intra-assay precision of the GenoType NTM-DR, the same samples as described for the intra-assay precision were applied. These samples and a negative control were tested in nine runs on three different days, using three different sets of instruments, and conducted by three different operators. DNA extraction was performed using the GenoLyse® DNA extraction kit. All strains showed the expected signals and the negative controls were negative. No deviations were detected within the parallels, the banding patterns were identical and the signals strengths were comparable. Hence, the intra-assay precision was 100%.

Inter-assay precision
In order to determine the inter-assay precision of the GenoType NTM-DR, the same samples as described for the intra-assay precision were applied. These samples and a negative control were tested in nine runs on three different days, using three different sets of instruments, and conducted by three different operators. DNA extraction was performed using the GenoLyse® DNA extraction kit. Apart from the varied parameter, all other testing conditions were identical. No deviations were detected between parallel samples, 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 NTM-DR, 6 different samples were cultured in 4 different media (solid media: Loewenstein–Jensen, Stonebrink, and Middlebrook–7H10; liquid medium: MGIT [BD Diagnostics, Franklin Lakes, USA]). DNA was extracted using the GenoLyse® DNA extraction kit and then tested with the GenoType NTM-DR.

All samples showed the same correct results. Hence, it can be excluded that the tested media import inhibitors into the GenoType NTM-DR test.

Stability

Shelf life of the GenoType NTM-DR test kit when stored as recommended: see box label.

Stability is determined according to DIN EN ISO 23640.

References
