GenoType Mycobacterium AS

VER 1.0

Instructions for Use

IFU-298-16

CE

IVD for in vitro diagnostic use only

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HAIN LIFESCIENCE

GenoType Mycobacterium AS
Molecular Genetic Assay for Identification of Nontuberculous Mycobacteria 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 Mycobacterium AS** is a qualitative in vitro test for the identification of the following nontuberculous mycobacterial species from cultured material: *M. simiae, M. mucogenicum, M. goodii, M. celatum, M. smegmatis, M. genavense, M. lentiflavum, M. heckeshornense, M. szulgai/M. intermedium, M. phlei, M. haemophilum, M. kansasii, M. ulcerans, M. gastri, M. asiaticum,* and *M. shimoidei*.
The test is indicated as an aid for diagnosis and intended for use in medical laboratories.

Summary and Explanation

Nontuberculous mycobacteria (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 [1]. Immunocompromised persons such as HIV or leukemia patients are most likely to develop a severe mycobacteriosis. Therapy of NTM infections is exceedingly difficult because of the relative resistance of nontuberculous mycobacteria to a wide range of antibiotics [1]. Since therapeutic and treatment measures must also be specifically adjusted to the infecting species, a quick and reliable differentiation within the NTM group is indispensable.

Principles of the Procedure

The **GenoType Mycobacterium AS** 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 the different sequences of the bacterial species. 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.
Store Internal Control DNA (IC) at –20°C in the same room where the DNA is extracted.
Store Control DNA (C+) at –20°C in the same room where the DNA is added to the tubes containing the aliquoted master mix.
Refreeze AM-A, AM-B, IC, and C+ immediately after use.
Avoid repeated freezing and thawing of AM-A, AM-B, IC, and C+; when processing only small sample numbers per run, aliquot AM-A, AM-B, IC, and C+.
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 3 control zones:
- a Conjugate Control zone (CC) to check the binding of the conjugate on the strip and a correct chromogenic reaction
- an Internal Control zone (IC) which documents a successful DNA extraction and amplification reaction
- a Genus Control zone (GC) which documents the presence of a member of the genus *Mycobacterium*

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.
The kit includes an Internal Control DNA (IC) which is added to each sample prior to DNA extraction. The amplicon of the Internal Control DNA binds to the Internal Control zone on the strip (see above).
A negative control sample for detection of possible contamination events should be part of each run and is included in the sample set during DNA extraction (see respective instructions for use). A valid negative control must exclusively show the CC and IC bands.
Additionally, a positive control sample containing the provided Control DNA (C+) may be included in the sample set during amplification. The Control DNA contains *M. kansasii* DNA and shows an *M. kansasii* banding pattern without IC band on the respective test strip.
IC and C+ must not be interchanged during the procedure because this may lead to erroneous results (see chapter Troubleshooting).

Specimen Requirements

Bacteria grown on solid medium or in liquid medium may be used as starting material for DNA extraction. The test must not be used for detection directly from patient specimens.

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 [2] or [3]). 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" [4], the "Clinical Microbiology Procedures Handbook" [5], 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 [6,7]. The specimens used for decontamination must not be older than 4 days.

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" [4]. 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

Mycobacteria grown on solid medium (e.g. Loewenstein-Jensen, Middlebrook) or in liquid medium (e.g. MGIT (BD Diagnostics, Franklin Lakes, USA)) can be used as starting material for DNA extraction. The working area must be free from contaminating DNA.
For DNA extraction the **GenoLyse®** kit (see chapter Ordering Information) is used according to protocol C.

The method described above was used for performance evaluation of the **GenoType Mycobacterium AS** 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. In a separate working area, add 5 µl DNA solution (or C+ for a positive control) to each aliquot. Refreeze AM-A, AM-B, and C+ immediately after use.

Amplification profile:
When using a thermal cycler from Hain Lifescience with the respective preinstallation, select protocol "MDR CUL".

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

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

Hybridization

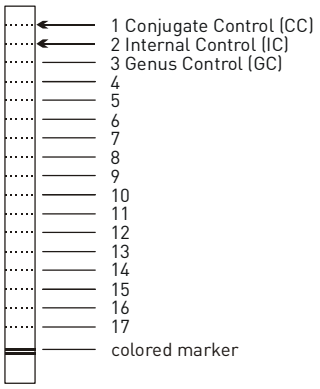
When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the hybridization protocol to be used.
The following protocol describes the manual hybridization using a water bath or a **TwinCubator**.

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

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

Evaluation and Interpretation of Results

Paste strips and store protected from light. An evaluation sheet is included in the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and IC with the respective lines on the sheet. Note down positive signals in the last but one column, determine species with the help of the interpretation chart and enter name of the identified species in the last column. The supplied template also serves as an aid for evaluation and must be aligned with the bands CC and IC of the strip as well. Each strip has a total of 17 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.

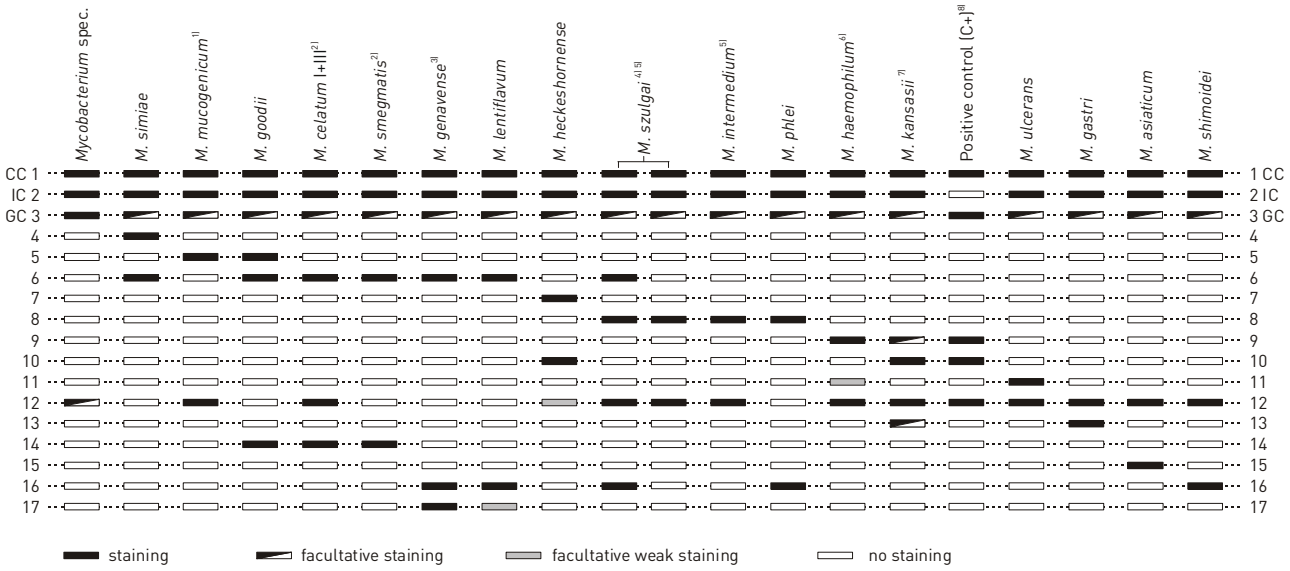
Internal Control (IC)
When the test is performed correctly, a control amplicon will bind to the Internal Control zone.
In case of a positive test result, the signal of the Internal Control zone can be weak or even vanish totally. This might be due to competition of the single reactions during amplification. In this case, the test was performed correctly and does not have to be repeated. Please note, that the positive control C+ does not show the IC band.
When only the CC and IC bands are developed, this represents a valid negative result. A missing IC band in case of a negative test result indicates mistakes during DNA extraction or during setup and/or performance of the amplification reaction, or the presence of amplification inhibitors. In this case, the test result is not valid and the test has to be repeated with the respective sample.

Genus Control (GC)
Staining of this zone documents the presence of a member of the genus *Mycobacterium*. The intensity of this band varies depending on the mycobacterial species.
When a species-specific banding pattern has developed, the GC band may be weak or even drop out completely due to competition of the single reactions during amplification. The test result, however, is to be assessed as valid.

Other bands
Specific probes, for evaluation see interpretation chart.

Please note:
Not all bands of a strip have to show the same signal strength. Generally, only those bands whose intensities are about as strong as or stronger than that of the Internal Control zone (IC) are to be considered (exceptions: see chapter Interpretation Chart).
An amplicon generated with the **GenoType Mycobacterium CM** VER 2.0 can directly be hybridized to **GenoType Mycobacterium AS** strips.
An amplicon generated with the **GenoType Mycobacterium CM** VER 1.0 must not be hybridized to **GenoType Mycobacterium AS** strips.

Interpretation Chart



Band No. 1 (CC): Conjugate Control
Band No. 2 (IC): Internal Control
Band No. 3 (GC): Genus Control

[1] For *M. mucogenicum*, the intensity of band 12 may be weaker than that of the IC band.

[2] When using liquid medium, contaminating bacteria may generate false-positive banding patterns for *M. celatum* or *M. smegmatis* (see chapter Performance Characteristics). The banding pattern indicating the presence of these two species is therefore only valid when the DNA was extracted from bacteria grown on solid medium (single colony/morphologically identical colonies).

[3] *M. triplex* shows the same banding pattern as *M. genavense*.

[4] Due to sequence variations two different *M. szulgai* banding patterns are possible.

[5] *M. szulgai* and *M. intermedium* can be differentiated using the **GenoType Mycobacterium CM** kit. *M. szulgai* will display the banding pattern 1, 2, 3, 10, and 11, *M. intermedium* will display the banding pattern 1, 2, 3, and 10.

[6] *M. nebraskense* shows the same banding pattern as *M. haemophilum*.

[7] Due to sequence variations four different *M. kansasii* banding patterns are possible.

[8] The positive control (C+) shows an *M. kansasii* banding pattern without IC band; the intensity of band 9 may be weaker than that of bands 10 and 12.

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 results of this test may only be interpreted in combination with additional laboratory and clinical data available to the responsible physician.
The test reflects the current state of knowledge of Hain Lifescience.

If more than one species is assigned to a banding pattern, these species cannot be discriminated with this test system.
In case a bacterial strain does not belong to one of the species identifiable with the **GenoType Mycobacterium AS** but is closely related to one of them, it may, in rare cases, generate the banding pattern of the closely related species detectable with the test.
The presence of multiple bacterial species in the sample to be analyzed might hamper the interpretation of the test.

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 with the **GenoLyse®** kit for DNA extraction from cultured material. Until the present edition of the instructions on hand, the performance of the test has not been validated with other DNA extraction methods or sample materials.

Troubleshooting

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

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

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

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

Unexpected result
- Wrong incubation temperature.
- Hybridization Buffer and/or Stringent Wash Solution were not properly prewarmed or mixed.
- Contamination of neighboring wells by spillage during addition of Hybridization Buffer.
  Repeat reverse hybridization.
- Contamination of extracted DNA with previously extracted or amplified DNA. Repeat extraction.
- Contamination of amplification reagents. In this case, a negative control sample shows additional bands besides CC and IC. Repeat amplification using fresh reagents.
- Depending on the amount of amplified DNA used and on the specific reaction conditions, a strong and fast color development may occur. In such cases, discontinue the substrate incubation as soon as the signals are clearly visible in order to prevent the development of cross-hybridizing bands. If necessary, the amount of amplicon used for reverse hybridization may be reduced down to 5 µl.
- No pure culture as starting material. Re-culture in order to exclude contamination.
- Error during DNA extraction. Repeat extraction.
- IC and C+ interchanged. In this case, the negative control and negative samples show an *M. kansasii* banding pattern without IC band and the positive control (if included) does not show the *M. kansasii* banding pattern, but only bands CC and IC. The banding pattern of positive samples is mostly not interpretable. Repeat extraction.

Material Required but not Included in the Kit

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

Kit Contents

Order no.	298	29896
Tests	**12**	**96**
Kit Component 1 of 2 (store at 2-8°C)		
Membrane strips coated with specific probes (Mycobacterium AS STRIPS)	12	2x 48
Denaturation Solution (DEN) contains <2% NaOH, dye	0.3 ml	2x 1.2 ml
Hybridization Buffer (HYB) contains <10% anionic tenside, dye	20 ml	120 ml
Stringent Wash Solution (STR) contains >25% of a quaternary ammonium compound, <1% anionic tenside, dye	20 ml	120 ml
Rinse Solution (RIN) contains buffer, <1% NaCl, <1% nonionic tenside	50 ml	3x 120 ml
Conjugate Concentrate (CON-C) contains streptavidin-conjugated alkaline phosphatase, dye	0.2 ml	1.2 ml
Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, <1% NaCl	20 ml	120 ml
Substrate Concentrate (SUB-C) contains <70% dimethyl sulfoxide, <10% 4-nitro blue tetrazolium chloride, <10% 5-bromo-4-chloro-3-indolyl phosphate	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 Mycobacterium AS) contains buffer, nucleotides, Taq polymerase	0.15 ml	4x 0.3 ml
Amplification Mix B (AM-B GT Mycobacterium AS) contains salts, specific primers, dye	0.53 ml	4x 1.05 ml
Internal Control DNA (IC GT Mycobacterium AS) contains bacterial DNA	0.25 ml	0.25 ml
Control DNA (C+ GT Mycobacterium AS) contains bacterial control DNA	0.1 ml	0.1 ml

Ordering Information

	Order no.
GenoType Mycobacterium AS (kit for analysis of 12 samples)	298
GenoType Mycobacterium AS (kit for analysis of 96 samples)	29896
GenoLyse® (kit for manual DNA extraction of 12 samples)	51612
GenoLyse® (kit for manual DNA extraction of 96 samples)	51610

Performance Characteristics

For the performance evaluation of the **GenoType Mycobacterium AS**, the test was carried out according to the instructions on hand.

Diagnostic performance

DNA extraction with quick method

The **GenoType Mycobacterium AS** (with Primer Nucleotide Mix) was tested in two studies [8,9] with a total of 319 isolates. All samples were previously characterized with at least one of the following methods: conventional biochemical and cultural methods, 16S rRNA sequence analysis of the first 500 bp of the 5' region, HPLC, INNO-LiPA Rif.TB line probe assay (Innogenetics, Gent, Belgium), AccuProbe Hybridization Protection Assays: Mycobacterium Tuberculosis Complex / Avium / Avium Complex / Intracellulare / Gordonae / Kansasii Culture Identification (all Gen-Probe, San Diego, USA).

The test panel consisted of 76 strains identifiable with the **GenoType Mycobacterium AS** test kit (covering all species identifiable with the test system), 232 *Mycobacterium* strains not identifiable with the test kit, and 11 nonmycobacterial strains. In total, 304 out of 319 results were correct.

All of the 76 identifiable strains were correctly detected by the **GenoType Mycobacterium AS**.

The 232 not-identifiable strains covered 73 mycobacterial species not identifiable with the **GenoType Mycobacterium AS**. Out of these 232 strains, 217 showed the expected banding pattern (CC, UC, GC). One strain was not identified as *Mycobacterium* spec. but as one of the detectable species and was hence rated as false-positive. 14 strains were identified as gram-positive bacteria with high G+C content because of a missing GC band.

All of the 11 nonmycobacterial strains (representing 6 different nonmycobacterial species) were correctly identified as gram-positive bacteria with high G+C content.

Table 1: Sensitivity and specificity of the species-specific probes of the **GenoType Mycobacterium AS** (with Primer Nucleotide Mix, DNA extraction with quick method[1])

		Methods of comparison			
Species-specific probes		Positive	Negative	Total	
GenoType Mycobacterium AS	Positive	76	1	77	Diagnostic sensitivity: 100%
	Negative	0	242	242	Diagnostic specificity: 99.6%
	Total	76	243	319	

Table 2: Sensitivity and specificity of the genus-specific probe (GC) of the **GenoType Mycobacterium AS** (with Primer Nucleotide Mix, DNA extraction with quick method[1])

		Methods of comparison			
Genus-specific probe		Positive	Negative	Total	
GenoType Mycobacterium AS	Positive	292	0	292	Diagnostic sensitivity: 94.8%
	Negative	16	11	27	Diagnostic specificity: 100%
	Total	308	11	319	

DNA extraction with **GenoLyse®**

In a study comprising 52 *Mycobacterium*-positive cultures (growth on Loewenstein-Jensen medium or in MGIT (BD Diagnostics, Franklin Lakes, USA)), DNA was extracted with the **GenoLyse®** kit and then tested with the **GenoType Mycobacterium AS** (with Primer Nucleotide Mix). For comparison, DNA was extracted with the quick method[1] in parallel and then tested with the **GenoType Mycobacterium AS** (with Primer Nucleotide Mix).

With both extraction methods, identical results were obtained (see table 3).

Table 3: Results of the *Mycobacterium* identification by **GenoType Mycobacterium AS** (with Primer Nucleotide Mix, DNA extraction with quick method[1] compared to DNA extraction with **GenoLyse®**)

Result after DNA extraction with quick method	Number of isolates		Result after DNA extraction with **GenoLyse®**
Mycobacterium spec.	37	37	*Mycobacterium* spec.
M. celatum	2	2	*M. celatum*
High GC gram-positive bacterium	13	13	High GC gram-positive bacterium
Total	52	52	Total

The Primer Nucleotide Mix (PNM) has been replaced by new kit constituents, namely Amplification Mixes A and B (AM-A and AM-B). In order to check if the change of kit constituents impacts test results, 39 culture samples were tested with both kit variants. DNA was extracted using the **GenoLyse®** kit and the isolates were analyzed with the **GenoType Mycobacterium AS** applying the "MDR CUL" PCR protocol. Method of comparison was the **GenoType Mycobacterium AS** (with Primer Nucleotide Mix). For discrepant samples, the result of the **GenoType Mycobacterium CM** VER 2.0 was used as reference. The results were correct for all samples (see table 4).

Table 4: Sensitivity and specificity of the species-specific probes of the **GenoType Mycobacterium AS** (with Amplification Mixes, DNA extraction with **GenoLyse®**)

		GenoType Mycobacterium AS (with PNM) + reference method			
Species-specific probes		Positive	Negative	Total	
GenoType Mycobacterium AS (with Amplification Mixes)	Positive	38	0	38	Diagnostic sensitivity: 100%
	Negative	0	1	1	Diagnostic specificity: 100%
	Total	38	1	39	Positive predictive value: 100%
					Negative predictive value: 100%

[1] For performance evaluation of the **GenoType Mycobacterium AS** with Primer Nucleotide Mix, a quick method was used for DNA extraction from cultured material as an alternative to the **GenoLyse®** kit, whereas for performance evaluation of the **GenoType Mycobacterium AS** with Amplification Mixes, solely the **GenoLyse®** kit was used. Until the present edition of the instructions on hand, the performance of the **GenoType Mycobacterium AS** with Amplification Mixes has not been validated with other DNA extraction methods.

Analytical performance

Analytical specificity

The specificity of the **GenoType Mycobacterium AS** 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 Mycobacterium AS** (with Primer Nucleotide Mix) was determined with strains of all identifiable *Mycobacterium* species and with strains of 73 species not detectable with the test system. Additionally, several isolates that could not yet be assigned to a certain species and 190 strains of the following non-*Mycobacterium* genera were tested: *Actinomyces*, *Campylobacter*, *Capnocytophaga*, *Corynebacterium*, *Gordonia*, *Legionella*, *Nocardia*, *Nocardioides*, *Nocardiopsis*, *Rhodococcus*, *Saccharomonospora*, *Streptomyces*, *Tsukamurella*, and *Yersinia*.

One isolate from a species technically identifiable with the **GenoType Mycobacterium AS** was not detected. All other species identifiable with the assay generated a positive result. In case a bacterial strain does not belong to one of the species identifiable with the **GenoType Mycobacterium AS** but is closely related to one of them, the strain to be tested may generate the banding pattern of the closely related species detectable with the test. In the studies, this was the case with one isolate that could not be allocated to an (accepted) mycobacterial species. In one study, false-positive results for *M. celatum* or *M. smegmatis* were found in samples from liquid cultures. This phenomenon did not occur with samples from solid culture. In order to avoid this negative influence from accompanying bacterial flora the *M. celatum* and *M. smegmatis* banding patterns are only valid when the DNA was extracted from single colonies or morphologically identical colonies. This is stated accordingly in the chapter Interpretation Chart. The isolates from mycobacterial species not identifiable with the test system and the isolates from non-*Mycobacterium* species displayed no species-specific banding pattern. Hence, the analytical specificity for the species-specific bands of the **GenoType Mycobacterium AS** (with Primer Nucleotide Mix) was 99.8%.
The same samples as described above were also evaluated for performance of the Genus-specific probe (GC). An analytical specificity of 100% was determined for this probe.

The Primer Nucleotide Mix (PNM) has been replaced by new kit constituents, namely Amplification Mixes A and B (AM-A and AM-B). In order to check if the change of kit constituents impacts test results, the analytical specificity of the **GenoType Mycobacterium AS** (with Amplification Mixes) was determined with strains of all identifiable *Mycobacterium* species, i.e.: *M. asiaticum, M. celatum* I+III, *M. gastri*, *M. genavense*, *M. goodii*, *M. haemophilum*, *M. heckeshornense*, *M. intermedium*, *M. kansasii*, *M. lentiflavum*, *M. mucogenicum*, *M. nebraskense*, *M. phlei*, *M. shimoidei, M. simiae*, *M. smegmatis*, *M. szulgai*, *M. triplex* and *M. ulcerans*.
Additionally, strains of the following species not detectable with the test system were analyzed:

M. abscessus	*M. canettii*	*M. intracellulare*	*M. peregrinum*
M. africanum	*M. chelonae*	*M. mageritense*	*M. pinnipedii*
M. alvei	*M. chimaera*	*M. malmoense*	*M. scrofulaceum*
M. avium	*M. fortuitum*	*M. marinum*	*M. tuberculosis*
M. bovis BCG	*M. gordonae*	*M. microti*	*M. xenopi*
M. bovis subsp. *bovis*	*M. immunogenum*	*M. palustre*	
M. bovis subsp. *caprae*	*M. interjectum*	*„M. paraffinicum"*	

Furthermore, strains of the following nonmycobacterial species were analyzed:
Bordetella pertussis, Corynebacterium ulcerans, C. xerosis, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Nocardia amarae, N. asteroides, N. farcinica, N. otidiscaviarum, Pseudomonas aeruginosa, Rhodococcus erythropolis, R. rhodochrous, R. ruber, Staphylococcus aureus, S. pneumoniae, Streptomyces somaliensis, Tsukamurella inchonensis, T. paurometabola, T. pulmonis.

All species identifiable with this assay generated a positive result. The isolates from mycobacterial species not identifiable with this test system and the isolates from non-*Mycobacterium* species displayed no species-specific banding pattern. Hence, the analytical specificity for the species-specific bands of the **GenoType Mycobacterium AS** (with Amplification Mixes) was 100%.
The same samples as described above were also evaluated for performance of the Genus-specific probe (GC). One isolate of a mycobacterial species not identifiable with the **GenoType Mycobacterium AS** displayed no GC band. All other isolates generated a correct result. Hence, the analytical specificity for the Genus-specific probe was 98.5%.

Analytical sensitivity (Limit of Detection, LOD)

For determination of analytical sensitivity of the **GenoType Mycobacterium AS** (with Amplification Mixes), four BCG culture dilutions (1.65x 10^6, 1.65x 10^5, 1.65x 10^4 and 1.65x 10^3 CFU/ml) were set up in triplicate. Including a negative control, DNA was extracted using the **GenoLyse®** kit and analyzed with the **GenoType Mycobacterium AS** applying the "MDR CUL" PCR protocol. The limit of detection was 1.65x 10^5 CFU/ml.

Reproducibility

In order to determine the intra-assay precision of the **GenoType Mycobacterium AS** (with Amplification Mixes), two BCG culture dilutions (one above and one at the cutoff concentration), one *B. pertussis* positive DNA sample and one negative control were set up in four parallels and tested under identical conditions in one PCR run. DNA was extracted using the **GenoLyse®** kit and analyzed with the **GenoType Mycobacterium AS** applying the "MDR CUL" PCR protocol. All strains showed the expected signals (*Mycobacterium* spec.) and the negative control was negative. No deviations were detected within the parallels, the banding patterns were identical and the signal strengths were comparable. Hence, the intra-assay precision was 100%.

In order to determine the inter-assay precision of the **GenoType Mycobacterium AS** (with Amplification Mixes), the same samples as described for the intra-assay precision were set up under identical conditions at three different points of time. DNA was extracted using the **GenoLyse®** kit and analyzed with the **GenoType Mycobacterium AS** applying the "MDR CUL" PCR protocol. Apart from the varied parameter, all other testing conditions were identical. All strains showed the expected signals (*Mycobacterium* spec.) and the negative control was negative. No deviations were detected within the parallels and between the runs, the banding patterns were identical and the 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 Mycobacterium AS** (with Amplification Mixes), 6 different *M. tuberculosis* complex samples were cultured in 4 different media (solid media: Loewenstein-Jensen, Stonebrink, and Middlebrook-7H10, liquid medium: MGIT (BD Diagnostics, Franklin Lakes, USA)). Then the culture samples were tested with the **GenoType Mycobacterium AS**. All samples showed correct results with all tested media. Hence, it can be excluded that the tested media import inhibitors into the **GenoType Mycobacterium AS** test (with Amplification Mixes).

Stability

Shelf life of the **GenoType Mycobacterium AS** test kit when stored as recommended: see box label.
Stability is determined according to DIN EN ISO 23640.

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Important Changes in IFU-298-16

Chapter	Change
Several (especially Quality Control, Amplification, Evaluation and Interpretation of Results, Troubleshooting, Kit Contents)	The kit now includes an Internal Control DNA (IC) which is added to each sample prior to DNA extraction. It serves as an extraction control, as an inhibition control for the amplification, and as a control of the correct performance of the test and the proper functioning of kit constituents. The Universal Control zone on the strip was replaced with an Internal Control zone to which the amplicon of the IC hybridizes. A positive control sample containing the provided Control DNA (C+) may be included in the sample set during amplification. The Control DNA contains *M. kansasii* DNA and shows a respective banding pattern (without IC band) on the strip.
Storage and Disposal of Kit Constituents	All constituents delivered as Kit Component 2 must be stored at –20°C.
DNA Extraction	As of now, the **GenoLyse®** kit is used for DNA extraction according to protocol C. The quick protocol (heating step followed by sonication) may no longer be applied for DNA extraction.
Several (especially Amplification)	In order to obtain a fully reactive master mix, now only two pipetting steps have to be performed combining the two Amplification Mixes A and B (AM-A and AM-B). AM-A already includes Taq polymerase and buffer; hence, the enzyme does not have to be purchased and added separately anymore.
Amplification	As of now, the "MDR CUL" PCR protocol (instead of the "HOT 30" protocol) is used for amplification.
Evaluation and Interpretation of Results	An amplicon generated with the **GenoType Mycobacterium CM** VER 2.0 can directly be hybridized to **GenoType Mycobacterium AS** strips. An amplicon generated with the **GenoType Mycobacterium CM** VER 1.0 must not be hybridized to **GenoType Mycobacterium AS** strips.
Interpretation Chart	The four different banding patterns that are possible for *M. kansasii* are now summarized in one column. The banding patterns have not changed.
Performance Characteristics	Generally revised and supplemented.

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