GenoType Mycobacterium CM

VER 1.0

Instructions for Use

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CE

IVD for in vitro diagnostic use only

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HAIN LIFESCIENCE

GenoType Mycobacterium CM
Molecular Genetic Assay for Identification of Clinically Relevant Mycobacterial Species 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 CM** is a qualitative in vitro test for the identification of the *Mycobacterium tuberculosis* complex as well as the following nontuberculous mycobacterial species from cultured material: *M. avium, M. chelonae, M. abscessus, M. fortuitum, M. gordonae, M. intracellulare, M. scrofulaceum, M. interjectum, M. kansasii, M. malmoense, M. marinum/M. ulcerans, M. peregrinum,* and *M. xenopi*.
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. In 2012, there were an estimated 8.6 million incident cases of TB globally, and an estimated 1.3 million deaths occurred [1].
The TB pathogens are immobile, obligate aerobic, acid-fast bacilli belonging to the family of *Mycobacteriaceae*. They are gram-positive with a high genomic G+C content (59-66%). The genus *Mycobacterium* comprises numerous species which are divided into three groups: (i) the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. africanum, M. bovis* subsp. *bovis, M. bovis* subsp. *caprae, M. bovis* BCG, *M. microti, M. canettii, and M. pinnipedii*), (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 [2]. Immunocompromised persons such as HIV or leukemia patients are most likely to develop a severe mycobacteriosis. The **GenoType Mycobacterium CM** permits the rapid and reliable differentiation of relevant mycobacteria and therefore the fast application of specific treatment and preventive measures. If it was not possible to identify a single species with this test, a specification may be achieved using the **GenoType Mycobacterium AS** kit (see Interpretation Chart). For this, the amplicon already generated for the **GenoType Mycobacterium CM** can directly be hybridized to the **GenoType Mycobacterium AS** strip.

Principles of the Procedure

The **GenoType Mycobacterium CM** 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 (the necessary thermostable DNA polymerase is not included in the kit), and (iii) a reverse hybridization.
The Primer Nucleotide Mix (PNM) contains biotinylated primers for the amplification of specific regions of the bacterial chromosome. 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

Store Primer Nucleotide Mix (PNM) at 2-8°C upon arrival isolated from contaminating DNA. If longer storage (more than 4 weeks) is required, store at –20°C. Avoid repeated freezing and thawing of PNM; when processing only small sample numbers per run, aliquot PNM. Store all other kit components at 2-8°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:
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 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
- 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
- a Genus Control zone (GC) which documents, as far as is known, 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.
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 culture plates (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 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 [3] or [4]). Always wear suitable protective clothing and gloves. Samples from patients at risk (infected by pathogenic microorganisms including Hepatitis B and Human Immunodeficiency Virus (HIV)) and cultures made from those samples must always be labeled and handled under suitable safety conditions according to institutional guidelines.
Handling of potentially infectious specimens must be carried out in a class II safety cabinet. Potentially infectious samples must be centrifuged in a class II safety cabinet or in an aerosol-tight rotor. Open aerosol-tight rotor in safety cabinet only. For inactivated samples, a standard rotor can be used for centrifugation outside the safety cabinet.
Discard used pipette tips immediately after use in a container for biohazardous waste. After finishing the assay, discard all used disposables in a container for biohazardous waste.

Storage and transport
All specimens should be collected and transported as recommended in the CDC publication "Public Health Mycobacteriology: A Guide for the Level III Laboratory" [5], the "Clinical Microbiology Procedures Handbook" [6], or your laboratory procedure manual.
It must be ensured that until decontamination takes place, specimens are kept in sterile plastic containers at a temperature of 2-8°C. The transport of specimens at room temperature has to be carried out as soon as possible and should be done within 1-2 days. 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" [5]. 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 culture plates (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 culture plates (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.
The following quick protocol can be used for DNA extraction from cultured material:

1a. When using bacteria grown on solid medium, collect bacteria with an inoculation loop and suspend in approximately 300 µl of water (molecular biology grade).
1b. When using bacteria grown in liquid media, directly apply 1 ml. Pellet bacteria by spinning for 15 min in a standard table top centrifuge with an aerosol-tight rotor or in a class II safety cabinet at approximately 10,000 x g. Discard supernatant and resuspend bacteria in 100-300 µl of water (molecular biology grade) by vortexing.
2. Incubate bacteria from 1a or 1b for 20 min at 95°C in a water bath.
3. Incubate for 15 min in an ultrasonic bath.
4. Spin down for 5 min at full speed and directly use 5 µl of the supernatant for PCR. In case DNA solution is to be stored for an extended time period, transfer supernatant to a new tube.

The assay on hand was also validated with the **GenoLyse®** kit (see chapter Ordering Information) which can alternatively be used for DNA extraction. For handling instructions, please refer to the instructions for use of the **GenoLyse®** kit.

The methods described above were used for performance evaluation of the **GenoType Mycobacterium CM** 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
Prepare the amplification mix (45 µl) in a room free from contaminating DNA. The DNA solution should be added in a separate working area.

Prepare for each sample:
- 35 µl PNM – included in the kit
- 5 µl 10x polymerase incubation buffer – not included in the kit
- x µl $MgCl_2$ solution[1)] – not included in the kit
- 1-2 unit(s) thermostable DNA polymerase (refer to manual) – not included in the kit
- y µl water (molecular biology grade) to obtain a volume of 45 µl (not considering volume of enzyme) – not included in the kit
- In a separate working area, add 5 µl DNA solution (20-100 ng DNA) leading to a final volume of 50 µl (not considering volume of enzyme).

[1)] Depending on the enzyme/buffer system used, the optimal $MgCl_2$ concentration may vary between 1.5 and 2.5 mM. Please note that some incubation buffers already contain $MgCl_2$.

The performance evaluation of the **GenoType Mycobacterium CM** assay was carried out using the HotStarTaq DNA Polymerase from Qiagen. When using this enzyme, the following amounts are required per sample:

- 35 µl PNM– included in the kit
- 5 µl 10x PCR Buffer for HotStarTaq (contains 15 mM $MgCl_2$) – not included in the kit
- 2 µl 25 mM $MgCl_2$ solution – not included in the kit
- 0.2 µl (1 U) HotStarTaq – not included in the kit
- 3 µl water (molecular biology grade) – not included in the kit
- 5 µl DNA solution (add in a separate working area)

The final $MgCl_2$ concentration in this amplification mix is 2.5 mM.

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 all reagents except for DNA solution and mix carefully but thoroughly (do not vortex). 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[2]:
When using a thermal cycler from Hain Lifescience with the respective preinstallation, select protocol "HOT 30".

15 min 95°C	1 cycle
30 sec 95°C 2 min 58°C	10 cycles
25 sec 95°C 40 sec 53°C 40 sec 70°C	20 cycles
8 min 70°C	1 cycle
Heating rate	≤ 2.2°C/sec

[2] Applies to the Taq polymerase used for validation. When using other hot start DNA polymerases, the time interval of the first step might have to be reduced (please refer to manual of the enzyme).

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

Hybridization

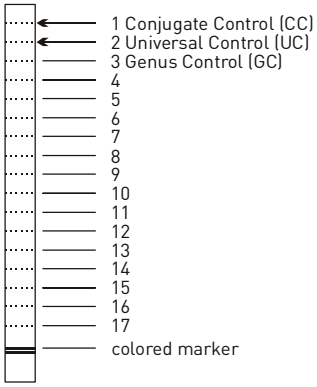
When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the hybridization protocol to be used.
The following protocol describes the manual hybridization using a water bath or a **TwinCubator**.

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 UC 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 UC 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.

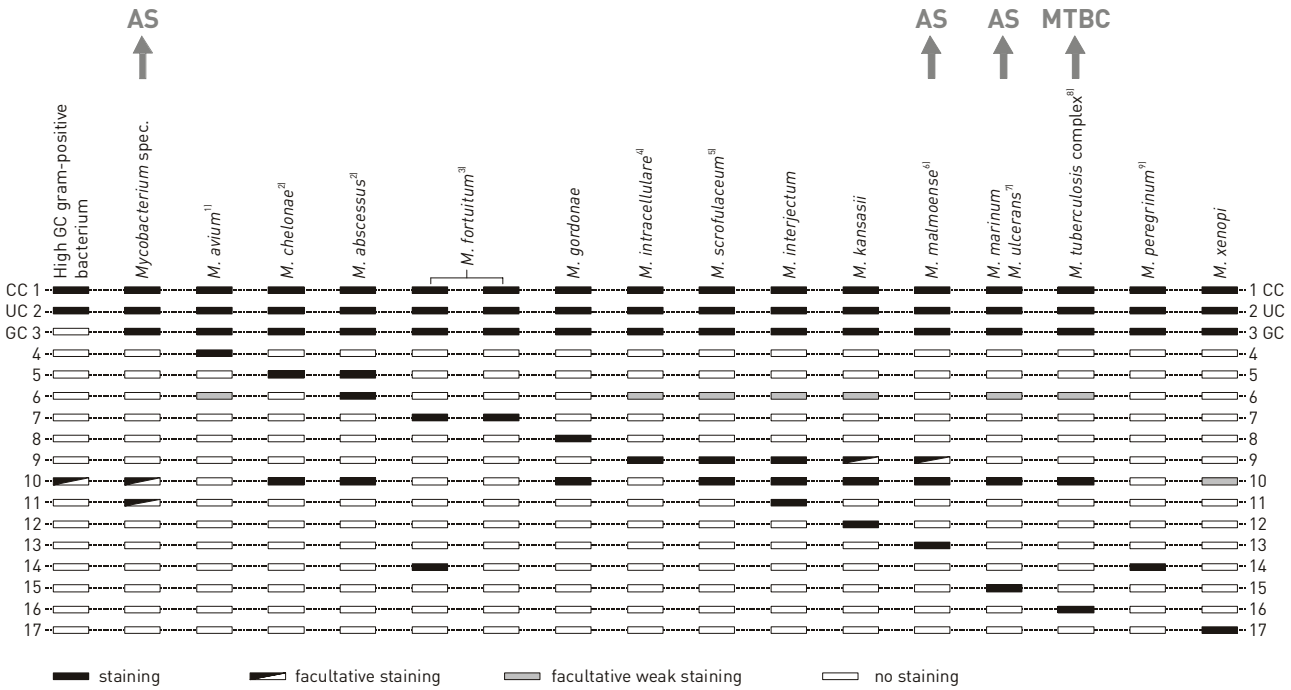
Universal Control (UC)
This zone detects, as far as is known, all mycobacteria and members of the group of gram-positive bacteria with a high G+C content. If this zone and the Conjugate Control zone stain positive but the remaining banding pattern cannot be assigned to a specific mycobacterium, additional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

Genus Control (GC)
Staining of this zone documents, as far as is known, 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. Only those bands whose intensities are about as strong as or stronger than that of the Universal Control zone (UC) are to be considered.
If a large amount of amplicon was used, additional bands may occur (see chapter Troubleshooting).
Additional mycobacteria species can be identified with the **GenoType Mycobacterium AS** kit; the amplicon generated with the **GenoType Mycobacterium CM** can directly be hybridized to the **GenoType Mycobacterium AS** strip.

Interpretation Chart



Band No. 1 (CC): Conjugate Control
Band No. 2 (UC): Universal Control
Band No. 3 (GC): Genus Control
AS: Species may possibly be identified with the **GenoType Mycobacterium AS** kit.
MTBC: For further differentiation use the **GenoType MTBC** kit.

1) Does not include other species of the *M. avium* complex.
2) *M. immunogenum* (belongs to the *M. abscessus/M. chelonae* group) shows the same banding pattern as *M. chelonae* or *M. abscessus*.
In case the quality and/or quantity of the extracted DNA does not allow an efficient amplification, the amplicon hybridizing both to the Genus Control and to band 6 may have been supplanted due to competition of the single reactions during amplification. In this case, *M. abscessus* shows the banding pattern identifying *M. chelonae*. However, as long as the specifications given in these instructions for use are observed and the DNA polymerase used for performance evaluation is applied, this does not occur.
3) Due to sequence variations within the species two different *M. fortuitum* banding patterns do occur.
M. mageritense shows the *M. fortuitum* banding pattern as depicted in the right column.
4) *M. chimaera* shows the same banding pattern as *M. intracellulare*.
5) *M. paraffinicum* and *M. parascrofulaceum* show the same banding pattern as *M. scrofulaceum*.
6) *M haemophilum*, *M. palustre*, and *M. nebraskense* show the same banding pattern as *M. malmoense*. *M. haemophilum/M. nebraskense* can be identified with the **GenoType Mycobacterium AS** kit.
7) *M. ulcerans* can be identified with the **GenoType Mycobacterium AS** kit.
8) If band 15 has also stained positive, additional detection methods must be applied.
9) *M. alvei* and *M. septicum* show the same banding pattern as *M. peregrinum*.

Limitations

Strictly adhere to the established protocols and procedures in order to obtain correct test results and to avoid contaminations.

Members of the *M. tuberculosis* complex cannot be differentiated.
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 CM** 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. The test reflects the current 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 methods for DNA extraction from cultured material which are described in chapter DNA Extraction and using the HotStarTaq DNA Polymerase from Qiagen (Hilden, Germany). Until the present edition of the instructions on hand, the performance of the test has not been validated with other DNA extraction methods, sample materials, or polymerases.

The results of this test may only be interpreted in combination with additional laboratory and clinical data available to the responsible physician.

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.
- 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. 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.
- Error during DNA extraction. This may manifest in a frequently missing GC band. Repeat extraction using the methods described in the chapter DNA Extraction.
- The bacterial species present in the sample cannot be detected with this test. Apply additional detection methods.

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 reagents for amplification use 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 platform + shaking water bath ***or*** **TwinCubator** (instrument for manual hybridization) ***or*** automated hybridization instrument
- Table top centrifuge, if applicable with aerosol tight rotor
- Thermal cycler
- Thermostable DNA polymerase with buffer (recommendation: HotStarTaq DNA Polymerase from Qiagen, Hilden, Germany)
- Timer
- Tweezers
- Ultrasonic bath
- Water (molecular biology grade)
- Water bath

Kit Contents

Order no.	299	29996
Tests	**12**	**96**
Membrane strips coated with specific probes (Mycobacterium CM STRIPS)	12	2x 48
Primer Nucleotide Mix (PNM Mycobacterium CM/AS) contains specific primers, nucleotides, dye	0.5 ml	4 ml
Denaturation Solution (DEN) contains <2% NaOH, dye	0.3 ml	2x 1.2 ml
Hybridization Buffer (HYB) contains 8-10% anionic tenside, dye	20 ml	120 ml
Stringent Wash Solution (STR) contains >25% of a quaternary ammonium compound, <1% anionic tenside, dye	20 ml	120 ml
Rinse Solution (RIN) contains buffer, <1% NaCl, <1% anionic tenside	50 ml	3x 120 ml
Conjugate Concentrate (CON-C) contains streptavidin-conjugated alkaline phosphatase, dye	0.2 ml	1.2 ml
Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, <1% NaCl	20 ml	120 ml
Substrate Concentrate (SUB-C) contains dimethyl sulfoxide, substrate solution	0.2 ml	1.2 ml
Substrate Buffer (SUB-D) contains buffer, <1% $MgCl_2$, <1% NaCl	20 ml	120 ml
tray, evaluation sheet	1 of each	4 of each
instructions for use, template	1 of each	1 of each

Ordering Information

	order no.
GenoType Mycobacterium CM (kit for analysis of 12 samples)	299
GenoType Mycobacterium CM (kit for analysis of 96 samples)	29996
GenoLyse® (kit for manual DNA extraction of 12 samples)	51612
GenoLyse® (kit for manual DNA extraction of 96 samples)	51610
GenoType Mycobacterium AS (kit for analysis of 12 samples)	298
GenoType Mycobacterium AS (kit for analysis of 96 samples)	29896
GenoType MTBC (kit for analysis of 12 samples)	301
GenoType MTBC (kit for analysis of 96 samples)	30196

Performance Characteristics

Diagnostic performance

DNA extraction with quick method

The **GenoType Mycobacterium CM** was tested in two studies [7,8] with a total of 340 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 (Innogenetics, Ghent, Belgium), AccuProbe: Mycobacterium Tuberculosis Complex / avium / avium Complex / intracellulare / gordonae / kansasii Culture Identification (all Gen-Probe, San Diego, USA).

The test panel consisted of 192 strains identifiable with the **GenoType Mycobacterium CM** test kit (covering all species identifiable with the test system), 137 *Mycobacterium* strains not identifiable with the test kit, and 11 nonmycobacterial strains. In total, 319 from 340 results were correct.

From 192 identifiable strains, 189 were correctly identified by the **GenoType Mycobacterium CM** and 3 did not show the expected pattern.

137 not-identifiable strains covered 58 mycobacterial species not identifiable with the **GenoType Mycobacterium CM**. From these 137 strains, 120 showed the expected banding pattern (CC, UC, GC). 7 strains were false-positive and were not identified as *Mycobacterium* spec., but as one of the identifiable species. The remaining 10 isolates were identified as gram-positive bacteria with high G+C content because of the missing GC band.

From 11 strains representing 6 nonmycobacterial species, 10 were correctly identified as gram-positive bacteria with high G+C content and one isolate was false-positive.

Table 1: Sensitivity and specificity of the species-specific probes of the **GenoType Mycobacterium CM** (DNA extraction with quick method)

		Methods of comparison			
Species-specific probes		Positive	Negative	Total	
GenoType Mycobacterium CM	Positive	189	8	197	Diagnostic sensitivity: 98.4%
	Negative	3	140	143	Diagnostic specificity: 94.6%
	Total	192	148	340	

Table 2: Sensitivity and specificity of the genus-specific probe of the **GenoType Mycobacterium CM** (DNA extraction with quick method)

		Methods of comparison			
Genus-specific probe (GC)		Positive	Negative	Total	
GenoType Mycobacterium CM	Positive	312	1	316	Diagnostic sensitivity: 94.8%
	Negative	17	10	27	Diagnostic specificity: 90.9%
	Total	329	11	340	

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 CM**. For comparison, DNA was extracted with the quick method in parallel and then tested with the **GenoType Mycobacterium CM**.

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

Table 3: Results of the Mycobacterium identification by **GenoType Mycobacterium CM** (DNA extraction with quick method compared to DNA extraction with **GenoLyse®**)

Result after DNA extraction with quick method	Number of isolates		Result after DNA extraction with **GenoLyse®**
M. tuberculosis complex	49	49	*M. tuberculosis* complex
M. chelonae	1	1	*M. chelonae*
M. gordonae	1	1	*M. gordonae*
High GC gram-positive bacterium	1	1	High GC gram-positive bacterium
Total	52	52	Total

Analytical performance

Analytical specificity

The specificity of the **GenoType Mycobacterium CM** 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 CM** was determined with strains of all *Mycobacterium* species identifiable by this test, i.e.: *M. avium, M. chelonae*, *M. abscessus*, *M. immunogenum*, *M. fortuitum, M. mageritense*, *M. gordonae, M. intracellulare*, *M. chimaera*, *M. scrofulaceum*, *M. parascrofulaceum*, *M. paraffinicum*, *M. interjectum*, *M. kansasii*, *M. malmoense*, *M. haemophilum*, *M. palustre*, *M. nebraskense*, *M. marinum*, *M. ulcerans*, *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, *M. bovis* BCG, *M. africanum*, *M. microti*, *M. canettii, M. pinnipedii*), *M. peregrinum*, *M. alvei*, *M. septicum*, and *M. xenopi*.

Additionally, strains of the following species not detectable with the test system were analyzed:

M. agri	*M. conspicuum*	*M. goodii*	*M. neoaurum*	*M. sphagni*
M. asiaticum	*M. diernhoferi*	*M. hassiacum*	*M. nonchromogenicum*	*M. terrae* group
M. aurum	*M. duvalii*	*M. heckeshornense*	*M. novocastrense*	*M. thermoresistibile*
M. bohemicum	*M. elephantis*	*M. heidelbergense*	*M. obuense*	*M. tokaiense*
M. botniense	*"M. engbaekii"*	*M. hiberniae*	*M. parafortuitum*	*M. triplex*
M. branderi	*M. fallax*	*M. hodleri*	*M. phlei*	*M. triviale*
M. brumae	*M. farcinogenes*	*M. holsaticum*	*M. porcinum*	*M. tusciae*
M. celatum	*M. flavescens*	*M. intermedium*	*M. poriferae*	*M. vaccae*
M. chitae	*M. florentinum*	*M. kubicae*	*M. rhodesiae*	
M. chlorophenolicum	*M. gadium*	*M. lactis*	*M. senegalense*	
M. chubuense	*M. gastri*	*M. lentiflavum*	*M. shimoidei*	
M. confluentis	*M. genavense*	*M. mucogenicum*	*M. simiae*	
M. cookii	*M. gilvum*	*M. murale*	*M. smegmatis*	

Additionally, several isolates that could not yet be assigned to a certain species were tested. Furthermore, 190 strains of the following non-*Mycobacterium* genera were also tested: *Actinomyces*, *Campylobacter*, *Capnocytophaga*, *Corynebacterium*, *Gordonia*, *Legionella*, *Nocardia*, *Nocardioides*, *Nocardiopsis*, *Rhodococcus*, *Saccharomonospora*, *Streptomyces*, *Tsukamurella*, and *Yersinia*.

Three isolates from species technically identifiable with the **GenoType Mycobacterium CM** were not detected with this test version. All other species identifiable with this assay generated a positive result. In case a bacterial strain does not belong to one of the species identifiable with the **GenoType Mycobacterium CM** 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 seven isolates. The mycobacterial species not identifiable with the test system and all but one of the non-*Mycobacterium* species displayed no species-specific banding pattern. Hence, the analytical specificity for the species-specific probe of the **GenoType Mycobacterium CM** strip was 97.6%.

The same samples as described above were also evaluated for performance of the Genus-specific probe (GC). An analytical specificity of 99.2% was determined for this probe.

Reproducibility

Intra-assay precision

In order to determine the intra-assay precision of the **GenoType Mycobacterium CM**, one *M. tuberculosis* complex strain, one *M. malmoense* strain, one *M. fortuitum* strain, one *M. goodii* strain, and one negative control (water instead of DNA in the amplification reaction) were set up in five parallels and tested under identical conditions. DNA extraction was performed using the quick method described in chapter DNA Extraction. 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 Mycobacterium CM**, one *M. tuberculosis* complex strain, one *M. malmoense* strain, one *M. fortuitum* strain, one *M. goodii* strain, and one negative control (water instead of DNA in the amplification reaction) were set up in five parallels and tested at three different points in time. The other experimental conditions (instruments, lot numbers, operator, etc.) were identical. DNA extraction was performed using the quick method described in chapter DNA Extraction. All strains showed the expected signals and the negative controls were 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 CM**, 5 different *M. kansasii* strains and 5 different *M. gordonae* strains 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 and 5 non-inoculated media samples were tested with the **GenoType Mycobacterium CM**. All *M. kansasii* samples and all *M. gordonae* samples showed the correct results and the non-inoculated controls were negative. Hence, it can be excluded that the tested media import inhibitors into the **GenoType Mycobacterium CM** test.

Other inhibitors may be imported by reagents used for DNA extraction. When using the recommended DNA extraction methods (see chapter DNA Extraction) according to the respective instructions for use, however, the DNA does not contain inhibiting substances in relevant concentrations.

Stability

Shelf life of the **GenoType Mycobacterium CM** 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-299-22

Chapter	Change
	generally revised and restructured: – former chapter "Methodology" extended and split in new chapters "Intended Use" and "Principles of the Procedure" – former chapter "Storage and Precautions" extended and split in new chapters "Storage and Disposal of Kit Constituents","Precautions for Handling Kit Constituents", and "Specimen Requirements" – new chapters: "Summary and Explanation","Ordering Information", "Performance Characteristics","References", "Important Changes"
DNA Extraction	DNA extraction can be performed using the described quick protocol or the **GenoLyse®** kit.
Amplification	New: Heating rate for thermal cyclers ≤2.2°C/sec
Interpretation Chart	For better clarity, the interpretation chart was revised.

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