GenoType Mycobacterium AS

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HAIN LIFESCIENCE

GenoType Mycobacterium AS
Molecular Genetic Assay for Identification of Mycobacterial Species from Cultured Material

Methodology

The **GenoType Mycobacterium AS** (Additional Species) test is based on the **DNA•STRIP** technology and permits the identification of the following mycobacterial species: *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 whole procedure is divided into three steps: DNA extraction from cultured material (culture plates/liquid medium; the necessary reagents are not provided), a multiplex amplification with biotinylated primers (the necessary thermostable DNA polymerase is not provided), and a reverse hybridization.

The hybridization includes the following steps: chemical denaturation of the amplification products, hybridization of the single-stranded, biotin-labeled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate, and an AP mediated staining reaction. A template ensures the easy and fast interpretation of the banding pattern obtained.

Storage and Precautions

Store Primer Nucleotide Mix (PNM) at 2-8°C upon arrival isolated from any potential source of contaminating DNA. If longer storage (more than 4 weeks) is required, store at –20°C. In order to avoid repeated freezing and thawing, aliquot PNM. Store all other kit components at 2-8°C. Do not use the reagents beyond their expiry date.

Patient specimens and cultures made from patient specimens must always be considered as potentially infectious. Samples from risk patients and cultures made from those samples must always be labeled and handled under suitable safety conditions. Observe all federal, state, and local safety and environmental regulations. Always wear suitable protective clothing and gloves.

Specimen treatment and sample preparation up to and including the heat inactivation step must be carried out in a class II safety cabinet. Before the heat inactivation step samples must be centrifuged in an aerosol-tight rotor. Open aerosol-tight rotor in

safety cabinet only. After heat inactivation a standard rotor can be used for spinning the samples outside the safety cabinet.
Observe the usual precautions for amplification set-up. It is essential that all reagents and materials used for DNA extraction and amplification set-up are free from DNases.

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 (R 36/38 and S 26-37/39-45).
The **Substrate Concentrate** (SUB-C) contains Dimethyl Sulfoxide and is irritating (R 36/37/38, S 23-26-36).

For additional information, please refer to material safety data sheets which can be downloaded from: www.hain-lifescience.com/products/msds.html

Quality Control

In order to validate the correct performance of the test and the proper functioning of reagents, each strip includes 3 control zones:

- a Conjugate Control zone to check the binding of the conjugate on the strip and a correct chromogenic reaction
- a Universal Control zone which detects, as known, all mycobacteria and members of the group of gram-positive bacteria with a high G+C content
- a Genus Control zone which documents, as known, the presence of a member of the genus *Mycobacterium*

DNA Extraction

Bacteria grown on culture plates (e. g. Loewenstein-Jensen, Middlebrook) or in liquid medium (e. g. BACTEC, MB-Check) may be used. The test must not be used to detect mycobacteria directly from patient material. The working area must be free from amplified DNA. It is crucial to heat samples to 95°C for at least 20 min in order to inactivate vegetative bacteria. Any DNA extraction procedure producing amplifiable DNA from bacteria can be used.

The following quick protocol normally yields DNA suitable for amplification:

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 in a class II safety cabinet at approximately 10000 x g. Discard supernatant and resuspend bacteria in 100-300 µl of water (see above) 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.

Detailed protocols can be requested from your local distributor or through:
www.hain-lifescience.com

Amplification

Prepare the amplification mix (45 µl) in a DNA-free room. The DNA sample should be added in a separate area.

Per tube mix:

- 35 µl PNM – provided
- 5 µl 10x polymerase incubation buffer – not provided
- x µl $MgCl_2$ solution[1)] – not provided
- 1-2 unit(s) thermostable DNA polymerase (refer to manual) – not provided
- y µl water to obtain a volume of 45 µl (not considering volume of enzyme) – not provided
- 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 AS** 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 – provided
- 5 µl 10x PCR Buffer for HotStarTaq (contains 15 mM $MgCl_2$) – not provided
- 2 µl 25 mM $MgCl_2$ solution – not provided
- 0.2 µl (1 U) HotStarTaq – not provided
- 3 µl water (molecular biology grade) – not provided
- 5 µl DNA solution (add in a separated 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). A contamination control sample, for example, contains 5 µl of water instead of DNA solution. Prepare a master mix containing all reagents except for DNA solution and mix well (do not vortex). Aliquot 45 µl in each of the prepared PCR tubes.

Amplification profile[2]:

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

[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.
For checking the amplification reaction, 5 µl of each sample might be directly applied to a 2% agarose gel without the addition of loading buffer. The amplicons have a length of approximately 230 bp (Genus Control) and 200 bp (Universal Control/species specific fragment), respectively.

Hybridization

Preparation

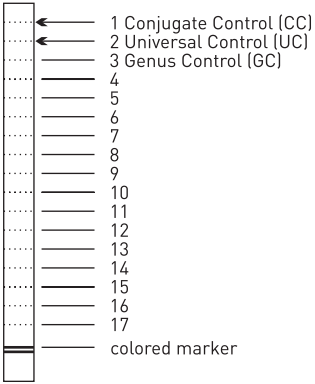
Prewarm shaking water bath/**TwinCubator** to **45°C**; the maximum tolerated deviation from the target temperature is +/–1°C. Prewarm solutions HYB and STR to 37-45°C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary. Warm the remaining 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/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/Twin**Cubator **(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/Twin**Cubator**.**
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/Twin**Cubator **(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 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 by briefly rinsing twice with distilled water.**
14. **Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.**

Evaluation and Interpretation of Results

Paste strips and store protected from light. An evaluation sheet is provided with the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and 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.

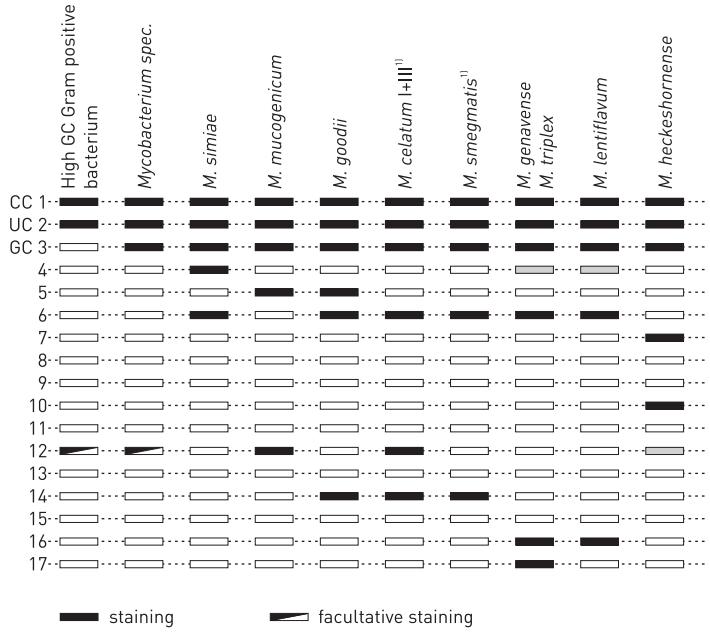
Universal Control (UC)
This zone detects, as known, all known mycobacteria and members of the group of gram-positive bacteria with a high G+C content. If this zone and the Conjugate Control zone stain positive but the remaining banding pattern can not be assigned to a specific mycobacterium, additional methods have to be applied to identify the respective bacterial species.
Only those bands whose intensities are about as strong as or stronger than that of the Universal Control zone are to be considered.

Genus Control (GC)
Staining of this zone documents, as known, the presence of a member of the genus Mycobacterium. The intensity of this band varies depending on the mycobacterial species. The Genus Control band may drop out in spite of the presence of mycobacterial DNA; as long as a species-specific banding pattern is present, however, the amplification reaction was performed properly and the test result is valid.

Other bands
Specific probes, for evaluation see interpretation chart.

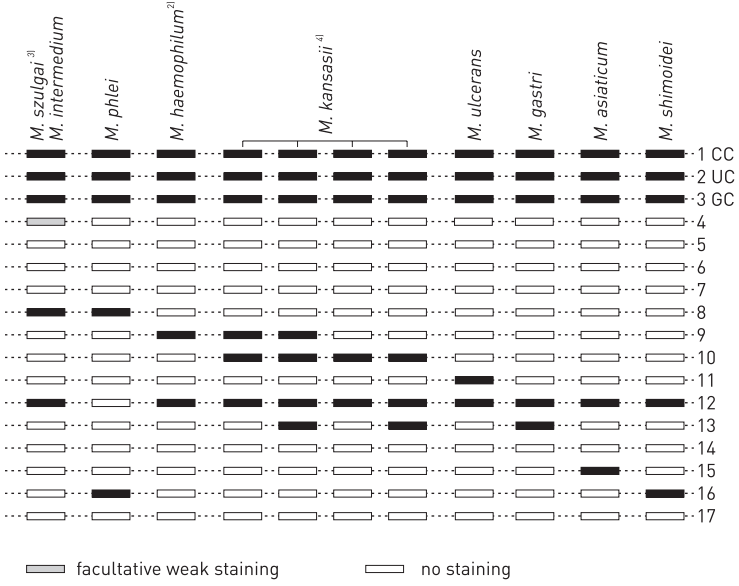
Not all bands of a strip have to show the same signal strength.
When no species-specific banding pattern is present, a pattern indicating the presence of a gram-positive bacterium with a high G+C content might, in rare cases, still originate from a mycobacterium that can not be detected by this kit and additional methods have to be applied to identify the respective bacterial species.
If a large amount of amplicon was used, additional bands may occur (see chapter Troubleshooting).

Interpretation Chart



Band No. 1 (CC): Conjugate Control
Band No. 2 (UC): Universal Control
Band No. 3 (GC): Genus Control

[1)] When using bacteria grown in liquid media, contaminating bacteria might possibly generate the same banding pattern. This result, therefore, is only valid when the DNA was extracted from bacteria grown on solid medium (single colony/morphologically identical colonies).



2) *M. nebraskense* shows the same banding pattern as *M. haemophilum*.

3) If this banding pattern is generated with the **GenoType Mycobacterium AS** kit, a differentiation of *M. szulgai* and *M. intermedium* can be performed using the **GenoType Mycobacterium CM** test. *M. szulgai* will display the banding pattern 1, 2, 3, 10, and 11, *M. intermedium* will display the pattern 1, 2, 3, 10.

4) Due to sequence variations 4 different *M. kansasii* banding patterns are possible.

Limitations

Prior to amplification, DNA has to be extracted from cultured bacteria using a suitable method. It must be ensured that the template DNA is efficiently amplified during the amplification reaction.

The test only works within the limits of the genomic regions the primers and probes were chosen from. Potential sequence analysis remains to further investigations.

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 sub-types might not be detected. The test reflects the state of knowledge of Hain Lifescience.

Use of this assay is limited to qualified personnel well trained in the test procedure and familiar with molecular biological methods.

Performance evaluation of this assay was carried out using the HotStarTaq DNA Polymerase from Qiagen (Hilden, Germany). Since the performance characteristics of this assay have not been validated for all polymerases commercially available, the user is in charge of validating the applicability of polymerases other than that mentioned above.

Performance data of the assay can be requested through:
www.hain-lifescience.com

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.

Weak or no signals except for Conjugate Control zone

- Quality and/or quantity of extracted DNA do not allow an efficient amplification. Check amplicon on a 2% agarose gel. In case no amplicon is visible, repeat DNA extraction and amplification. If necessary, try a different DNA extraction method (see chapter DNA Extraction).
- Incubation temperature too high.

– The extracted bacterial species can not be detected by the Universal Control and Genus Control.

No homogeneous staining
– Strips were not completely immersed during incubation steps.
– Tray was not shaken properly.

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.

Unexpected result
– Wrong incubation temperature.
– Hybridization Buffer and/or Stringent Wash Solution were not properly prewarmed or mixed.
– Contamination of extracted DNA and/or amplification agents with extracted and/or amplified DNA. In case amplification agents are contaminated a negative control sample also shows the respective banding pattern.
– Contamination of neighboring wells by spillage during addition of Hybridization Buffer.
– 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.
– The bacterial species present in the sample can not be detected with this test.

Material Required but not Provided

- Absorbent paper
- Adjustable pipettes for 10, 20, 200, and 1000 µl
- Calibrated thermometer
- 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
- Shaking water bath/**TwinCubator**
- Shaking platform/**TwinCubator**
- Table top centrifuge
- Thermal cycler (heating rate: 3°C/sec, cooling rate: 2°C/sec, precision: +/–0.2°C)
- Thermostable DNA polymerase with buffer (recommendation: hot start enzyme, extension rate: 2-4 kb/min at 72°C, half-life: 10 min at 97°C, 60 min at 94°C, amplification efficiency: $>10^5$ fold)
- Timer
- Tweezers
- Ultrasonic bath
- Water (molecular biology grade)
- Water bath

Kit Contents

	Supplied	
Membrane strips coated with specific probes (STRIPS)	12	96
Primer Nucleotide Mix (PNM) contains specific primers, nucleotides, dye	0.5 ml	4 ml
Denaturation Solution (DEN) ***ready to use*** contains <2% NaOH, dye	0.3 ml	2.4 ml
Hybridization Buffer (HYB) ***ready to use*** contains 8-10% anionic tenside, dye	20 ml	120 ml
Stringent Wash Solution (STR) ***ready to use*** contains >25% of a quaternary ammonium compound, <1% anionic tenside, dye	20 ml	120 ml
Rinse Solution (RIN) ***ready to use*** contains buffer, <1% NaCl, <1% anionic tenside	50 ml	360 ml
Conjugate Concentrate (CON-C) ***concentrate*** contains streptavidin-conjugated alkaline phosphatase, dye	0.2 ml	1.2 ml
Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, <1% NaCl	20 ml	120 ml
Substrate Concentrate (SUB-C) ***concentrate*** contains Dimethyl Sulfoxide, substrate solution	0.2 ml	1.2 ml
Substrate Buffer (SUB-D) contains buffer, <1% $MgCl_2$, <1% NaCl	20 ml	120 ml
tray, evaluation sheet	1 of each	4 of each
manual, template	1 of each	1 of each

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