MTBC-NTM Real Time PCR kit

Kit for the detection of DNA of species belonging to the genus *Mycobacterium, Mycobacterium tuberculosis* complex and *Mycobacterium tuberculosis* species by Real-Time PCR.

REF **Ref. MAD-003963M-50W**
Ref. MAD-003963M-W

Σ **50 determinations**
100 determinations

For in vitro diagnostic use only
Directive 98/79/EC

Rev.: 2022/12/19

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

TABLE OF CONTENTS

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

1 INTENDED USE

The MTBC-NTM Real Time PCR kit is an in vitro diagnostic kit for the qualitative detection of DNA of *Mycobacterium spp., Mycobacterium* tuberculosis complex (MTBC) and *Mycobacterium tuberculosis* species from human pulmonary and extra pulmonary clinical specimens such as sputum, bronchoalveolar lavage, pleural fluid, skin, cerebrospinal fluid and bacterial cultures. It is based on the multiplex real-time PCR technique, using primers and specific fluorescent probes for target genes.

Specific primers and fluorescent probe are included for the simultaneous detection of the human hemoglobin beta gene as internal quality control of the starting and amplification material. The detection channels of the different targets are:

Target	Fluorophore
Mycobacterium spp.	Cy5
MTBC	ROX
Mycobacterium tuberculosis	FAM
Beta globin	HEX/JOE/VIC

Table 1. Detection channels for the different targets of MTBC-NTM Real Time PCR kit.

This test must be carried out at hospital level in clinical microbiology laboratories to those patients who show symptoms compatible with *Mycobacterium* infection. The intended end use is as aid in the diagnosis of this infection in combination with clinical risk and epidemiological factors.

Microbiological status: Non-sterile product.

2 PRINCIPLE OF THE METHOD

The **MTBC-NTM Real Time PCR** kit is a multiplex assay based on the real-time polymerase chain reaction. The Master mix contains a set of primers and probes that allow the detection of DNA from species of the genus *Mycobacterium*, *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium tuberculosis* species. It also includes a set of primers and probe for the detection of the human gene of the beta globin in clinical or control specimens. The oligonucleotides used as primers and probes were selected in evolutionary conserved regions of the bacterial genome.

In the presence of any of these microorganisms in clinical samples, bacterial DNA is amplified by polymerase chain reaction (PCR). The detection of the amplicons obtained is based on the TaqMan probe technology. These probes are modified single-stranded DNA oligonucleotides that have a fluorophore (reporter) covalently attached to the 5' end and a quencher attached to the 3' end If the target nucleic acids are present, these are amplified and, during the PCR process, the probes will bind specifically in the complementary regions located between the forward and reverse primers.

While the extension phase of the PCR occurs, the 5’ nuclease activity of the polymerase ADN degrades the probes bound specifically to their targets, causing the split between the reporter and the quencher, and a

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

fluorescent signal will generate. The specific probes for the different targets and the internal control are labeled with different fluorophores, so that in each case a fluorescent signal will be generated at different wavelengths, allowing the Real Time PCR platform to simultaneously detect and differentiate the different signals in a single reaction. In each denaturation-extension cycle, the split of new molecules of reporter occurs, and, consequently, the intensity of the fluorescent signal increases. The intensity of the fluorescence is monitored on the real-time PCR instruments in each of the cycles and the data are analyzed with a specific analysis software for each platform.

The detection of bacterial DNA is of great usefulness in the diagnosis and follow-up of infections caused by these microorganisms.

3 COMPONENTS

The **MTBC-NTM Time PCR** kit is commercialized as a ready-to-use Master Mix which includes all the necessary reagents to perform the real-time PCR.
Furthermore, in order to avoid contamination with previous PCR products, the Mix contains the enzyme *Uracil-ADN Glycosylase* (UDG), which degrades PCR products containing dUTP.
A tube as positive control (PC) and DNase/RNase-free water to include in the negative controls (NTC) are supplied along with the RT-PCR Mix.

REFERENCE (DESCRIPTION)		CONTENT	AMOUNT
MAD-003963M-100-W (MTBC-NTM MMIX)	**MAD-003963-MIX-W (MTBC-NTM MMix)**	Hot Start ADN Polymerase, Uracil DNA glycosylase, primers, fluorescent probes, reaction buffer, dNTPs (dATP, dCTP, dGTP, dTTP, dUTP)	**2 vials with 50 test/vial**
	MAD-DDW-200 (RNAse/DNAse free water)	---	**1 vial (200 µl)**
MAD-MTBC-NTM (MTBC PC)		Synthetic non-infectious DNA containing parts of the *Mycobacterium tuberculosis* genome and human DNA	**1 vial (100 µl)**

Table 2. Reagents supplied in the MTBC-NTM Real Time PCR kit (100 test format).

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

REFERENCE (DESCRIPTION)		CONTENT	AMOUNT
MAD-003963M-50-W (MTBC-NTM MMIX)	**MAD-003963-MIX-W (MTBC-NTM MMix)**	Hot Start ADN Polymerase, Uracil DNA glycosylase, primers, fluorescent probes, reaction buffer, dNTPs (dATP, dCTP, dGTP, dTTP, dUTP)	**1 vial with 50 tests**
	MAD-DDW-200 (RNAse/DNAse free water)	---	**1 vial (200 µl)**
MAD-MTBC-NTM (MTBC PC)		Synthetic non-infectious DNA containing parts of the *Mycobacterium tuberculosis* genome and human DNA	**1 vial (100 µl)**

Table 3. Reagents supplied in the MTBC-NTM Real Time PCR kit (50 test format).

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and materials

- Disposable gloves.
- Pipette tips with DNase/RNase-free filters.
- DNA extraction kit.
- Tube strips/plates/optical adhesive films specific for each equipment of Real-Time PCR

4.2 Equipment

- Laminar flow cabinet
- Microcentrifuge for tubes of 1.5ml.
- Microcentrifuges of PCR tube strips or 96-well plates.
- Vortex.
- Automatic micropipettes: P1000, P200, P20 and P2.
- Real-Time PCR instrument.

5 STORAGE AND STABILITY CONDITIONS

The **MTBC-NTM Real Time PCR** kit must be transported and stored at -10/-30 °C*. Nonetheless, besides the recommended transport at -10/-30 °C, it is also possible to transport it at refrigeration temperature (2 °C– 8 °C), as long as the transit period does not exceed a maximum of ten days. In any case, the kit must be stored at a temperature of -10/-30 °C upon receipt.

The reaction mix **MTBC-NTM MMix** is sensitive to physical state changes and it has been proven that it supports up to five freeze-thaw cycles. If a run is performed with a low number of samples, it is recommended to aliquot the reagent in advance. The mix contains fluorescent molecules and it must be kept away from direct light.

The positive control is sensitive to physical state changes and it must not undergo more than eight freeze-thaw cycles. It is advisable to handle the positive control vial separately from the clinical samples to avoid potential contamination which might yield false positives.

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

If stored at recommended temperature, the PCR reagents are stable until the expiration date indicated. The PCR reagents must be stored in areas free of DNA or PCR products contamination.

*A temperature indicator is included in the package to control the conditions during the shipment. In case the cold chain is interrupted, it is recommended to contact the manufacturer before using the reagents.

6 WARNINGS AND PRECAUTIONS

- Read the instructions for use before using this product.
- The kit must be handled by qualified technicians in molecular biology techniques applied to diagnosis.
- Do not use any component of the kit after the expiration date.
- The MTBC-NTM MMix must be thawed before use and handled on ice or cold plate and away from light. Mix the solutions by inverting the tubes several times without shaking in vortex, and centrifuge briefly.
- The positive control must be thawed at room temperature, mixed well and centrifuged briefly before use.
- The safety and disposal precautions are described in the Safety Data Sheet of this product. This product is only intended for professional laboratory purposes, and it is not intended for pharmacological, home or any other type of use. The current version of the Safety Data Sheet of this product can be downloaded in the web page www.vitro.bio or requested at regulatory@vitro.bio.
- The MTBC-NTM Real Time PCR kit uses nucleic acids previously extracted and purified as starting material. It is the client's responsibility to include the necessary controls to verify that the system of extraction of the used genetic material works properly.

- **General considerations to avoid the contamination with PCR product**
  The most important contamination source is usually the same amplified PCR product. Therefore, it is recommended to carry out the amplification and handling of the amplified products in a different area to the one where the DNA extraction and PCR preparation are performed. It is recommended to work in different pre- and post-PCR areas where the handling of the test DNA and preparation of the PCR tubes (pre-PCR), and the amplification and handling of the amplified products (post-PCR) are performed. These areas must be physically separated and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. Furthermore, in order to avoid the contamination with previous PCR products, the enzyme *Uracil-ADN Glycosylase (UDG),* which degrades the PCR products containing dUTP, is included in the kit.

  It is recommended to include negative amplification controls replacing the DNA specimen with RNase/DNase-free water, in order to detect and control any possible contamination of the reagents with test samples or amplified products.

- **Waste disposal**
  The handling of wastes generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable law in the country in which these products are being used. As

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

reference, the following table indicates the classification of waste generated by this kit according to the European Law, specifically according to the European Commission Decision of December 18, 2014 amending decision 2000/532/EC on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

POTENTIAL WASTE GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW*
1. Liquid waste disposal	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, this waste must be considered as "waste whose storage and disposal is subjected to special requirements in order to prevent infection"
2. Perishable material (tubes, tips, etc.) 3. Any element that has been in contact with the starting genetic material	180103	"Waste whose collection and disposal is subject to special requirements in order to prevent infection"
4. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers containing waste or contaminated by dangerous substances"

Table 4. Classification of wastes generated by this kit according to the European Legislation. *ELW: *European Legislation of Waste.*

*Note: This classification is included as a general guideline of action, being under the final responsibility of the user the accomplishment of all the local, regional and national regulations on the disposal of this type of material

7 PREPARATION OF THE CLINICAL SAMPLE FOR ANALYSIS

The MTBC-NTM Real Time PCR kit has been validated for use with purified genetic material from different types of pulmonary and extra pulmonary clinical samples, such as sputum, bronchoalveolar lavage, pleural fluid, skin, cerebrospinal fluid and bacterial cultures.

This kit has been validated with starting genetic material from the following DNA/RNA purification kits and extraction equipment*:

EXTRACTION KITS	EXTRACTION EQUIPMENT
MagCore® Genomic DNA Bacterial kit (Cartridge code 502)	MagCore® HF16 Plus System
RNA/DNA pathogen extraction kit (Robot Opentrons OT2) (Vitro, ref. MAD-003955M)	Opentrons OT-2

Table 5. Extraction kits and instruments used for the purification of DNA/RNA from clinical samples.

*Note: The system has not been validated with other DNA/RNA extraction systems. Therefore, if any other purification system is used, this must be verified beforehand.

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

8 PCR PROTOCOL

8.1 Preparation of the Reaction mix

The PCR is carried out in a final volume of 20 µl. Prepare the Master Mix as indicated below:

1. Thaw and homogenize MTBC-NTM MMix (do not use vortex). Once it is thawed, centrifuge briefly.
2. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
MTBC-NTM MMix	12 µl
Sample	8 µl

Table 6. Preparation of the mix of the MTBC-NTM Real Time PCR kit.

3. Include a negative control by adding 8 µl of the water included in the kit.
4. Include a positive control by adding 8 µl of the positive control DNA MTBC-NTM included in the kit.
5. Centrifuge briefly to make sure there are no air bubbles in the wells.

It is recommended to keep the MMix on cold plate during the preparation of the samples and not thaw the vial more than five times.

8.2 Configuration of the instrument for real-time PCR

Enter the different targets and detection channels for each of them in the instrument's software. Create the samples, the positive control (PC), the PCR targets (NTC) and allocate the positions of the samples in the PCR plate.

Set the real-time PCR instrument following the steps below:

PCR PROGRAM		
25°C	5 min	1 cycle
95°C	5 min	1 cycle
95°C	15 sec	5 cycles
75°C	30 sec	
95°C	15 sec	45 cycles
60°C*	40 sec	

Table 7. PCR program of the MTBC-NTM Real Time PCR kit.

*Fluorescence data should be collected during the extension stage (*) through FAM (*Mycobacterium tuberculosis*), Cy5 (*Mycobacterium*), ROX (MTBC) and HEX, JOE or VIC (Internal Control) channels.

This kit has been validated with the platforms:

- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- Vitrocycler (Vitro S.A.)

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

For use in other platforms, it is recommended to verify the compatibility of the fluorochromes with the detection channels of each platform, although the fluorochromes included in the kit are compatible with the majority of the most-used real-time instruments available on the market.

In the Applied Biosystems QuantStudio™ 5 Real-Time PCR System thermal cycler the ROX passive control option must be disabled.

In the thermal cycler Applied Biosystems QuantStudio™ 5 Real-Time PCR System select Ramp Speed Standard in the menu "Select New Experiment/Advanced Setup/Experiment Properties".

9 INTERPRETATION OF RESULTS

Before interpreting the results of the clinical samples, it is necessary to follow the interpretation guide of the positive and negative controls as in the table below:

	RESULT	INTERPRETATION
Positive Control MTB-MDR	Signal for the channels FAM, ROX, Cy5 and JOE*	The control/reaction is correct
	No signal for FAM and/or ROX and/or Cy5 and/or JOE	Problem in the amplification: repeat analysis
Negative control:	Signal for the channels FAM and/or ROX and/or Cy5 and/or JOE	Contamination, repeat analysis
	No signal	The control/reaction is correct

Table 8. Interpretation guide of the controls of the MTBC-NTM Real Time PCR kit.

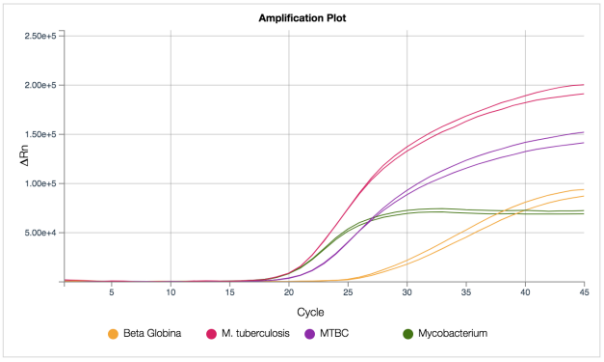
*The amplification signal must be determined by a rapid and steady increase in the fluorescence values and not by peak phenomena or gradual increase of the background signal (irregular background or increased background noise) (Fig 1).

The run is considered valid when adequate results have been obtained for all reaction controls and the Cts values obtained in the positive control for the different targets are within the range of expected values, being these:

- 20±2 for *Mycobacterium tuberculosis* (FAM)
- 20±2 for MTBC (ROX)
- 20±2 for *Mycobacterium* spp. (Cy5)
- 25±2 for Betaglobin (JOE/VIC/HEX)

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

A



B

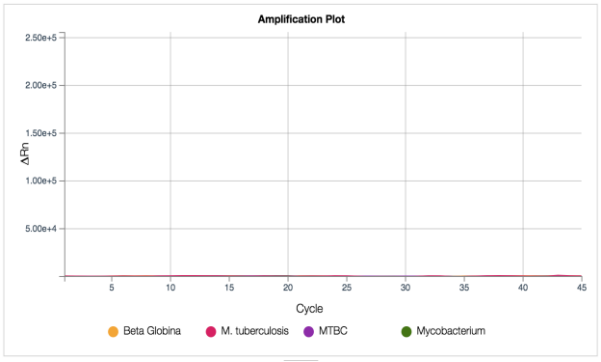


Figure 1: Graphs of amplification of a positive control PC (A) and of a negative control with NTC water (B). Experiment performed in Applied Biosystems QuantStudio™ 5 Real-Time PCR System.

If the run has been validated, interpret the results of the clinical samples according to the following table:

FAM	ROX	Cy5	JOE	Result
+	+	+	+/-	*Mycobacterium tuberculosis*
-	+	+	+/-	*Mycobacterium tuberculosis* complex mycobacteria
-	-	+	+/-	Non Tuberculous Mycobacterium (NTM)*.
-	-	-	+	Negative sample
-	-	-	-	Invalid sample**

Table 9. Interpretation guide of the results obtained with the MTBC-NTM Real Time PCR kit.

*The presence of signal in the Cy5 channel together with the absence of signal in the FAM and ROX channels is
indicative of a Mycobacterium species not belonging to the MTBC complex, and would therefore be considered a nontuberculous mycobacterium (NTM).

**Problems in the extraction or amplification.

A sample is considered positive if there is signal in the Cy5 and/or ROX and/or FAM channel, even if the internal control does not show an amplification graph. Sometimes, it might occur that the internal control is not amplified correctly due to the presence of a high initial number of copies of target bacterial nucleic acid, which can cause a preferential amplification of the latter.

A sample is negative if an amplification curve is not detected over the threshold value, and if the internal control does show it. The inhibition of the PCR reaction can be excluded by the amplification of the internal control.

The figures below show the expected amplification curves in each case:

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

Mycobacterium tuberculosis

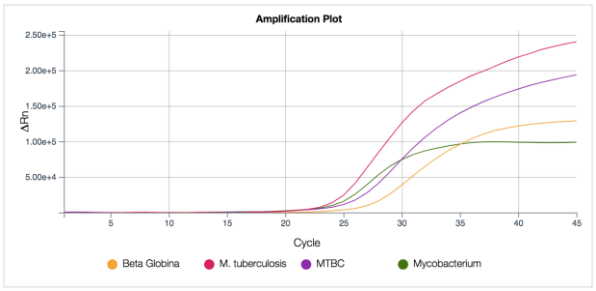


Figure 2: Amplification curves of *M. tuberculosis.*

MTBC

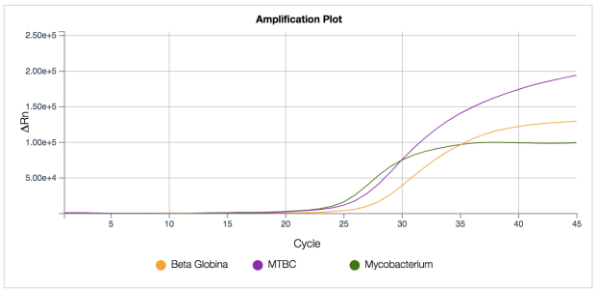
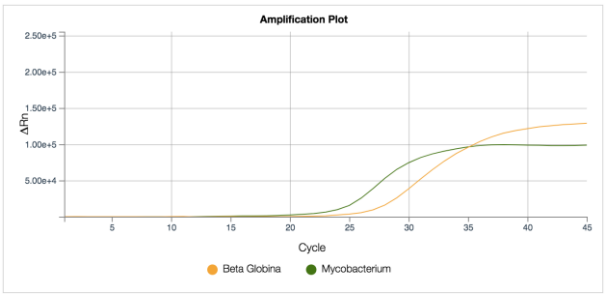


Figure 3: Amplification curves of mycobacteria belonging to the MTBC complex.

NTM



Negative sample

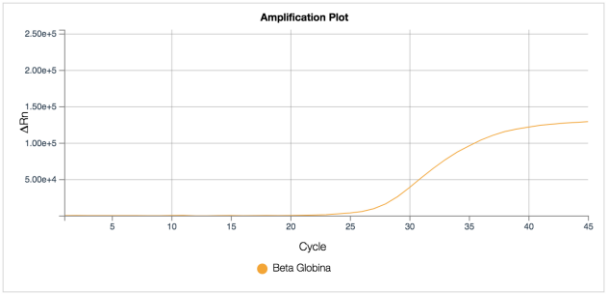


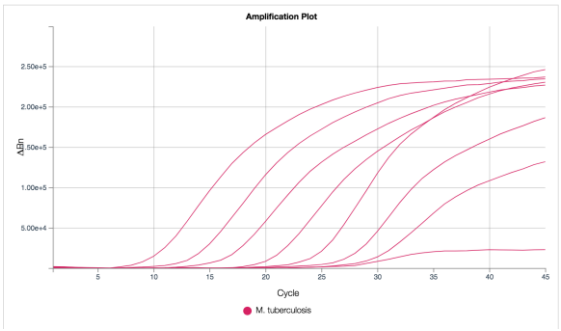
Figure 4: Amplification curves of mycobacteria Figure 5. Tuberculous negative sample (MNT).

10 PERFORMANCE CHARACTERISTICS

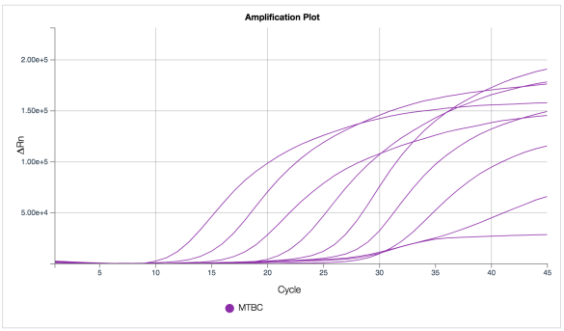
10.1 Analytical sensitivity

The analytical sensitivity of the MTBC-NTM Real Time PCR kit was determined by performing serial dilutions of synthetic fragments containing different parts of the *Mycobacterium tuberculosis* genome. It has been established that the MTBC-NTM Real Time PCR kit has a detection limit of 10 copies/reaction for the *M. tuberculosis* and *Mycobacterium* targets and 1 copy/reaction for the MTBC target (Figure 6).

A



B



Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

C

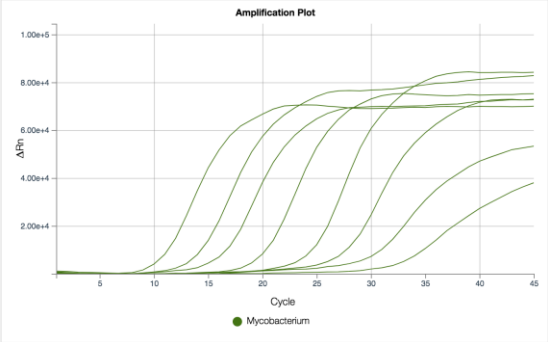


Figure 6: Serial dilutions from 10^8 copies/reaction to 1 copies/reaction of synthetic fragments of the *Mycobacterium tuberculosis* genome in the FAM (A), ROX (B) and Cy5 (C) channel.

The amplification efficiency for each of the targets was evaluated with 9 serial dilutions of a standard from 10^8 copies/rxn to 1 copy/rxn. By adjusting the Cts data to a line, the amplification efficiency, R^2 and the slope were determined for each of the genes.

The target for *M. tuberculosis* had an amplification efficiency of 100.47%, an R^2 of 0.994 and a slope of -3.321. The target for MTBC presented an amplification efficiency of 106.247%, an R^2 of 0.997 and a slope of -3.181. The target for Mycobacterium showed an amplification efficiency of 97.201%, an R^2 of 0.994 and a slope of -3.391 (Figures 7 to 9).

The MTBC-NTM Real Time PCR kit has a detection sensitivity for the genus *Mycobacterium*, and the species *Mycobacterium tuberculosis* of 10 copies/reaction and 1 copy/reaction for the *Mycobacterium tuberculosis* complex.

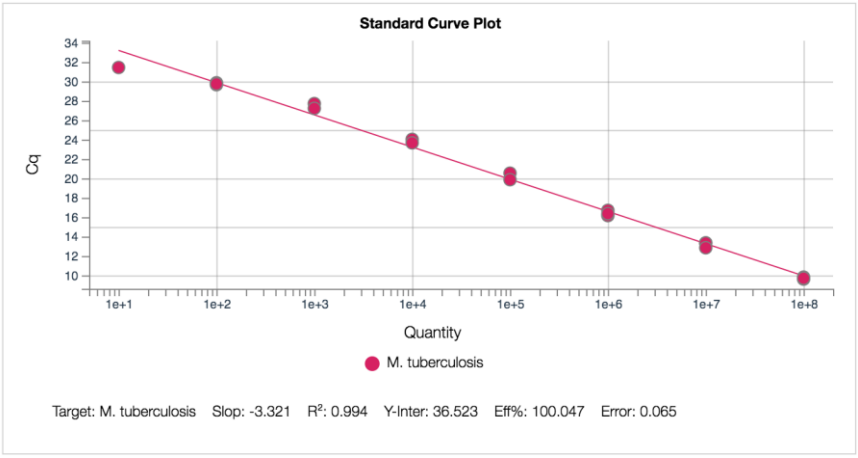


Figure 7: Calibration line for the *M. tuberculosis* target in the FAM channel.

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

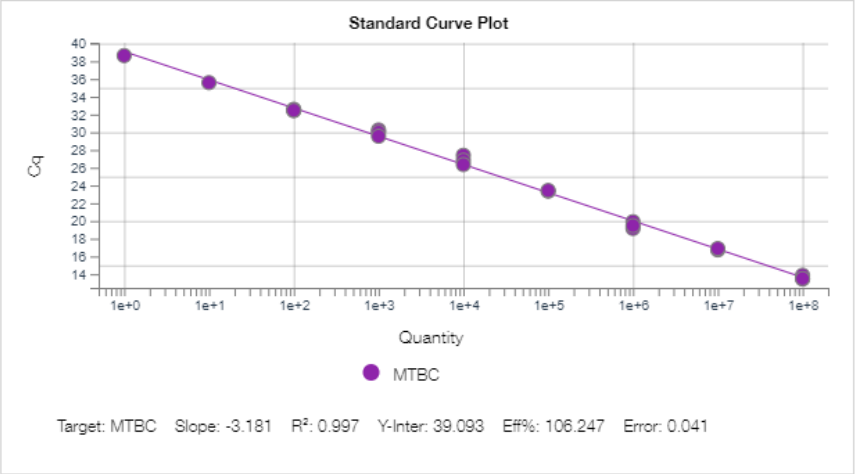


Figure 8: Calibration line for the MTBC target in the ROX channel.

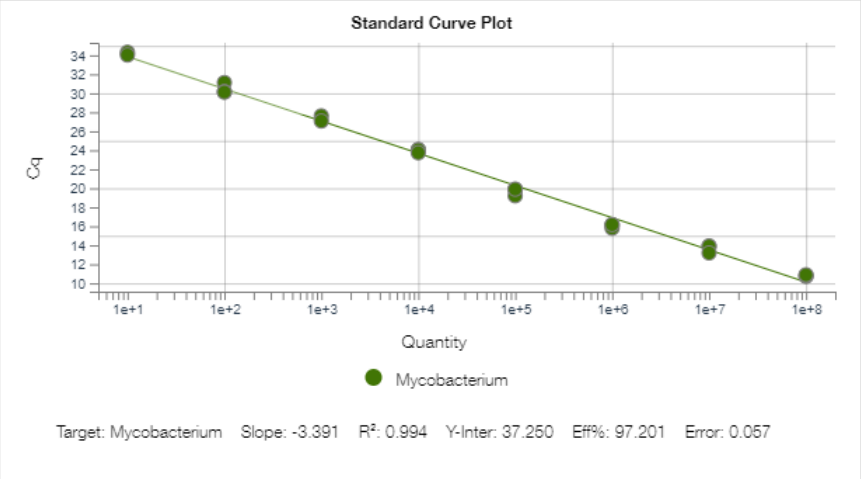


Figure 9: Calibration line for the *Mycobacterium* target in the Cy5 channel.

10.2 Analytical specificity

The specificity of the MTBC-NTM Real Time PCR kit was confirmed by testing a panel composed of the genome of different microorganisms (bacteria, viruses and fungi) representing the most common respiratory pathogens. The full list of organisms tested for cross-reactivity is detailed in the following table.

Cross-reactivity test	
Microorganism	**Results**
Candida albicans	Negative
Chlamydophila pneumoniae	Negative
Coronavirus	Negative
Influenza A (H1N1)	Negative
Influenza A (H3)	Negative

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

Influenza B	Negative
Klebsiella pneumoniae	Negative
Mycoplasma pneumoniae	Negative
SARS-CoV-2	Negative
Rhinovirus	Negative
Staphylococcus aureus	Negative
Streptocccus pneumoniae	Negative
Parainfluenza virus type 1	Negative
Parainfluenza virus type 2	Negative
Parainfluenza virus type 3	Negative
Parainfluenza virus type 4	Negative
Respiratory syncytial virus subtype A	Negative
Respiratory syncytial virus subtype B	Negative

Table 10. Microorganisms tested in the specificity test.

No cross-reactions were detected with any of the pathogens tested.

10.3 Analytical reactivity

The MTBC-NTM Real Time PCR kit has been validated for the detection of the following species:

- Species belonging to the MTBC complex.

Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, *Mycobacterium bovis* BCG, *Mycobacterium caprae.*

- Non-tuberculous mycobacteria*:*

Mycobacterium abscessus subsp. abscessus, Mycobacterium abscessus subsp. bolletii, Mycobacterium abscessus subsp. massiliense, Mycobacterium avium, Mycobacterium celatum, Mycobacterium chelonae, Mycobacterium chimaera, Mycobacterium fortuitum, Mycobacterium gordonae, Mycobacterium interjectum, Mycobacterium intracellulare, Mycobacterium kansassii, Mycobacterium lentiflavum, Mycobacterium malmoense, Mycobacterium marinum, Mycobacterium mucogenicum, Mycobacterium scrofulaceum, Mycobacterium simiae, Mycobacterium smegmatis, Mycobacterium specie, Mycobacterium szulgai y Mycobacterium xenopi.

10.4 Repeatability

The repeatability of the MTBC-NTM Real Time PCR kit was analyzed by assaying the method 7 times for each of the targets included in the panel. For this purpose, a known concentration of synthetic DNA fragments

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

mimicking each of the targets to be amplified was used. The test was performed by the same operator, in a single location and using the same reagent lot and the same platform. The platform used was Applied Biosystems QuantStudio™ 5 Real-Time PCR System and the results were analyzed with the version v. 2.4.3 of the software Design and Analysis (Applied Biosystems). Inter-trial variability was determined from the Cts values obtained from the replicates. The coefficient of variation (CV) was calculated as the standard deviation divided by the mean of the Cts, being 1.12% for *Mycobacterium*, 1.39% for MTBC and 1.47% for *Mycobacterium tuberculosis*.

10.5 Reproducibility

The reproducibility of the MTBC-NTM Real Time PCR kit was analyzed by simulating inter-laboratory variability, varying the operator, the equipment used in the process and the PCR mix lots. Sixty samples of DNA from bacterial cultures purified with the RNA/DNA pathogen extraction kit (Robot Opentrons OT2) (Vitro, ref. MAD-003955M) were assayed. Of the 60 samples, 53 were positive for any of the targets included in the kit and 7 samples were negative. The results obtained are shown in Table 9.

	Laboratory 2		
Laboratory 1	Positive	Negative	Total
Positive	51	0	51
Negative	2	7	9
Total	53	7	60

Table 11. Reproducibility test for the targets included in the MTBC-NTM Real Time PCR kit.

With these results we obtained a concordance of 96.7% and a kappa index of 0.856, demonstrating a very good concordance strength for the MTBC-NTM Real Time PCR kit.

10.6 Measurement range

It has been established that the MTBC/NTM Real Time PCR kit has a measurement range of 10^9 copies/reaction to 10 copies/reaction for the *Mycobacterium* and *Mycobacterium tuberculosis* targets and 109 copies/reaction to 1 copy/reaction for the MTBC target.

10.7 Clinical sensitivity and specificity

The diagnostic capability of the MTBC-NTM Real Time PCR kit was evaluated by studying its diagnostic sensitivity and specificity. These two parameters are defined and calculated as follows:

- The **diagnostic specificity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 x the number of true negative values (TN) divided by the sum of true negative values (TN) plus the number of false positive (FP) values, or 100 × TN/ (TN + FP).

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

- The **diagnostic sensitivity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of true positive values (TP) plus the number of false negative values (FN), or 100 × TP/ (TP + FN).

The study consisted of a retrospective analysis of 149 samples (pulmonary and extrapulmonary). The samples had been previously characterized using the GeneXpert® (Cepheid) and Genotype Mycobacterium CM (HAIN Lifescience) systems, which were taken as the reference method for the comparative study. Of the 149 samples, 36 were sputum or bronchoalveolar lavage (BAL) smear-positive samples from which Mycobacterium tuberculosis was isolated and 113 were bacterial cultures of which 78 were characterized as NTM, 29 as MTBC, 5 as M. tuberculosis and 1 was positive for MTBC and M. tuberculosis. Pretreatment and purification of DNA from bacterial cultures was fully automated on the Opentrons OT-2 Platform using the RNA/DNA pathogen extraction kit (Opentrons OT2 Robot) (Vitro, ref. MAD-003955M). DNA purification from sputum and BAL samples was performed with the MagCore® Genomic DNA Bacterial kit (Cartridge code 502).

The results of diagnostic specificity and sensitivity, as well as positive and negative predictive value, concordance and kappa index obtained from the analysis of samples from bacterial cultures are shown in the following tables:

Total 113	TP	TN	FP	FN	SENSITIVITY (%)	95% CI	SPECIFICITY (%)	95% CI
NTM	78	35	0	0	100	0.942 - 1	100	0.877 - 1
MTBC	28	83	0	2	93.3	0.765 - 0.988	100	0.945 - 1
TB	6	107	0	0	100	0.517 - 1	100	0.957 - 1
GLOBAL	112	225	0	2	98.3	0.932 - 0.997	100	0.979 - 1

Table 12. Clinical sensitivity and specificity of the MTBC-NTM Real Time PCR kit.

Total 113	TP	TN	FP	FN	VPP (%)	95% CI	VPN (%)	95% CI
NTM	78	35	0	0	100	0.942 - 1	100	0.877 - 1
MTBC	28	83	0	2	100	0.85 - 1	97.7	0.91 - 0.996
TB	6	107	0	0	100	0.517 - 1	100	0.957 - 1
GLOBAL	112	225	0	2	100	0.959 - 1	99.1	0.965 - 0.998

Table 13. Positive predictive value and negative predictive value of MTBC - NTM Real Time PCR kit.

Total 113	TP	TN	FP	FN	CONCORDANCE (%)	Kappa coefficient	Standard error	95% IC	Force of concordance
NTM	78	35	0	0	100	1	0	1 - 1	Very good
MTBC	28	83	0	2	98	0.954	0.032	0.89 - 1.017	Very good
TB	6	107	0	0	100	1	0	1 - 1	Very good
GLOBAL	112	225	0	2	99	0.987	0.009	0.968 - 1.005	Very good

Table 14. Concordance values and kappa index of the MTBC-NTM Real Time PCR kit.

Preliminary results obtained with sputum and BAL samples showed a clinical sensitivity of 100% for *M. tuberculosis*.

11 LIMITATIONS OF THE TEST

1. The results of the test must be evaluated by a healthcare professional in the context of medical history, clinical symptoms, and other diagnostic tests.

2. This test can be used with different types of samples, although it has only been validated with DNA extracted from sputum, bronchial lavage, cerebrospinal fluid and cell cultures.

3. The correct functioning of the test depends on the quality of the sample; the nucleic acid must be properly extracted from the clinical samples. Improper collection, storage and/or transport of samples can result in false negatives.

4. A low number of target copies below the detection limit can be detected, but the results may not be reproducible.

5. A positive test for Mycobacterium does not exclude the possibility that other pathogens are present in the clinical sample.

6. The test works within the genomic regions in which the probes have been designed.

12 LABEL AND BOX SYMBOLS

IVD	In vitro diagnostic medical device		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions for use		Sufficient content for <n> assays
	Safety data sheet		Keep away from sunlight

13 CHANGELOG

Date	Description
2022-12-19	Creation date

Vitro S.A.
Calle Luis Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Seville (Spain).
Phone: +34 954 933 200. vitro@vitro.bio; www.vitro.bio