MYCOBACTERIUM REALTIME PCR KIT (Ref: RTPCR016-LP AND RTPCR016-LPD)

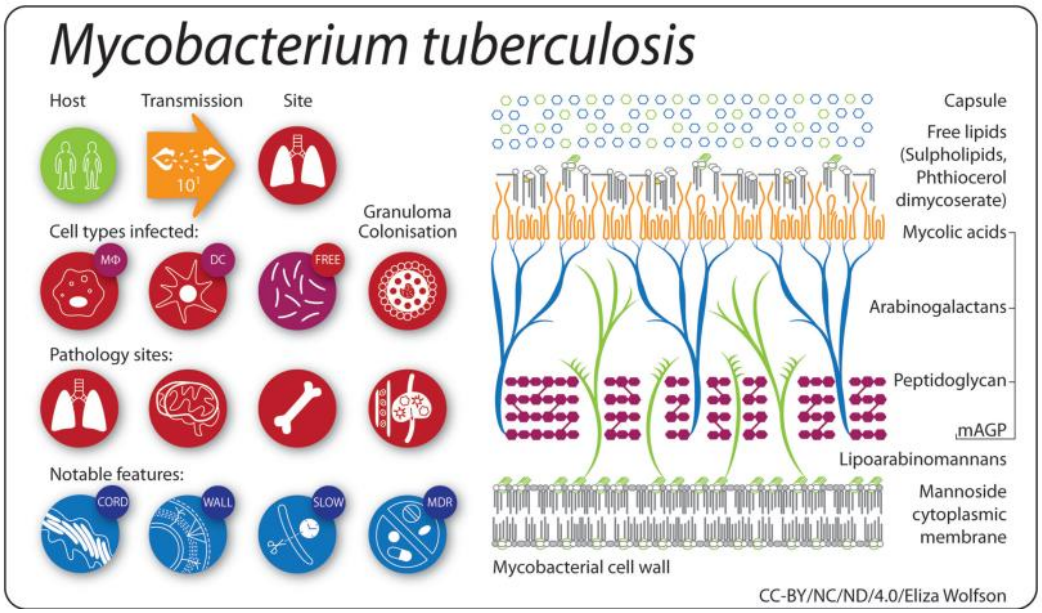
Introduction

Tuberculosis (TB) is caused by the bacillus *Mycobacterium tuberculosis*, bacillus discovered by Robert Koch in 1882. This is still considering a major event in the history of medicine.

Mycobacterium tuberculosis causes tuberculosis in both humans and animals.

They are found in various sources, such as soil, water and animals.

Mycobacterium tuberculosis is a slow-growing, chemoorganotrophic, non-motile, non-spore-forming, aerobic bacillus (rod-shaped bacteria) with a size of 0.2–0.6 µm wide and 1.0–10 µm long.



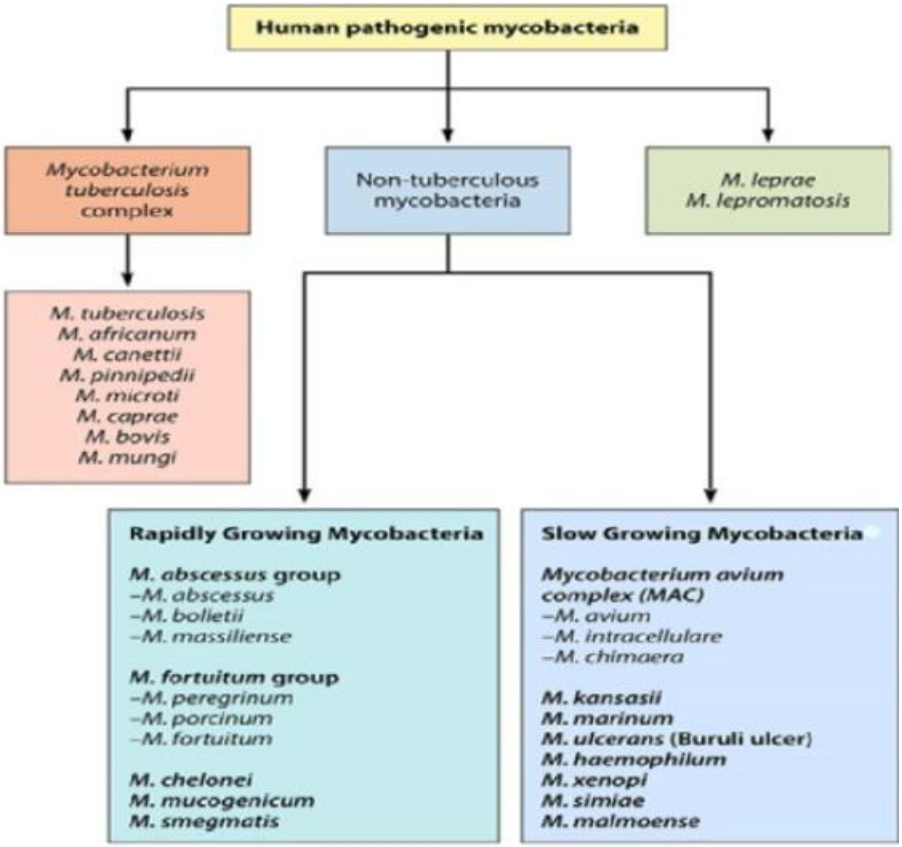
MΦ, Macrophage; DC, Dendritic cell; CORD, Cord factor; MDR, Multidrug resistance

The cell wall of *M. tuberculosis* is characteristic of the mycobacteria; it has a structure similar to that of Gram-negative bacteria with a "second outer membrane" containing the mycolic acids. This wall makes *M. tuberculosis* to have a relative impermeability to antibiotics. Cord factor (trehalose dimycolate) is required for virulence and responsible for the characteristic growth of *M. tuberculosis* in culture as long snake-like cords. The elaborate cell wall is however the Achilles heel of the bacillus, since its synthesis is the target of several frontline anti-tubercular drugs (isoniazid, ethambutol, ethionamide).

Depending on the ability of the *Mycobacteria* to produce Tuberculosis disease, they are classified as:

- Tuberculosis *Mycobacteria*: Due to *M. tuberculosis* complex. There are different species within this complex causing a chronic granulomatous disease that mainly affects the lungs.

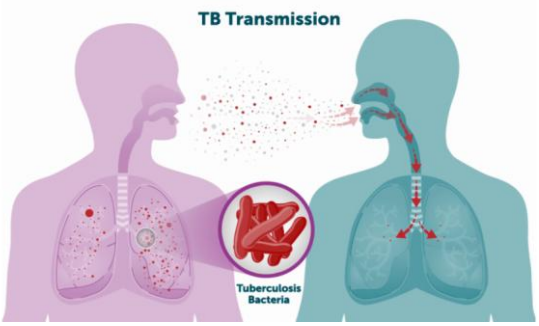
- Non-tuberculous *Mycobacteria* (NTMs): Due to Non tuberculosis complexes such as *Mycobacterium avium* complex *(MAC),* or *Mycobacterium abscessus (MABSC)* complex among others.



The disease is spread when people who are sick with TB expel bacteria into the air from one person to another (e.g. by coughing, sneezing, spitting). TB typically affects the lungs (pulmonary TB) but can also affect other sites (extrapulmonary TB: such as the kidney, spine or brain). Most people who develop the disease (about 90%) are adults and there are more cases among men than women.

Not everyone who is exposed to a person with infectious TB disease becomes infected. There are four factors that determine the probability of transmission of TB bacteria.

- **Susceptibility** (immune status) of the exposed individual.
- **Infectiousness** of the person with TB disease, which is directly related to the number of tubercle bacilli that they expel into the air.
- **Environmental** factors (such as space, ventilation, circulation, etc.) that affect the concentration of TB bacteria in the air.
- **Exposure** factors, including duration, frequency, and physical proximity to the person with TB disease



About a quarter of the global population is estimated to have been infected with TB. Following infection, the risk of developing TB disease is highest in the first 2 years (approximately 5%), after which it is much lower.

TB is a preventable and usually curable disease. Data of 2022, TB was the world's second leading cause of death from a single infectious agent, after coronavirus disease (COVID-19). TB is a major infection disease of global public health concern with high mortality and morbidity.

Vaccination stands as the most economically efficient method for the prevention and management of TB, serving as a crucial approach towards realizing the WHO's Global End TB Strategy by 2035.
One of the most successful measures in this regard is the Bacillus Calmette–Guérin (BCG) vaccine, which has been widely deployed worldwide for 140 years since its inception.
BCG provides significant protection against severe TB in infants and young children, such as disseminated tuberculosis and meningeal tuberculosis, its protection against adult pulmonary tuberculosis (PTB) is limited, with varying efficacy and no effective protection against primary or latent TB infection caused by MTB.

Not everyone infected with TB bacteria becomes sick. As a result, two TB-related conditions exist: latent TB infection and TB disease.

After TB infection occurs, within 2 to 8 weeks, special immune cells called macrophages ingest and surround the tubercle bacilli. The cells form a barrier shell called a granuloma that keeps the bacilli contained and under control. This condition is known as latent TB infection.
In the latent TB infection, bacteria can live in the body without making you sick. Moreover, people will never develop TB disease. They are not able to spread the TB disease. However, they need to receive an appropriate treatment in order to prevent TB disease.
TB disease occurs when tubercle bacilli overwhelm the immune system of someone with latent TB infection and the bacilli will rapidly multiply. This process can occur in different areas in the body, such as the lungs, kidneys, brain, or bone.
The progression from latent TB infection to TB disease may occur at any time, but it is most common within the first two years of infection, or in people who have weaker immune systems because of certain medications or medical conditions (such as diabetes, cancer, or HIV).

PEOPLE WITH LATENT TB	**PEOPLE WITH TB DISEASE**
Have a small number of TB bacteria in their body that are alive but **inactive**	Have a large amount of active TB bacteria in their body
Cannot spread TB bacteria to others	May spread TB bacteria to others
Usually have a positive TB blood test or TB skin test results indicating TB infection	Usually have a positive TB blood test or TB skin test result indicating TB infection
Have typically normal chest radiographs	May have abnormal chest radiographs
Have negative sputum smears and cultures	May have positive sputum smears and cultures
Should consider treatment for latent TB infection to prevent TB disease	Need treatment for TB disease
Do not require respiratory isolation	May require respiratory isolation

The most common symptoms of TB disease include:

- Prolonged cough
- Chest pain
- Weakness or fatigue

- Weight loss
- Fever
- Night sweats

Due to these symptoms could be mild for many months, there is delay in seeking care as well as an increasing risk of spreading the infection to others.
In the case of suspected lung TB disease, patients will be asked to give a sputum sample for testing for TB bacteria.
For non-lung TB disease, samples of affected body fluids and tissue can be tested.

Diseases caused by non-tuberculous mycobacteria (NTM) have global importance in the public health worldwide. There are increases in incidence and prevalence of these diseases mainly linked with the increasing numbers of patients with pulmonary *Mycobacterium avium* complex (MAC) disease in many countries. NTM are found worldwide and cause infections that are easily missed, difficult to diagnose, and difficult to treat.

The natural habitats for NTM range from natural brackish and marshy waters to municipal water distribution systems and household plumbing including shower heads. NTM are also found in potting soil and other peat rich soils. This overlap of bacterial habitat with human habitation provides an ideal opportunity for human infection.

It is believed that NTM are generally acquired from the environment via ingestion, inhalation, and dermal contact, which results in lymphadenitis, pulmonary and disseminated infections, and skin and soft tissue infections. There is no evidence for human-to-human transmission of NTM infection. However, *M. abscessus* subsp. *massiliense* clones are widely dispersed globally in patients with cystic fibrosis and there have been reports of transmission within a hospital or clinic setting although whether transmission occurred directly or indirectly between patients has not been established unequivocally.

Although it is not clear why NTM diseases have been increasing, there are several contributing factors, such as, (i) an increase of mycobacterial infection sources in the environment, (ii) an increase in the number of susceptible individuals, (iii) improvements of laboratory detection techniques, and (iv) increased awareness of NTM diseases.

Moreover, as opposed to tuberculosis caused by *M. tuberculosis* infection, NTM infection reporting is not mandatory; therefore, the incidence and prevalence of the different species of NTM are difficult to determine. However, the prevalence of NTM infection is growing in the United States, Europe, and other developed countries in the Western world. The increased occurrences of NTM infections are associated with declining tuberculosis rates in areas of higher socioeconomic standards.

An 81% declining tuberculosis (TB) incidence rate, while NTM disease rose by 94% in almost every geographic area has been shown in a review of 22 studies between 1946 and 2014. Their importance is due to their growing emergence as human pathogens causing opportunistic infections in severely immune-compromised individuals, people with congenital or acquired anatomical lung diseases, and healthcare-associated infections. However, there is also a noted increased incidence in NTM disease in people who are not immunocompromised and without any preexisting lung diseases.

- **Diagnosis**

Diagnostic tests for TB disease have improved substantially in recent years. WHO recommends rapid molecular diagnostic tests as initial tests for people showing signs and symptoms of TB.

Some of which can detect drug resistance to a variety of first- and second-line anti-TB drugs.

The older method of sputum smear microscopy (developed >100 years ago) is still widely used for TB diagnosis in low and middle-income countries but is increasingly being replaced with rapid tests.

Culture testing remains the reference standard for TB diagnosis. In addition, culture is required for the detection of resistance to newer anti-TB drugs and may also be used as a confirmatory test in settings and situations in which people have a low pre-test probability of having TB disease.
Following diagnosis, culture or smear (as opposed to rapid molecular tests) are necessary to monitor an individual's response to treatment.

Molecular techniques have gained prominence in the diagnosis of TB as well as in the identification of different species, allowing at early stages differentiate between MTB and NTM.
PCR-based assays targeting specific genes or regions are increasingly utilized for their accuracy and high sensitivity and specificity and the ability to provide rapid results directly from different kind of samples facilitating early diagnosis which is crucial for tailoring effective treatment regimens in a timely way.
PCR-based assays are precious in cases where traditional culture methods may be slow or challenging due to the slow growth characteristics of some *Mycobacteria* or even when Ziehl Neelsen stain is positive but it is not possible to distinguish between MTB and NTM.

Other complementary diagnostic tools could include Radiological techniques such as chest X-rays or even high-resolution computed tomography (HRCT) chest scan in due cases.

- **Treatment**

According to the WHO current guidelines for TB treatment consist of a 2-month period of four antibiotics, isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA), followed by a 4-month period of INH and RIF.
The duration of this treatment challenges patient adherence and consequently limits its overall effectiveness.

Drug	Recommended dose			
	Daily		Three times weekly	
	Dose and range (mg/kg body weight)	Maximum (mg)	Dose and range (mg/kg body weight)	Daily maximum (mg)
isoniazid	5 (4–6)	300	10 (8–12)	–
rifampicin	10 (8–12)	600	10 (8–12)	600
pyrazinamide	25 (20–30)	–	35 (30–40)	–
ethambutol	children 20 (15–25) adults 15 (15–20)	–	30 (25–35)	–
streptomycin	15 (12–18)	–	15 (12–18)	–

However, there are situations of resistance to these drugs (multidrug-resistant tuberculosis or MDR-TB), which requires a more complex and prolonged treatment regimen, generally involving a combination of second-line drugs (fluoroquinolones, second-line injectables, bedaquiline, linezolid, etc), and the length of treatment is 18–24 months, depending on the individual case and the drugs available.

On the other hand, NTM present a high variability in their drug resistance, which is why they do not respond to the same standard TB treatment.
Some NTM species, such as *Mycobacterium avium* and *Mycobacterium abscessus*, are resistant to common anti-tuberculosis drugs and may require personalized treatments with antibiotic combinations such as clarithromycin, amikacin, and linezolid, among others. Treatments for NTM infections are usually longer and vary depending on the species of mycobacteria, the severity of the infection, and the patient's immune status.
Normally these treatments include macrolides as a main difference compared to TB treatment.
Macrolides (clarithromycin and azithromycin) are the cornerstones of treatment for MAC lung disease. The standard optional regimen includes a rifamycin (rifampin or rifabutin), ethambutol, and a macrolide administered for 18–24 months, including 12 months of sputum culture negativity.
M. abscessus complex infections are extremely difficult to treat due to their high antimicrobial drug and disinfectant resistance. There is no standard treatment, but current guidelines recommend a macrolide-containing multidrug treatment regimen and may require adjustment depending on de specie identified and the status of the patient.
Just an example of treatment is shown below:

Mycobacterium Species	**Established Regimens**	**Additional or Suggested Agents**
M. avium complex	rifampin, ethambutol, isoniazid, streptomycin or amikacin	clarithromycin (azithromycin), ciprofloxacin, clofazimine
M. abscessus	amikacin, streptomycin, cefoxitin	clofazimine, clarithromycin, a cocktail of azithromycin, imipenem, clarithromycin,

Differentiating between tuberculous and non-tuberculous mycobacteria is essential to ensure effective and adequate treatment, prevent the spread of tuberculosis and correctly manage cases of NTM, which although not transmitted from person to person, can be serious in vulnerable populations.

In this context, VIRCELL S.L is launching into the market MYCOBACTERIUM REALTIME PCR KIT to detect nucleic acids from mycobacterial species belonging to the *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* complex (MABSC), as well as other non-tuberculous mycobacterial (NTM) species in sputum samples.

PRODUCT INFORMATION

1. Name / references / Indicated use / Regulatory status.

Name: MYCOBACTERIUM REALTIME PCR KIT

References: RTPCR016-LP and RTPCR016-LPD.

Indicated use: Real-time PCR kit to detect nucleic acids from *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* complex (MABSC) species and other non-tuberculous mycobacteria (NTM) in human sputum samples.

Regulatory status: CE_{0123} (CE-IVDR).

2. Main features of the test.

- Targets included in the kit are as follow: a specific fragment of the *IS1081* insertion sequence for MTBC (labelled with Texas Red/ROX), a specific fragment of the intergenic spacer region (*ITS)* (*16S-23S rRNA*) for MAC (labelled with Cy5) and MABSC (labelled with HEX) and a specific fragment of the *16S rRNA* gene for genus *Mycobacterium* (labelled in FAM).

- An amplification control is included to check quality of the sample, absence of carry-over of amplification inhibitors and the correct amplification set-up. This control consists of human *RNAse P* gene (labelled with Q705/Cy5.5).

- One lyophilized vial contains all the necessary reagents: Taq polymerase, buffer and specific oligonucleotides/probes for the detection of the different TB and non-TB complexes as well as primers and probe for internal control are included.

- Presentation in pre-dispensed format in white low-profile PCR tubes (0,1ml). 2 different presentations for a great customer´s convenience.

3. Advantages.

- Unique kit using RTPCR for detection MTBC, MAC, MABSC complexes and the genus *Mycobacterium* in the same assay (to date).

- Detection of all species and subspecies within the MTBC, MAC and MABSC complexes.

- Differentiate between *Mycobacterium tuberculosis* complex and non-tuberculosis *Mycobacteria* complexes.

- Differentiation of these complexes is important for better address treatment options.

- Fast and reliable results in less than 2 hours.

- Lyophilized Master Mix and positive control to ensure stability and reduce transportation costs compared to products in a frozen format.

4. Examples of amplification results obtained with MYCOBACTERIUM REALTIME PCR KIT

Color legend:
Blue (FAM): Mycobacteria
Green (HEX/VIC): *Mycobacterium abscessus* complex (MABSC)
Purple (Cy5): *Mycobacterium avium* complex (MAC)
Red (Texas/ROX): *Mycobacterium tuberculosis* complex (MTBC)
Brown (Q705/Cy5.5): IC

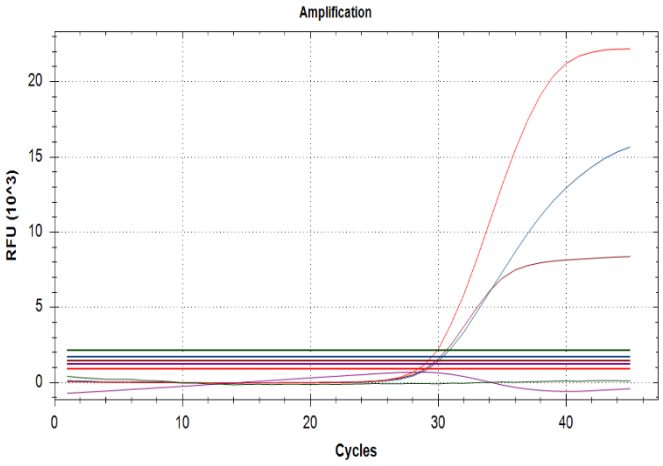


Figure 1: Mycobacterium tuberculosis complex (MTBC) positive result.

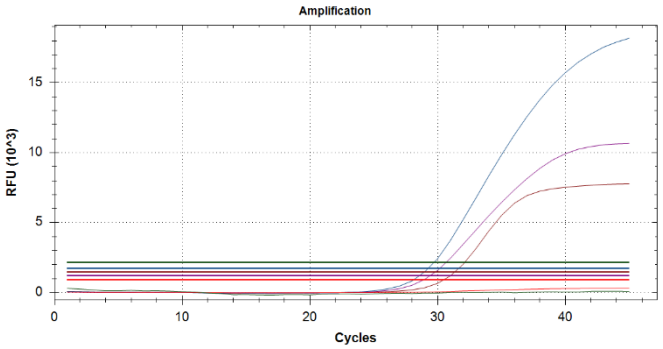


Figure 2. Mycobacterium avium complex (MAC) positive result.

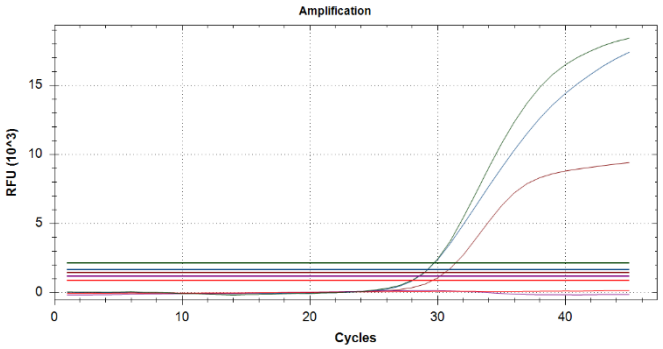


Figure 3. Mycobacterium abscessus complex (MABSC) positive result

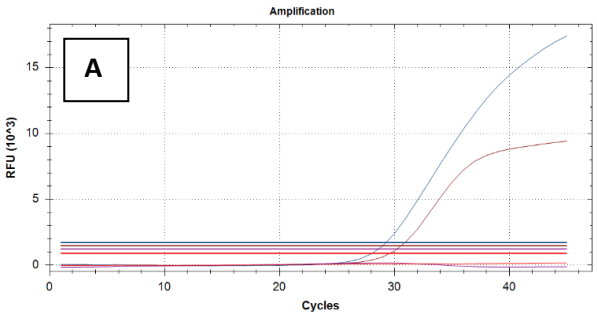


Figure 4 A.: Mycobacteria positive result (CT value < 37)

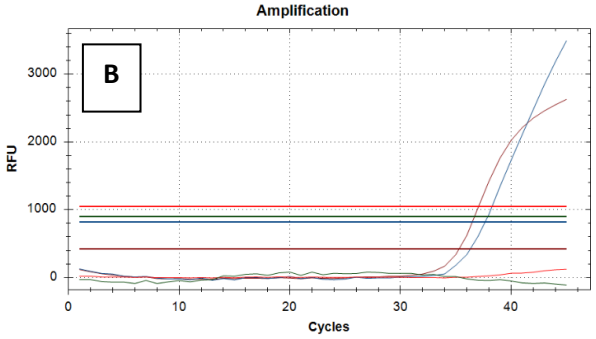


Figure 4 B.: Mycobacteria negative result (CT value > 37)

Examples with coinfections with two or three complexes:

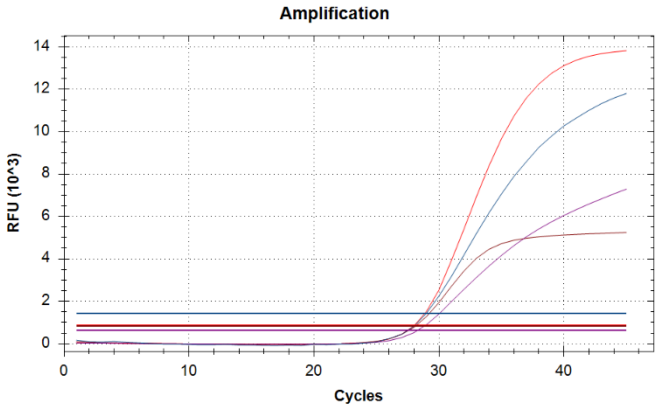


Figure 5. MTBC + MAC positive result.

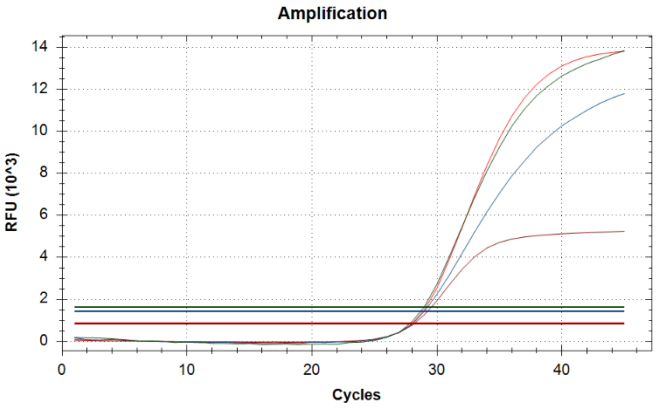


Figure 6. MTBC + MABSC positive result.

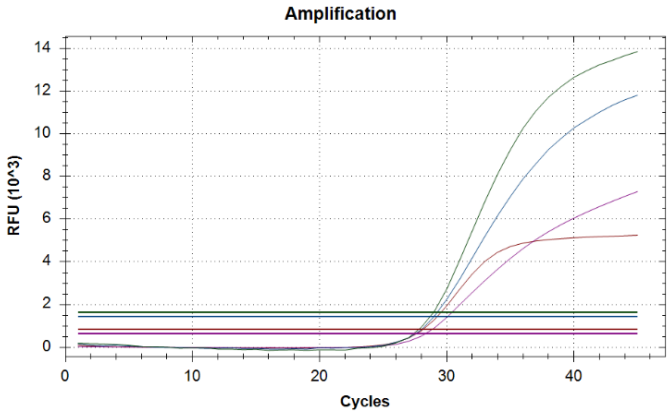


Figure 7. MAC + MABSC positive result

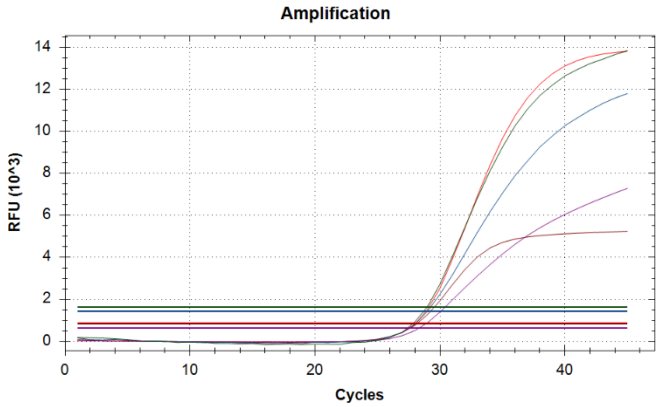


Figure 8. MTBC + MAC + MABSC positive result

5. Sample type.

The sample validated with the MYCOBACTERIUM REALTIME PCR KIT is sputum.
For a secure manipulation it is necessary that sputum samples are inactivated (30 minutes at 95 °C) before extraction step.

6. Technology.

Real-time PCR in one step, suitable for thermal cyclers with five fluorescent detection channels (FAM and HEX/VIC, Texas Red/ROX, Cy5 and Q705/Cy5.5) compatible with low profile white PCR tubes (0.1ml).

7. Test format.

Pre-dispensed format in white low-profile 0,1ml PCR tubes (RTPCR016-LP and RTPC016-LPD with 24 and 96 tests respectively).

RTPCR016-LPD: 1 Divisible 96-well PCR plate with pre-dispensed Lyophilized Master mix which could easily be separated into 12 strips of 8 tubes. Users can separate the necessary strips for the run and store the rest of them without affecting expire date.

RTPCR016-LP: 3 separate strips with pre-dispensed Lyophilized Master mix. Users can use the necessary strips for the run and store the rest of them without affecting expire date.

8. Validation and Compatibility with real-time PCR systems.

Reference	Real-time PCR thermal cycler (5 detection channels)
RTPCR016-LP RTPCR016-LPD	• CFX96 Touch™/CFX OPUS™ (Bio-Rad Laboratories)- **Validated** • Azure Cielo 6 (Azure Biosystems)- **Compatible** • QuantStudio 5 (QS5) (ThermoFischer)- **Compatible**

9. Validation and Compatibility with extraction systems.

- **Tanbead** (Maelstrom 4800)- **Validated**

- Other standard systems are expected to be compatible such as:
    - **Chroma (**MagXtract 3200**)**
    - **Tanbead** (Maelstrom 9600)
    - **BioMérieux** (NucliSENS® easyMag®)
    - **Roche** (MagNA Pure System)
    - **ThermoFisher** (KingFisher Flex)
    - **Bruker** (GenoXtract 12)
    - Others

10. Other material needed, but not supplied.

- Laminar flow chamber
- Compatible qRT-PCR-Thermocycler
- RNA extraction kit
- Pipettes / Tips with filter
- Microcentrifuge
- PCR cabinet (recommended)
- Vortex

11. Duration of the technique.

The test time after obtaining the purified DNA is less than 2 minutes since the product is lyophilized and only requires reconstitution and addition of the DNA of the sample. The time of the thermal cycler will depend on the model, although it is usually less than 2 hours.

12. Programming the Real Time PCR instrument.

Insert the PCR tubes/strips into the thermal cycler in real time and run the following program*:

1 cycle:	95°C 3 minutes
45 cycles:	95°C 15 seconds 60°C 45 seconds*

*Fluorescence data (FAM, HEX/VIC, Texas/ROX, Cy5 and Q705/Cy5.5) must be captured.

13. Interpretation of results

It is recommended to include one negative control in each run performed. The negative control will monitor reagent or environmental contamination.
The positive control is recommended to be included on each run. The positive control monitors for reagent failures and for correct operation of essential procedure.
The thermocycler software is likely to automatically calculate the baseline fluorescence value (threshold) based on the amplification curve for each target (fluorescence detection). Nevertheless, it is recommended to set the thresholds for the different detection channels individually. In order to set a threshold for each target, it is recommended to use as a reference the amplification curves of the positive and negative controls. The threshold should be fixed at the beginning of the exponential reading of fluorescence and above the background signal.

The controls result interpretation is as follows:

CONTROL	Mycobacteria (FAM)	MABSC (HEX/VIC)	MTBC (Texas/ROX)	MAC (Cy5)	IC (Q705/Cy5.5)	Interpretation
VIRCELL MTBAVAB POSITIVE CONTROL	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Correct
	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	Invalid
VIRCELL NEGATIVE CONTROL	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	Correct
	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	No Amplification or Ct >40	Invalid

Possible results which can be obtained with the MYCOBACTERIUM REALTIME PCR KIT:

RESULT	Mycobacteria (FAM) [2]	MABSC (HEX/VIC)	MTBC (Texas/ROX)	MAC (Cy5)	IC (Q705/Cy5.5)[1]	Interpretation
1	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	Invalid (sample/kit/setup related)
2	No Amplification or **Ct >37**	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	Amplification (Ct < 40)	Negative
3	**Amplification (Ct < 37)**	No Amplification or Ct >40	No Amplification or Ct >40	No Amplification or Ct >40	Amplification (Ct < 40) or No amplification	NTM
4	Amplification (Ct < 40)	Amplification (Ct < 40)	No Amplification or Ct >40	No Amplification or Ct >40	Amplification (Ct < 40) or No amplification	MABSC
5	Amplification (Ct < 40)	No Amplification or Ct >40	No Amplification or Ct >40	Amplification (Ct < 40)	Amplification (Ct < 40) or No amplification	MAC
6	Amplification (Ct < 40)	No Amplification or Ct >40	Amplification (Ct < 40)	No Amplification or Ct >40	Amplification (Ct < 40) or No amplification	MTBC
7	Amplification (Ct < 40)	Amplification (Ct < 40)	No Amplification or Ct >40	Amplification (Ct < 40)	Amplification (Ct < 40) or No amplification	MABSC + MAC
8	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	No Amplification or Ct >40	Amplification (Ct < 40) or No amplification	MABSC + MTBC
9	Amplification (Ct < 40)	No Amplification or Ct >40	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40) or No amplification	MAC + MTBC
10	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40) or No amplification	MABS + MAC + MTBC

[1] In case of a high copy number of the target nucleic acid, the amplification of the internal control (IC) in results 3 to 10 may be affected. The late amplification or absence of IC amplification does not change the interpretation of the result.

[2] In borderline positive samples, there may be amplification in the HEX/VIC (MABSC), Cy5 (MAC) and/or Texas/ROX (MTBC) channels and no amplification in the FAM (Mycobacteria) channel or amplification with Ct >37. The result will be considered valid.

Amplification curves with Ct >37 could be observed in the FAM channel due to environmental Mycobacteria.

In case of invalid or inconclusive result, it is recommended to re-extract DNA/RNA from original specimen and re-test it. In the case of failure of amplification of internal control, improper extraction of nucleic acids or inhibition of amplification could be assumed. Testing a new sample is recommended.

14. Number of tests per Kit.

RTPCR016-LP: 24 tests
RTPCR016-LPD: 96 tests

15. Transportation needs.

Room temperature. Lyophilized.

16. Reagents included in the kit.

Ref. RTPCR006-LP (24 tests)	Ref. RTPCR006-LPD (96 tests)
3 x 8-well PCR strip tubes	12 x 8-well PCR strip tubes
Reconstitution solutions	Reconstitution solutions
Positive Control	Positive Control
Negative Control	Negative Control
6 x 8 Strip tube caps	12 x 8 Strip tube caps

17. Clinical sensitivity and specificity

***Mycobacterium abscessus* complex**

Positive processed human sputum samples (n=50) and previously confirmed negative human sputum samples (n=51) were analysed. Samples were tested against a commercial Real-time PCR kit.
Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and amplified in CFX96 (Bio-Rad).
The results were as follows:

Samples No.		101
Sensitivity (%)		100
	95% CI	93-100
Specificity (%)		100
	95% CI	93-100
PPV (%)		100
NPV (%)		100
LR+/LR-		-1.01/-0.99
True Positive		50
True Negative		51
False Positive		0
False Negative		0
Borderline		0

***Mycobacterium avium* complex**

Positive processed human sputum samples (n=49) and previously confirmed negative human sputum samples (n=51) were analysed. Samples were tested against a commercial Real-time PCR kit.
Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and amplified in CFX96 (Bio-Rad).
The results were as follows:

Samples No.		100
Sensitivity (%)		100
	95% CI	93-100
Specificity (%)		100
	95% CI	93-100
PPV (%)		100
NPV (%)		100
LR+/LR-		-1.01/-0.99
True Positive		49
True Negative		51
False Positive		0
False Negative		0
Borderline		0

***Mycobacterium tuberculosis* complex**

Positive processed human sputum samples (n=58) and previously confirmed negative human sputum samples (n=51) were analysed. Samples were tested against a commercial Real-time PCR kit.
Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and amplified in CFX96 (Bio-Rad).
The results were as follows:

Samples No.		109
Sensitivity (%)		100
	95% CI	94-100
Specificity (%)		100
	95% CI	93-100
PPV (%)		100
NPV (%)		100
LR+/LR-		-1.01/-0.99
True Positive		58
True Negative		51
False Positive		0
False Negative		0
Borderline		0

Non-tuberculous micobacteria

Positive processed human sputum samples (n=51) and previously confirmed negative human sputum samples (n=51) were analysed. Samples were tested against a commercial Real-time PCR kit.
Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and amplified in CFX96 (Bio-Rad).
The results were as follows:

Samples No.		102
Sensitivity (%)		98
	95% CI	90-100
Specificity (%)		100
	95% CI	93-100
PPV (%)		100
NPV (%)		98
LR+/LR-		-0.99/-0.97
True Positive		50
True Negative		51
False Positive		0
False Negative		1
Borderline		0

CI: Confidence intervals
PPV: Positive predictive value
NPV: Negative predictive value
LR+: Positive likelihood ratio
LR-: Negative likelihood ratio

18. Precision

6 samples (4 positive and the positive and negative controls) were amplified twice in 2 runs per day in 2 different qRT-PCR thermocyclers on 20 consecutive days. Samples were run in CFX96 (Bio-Rad). Within-run precision, between-run precision, between-day precision and within-laboratory precision were determined.
The results were as follows:

***Mycobacterium abscessus* complex**

Sample	Within-run precision %CV	Between-run precision %CV	Between-day precision %CV	Within-laboratory precision %CV
Positive control	0.6	0.8	0.1	1.0
Positive sample 1	0.7	1.0	0.6	1.4
Positive sample 2	1.7	0.8	1.0	2.1
Positive sample 3	0.7	1.0	1.0	1.6
Positive sample 4	0.8	0.9	0.3	1.2
Negative control	No amplification	No amplification	No amplification	No amplification

***Mycobacterium avium* complex**

Sample	Within-run precision %CV	Between-run precision %CV	Between-day precision %CV	Within-laboratory precision %CV
Positive control	0.4	1.1	0.7	1.3
Positive sample 1	1.0	1.1	0.7	1.7
Positive sample 2	1.3	1.4	0.7	2.1
Positive sample 3	0.7	0.6	0.6	1.2
Positive sample 4	1.9	0.8	0.3	2.1
Negative control	No amplification	No amplification	No amplification	No amplification

***Mycobacterium tuberculosis* complex**

Sample	Within-run precision %CV	Between-run precision %CV	Between-day precision %CV	Within-laboratory precision %CV
Positive control	0.3	0.6	0.3	0.7
Positive sample 1	0.6	0.5	0.7	1.1
Positive sample 2	0.9	0.2	0.6	1.1
Positive sample 3	0.6	0.3	0.7	1.0
Positive sample 4	0.9	0.7	0.3	1.2
Negative control	No amplification	No amplification	No amplification	No amplification

Non-tuberculous micobacteria

Sample	Within-run precision %CV	Between-run precision %CV	Between-day precision %CV	Within-laboratory precision %CV
Positive control	0.5	1.0	0.6	1.3
Positive sample 1	0.8	1.2	0.7	1.6
Positive sample 2	0.8	0.8	1.1	1.6
Positive sample 3	0.8	1.5	1.1	2.0
Positive sample 4	1.1	1.5	0.5	2.0
Negative control	No amplification	No amplification	No amplification	No amplification

19. Interference

A study has been performed to evaluate the effect of potentially interfering substances.
Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and run in CFX96 (Bio-Rad).
The results were as follows:

Interfering substances	Samples No.	Maximum added concentration without interference
Mucin	2	2.5 mg/mL
Paracetamol	2	1324 µmol/L
Rifampicin	2	25 µg/mL
Ibuprofen	2	2425 µmol/L
Nicotine	2	6,2 µmol/L
Acetylcystein	2	10,2 mmol/L
Ebastine	2	0,78 µmol/L
Human whole blood	2	2% (v/v)
Saline nasal spray	2	10% (v/v)

20. Cross reactivity

A study has been performed to evaluate the effect of potentially cross-reactive microorganisms. Samples were run in CFX96 (Bio-Rad).
The results were as follows:

Microorganism	Samples No.	Positives No.
Acinetobacter baumannii	1	0
Adenovirus 31	1	0
Adenovirus 7	1	0
Adenovirus 8	1	0
Aspergillus fumigatus	1	0
Bordetella bronchiseptica	1	0
Bordetella holmesii	1	0
Bordetella parapertussis	1	0
Bordetella pertussis	1	0
Candida albicans	1	0
Chlamydophila pneumoniae	1	0
Chlamydophila psittaci	1	0
Citrobacter freundii	1	0
Corynebacterium diphteriae	1	0
Cytomegalovirus	1	0
Enterobacter cloacae	1	0
Epstein-barr virus	1	0
Escherichia coli (EIEC)	1	0
Fusobacterium nucleatum	1	0
Haemophilus influenzae	1	0
HCoV-NL63	1	0
Human metapneumovirus	1	0
Influenza A virus H1N1	1	0
Influenza B virus	1	0
Klebsiella pneumoniae	1	0
Legionella bozemanii (Fluoribacter bozemanae)	1	0

Microorganism	Samples No.	Positives No.
Legionella dumoffii (Fluoribacter dumoffii)	1	0
Legionella longbeachae	1	0
Legionella micdadei	1	0
Legionella pneumophila	1	0
Measles virus	1	0
MERS-CoV	1	0
Moraxella catarrhalis	1	0
Mycoplasma pneumoniae	1	0
Neisseria meningitidis serogroup A	1	0
Nocardia asteroides	1	0
Parainfluenza 1 virus	1	0
Parainfluenza 2 virus	1	0
Parainfluenza 3 virus	1	0
Parainfluenza 4 virus	1	0
Prevotella melaninogenica	1	0
Propionibacterium acnes	1	0
Pseudomonas aeruginosa	1	0
Respiratory syncytial virus subtype A	1	0
Respiratory syncytial virus subtype B	1	0
Rhinovirus	1	0
Rhodococcus equi	1	0
*Staphylococcus aureus (*mecA-)	1	0
Streptococcus agalactiae	1	0
Streptococcus constellatus	1	0
Streptococcus pneumoniae	2	0
Streptococcus pyogenes	1	0
TOTAL	53	0

In addition, an in-silico analysis of the primers/probe's sequences comparing to other microorganisms that could be found in clinical samples was performed.
The results were as follows:

	Homology >80%			
Microorganism	**MABSC**	**MAC**	**MTBC**	**Mycobacteria**
Actinomyces meyeri	No	No	Yes	No
Actinomyces naeslundii	No	Yes	Yes	Yes
Actinomyces pyogenes (Trueperella pyogenes)	No	Yes	No	No
Bocavirus	No	No	No	No
Chlamydia caviae	No	No	No	Yes
Corynebacterium pseudodiphtheriticum	No	No	Yes	Yes
Corynebacterium xerosis	No	Yes	Yes	Yes
Eikenella corrodens	No	No	Yes	Yes

	Homology >80%			
Microorganism	**MABSC**	**MAC**	**MTBC**	**Mycobacteria**
Pasteurella multocida	Yes	No	No	Yes
Porphyromonas gingivalis	Yes	Yes	Yes	No
Stenotrophomonas maltophilia	Yes	Yes	Yes	Yes
Streptococcus mitis	No	Yes	No	No
Streptococcus mutans	No	No	No	Yes

"YES" indicates microorganisms that showed > 80% homology with respect to one of the primers but not with any other primers included in the assay. Cross-reaction and/or interference with the assay due to the presence of these organisms could not be tested, but it is unlikely to occur.

21. Analytical sensitivity

A preliminary LoD (limit of detection) was determined by testing serial dilutions of quantified MTBC, MAC, MABSC and NTM samples. Samples were extracted using OptiPure Viral kit on Maelstrom 4800 instrument (TANBead) and amplified in CFX96 (Bio-Rad).
Once an approximated LoD is determined, the final concentration was confirmed by testing 3 serial dilutions. A minimum of 20 replicates is tested for each dilution.
The LoD is determined as the lowest concentration where ≥ 95% of the replicates are positive.

	MABSC	**MAC**	**MTBC**	**NTM**
LoD (copies/µl)	3	3	0.2	6
LoD (copies/ml)	400	400	23.1	800
LoD (copies/reaction)	15	15	0.9	30

22. Analytical inclusivity

An in-silico analysis for the primered genes included in the assay was performed to determine the inclusivity for the different MTBC, MAC, MABSC and NTM species/subspecies sequences available.
The criteria selected for including the different sequences in the analysis was geographic and the date when the sequence was deposited. Different lineages, types or subtypes were included in the analysis of each microorganism.
GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) was used for accessing sequences.
The results of the in-silico analysis show that the kit is predicted to detect all genome variants included in the analysis.

The analytical inclusivity of the kit for MTBC, MAC, MABSC and NTM detection was analysed by testing representative samples of the different species and subspecies. Samples were amplified in CFX96 (Bio-Rad).
The results were as follows:

Microorganism	**Samples No.**	**Positives No.**	**Result Interpretation**
Mycobacterium tuberculosis	1	1	MTBC
Mycobacterium microti	1	1	MTBC
Mycobacterium bovis BCG	1	1	MTBC

Microorganism	Samples No.	Positives No.	Result Interpretation
Mycobacterium caprae	1	1	MTBC
Mycobacterium bovis	1	1	MTBC
Mycobacterium africanum	1	1	MTBC
Mycobacterium avium	1	1	MAC
Mycobacterium intracellulare	1	1	MAC
Mycobacterium colombiense	1	1	MAC
Mycobacterium avium subsp. *silvaticum*	1	1	MAC
Mycobacterium abscessus	1	1	MABSC
Mycobacterium abscessus subsp. *bolletii*	1	1	MABSC
Mycobacterium abscessus subsp. *massiliense*	1	1	MABSC
Mycobacterium kansasii	1	1	NTM
Mycobacterium ulcerans	1	1	NTM
Mycobacterium smegmatis	1	1	NTM
Mycobacterium malmoense	1	1	NTM
Mycobacterium marinum	1	1	NTM
Mycobacterium gastri	1	1	NTM
Mycobacterium peregrinum	1	1	NTM
Mycobacterium scrofulaceum	1	1	NTM
Mycobacterium terrae	1	1	NTM
Mycobacterium xenopi	1	1	NTM
Mycobacterium celatum	1	1	NTM
Mycobacterium genavense	1	1	NTM
Mycobacterium chelonae	1	1	NTM
Mycobacterium lentiflavum	1	1	NTM
Mycobacterium mageritense	1	1	NTM
Mycobacterium chimaera	1	1	MAC
Mycobacterium fortuitum	1	1	NTM
Mycobacterium phlei	1	1	NTM
Mycobacterium mucogenicum	1	1	NTM
Mycobacterium elephantis	1	1	NTM
TOTAL	33	33	

All species and subspecies tested resulted in positive amplification.

23. Recommended external controls

Controls that are suggested to use but not included in the kit are the following:

- As a positive extraction control:
  - AMPLIRUN® TOTAL MTB CONTROL (SPUTUM): Cat. MBTC013-R
  - AMPLIRUN® TOTAL MTB RIF RESISTANT CONTROL (SPUTUM) Cat. MBTC014-R
  - AMPLIRUN® TOTAL MTB INH RESISTANT (SPUTUM) Cat. MBTC015-R

External controls are used to monitor any cross-contamination that occurs during the extraction process, as well as to validate the extraction reagents used.

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