For professional use only

MTB MDR Resistance Real-TM

HANDBOOK

Real Time PCR kit for the detection of Rifampicin and Isoniazid drug resistance in MTB positive samples

REF H3611-50FRT

50

NAME

MTB MDR Resistance Real-TM

INTRODUCTION

Tuberculosis (abbreviated as **TB** for *tubercle bacillus*) is a common and deadly infectious disease caused by mycobacteria, mainly Mycobacterium tuberculosis. Tuberculosis most commonly attacks the lungs (as pulmonary TB) but can also affect the central nervous system, the lymphatic system, the circulatory system, the genitourinary system, bones, joints and even the skin. Other mycobacteria such as Mycobacterium bovis, Mycobacterium africanum and Mycobacterium microti can also cause tuberculosis. Over one-third of the world's population has been infected by the TB bacterium, and new infections occur at a rate of one per second. Not everyone infected develops the full-blown disease; asymptomatic, latent TB infection is most common. However, one in ten latent infections will progress to active TB disease, which, if left untreated, kills more than half of its victims.

Early diagnosis of tuberculosis makes effective treatment possible and increases the probability of clinical outcome owing to quite effective antituberculosis therapy, however the tuberculosis diagnosis has certain difficulties. Effective first line anti-tuberculosis drugs like isoniazid (INH) were developed but shortly after their introduction a resistance was reported (Crofton and Mitchison, 1948). In the 60s rifampicin (RIF) was introduced and this led, using combination therapy, to a decline in both drug resistant and drug susceptible TB. As a consequence, funding and interest in TB control programs declined: no effective monitoring of drug resistance was carried out for the following 20 years (Espinal, 2003). It is now known that resistance to first-line drugs is linked to specific mutations in MTB genes: the most significant ones are katG and inhA for INH resistance, rpoB for RIF resistance.

Up to date it is well known that 95% rifampin (RMP) resistance is associated with rpoB gene mutations and most of mutations frequency, associated with isoniazid (INH) resistance, are discovered in 315 codon of katG gene and in the regulatory region of inhA gene.

Drug resistance surveillance was restored in the late 90s, with reports showing also multidrug resistance (MDR) to both RIF and INH. A WHO study showed that in the period 2000-2004 about 20% of clinical samples tested showed MDR.

Second line drugs are now available but they are less efficient and more expensive. So it becomes critical to detect drug resistant TB early, allowing an appropriate and effective treatment for the disease, avoiding wasting time and resources using non effective drugs. Moreover, the spread of multidrug resistant strains needs to be constantly monitored to avoid a decrease in treatment efficiency. Due to MTB slow growth rate, cultural methods of detection usually used are really slow, taking up to 20 days to have a valid result on drug resistance of the sample tested. The application of a molecular biology technique such as real-time PCR, makes this detection much faster than cultural methods (valid result in 1-3 day), highly precise, specific and sensitive.

INTENDED USE

The kit **MTB MDR Resistance Real-TM** is a test for Real Time PCR qualitative detection of Mycobacterium tuberculosis resistance to Rifampicin and Isoniazid in MTB positive samples.

PRINCIPLE OF ASSAY

kit **MTB MDR Resistance Real-TM** is a Real-Time Amplification PCR test which detects mutations in RpoB region for Rifampicin resistance and mutations in katG and inhA regions for Isoniazid resistance. Results are analysed using automated spreadsheet file supplied with CD inside the kit's box.

MATERIALS PROVIDED

"MTB MDR Resistance Real-TM": Real Time PCR amplification and controls

- **PCR-mix-RIF N°1**, 1 x 0,6 ml;
- **PCR-mix-RIF N°2**, 1 x 0,6 ml;
- **PCR-mix-INH**, 1 x 0,6 ml;
- **PCR-mix-2**, 3 x 0,3 ml;
- **DNA Polymerase (TaqF),** 3 x 0,03 ml;
- **C+ MTB-wt,** 2 x 0,2 ml;
- **C+ MTB-mut,** 2 x 0,2 ml;
- **TE-buffer,** 2 x 0,2 ml;

Contains reagents for 55 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Vortex
- 65°C ± 2°C dry heat block
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- Tube racks
- Microcentrifuge tubes, 1,5 - 2,0 ml
- Pipettes with sterile, RNase-free filters tips
- Biohazard waste container
- Disposable gloves, powderless
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Real Time Thermalcycler
- Tubes or PCR plate
- Workstation
- Pipettes with sterile, RNase-free filters tips
- Tube racks

STORAGE INSTRUCTIONS

“MTB MDR Resistance Real-TM” must be stored at 2-8°C and -20°C (see labels inside the kit’s box). The kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

MTB MDR Resistance Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

RUO For Research Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

MTB MDR Resistance Real-TM can analyze DNA extracted from:

- *Sputum, bronchial or tracheal lavage* must be treated with the following procedure:
  - Collect sputum into 50 mL single-use PP tubes with a screw cap.
  - In a biological safety cabinet, homogenize samples after mixing with equal volume of 4% NaOH solution. (*N-acetyl-L-cysteine may be added if required in the amount of 50-70 mg per sample).* Mix intensely with a tube rotator for 5-20 minutes (depending on the density of a sample).
  - Centrifuge samples at 3000 rpm (2800-3000 g) for 15 min and carefully discard the supernatant leaving 500-1000 µl in the tube. Resuspend sediment and transfer it into a 1.5 ml tube.
  - Centrifuge samples at 12000 rpm for 5-10 min, discard the supernatant and use the same 1,5 ml sample tube for DNA isolation from sample sediment.
- *tissue* (~1,0 gr) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile (1 volume of tissue to 1 volumes of saline solution). Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
- *whole blood* collected in either ACD or EDTA tubes;
- *liquor* stored in “Eppendorf” tube;
- *sinovial liquid* stored in “Eppendorf” tube;
- *urine sediment* (use the intermedium part of stream);
- *pleuric versament* stored in “Eppendorf” tube;
- *mycobacterium liquid culture* conserved in Trilon-B;

Specimens can be stored at +2-8°C for no longer than 48 hours, or freeze at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kit:

- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);
- ⇨ **SaMag TB DNA Extraction kit** (Sacace, REF SM008).

Please carry out DNA extraction according to the manufacture’s instruction.

PROTOCOL:

1. Prepare required quantity of reaction tubes (or PCR plate) for samples and controls.
2. Briefly vortex and spin all reagent tubes before opening.
3. Prepare in new sterile tubes 3 mastermixes with the following: **10*(N+1) µl** of **PCR-mix-RIF N°1 or 10*(N+1) µl** of **PCR-mix-RIF N°2 or 10*(N+1) µl** of **PCR-mix-INH**, **5*(N+1) µl** of **PCR-mix-2** and **0,5*(N+1) µl** of **DNA Polymerase (TaqF)**. Vortex and centrifuge briefly.
4. For each sample prepare 3 PCR tubes with **15 µl** of the **Reaction Mastermix RIF N°1 or RIF N°2** or **INH**
5. For each sample add **10 µl** of **extracted DNA** to each of the Mastermix tubes.
6. Prepare for each mastermix 3 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **C+ MTB-wt** to the tube labeled Wild Type Control;
   - add **10 µl** of **C+ MTB-mut** to the tube labeled Mutant Control;
7. Insert the tubes in the thermalcycler.

Amplification

1. Create a temperature profile on your instrument[1] as follows:

Stage	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***
Hold	**95**	15 min	–	1
Cycling	**95**	15 s	–	5
	65	30 s	–	
	72	15 s	–	
Cycling 2	**95**	15 s	–	40
	65	30 s	FAM(Green), JOE(Yellow), ROX (Orange), Cy5 (Red)	
	72	15 s	–	

[1] For example SaCycler-96™ (Sacace), Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen), CFX96™/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems)

Fluorescence is detected at the 2nd step of Cycling 2 stage (65 °C) in FAM/Green, JOE/Yellow/Hex/Cy3, ROX/Orange, Cy5/Red fluorescence channels.

INSTRUMENT SETTINGS

Rotor-type instruments

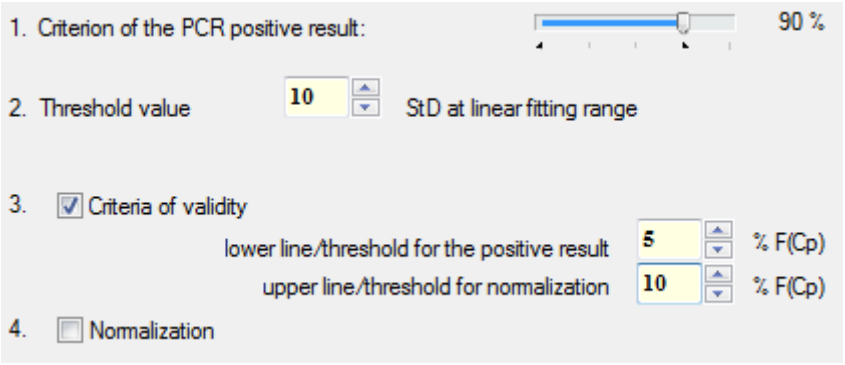
Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Dynamic tube/ Slope Correct
	Settings for PCR-mix-RIF N°1 and PCR-mix-INH			
FAM/Green JOE/Yellow ROX/Orange Cy5/Red	*from 2 Fl to 5 Fl*	*0.1*	*15 %*	*On*
	Settings for PCR-mix-RIF N°2			
FAM/Green ROX/Orange Cy5/Red	*from 2 Fl to 5 Fl*	*0.1*	*10 %*	*On*
JOE/Yellow	*from 2 Fl to 5 Fl*	*0.1*	*10-15 %*	*On*

Plate-type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and be set to 10-20% of the fluorescence level reached by the positive control in the last cycle of amplification. Set the threshold in the log-linear phase of the amplification.

SaCycler-96 instrument settings

Click on the icon  to set the parameters of data analysis as in the following picture:



DATA ANALYSIS

The fluorescent signal intensity is detected in four channels:

Fluorescence channel	FAM/Green	JOE/Yellow	ROX/Orange	Cy5/Red
PCR Mix	Detected target DNA (amplification region)			
PCR-mix-RIF N°1	RRDR *rpoB* gene region (wild type)		S 531 L mutation in RRDR region of *rpoB* gene	RRDR gene region *rpoB* (wild type)
PCR-mix-RIF N°2	RRDR gene region *rpoB* (wild type)			
PCR-mix-INH	gene *katG* (region of codon 315) (wild type)	region outside the RRDR gene *rpoB* (codon region 572) (wild type)	Internal Control	promoter region *inhA* gene (wild type)

Interpretation of results

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

Analysis of result for clinical samples

The results are analysed automatically by pasting Ct results from Real Time PCR software to the Excel spreadsheet Macro provided by Sacace (CD inside the kit's box).

To do the analysis manually, follow the below procedure.

Rifampicin Resistance

a) **Absence of MTB Resistance to rifampicin** if **all** the 3 conditions below are met:
   1. **Ct values** are defined **for channels FAM, JOE, Cy5** and there is **no Ct value for channel ROX** in mix **PCR-mix-RIF N°1**
   2. **Ct values** are defined **for channels FAM, JOE, ROX, Cy5** in mix **PCR-mix-RIF N°2**
   3. **Ct value** is defined **for channel JOE** in mix **PCR-mix-INH**

b) **Presence of MTB Resistance to rifampicin if at least one of the following conditions is met:**
   1. **There is no Ct value defined for one or more channels in FAM, Joe, Cy5 and/or there is a Ct value defined for ROX channel** in mix **PCR-mix-RIF N°1**
   2. **There is no Ct value defined for one or more channels in FAM, Joe, ROX, Cy5** in mix **PCR-mix-RIF N°2**
   3. **There is no Ct value defined for channel Joe** in mix **PCR-mix-INH**

Isoniazid Resistance

a) **Absence of MTB Resistance to Isoniazid if there are Ct values defined for FAM and Cy5 channel** in mix **PCR-mix-INH**

b) **Presence of MTB Resistance to Isoniazid if there is no Ct value defined for FAM channel (or for FAM and Cy5 channels)** in mix **PCR-mix-INH**

c) **Presence of MTB Resistance to low level Isoniazid if there is no Ct value defined for Cy5 channel and there is a Ct value defined for FAM channel** in mix **PCR-mix-INH**

ANALYSIS LIMITATIONS

The above results of detection of mutations associated with resistance to rifampicin and isoniazid are not considered valid in the following cases:

a) There is not enough MTB DNA for analysis. This happens if one of the following options is observed:

- Ct values are missing simultaneously for five or more of the following channels: in PCR mix-RIF N°1 channels for fluorophores FAM, JOE and Cy5; in PCR-mix-RIF N°2 - FAM, JOE, ROX and Cy5; in PCR-mix-INH - JOE, and the Ct value is less than the boundary value in PCR mix-INH for the ROX fluorophore (detection of IC);

- Ct values for one or several channels are missing , and Ct values exceed the boundary for four of the following channels: in PCR-mix-RIF N°1 channels for fluorophores FAM, JOE and Cy5; in PCR mix-RIF N°2 - FAM, JOE, ROX and Cy5; in PCR mix-INH - JOE, and the Ct value is less than the limit in PCR-mix-INH through the channel for the ROX fluorophore (detection of IC);

- Ct values are missing simultaneously for all four channels in PCR mix-RIF N°1 and / or in PCR-mix-RIF N°2, the present Ct values for the remaining channels in these PCR-mixes exceed the boundary values, and the Ct value is determined to be less than the boundary value in PCR-mix-INH in the ROX channel (Internal Control detection).

If the result is “there is not enough MTB DNA for analysis,” then this DNA sample cannot be analyzed using this reagent kit, since the DNA content of the MTB in it is lower than the analytical sensitivity of the reagent kit or the MBT DNA is missing.

b) The result is not valid if for this sample the Ct value is missing or exceeds the boundary value for the ROX channel in PCR-mix-INH (detection of Internal Control), and there are no Ct values for five or more of the listed below channels: in PCR-mix-RIF N°1 for channels FAM , JOE and Cy 5; in PCR-mix-RIF N°2 - FAM , JOE , ROX and Cy5; in PCR-mix-INH - JOE . It is necessary to conduct a repeated PCR study of the corresponding test sample, starting from the stage of DNA extraction.

ATTENTION! Boundary value Ct are found in the below table.

Expected results for controls

PCR mix RIF N°1

Controls	Rotor Type instruments				Plate type instruments			
	Expected Ct values							
	FAM	JOE	ROX	Cy5	FAM	JOE	ROX	Cy5
C+ wt	28	29	-	30	31	32	-	33
C+ mut	-	-	29	-	-	-	31	-
NCA	-	-	-	-	-	-	-	-
NCE	-	-	-	-	-	-	-	-
Samples	30,5	30,5	40	31	33,5	33,5	40	33,5

PCR mix RIF N°2

Controls	Rotor Type instruments				Plate type instruments			
	Expected Ct values							
	FAM	JOE	ROX	Cy5	FAM	JOE	ROX	Cy5
C+ wt	31	30,5	30	32	33,5	33,5	33	34
C+ mut	40	-	31	Ignore	40	-	34	Ignore
NCA	-	-	-	-	-	-	-	-
NCE	-	-	-	-	-	-	-	-
Samples	33	31	32	33,5	34	33,5	33,5	34,5

PCR mix INH (N°3)

Controls	Rotor Type instruments				Plate type instruments			
	Expected Ct values							
	FAM	JOE	ROX	Cy5	FAM	JOE	ROX	Cy5
C+ wt	28	28	26	31	32	32	28	34
C+ mut	-	-	26	-	-	-	28	-
NCA	-	-	-	-	-	-	-	-
NCE	-	-	27	-	-	-	29	-
Samples	37	31	27	37	39	34,5	29	39

ANALYTICAL CHARACTERISTICS

ANALYTICAL SPECIFICITY

The analytical specificity of the MTB Resistance MDR kit has been proven in DNA testing of the following microorganism strains: *Corynebacterium jeikeium, Corynebacterium xerosis, Corynebacterium minutissimum, Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Klebsiella oxytoca, E. coli, Neisseria meningitidis, Haemophilus influenzae, Stenotrophomonas maltophilia, Pseudomonas stutzeri, Pseudomonas putida, Pseudomonas aeruginosa, Pseudomonas koreensis, Enterococcus faecium*, clinical isolates DNA following non-tuberculous mycobacteria: *M. avium complex, M. kansasii, M. fortuitum group* , as well as human genomic DNA.

ANALYTICAL SENSITIVITY

The analytical sensitivity of the **MTB MDR Resistance Real-TM** is not less than $1x10^3$ GE/ml.

DIAGNOSTICS CHARACTERISTICS

Diagnostic performance of the kit were evaluated by comparing the results of the study 87 samples of different types of biomaterial obtained from patients with pulmonary tuberculosis and tuberculosis in other localization, with the data of phenotypic determination of drug resistance of MTB to rifampicin and isoniazid. As a reference method for determining the drug resistance, an absolute concentration method on Löwenstein–Jensen solid nutrient medium was used.

Results of Sacace MTB Resistance MDR kit in comparison with the reference method

Sample type	Drug	Sample tested	Results with Sacace MTB Resistance MDR kit	Reference method test	
				Resistant MTB (positive)	Sensitive MTB (negative)
DNA samples extracted from biological materials	Rifampicin	87	**Resistance positive**	41	2
			Resistance Negative	0	44
	Isoniazid	87	**Resistance positive**	46	2
			Resistance Negative	0	39

Diagnostics characteristics of Sacace MTB Resistance MDR kit

Samples	Drug	Diagnostic sensitivity (%)	Diagnostic specificity(%)
DNA samples extracted from biological materials	Rifampicin	94,5	89,0
	Isoniazid	95,0	87,5

PRECAUTIONARY MEASURES AND DISPOSAL CONSIDERATIONS

Tests using this kit should be carried out in a laboratory setup for molecular biology (PCR) studies of biological material for the presence of infectious disease pathogens, in compliance with the sanitary and epidemiological rules.

When working you should always fulfill the following requirements:

- The temperature in the laboratory from 20 to 28 ° C, relative humidity from 15 to 75%.
- Consider the tested samples as infectious-hazardous, so organize work and storage with proper Good Molecular Biology Laboratory Diagnostics practices for level III –IV of pathogenicity
- Clean up and disinfect spilled samples using disinfectants
- The laboratory process must be unidirectional. The analysis is carried out in separate rooms (zones). Work should begin in Extraction zone, continue in the Amplification and Detection Zones. Do not return samples, equipment and reagents to the area in which the previous stage of the process was carried out.
- Unused reagents, expired reagents, as well as used reagents, biological material, including materials, tools and objects contaminated with biological material should be disposed of in accordance with good laboratory practices and local regulation.

ATTENTION! When removing the waste after amplification (tubes containing PCR products), opening the tubes and spraying the contents is strictly forbidden, as this may lead to contamination of the laboratory area, equipment and reagents with PCR products amplicon.

- Use and change at each operation disposable. Disposable plastic dishes (tubes, tips) must be disposed of in a special container containing a disinfectant that can be used to disinfect medical waste.
- The surfaces of the tables, as well as the cabinet in which the PCR setup is performed, must be subjected to ultraviolet irradiation for 30 minutes before and after completion of work.
- The reagent kit is designed for single use for PCR studies of a specified number of samples (see the “Composition” section).
- The reagent kit is ready for use according to this manual. Use the reagent kit strictly for its intended purpose.

- Only personnel trained in molecular diagnostic methods and working in a clinical diagnostic laboratory are allowed to work with the kit
- Do not use the kit if the inner packaging is broken or the reagent does not fit the description.
- Do not use the kit if transportation and storage conditions are not observed according to the instructions.
- Do not use reagents after expiration date.
- Use disposable powder-free gloves, lab coats, protect eyes while working with samples and reagents. Wash hands thoroughly after work. All operations are carried out only in gloves to prevent contact with the human body.
- Avoid inhalation of vapors, contact with skin, eyes and mucous membranes. Harmful if swallowed. Upon contact, immediately flush the affected area with water and, if necessary, seek medical attention.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
RUO	For Research Use Only	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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