IVD For *in Vitro* Diagnostic Use

CE

MTB Diff Real-TM

Handbook

Real-Time Amplification test for the qualitative detection and differentiation of *M.tuberculosis, M.bovis and M. bovis BCG*

REF **B41-50FRT**

Σ **50**

NAME

MTB Diff 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. According to international standards, tuberculosis diagnosis must be confirmed either by bacteriology or by histology studies, but the bacteriological methods do not always allow to detect Mycobacterium tuberculosis in people affected with pulmonary tuberculosis and especially with extrapulmonary tuberculosis.

The application of molecular biology methods allow to overcome the difficulties in the diagnosis of Mycobacterium tuberculosis, but due to the biological peculiarities of this microorganism and immune response of human organism, tuberculosis can not be diagnosed only by one method.

INTENDED USE

The development of a test to differentiate between infection with *Mycobacterium tuberculosis* or *Mycobacterium bovis* and vaccination with *M. bovis* BCG could greatly assist in the diagnosis of early infection as well as enhance the use of tuberculosis vaccines on a wider scale.

This kit is meant to be used with samples already tested and found positive for Mycobacterium Tuberculosis Complex with Sacace kit MTB Real-TM (**REF B15-50FRT**).

PRINCIPLE OF ASSAY

kit **MTB Diff Real-TM** is a Real-Time Amplification test for the qualitative detection and differentiation of *M.tuberculosis, M.bovis and M. bovis BCG* in the sputum, urine, blood, bronchial lavages, tissue and other biological materials.

DNA is amplified using Real Time Amplification and detected using fluorescent reporter dye probes specific for *M. tuberculosis, M.bovis, M. bovis BCG* and IC.

M. tuberculosis is detected on the Fam (Green) channel, *M.bovis* and *M. bovis BCG* on the Joe (Yellow)/HEX/ TET/Cy3 channel, *M. bovis BCG* on the Rox (Orange)/TexasRed channel and IC on the Cy5 (Red) channel.

MATERIALS PROVIDED

"MTB Diff Real-TM": Real Time amplification

- **PCR-mix-1 Diff**, 2 x 0,28 ml;
- **PCR-mix-2**, 1 x 0,3 ml;
- **TaqF Polymerase**, 1 x 0,03 ml;
- **UDG Enzyme,** 1 x 0,03 ml;
- **MTB Diff C+**, 1 x 0,1 ml;
- **DNA-buffer**, 1 x 0,5 ml;

Contains reagents for 55 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

The kits can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt. Store kit at 2-8°C. **PCR-mix-1, UDG enzyme** and **TaqF Polymerase** must be stored at -20°C.

STABILITY

MTB Diff 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

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic 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.

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 Diff 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;
- *feces:* prepare 20% feces suspension by adding in 5 ml tube of 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces. Vortex to get the homogeneous suspension and centrifuge for 5 min to 7000-12000g and using a micropipette with a plugged aerosol barrier tip transfer in a new sterile 1,5 ml tube 100 µl of the supernatant
- *sinovial liquid* stored in “Eppendorf” tube;
- *urine sediment* (use the intermedium part of stream);
- *prostatic liquid* stored in “Eppendorf” tube;
- *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 kits:

- ⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B);
- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);
- ⇨ **SaMag TB DNA Extraction kit** (Sacace, REF SM008);

Please carry out DNA extraction according to the manufacturer's instruction.

REAL TIME PCR PROTOCOL:

1. Prepare required quantity of reaction tubes (or PCR plate) for samples and controls.
2. Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1 Diff**, **5*(N+1) µl** of **PCR mix-2, 0,5*(N+1) µl** of **TaqF Polymerase** and **0,5*(N+1) µl** of **UDG Enzyme.** Vortex and centrifuge briefly.
3. Add to each tube **15 µl** of **Reaction Mix**.
4. Add **10 µl** of **extracted DNA** to appropriate tube.
5. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **MTB Diff C+** to the tube labeled Amplification Positive Control;
6. Insert the tubes in the thermalcycler.

Amplification

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

Cycle	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycles*
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, JOE, ROX, Cy5	
	72	15 s	–	

[1] SaCycler-96™ (Sacace), Rotor-Gene™ 6000/Q (Corbett Research, Qiagen), iQ5™ (BioRad); Mx3005P™ (Stratagene), ABI® 7500 Real Time PCR (Applied Biosystems), EcoqPCR® (Illumina)

Step	Only for SmartCycler® (Cepheid) instrument		
	Temperature, °C	Time	Cycles
Hold	95	15 min	1
Cycling	95	30 s	45
	65	30 s (detection)	
	72	30 s	

Fluorescence is detected at 65 °C in FAM/Green, JOE/Yellow/HEX/Cy3, ROX/Orange/Texas Red, and Cy5/Red fluorescence channels.

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.03*	*10 %*	*On*
JOE/Yellow	*from 5 Fl to 10 Fl*	*0.05*	*10 %*	*On*
ROX/Orange	*from 5 Fl to 10 Fl*	*0.05*	*10 %*	*On*
Cy5/Red	*from 5 Fl to 10 Fl*	*0.05*	*30 %*	*On*

Plate-type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at 15% of the maximum fluorescence level of the C+ in the last amplification cycle.

For SmartCycler instrument set the threshold at level 30.

BOUNDARY CT VALUES

Controls	Rotor Type instruments				Plate type instruments			
	Expected Ct values							
	FAM	*JOE*	*ROX*	*Cy5*	*FAM*	*JOE*	*ROX*	*Cy5*
C+	≤30	≤30	≤30	≤30	≤34	≤34	≤34	≤34
Samples	≤35	≤35	≤35	≤35	≤37	≤37	≤37	≤37

RESULTS ANALYSIS

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

M. tuberculosis is detected on the Fam (Green) channel, *M.bovis* and *M. bovis BCG* on the Joe (Yellow)/HEX/TET/Cy3 channel, *M. bovis BCG* on the Rox (Orange)/TexasRed channel and IC on the Cy5 (Red) channel.

Results are accepted as relevant if positive and negative controls of amplification and extraction are passed (see table 1).

Table 1. Results for controls

Control	Stage for control	Fam (Green)	Joe (Yellow)/ HEX/Cy3	Rox/(Orange)/ TexasRed	Cy5 (Red)	Interpretation
NCA	Amplification	NEG	NEG	NEG	NEG	Valid result
MTB Diff C+	Amplification	POS	POS	POS	NEG	Valid result

The following results are possible:

Fam (Green)	Joe (Yellow) HEX/Cy3	Rox/(Orange)/ TexasRed	Cy5 (Red)	Interpretation
+	+	+	+ /-	Mix M. tuberculosis + M. bovis BCG
+	+	-	+ /-	Mix M. tuberculosis + M. bovis
+	-	+	+ /-	Mix M. tuberculosis + M. bovis BCG
+	-	-	+ /-	M. tuberculosis
-	+	+	+ /-	M. bovis BCG
-	-	+	+ /-	M. bovis BCG
-	+	-	+ /-	M. bovis
-	-	-	+	Negative for M. tub, M.bovis, M.bovis BCG
-	-	-	-	Not valid

QUALITY CONTROL PROCEDURE

A negative control of amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

TROUBLESHOOTING

1. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
2. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	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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