



DUPLIC α Real Time Mycobacterium Tuberculosis Detection KIT

Ref.EBR001032 – 32 test



Instruction Manual

IVD



Euroclone S.p.A. has a Quality System certified by Dasa-Raegister in compliance with EN ISO 9001 (2000)

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INTRODUCTION

Mycobacteria are Gram-positive bacteria (they lack of an outer cell membrane), they are not motile, they have pleomorphic appendix and they are part of the Actinomycete group. Most of the Mycobacteria are found in water or in earth, but some are intracellular pathogens of human and animals. *Mycobacterium tuberculosis*, together with *M. bovis*, *M. africanum*, and *M. microti* are all members of the same complex. The Mycobacterium tuberculosis is an etiological agent of tuberculosis in human and it is the cause of death for almost 2 million people every year all over the world.

Human are the only host for the bacteria. One third of the world population has become infected by the tuberculosis bacillus and every year more than eight million cases are diagnosed. TB is mainly transmitted by air, but it can also be transmitted by gastrointestinal tract. The infection happens if the inhalation of this aerosol gains the alveoli of lungs. Only a 10% of people infected by the bacterium develops the full-blown disease, the remaining 90% remain latent and cannot transmit the disease. Nonetheless, in many groups like children or people with immunodeficiencies (AIDS or malnutrition) the rate of people developing an active form of TB disease is much higher. When the mycobacteria reach the lungs it is captured by the alveolar macrophages and transported to the lymph nodes and from there it spreads to all parts of the body.

From two to eight weeks after the infection the cells mediating immunity (CMI) and hypersensitivity (DTH) bring to the development of the characteristic reaction to the tubercoline skin test and, in immunocompetent people, to the containment of the infection. An immunitary inflammatory reaction can eventually damage the lung.

High concentration of lipids in the cell wall of *Mycobacterium tuberculosis* has been associated to these properties of the bacterium:

- Impermeability to colourings
- Resistance to many antibiotics
- Resistance to the destruction through acid and alkaline components
- Resistance to osmotic lysis via complement deposition
- Resistance to fatal oxidations and surviving inside macrophages

For reasons still unknown people co-infected with HIV are particularly susceptible to TB.

The emergence of stocks resistant to many drugs and its association with epidemics inside and outside hospitals shows how important a fast diagnosis is. In recent years there has been (1) an increasing incidence of tuberculosis in countries of the developing world and a fatal synergy with viruses of human immunodeficiency was noticed in industrialized countries.

In the past techniques based on the amplification of nucleic acids became accessible to laboratories of clinical mycobacteriological. Protocols in PCR that amplify a great variety of chromosome DNA concentrated on the detection both on specific genes and on complex-specific DNA regions. Gene-specific protocols target is the rRNA 16S gene, where the use of at least one specific oligonucleotide allows the amplification of specific fragments for the *M.tuberculosis* stock.

TARGET SEQUENCE

DUPLIC α ^{RealTime} Mycobacterium tuberculosis was designed to distinguish the sequence of the gene 16S-23S from fluids or biopsies.

KIT DUPLIC α ^{RealTime} TECHNOLOGY

DUPLIC α ^{Real Time} Mycobacterium tuberculosis is a test with the DNA-based detection of the Mycobacterium tuberculosis. The reagents for the amplification are ready to use and provided with 3 reactions mix:

- AMPLIFICATION MIX: with Hot Start Taq DNA polymerase, (BlueTaq DNA polymerase, Euroclone), nucleotides, MgCl₂ e buffer.
- OLIGO MIX with a primers and fluorogenic probes.
- INTERNAL CONTROL OF AMPLIFICATION: it allows to value the success of the reaction of amplification, it is identified by a hydrolysis probe.

The polymerase chain reaction (PCR) is a technique that makes it possible to amplify a target piece of DNA generating several copies of a template.

Most commonly, PCR is carried out in three steps: **denaturation**, **annealing** (primers binding) and **extension**. These steps, which are carried out in a thermal cycler that allows heating and cooling, represent a basic cycle of amplification; the continual repetition of a basic cycle produces billions of fragments of the examined DNA. The denaturation of DNA which is carried out at high temperatures is followed by the annealing of specific primers to the target DNA: the Taq enzyme polymerase recognizes the 3'-ends of these primers and polymerizes the DNA using the dNTPs, thus creating several copies of the template. In all PCR-real time systems the amplified products are detected by using a fluorescent dye that in fluorogenic probes is called "*reporter*".

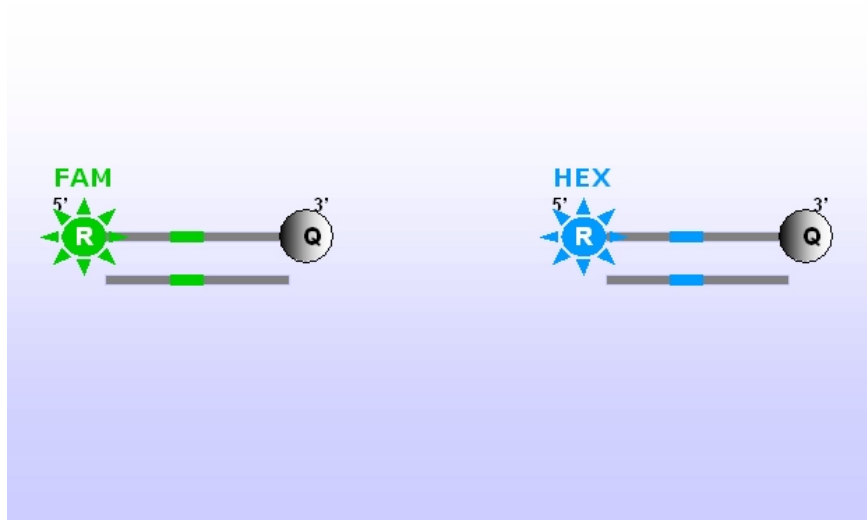


Fig. 1

The kit **DUPLIC α** ^{RealTime} **Mycobacterium tuberculosis** allows to find out bacterial DNA by using specific fluorogenic sequence probes. The fluorogenic probes are made of an oligonucleic sequence which presents at its 5'-end a fluorescent dye called «*reporter*», while at the 3'-end there is a second dye called «*quencher*». The test can detect the specific product of amplification by monitoring the increase of the fluorescence signal which is proportional to the quantity of the product amplified; the high specificity of the system allows the probe to choose between fragments which differ from only one nucleotide.

With the kit is used a probe dyed with a different fluoride for each investigated sequence; in particular one probe detects the allele target that bound covalently as a fluorophore at the 5'-end the FAM molecule (6-carboxyfluorescein) while the other probe detects the internal control (IC) bound as a fluorophore at 5'-end the HEX molecule (hexa-chloro-fluoresceine). The two probes have at their 3'-ends a quencher called BHQ-1 (fig.1). If excited, the integral probe does not emit fluorescence, because the closeness of the quencher to the reporter prevents this last from emitting fluorescence (quenching effect).

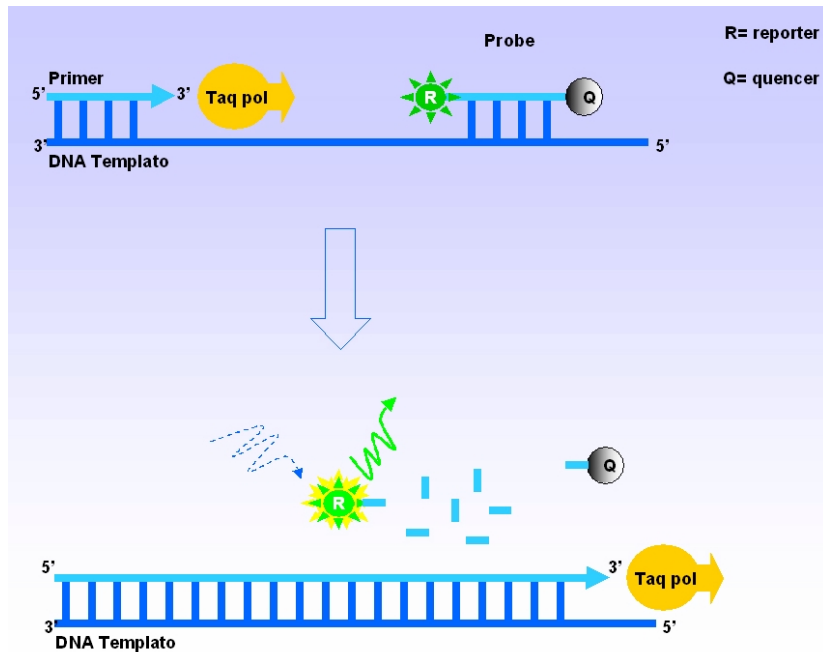


Fig.2

ADVANTAGES

- ◆ **Ease of handling:** The Oligo-Mix and Amplification mix of Kit contains all the reagents for PCR; you will need to add the DNA produced.
- ◆ **Real Time PCR:**
 1. increases the system sensibility;
 2. combines DNA amplification with detection of the products in a single tube;
 3. Reduces detection times (the detection takes place during the amplification phase);
 4. Minimized risk of cross contamination
 5. High throughput and the possibility to automatize the procedure
 6. removes the significant contamination risk caused by opening tubes for post-PCR-manipulation (Carry-over).

CONTENT OF THE KIT

Reagents	Colour code
Amplification mix	Blue Cap
Oligo Mix*	Green Cap
Control 1 (Positive C. of amplification)	Red Cap
Control 2 (Internal C. of amplification)	Yellow Cap
Reaction blank (B)	White Cap

*store the tube far from light

Controls

This kit was designed to execute 32 reactions, included an internal control of amplification that must be co-amplified with any sample and that allows to identify inhibitions in the reactions of amplification.

Storage and stability

Twelve month at 2-8°C.

Reagents required but not supplied

Equipments and materials required for DNA extraction:

- Tubes of 1.5 ml
- Micropipette 1000 µl
- Micropipette 10-100 µl
- Plugged tips
- Rack for 1.5 ml tubes
- Centrifuge (12 000 rpm)
- Incubator
- Vortex

Materials for amplification:

- Micropipette 1-10 µl
- Micropipette 10-100 µl
- Plugged tips
- Rack for 0.2 ml tubes
- Optical microplate for real Time PCR
- Tubes of 0.2 ml with optical caps
- Thermalcycler for Real Time PCR

Precautions and handling

- The kit can be used only after a training for instruction of use.
- Laboratory equipments (pipettes, pipes, etc.) cannot be moved from a working area to another.
- Don't use the reagents after the date of expiration.
- Mix the reagents of the kit before use.
- Periodically verify the calibration of micropipette and the operative of instruments..
- Change gloves frequently.
- Periodically wash the working area with hypochlorite 5%
- Use powder-free gloves, don't leave fingerprints on optical caps. Don't write on caps as this may cause an overlap with fluorescences detection.

OPERATING PROCEDURE

a) Treatment of the sample – PRE-EXTRACTION

Pre-treatment of the sample

The sample (bronchoalveolar washing, saliva, urine) is treated with alkali and neutralized following the standard protocols for mycobacteria.

a1) Sputum or bronchoalveolar washing.

Raise the sample to 10 ml with distilled water.

Add 10 ml of **Solution 1** to the diluted sample.

Shake using vortex.

Incubate at room temperature with constant shaking.

Raise the volume to 50 ml with **Solution 2**.

Shake using vortex.

Centrifuge the sample at 4000 RPM for 15 minutes.

Discard the supernatant .

Resuspend the pellet with 2 ml of **Solution 2**.

a2) Urine

Use 50 ml of fresh urine, it is better to use morning urines as Mycobacterium cells are more concentrated. DO NOT USE THE URINES OF THE 24 h. Centrifuge the sample and discard the supernatant. Dilute the sample with 10 ml of distilled water. If the sample is not processed in short time it is advisable to store it at 4°C before processing the centrifuge step.

Raise the sample to 10 ml with distilled water.

Add 10 ml of **Solution 1** to the diluted sample.

Shake using vortex.

Incubate at room temperature with constant shaking.

Raise the volume to 50 ml with **Solution 2**.

Shake using vortex.

Centrifuge the sample at 4000 RPM for 15 minutes.

Discard the supernatant .

Resuspend the pellet with 2 ml of **Solution 2**.

b) DNA extraction

The “Mycobacterium DNA Gene Releaser” (Euroclone, EMR058050) is a system used to extract DNA from *mycobacteria* presents in clinical samples: bronchoalveolar washing, saliva, urine. Protocol of extraction of the DNA from Mycobacteria

- 1 Transfer **200µl** of **decontaminated sample** in a sterile tube (included in the extraction kit)
- 2 Inactivate the sample at 80°C for 30 minutes
- 3 Add 500ul of sterile H₂O and mix carefully
- 4 Centrifuge at 12.000 RPM for 15 minutes and discard the supernatant
- 5 Add 500ul of sterile H₂O and mix carefully
- 6 Centrifuge at 12.000 RPM for 15 minutes and discard the supernatant

- 7** Add 50ul of **Solution A** to the pellet
- 8** Mix with VORTEX for 1 minute
- 9** Add 50 µl of **Solution B**
- 10** Mix with VORTEX for 1 minute
- 11** Centrifuge at 12.000 RPM for 15 minutes
- 12** Suck 4 µl of supernatant containing the mycobacteria DNA and use it for the amplification

The extracted DNA is contained in the supernatant

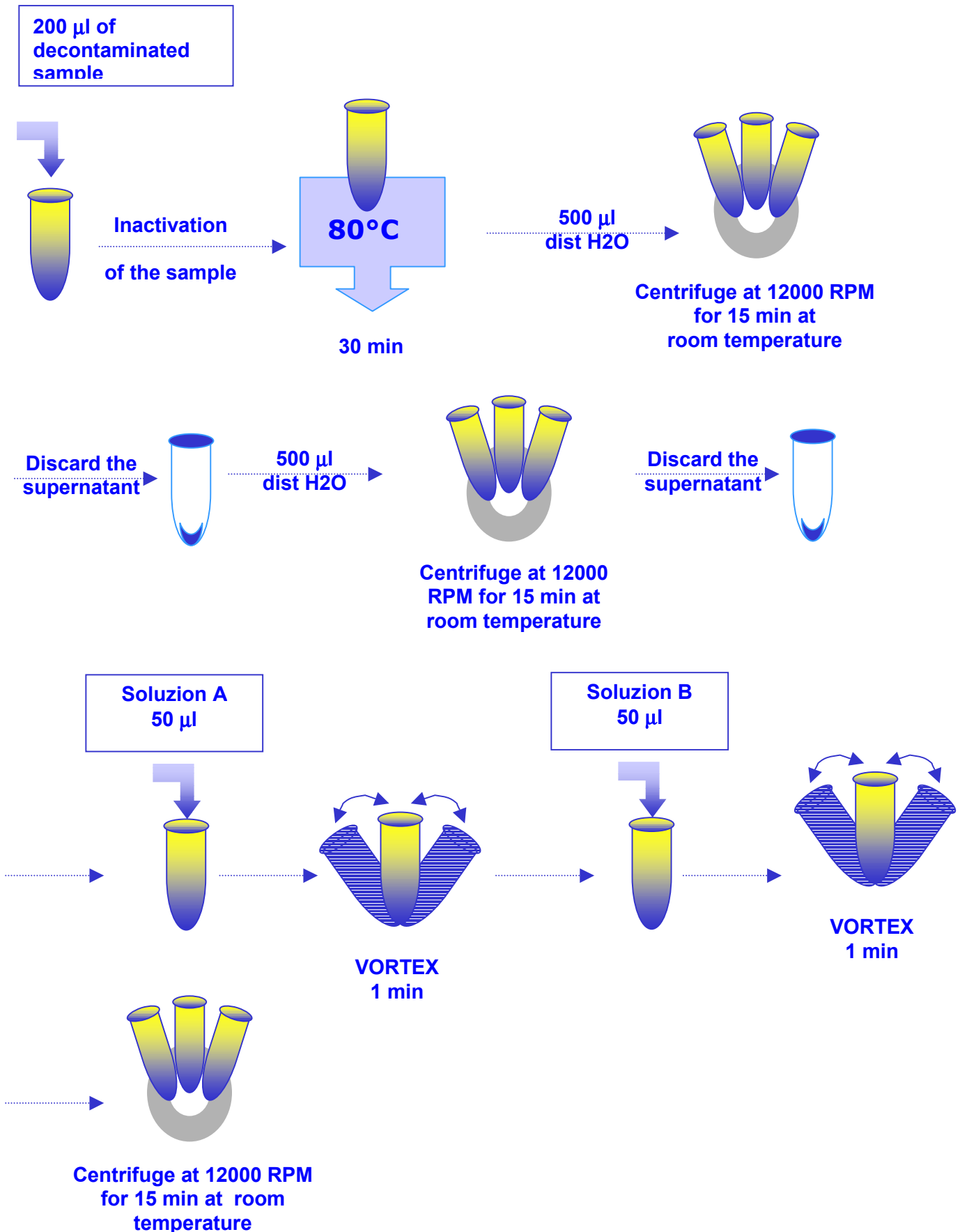
Notes and warnings

The extracted DNA must be separated immediately after the centrifuge, otherwise the sample must be newly centrifuged.

The extracted DNA can be frozen at -20°C and it is stable for about 15-30 days.

Mycobacterium DNA gene releaser (Euroclone, EMR058050)

Fig.3



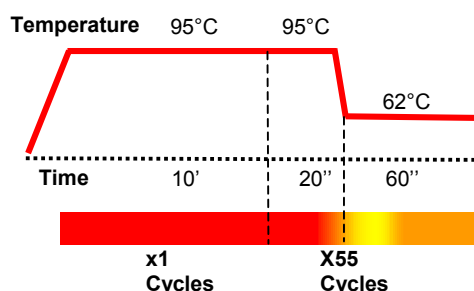
c) Thermal cycler set up

Refer to the specific handbook of the equipment used but be sure to set the following thermal profile.

Thermal Profile

Time	Temperature	N° Cycles
10 min	95°C	1
20 sec	95°C	55
60 sec	62°C	

Fig.4



We recommend to switch on the instrument, setting the thermal profile and having the plate ready before preparing the reaction mix.

d) Preparation of PCR mix

For each experiment prepare a PCR mix for the 2 controls (1 and 2), 1 reaction blank, n+1 samples. The reagents of the mix have to be mixed under this ratio:

REAGENT	VOLUME (μl)
Amplification mix	10
Oligo mix	10
Control 2 (Internal C.)	1
Extracted DNA	4

Once the mix is ready, aliquote 21ul of Master Mix in the tubes or in the microplates for PCR and add in each tube 4ul of extracted DNA and of controls; set the tubes inside the instrument and start the program of amplification you had set before. At the end of the program remove the tubes from the thermal cycler.

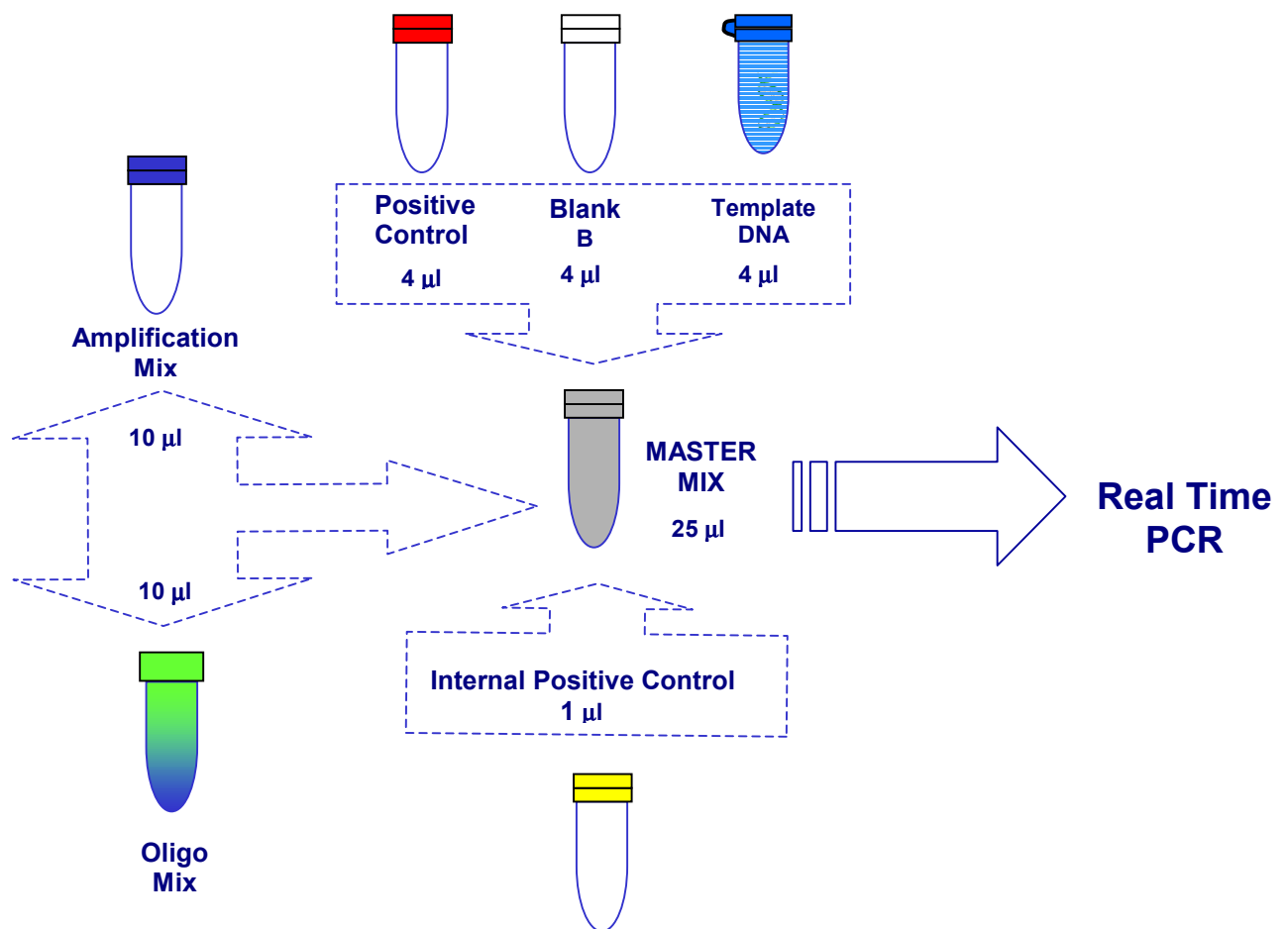


Fig.5

e) ANALYSIS and INTERPRETATION of the RESULTS

The fluorescence signal registered by the instrument detects the presence of the DNA, in particular the fluorescence detected in the FAM channel reveals the target DNA, while the fluorescence detected in the HEX channel reveals the amplification of the internal control (Control 2). When a signal at fluorophore FAM ($ct > 0$) is detected, the sample is surely positive and the signal detected by HEX fluorophore ($Ct \geq 0$) is not relevant. When no signal at fluorophore FAM ($Ct = 0$) is detected, to confirm the negativeness of the result, the completed amplification of internal control, and therefore the appearance of a fluorescent signal at HEX ($ct > 0$) fluorophore level, must be verified. Only in this case we can state that the sample is definitely negative. The absence of signal the amplification at the level of the two FAM-HEX ($Ct=0$)

fluorophores indicates the presence of inhibition of the PCR. The sample must be repeated, and a dilution 1:10 of the target DNA is suggested.

FAM Fluorophore (M.Tuberculosis)	HEX Fluorophore (I.C.)	RESULT
Ct > 0	Not relevant (Ct ≥ 0)	POSITIVE
Ct = 0	Ct > 0	NEGATIVE
Ct = 0	Ct = 0	INIBITION

TROUBLESHOOTING

Problem 1: No signal

1. Wrong channel/filter was chosen.
2. Pipetting error due to omitted reagents or samples.
3. Inhibitory effect of the sample: genomic DNA with a insufficient purification and/or insufficient extraction
4. Check for correct conservation of the kit.
5. Control the performances of the Thermal Cycler

Problem 2: Fluorescence intensity too low

1. Deterioration of dyes and or primers in the device due to unsuitable storage condition
2. Very low starting amount of genomic DNA and/or low purity.

Problem 3: Fluorescence intensity varies

1. The prepared PCR master mix is not well mixed
2. Air bubbles trapped in the PCR tubes

REFERENCE

1. Lachnik J et al. Rapid-cycle PCR and fluorimetry for detection of mycobacteria. J Clin Microbiol. 2002 Sep;40(9):3364-73.
2. Manabe YC and Bishai WR. Latent Mycobacterium tuberculosis-persistence, patience, and winning by waiting. Nat Med. 2000 Dec;6(12):1327-9. Review.
3. Kirschner P et al. Diagnosis of mycobacterial infections by nucleic acid amplification: 18-month prospective study. J Clin Microbiol. 1996 Feb;34(2):304-12.

RELATED PRODUCTS

EBR001032	Duplica RealTime Mycobacterium tuberculosis	32 tests
EBR010032	Duplica RealTime Mycobacterium Avium complex	32 tests
EBR008032	Duplica RealTime Adenovirus	32 tests
EBR007032	Duplica RealTime Legionella pneumophila	32 tests
EBR011032	Duplica RealTime Legionella ssp	32 tests
EBR006032	Duplica RealTime Chlamydia pneumoniae	32 tests
EBR012032	Duplica RealTime Mycoplasma pneumoniae	32 tests
EBR005032	Duplica RealTime Chlamydia trachomatis	32 tests
EBR009032	Duplica RealTime Thricomonas vaginalis	32 tests
EBR013032	Duplica RealTime Neisseria gonorrhoeae	32 tests
EBR014032	Duplica RealTime Mycoplasma genitalium	32 tests
EBR015032	Duplica RealTime Mycoplasma hominis	32 tests
EBR016032	Duplica RealTime Ureaplasma urealyticum	32 tests
EMR058050	Mycobacterium DNA Gene Releaser	50 test
EMR059050	EurocloneSpin Mycobacterium DNA purification Kit	50 test