
# 3B BIOTUB® QT Kit

## MTB/ NTM Real Time PCR Detection

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**3B BlackBio Biotech India Ltd**

A joint venture of Kilpest India Limited, 2B BlackBio, S.L. and Biotools B&M Labs, S.A. Madrid, Spain

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# 3B BIOTUB® QT Kit

## MTB/ NTM Real Time PCR Detection

For detection and differentiation of ***Mycobacterium tuberculosis complex*** **(MTBC)** and ***nontuberculous mycobacteria*** **(NTM)** from human specimens

For use with

ABI Prism 7500 SDS, 7500 Fast SDS, ViiA™ 7 and QuantStudio™ 12K Flex (Applied Biosystems)

Rotor-Gene Q5/6 plex Platform (Qiagen)

CFX384 Touch™, CFX96 Touch™ (BioRad)

PicoReal 24, PicoReal 96 (Thermo)

LightCycler® 96 (Roche)

LineGene K, LineGene 9600 (Bioer)

IVD FOR IN VITRO DIAGNOSTIC USE

REF Product No.: 111111

Σ 48 tests

Temperature limitation

April 2013

3B BlackBio Biotech India Ltd.

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## INTENDED USE

**3B MTB/NTM KIT** accurately differentiates Mycobacterium tuberculosis (MTB) from non-tuberculosis Mycobacterium species (NTM) in a qualitative/quantitative form from various sources of clinical samples using Real time PCR.

## PRINCIPLE

3B MTB/NTM detection is a Real-Time Amplification test for the qualitative/quantitative detection of *Mycobacterium tuberculosis* complex (MTC) and Non-*tuberculosis mycobacteria* (NTM) in clinical samples. Mycobacterium tuberculosis/ Non-tuberculosis DNA is extracted from samples, amplified using Real Time Amplification and detected using fluorescent reporter dye probes specific for *M. tuberculosis* complex and all mycobacterium genus. In real-time PCR, the fluorescent signal is generated from the presence of an oligonucleotide probe specific for target DNA sequence. The probe contains a fluorescent dye molecule on its 5' end and a quencher molecule on its 3' end. The probe hybridizes with one of the chains of the amplified fragment. During synthesis of a complementary chain, Taq DNA polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. As a result, the fluorescent dye and quencher dye are separated, and the total fluorescence of reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR. The fluorescent signal is measured in each cycle of reaction, and the threshold cycle value is determined from the obtained curve. The threshold cycle is proportional to the initial number of DNA copies in a sample and its value allows qualitative/quantitative comparisons of analyzed and control samples.

In 3B MTB/NTM detection kit is based on amplification of region upstream of the 65 kDa heat shock protein (65kDa hsp) gene[1, 2, 3] by primer and probes specific for *M. tuberculosis* complex and mycobacterium genus[4]. In this kit there are three independent reactions running in parallel in each tube: the first detects Mycobacterium tuberculosis complex (M. tuberculosis, M. africanum, M. bovis, M. bovis BCG, M. microti) (HEX channel), second detects all mycobacterium by genus specific probe (FAM channel) and the third detects internal control (IC) DNA (Tex Red channel) which allows excluding unreliable results.

## REAGENTS

The Kit contains amplification reagents for performance of 48/96 amplification reactions. Thaw and handle reagents on ice. Do not freeze/thaw Kit vials repeatedly. In case of frequent use, we recommend to aliquot the contents of the vials into 10 reactions each. This will also rule-out kit/ reagent contamination.

## 3B MTB/NTM REAL TIME KIT

| Reagent | Description | Volume in µL 48 reactions | Volume in µL 96 reactions |
|---|---|---|---|
| Multiplex Master Mix | • Hot-start DNA polymerase<br>• Reaction Buffer<br>• dNTPs (dATP, dCTP, dGTP, dTTP)<br>$MgCl_2$ and stabilizers | 500 µL | 500 µL X 2 |
| MTB/ NTM Primer probe mix | • Primer and probe mix for MTB and NTM detection | 100 µL | 100 µL X 2 |
| Internal control Primer probe mix | • Primer, probe and DNA mix for IC detection | 100 µL | 100 µL X 2 |
| Positive Control MTB | • MTB Positive Control | 50 µL | 100 µL |
| Positive Control NTM | • NTM Positive Control | 50 µL | 100 µL |
| Negative Control | • Sterilized water | 50 µL | 100 µL |

## MTB DNA EXTRACTION KIT

| MTB DNA Extraction Kit | 48 Reactions | 96 Reactions | 240 Reactions |
|---|---|---|---|
| **Reagent** | **Volume** | **Volume** | **Volume** |
| Buffer BT1 | 20 ml | 20 ml X 2 | 100 ml |
| Buffer BB1 | 12 ml | 12 ml X 2 | 60 ml |
| Reagent BB2 | 3 ml | 3 ml X 2 | 15 ml |
| Buffer BB5 (Concentrate) | 7 ml X 2 | 7 ml X 4 | 40 ml X 2 |
| Buffer BBW | 30 ml | 30 ml X 2 | 75 ml x 2 |
| Buffer BBE | 15 ml | 15 ml X 2 | 75 ml |
| Proteinase K (Lyophilized) | 30 mg | 30 mg X 2 | 75 mg x 2 |
| Proteinase Buffer | 1.8 ml | 1.8 ml X 2 | 8 ml |
| MTB DNA Spin Columns | 50 | 100 | 250 |
| 2 ml Collection Tubes | 100 | 200 | 500 |
| Label for Buffer BB3 | 1 | 1 | 1 |
| User Manual | 1 | 1 | 1 |

## INSTRUCTIONS FOR USE

### A. Sample collection

Sputum samples

- Collection of early morning sputum specimen is optimal.
- Rinse the mouth with water and then should cough deeply to expectorate sputum directly into the sterile, leak-proof container.
- Samples should be stored at temperatures of 2-8 $^{o}$C or freezing.

Tissues

- Fresh biopsies of up to 5 mm must be used. Any tissue must be collected aseptically into a sterile container without fixatives or preservatives. To keep moist add sterile saline to the dried specimen. Keep refrigerated until transport.
- Biopsy should be stored at -15±8 $^{o}$C.
- **Paraffin-embedded tissues** can also be used, in cases where the tissue fixation method does not degrade DNA and purification of DNA is performed with methods specific for this kind of sample.

Urine

- An early morning midstream specimen should be collected. Multiple specimens over several days are optimal to obtain a positive specimen.
- Samples should be stored at temperatures of 2-8 $^{o}$C or freezing.

Aseptic fluids (body fluid, bone marrow, blood), Bronchial washing

- Body fluids (spinal, pleural, pericardial, synovial, ascitic, bone marrow), bronchial secretions/washings should be aseptically collected in a sterile container using aspiration techniques or surgical procedures.

- For fluids that may clot, sterile potassium oxalate (0.01 ~ 0.02 ml of 10% neutral oxalate per ml fluid) or heparin (0.2 mg per ml) should be added.

Stool

- Stool should be passed into a clean and dry container.
- Carefully open the specimen vial and collect a small sample with the help of a sterile spoon in to a new sterile vial.
- Close the vial tightly and shake to mix well.

| Specimen Type | Quantity | |
|---|---|---|
| | Optimal | Minimum |
| Respiratory specimens (sputum, BAL, BW, tracheal aspirate, etc) | 5–10 mL | 1 mL |
| Blood/bone marrow | 5 mL | 1 mL |
| CSF | 5–15 mL | 1 mL |
| Abscess (wound)aspirates | 5–15 mL | 1 mL |
| Body fluids/ aspirates | 5–15 mL | 1 mL |
| Urine | 30–50mL | 10 mL |
| Stool | 2-5 g | 1 gm |
| Tissue | 5 mm | Visible |
| Pus | 1-10 mL | 1 mL |

## B. Preparation of Samples

- Prepare 2% N-Acetyl Cystein/ NaOH [Dissolve 2 g of NaOH, 1.45 g of Sodium citrate, 0.5 g N-acetyl cystein in 100 ml of sterile distilled water].
- Prepare 0.067M phosphate buffer solution by dissolving 2.37 g of Dibasic Sodium Phosphate ($Na_2HPO_4$) and 2.27 g Monobasic Potassium Phosphate in 500 ml of sterile distilled water. Adjust the final pH to 6.8.

***i) Viscous Fluids (sputum, body fluids, Pus):***

- Homogenize the sample by vigorous vortexing for 5-10 min.
- In the 15 ml falcon tube, add the 2% NALC-NaOH with the specimen (sputum/body fluids/urine) to an **equal volume (1:1)** and vortex for 1 min.
- Incubate the mixture for **15 min** at **room temperature** with occasional shaking.
- Adjust the **volume to 15 ml** with **sterile water or sterile phosphate buffer solution (pH 6.8)** and mix well.
- Transfer 1.5 ml sample to a 2 ml Eppendorf tube using a pipette with a tip with aerosol barrier, mark it, and centrifuge at 10,000 g or 15,000 rpm for 10 min. Discard the supernatant, add 1 ml of PBS solution and mix well.

*Note: (if the pellet is not visible, remove the supernatant leaving about 100 µl of the sample)*

- Centrifuge at 10,000 g or 15,000 rpm for 10 min, discard the supernatant and re-suspend the pellet as per DNA extraction step.

***ii) Non-viscous Fluids (aspiration, body fluids, urine, pleural fluid):***

- Homogenize the sample by vigorous vortexing for 5-10 min.
- Transfer 1.5 ml of the sample to a 2 ml Eppendorf tube using a pipette with a tip with aerosol barrier, mark it, and centrifuge at 10,000 g or 15,000 rpm for 10 min.
- Discard the supernatant and re-suspend the pellet as per DNA extraction step.

*Note: (if the pellet is not visible, remove the supernatant leaving about 100 µl of the sample)*

***iii) Fresh tissue (Biopsy, Endometrium tissue)***

- If RBCs are present then give PBS wash to remove RBC's totally.
- If tissues are soft then add NALC-NaOH solution and start processing as described below. If tissues are tough, then grind and homogenize it with PBS in sterile mortar-pestle and process as described below.
- Process sample in original sample container. If sample is in syringe or small container then transfer and process the sample in 50 ml Falcon tube (preferred for more surface area) or 15 ml falcon tube.
- To specimen add 2% NALC-NaOH solution in 1:1 ratio. Incubate for 15-20 min depending upon sample consistency at room temperature with intermittent vigorous vortexing.
- If the sample is very viscous or appears to be tough/thick then incubate it in water bath of temperature 40-50$^{o}$C.
- Neutralize the digested-decontaminated specimens by adding Phosphate buffer saline (PBS) up to 15 ml and mix by inverting. Centrifuge 1.5 ml neutralized sample for 15 min at 13000 rpm.
- Wash the pellet with 1ml PBS and centrifuged for 15 min at 13000 rpm.
- Check the pH of supernatant and discard it. If pH is neutral then wash pellet with 1ml water and if it is still alkali then give one more (or two till it become neutral) wash with PBS.
- After neutralization give 1 more PBS washing.
- Now add 1ml sterile water to the pellet, vortex and centrifuge at 13000 rpm for 10 min. and carefully discard the supernatant. Repeat this step once and proceed as per DNA Extraction step.

***iv) Paraffin- embedded tissue***

- Add 1 ml of n-Octane or Xylene to the tube containing several pieces of paraffin fragment.
- Vortex vigorously and incubate at room temperature for about 30 min. Vortex occasionally. Centrifuge at 13,000 rpm for 3 min. Pipette off supernatant. (If paraffin remains, repeat step 1 and 2)
- Add 1 ml of ethanol (96-100%) and then vortex it.
- Centrifuge for 3 min at 13,000 rpm. Pipette out supernatant.
- Repeat step 3 and 4. Pipette off as much of the ethanol as possible.
- Incubate the open tube at 37 $^{o}$C until the ethanol has evaporated (15 min).
- Discard the supernatant and re-suspend the pellet as per DNA extraction step.

*Note: (if the pellet is not visible, remove the supernatant leaving about 100 µl of the sample)*

***v) Stool***

- Homogenize the sample by vigorous vortexing for 5-10 min.
- Mix the stool (at least 1 g) with 10 ml of 2% **NALC-NaOH**, vortex vigorously for 30 sec and incubate for 15 min at room temperature.
- Centrifuge for 10 min at 1,500 rpm, transfer 1.5 ml of the supernatant to 2 ml Eppendorf tube using a pipette with a tip with aerosol barrier, mark it, and centrifuge at 10,000 g or 15,000 rpm for 10 min.
- Discard the supernatant and re-suspend the pellet as per DNA extraction step.

*Note: (if the pellet is not visible, remove the supernatant leaving about 100 µl of the sample)*

***vi) Blood, Bone marrow aspiration, Menstrual blood***

- Take 0.5 ml of whole blood (or Bone marrow aspiration) and mix well with 1 ml of sterile water.

- Centrifuge for 10 min at 10,000 g or 15,000 rpm.
- Discard the supernatant and add 1 ml sterile water to pellet.
- Mix well and centrifuge at 15,000 rpm for 10 min.
- Discard the supernatant and re-suspend the pellet as per DNA extraction step.

Note: (if the pellet is not visible, remove the supernatant leaving about 100 µl of the sample)

***vii) Broncho Alveolar Lavage (BAL)***

- Homogenize the sample by vigorous vortexing for 5-10 min. If sample is viscous dilute sample with water in 1:0.5 ratio and vortex to homogenize.
- Centrifuge whole sample at 13,000 rpm for 15 min. discard the supernatant.
- If RBCs are present then give PBS wash to remove RBCs.
- To pellet add 500-600 µl 2% NALC-NaOH solution and incubate for 15-20 min depending upon sample consistency at room temperature with intermittent vigorous vortexing.
- If the sample is very viscous or appears to be tough/thick then incubate it in water bath of temperature 40-50 $^{o}$C.
- Neutralize the digested-decontaminated specimens by adding Phosphate buffer saline (PBS) up to 15 ml and mix by inverting. Centrifuge whole neutralized sample for 15 min at 13000 rpm.
- Wash the pellet with 1ml PBS and centrifuged for 15 min at 13000 rpm.
- Check the pH of supernatant and discard it. If pH is neutral then wash pellet with 1ml water and if it is still alkali then give one more (or two till it become neutral) wash with PBS.
- After neutralization give 1 more PBS washing.
- Now add 1ml sterile water, vortex pellet and centrifuge at 13000rpm for 10 min. and carefully discard the supernatant. Repeat this step once and re-suspend the pellet as per DNA Extraction step.

## C. DNA Extraction Step

Before starting DNA extraction protocol prepare the following:

**Lysis Buffer BB3:** Transfer the total contents of Buffer BB1 to Buffer BB2 and mix well. Place the labels for Lysis Buffer BB3 on the bottle. The resulting Lysis Buffer BB3 is stable for up to one year at room temperature.

**Wash Buffer BB5:** Add the below indicated volume of ethanol (96 – 100 %) to Wash Buffer BB5 Concentrate. Mark the label of the bottle to indicate that ethanol was added. Store Wash Buffer BB5 at room temperature (18 – 25° C) for up to one year.

| Format | Volume of BB5 | Volume of Ethanol to be added |
|---|---|---|
| 48 rxns | 7 ml x 2 | 28 ml to each bottle |
| 96 rxns | 7 ml x 4 | 28 ml to each bottle |
| 240 rxns | 2 x 40 ml | 160 ml to each bottle |

**Proteinase K:** Add the below indicated volume of Proteinase Buffer (**PB)** to dissolve lyophilized Proteinase K. Proteinase K solution is stable at - 20 °C for up to 6 months.

| Format | Qty. of Proteinase K | Volume of PB to be added |
|---|---|---|
| 48 rxns | 30 mg | 1.35 ml to each vial |
| 96 rxns | 30 mg X 2 | 1.35 ml to each vial |
| 240 rxns | 75 mg X 2 | 3.35 ml to each vial |

- Set on incubator or water bath to 50$^{o}$C
- Preheat Elution Buffer BBE to 70$^{o}$C

***i) Resuspension:***

Resuspend the formed pellet in **0.2 – 1 ml Buffer BT1** (depending on sample viscosity).Transfer **200 µl** of the resuspended sample to a new microcentrifuge tube (not provided).

***ii) Pre-lyse sample:***

Add **180 µl Buffer BT1** and **25 µl Proteinase K** solution. Vortex to mix. Be sure that the samples are completely covered with lysis solution.

Note: *If processing several samples, Proteinase K and Buffer BT1 may be premixed directly before use.* ***Do not*** *mix Buffer BT1 and Proteinase K more than 10 – 15 min before addition to the sample.*

Incubate at **56 °C** until complete lysis is obtained **(at least 1 – 3 h)**. Vortex occasionally during incubation or use a shaking incubator.

Note: *Samples can be incubated overnight as well. If RNA-free DNA is crucial for downstream applications, an RNase digest may be performed: Add 20 µl RNase A (20 mg/ml) solution (not included and incubate for an additional 5 min at room temperature.*

***iii) Lyse sample:***

Vortex the samples. Add **200 µl Buffer BB3**, vortex vigorously and incubate at **70 °C** for **10 min**. Vortex briefly.

Note: *If insoluble particles are visible in Buffer BB3, centrifuge for 5 min at high speed (e.g., 10,000 rpm) and transfer the supernatant to a new micro-centrifuge tube (not provided)*

***iv) Adjust DNA binding conditions:***

Add 210 µl ethanol (96 – 100 %) to the sample and vortex vigorously**.**

***v) Bind DNA:***

For each sample, place one **MTB DNA Spin Column** into a Collection Tube. Apply the sample to the column. Centrifuge for 1 min at 10,000 rpm. Discard the flow-through and place the column back into the Collection Tube.

Note: *If the sample is not drawn completely through the matrix, repeat the centrifugation step at 10,000* rpm*. Discard flowthrough.*

***vi) Wash silica membrane***

**1st wash**

Add **500 µl Buffer BBW**. Centrifuge for **1 min** at **10,000 rpm**. Discard flow-through and place the column back into the Collection Tube.

**2nd wash**

Add **600 µl Buffer BB5** to the column and centrifuge for **1 min at 10,000 rpm**. Discard flow-through and place the column back into the Collection Tube.

***vii) Dry silica membrane***

Centrifuge the column for **1 min** at **10,000 rpm**. Residual ethanol is removed during this step.

***viii) Elute highly pure DNA***

Place the **MTB DNA Spin Column** into a 1.5 ml micro-centrifuge tube (not provided) and add **100 µl pre-warmed Buffer BBE (70° C)**. Incubate at room temperature for 1 min. Centrifuge **1 min** at **10,000 rpm**.

**xi)** 50 -100 ng of the eluted DNA should be used for the amplification.

# REAL TIME PCR PROTOCOL FOR MTB/NTM DETECTION

## 1. REACTION PREPARATION

**a)** Prepare the PCR Mix as follows:

| Name of the Reagent | For "1" rxn. |
|---|---|
| Multiplex Master Mix | 10 µl |
| MTB /NTM Primer probe mix | 2 µl |
| Internal control Primer probe mix | 2 µl |

***b)*** Mix well by inverting or quick vortexing and centrifuge briefly.

**c)** Transfer **14 µl** of the above prepared PCR Mastermix in 0.2 ml PCR tubes and close the tubes.

**d)** For **14 µl** of above reaction mix, add **50-100 ng** of DNA samples and make up the final volume **20 µl** with nuclease free water.

| Name of the Reagent | For "1" rxn. |
|---|---|
| DNA | 50-100 ng |
| Nuclease free Water | Make up to 20 µl |

## 2. PROGRAM SET UP

Define the following setting for Temperature Profile and Dye Acquisition

| Step | Temperature, °C | Time | Dye Acquisition | Cycles |
|---|---|---|---|---|
| 1 | 94 | 15 min | - | 1 |
| 2 | 94 | 15 sec | - | 35 |
| | 60 | 30 sec | - | |
| | 72 | 20 sec | Yes | |

## 3. CHANNEL SELECTION

Define the following setting for channel selection

| Detection | Detector Name | Reporter | Quencher | Gain Setup |
|---|---|---|---|---|
| MTB specific DNA | MTB | HEX | None | 43 |
| NTM specific DNA | NTM | FAM | None | 50 |
| Internal Control | IC | Tex Red | None | 50 |

## RESULT ANALYSIS

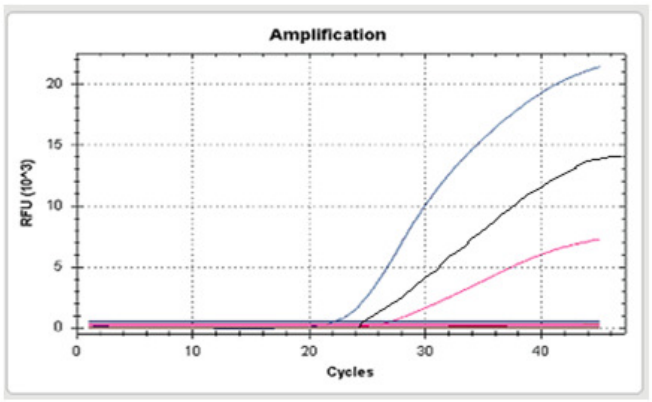
| Case | Amplification Signals in HEX Channel | Amplification Signals in FAM Channel | Amplification Signals in Texas Red Channel | Interpretation |
|---|---|---|---|---|
| 1 | Present | Present | Present/ Absent# | *Mycobacterium tuberculosis* (MTB) is present.<br><br>Test sample is positive for MTB or co-infection* of MTB and NTM |
| 2 | Absent | Present | Present/ Absent# | *Non-tuberculosis Mycobacterium species (NTM)* is present.<br><br>Test sample is negative for MTB but positive for NTM |
| 3 | Absent | Absent | Present | Test sample is negative for MTB and NTM |
| 4 | Absent | Absent | Absent | PCR inhibition, retest the sample |

*** Co-infection but must be confirmed by further tests, e.g. genotyping**

**# Detection of the Internal Control is not required for positive results in the FAM or HEX detection channels. High MTB or NTM-load in the sample can lead to reduced or absent Internal Control signals.**

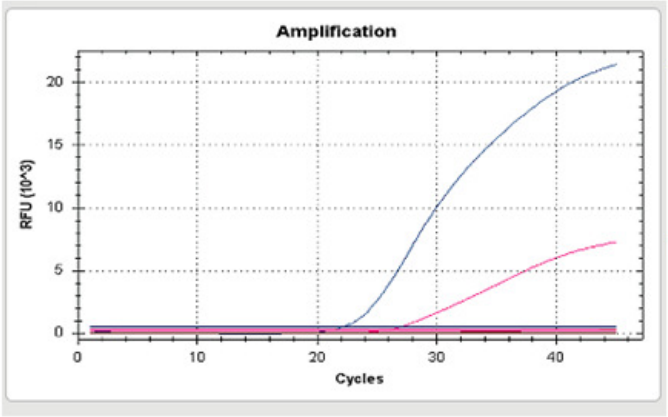
**Case 1**

MTB Positive



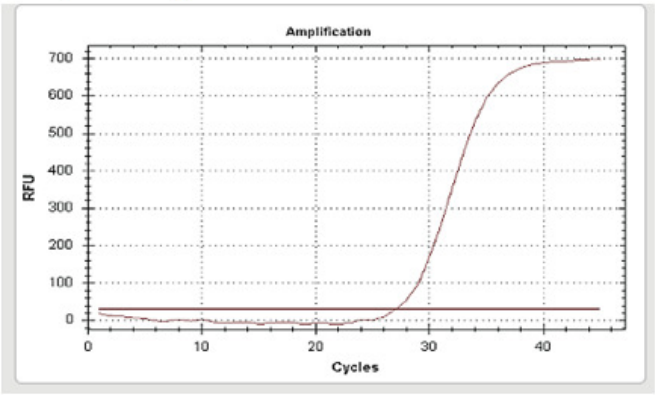

**Case 2**

NTM Positive




**Case 3**

MTB / NTM Negative



## SPECIFICATIONS

**A. Sensitivity:**

1) M. tuberculosis

The analytical sensitivity for M. tuberculosis complex of the **3B MTB/NTM KIT** was determined by analyzing dilution series of known copy number of plasmid containing MTB target gene. The 3B **MTB/NTM KIT** test demonstrated the ability to reproducibly detect the presence of M. tuberculosis at the level of ≥ 10 copies/µl.

| Target Concentration (copies/µl) | Average (Ct) |
|---|---|
| **5 X $10^6$** | 17.30 |
| **5 X $10^5$** | 21.03 |
| **5 X $10^4$** | 24.02 |
| **5 X $10^3$** | 27.03 |
| **5 X $10^2$** | 30.19 |
| **5 X $10^1$** | 33.25 |
| **10** | 34.89 |

**Table 1 Analytical sensitivity of the 3B MTB/NTM KIT for MTB detection**

2) NTM [Mycobacterium avium subsp.avium (MTCC1723)]

The analytical sensitivity for NTM of the **3B MTB/NTM KIT** was determined by analyzing dilution series of known copy number of plasmid containing NTM target gene. The **3B MTB/NTM KIT** tests demonstrated the ability to reproducibly detect the presence of NTM at the level of ≥ 50 copies/µl.

| Target Concentration (copies/µl) | Average (Ct) |
|---|---|
| **5 X $10^6$** | 17.97 |
| **5 X $10^5$** | 21.06 |
| **5 X $10^4$** | 24.66 |
| **5 X $10^3$** | 28.37 |
| **5 X $10^2$** | 31.61 |
| **5 X $10^1$** | 34.76 |

**Table 2 Analytical sensitivity of the 3B MTB/NTM KIT for NTM detection**

**B. Clinical Performance**

The clinical performance of the **3B MTB/NTM KIT** is evaluated regularly by analysing reference samples and diagnostic samples previously tested with a reference method (mycobacteria culture). 181 specimens derived from Sputum, Aseptic fluids, Broncho Alveolar Lavage, Aseptic tissues and Pus collected in different laboratories and hospitals were tested for determining the diagnostic sensitivity and specificity of the **3B MTB/NTM KIT.** Results were achieved by comparing the results obtained with the **3B MTB/NTM KIT** against results obtained by mycobacteria culture for the individual specimen.

| | | | Mycobacteria Culture | | | |
|---|---|---|---|---|---|---|
| | | | Positive | | Negative | Total |
| | | | MTB | NTM | | |
| 3B MTB/NTM KIT | Positive | MTB | 102 | 0 | 2 | 104 |
| | | NTM | 0 | 7 | 0 | 7 |
| | Negative | | 5 | 2 | 63 | 70 |
| | Total | | 107 | 9 | 65 | 181 |

**Table 3 Clinical performance of 3B MTB/NTM KIT**

**C. Clinical performance analysis**

| | | Results | Percentage (%) |
|---|---|---|---|
| **Sensitivity** | **MTB** | 102/107 | 95.32 |
| | **NTM** | 7/9 | 77.77 |
| **Specificity** | | 63/65 | 96.92 |
| **Positive Predictive Value** | **MTB** | 102/104 | 98.07 |
| | **NTM** | 7/7 | 100 |
| **Negative Predictive Value** | | 63/70 | 90 |

**Table 4 Analysis of clinical performance of 3B MTB/NTM KIT**

**D. Specificity.**

Specificity of **3B MTB/NTM KIT** is assured by selection of specific primers and probes as well as the selection of stringent reaction conditions. The primers and probes were checked for possible homologies and to ensure that all relevant MTB and NTM are detected, to all gene banks published sequences by sequence comparison analysis. Specificity of **3B MTB/NTM KIT** was confirmed in laboratory clinical trials too. It has been tested for cross-reactivity with several common human pathogenic bacteria other than MTB/NTM and as a result there was no case of real time PCR amplification was observed.

**E. Reproducibility**

The Reproducibility of specificity, sensitivity and accuracy of **3B MTB/NTM KIT** were evaluated by reproducibility tests carried out at different points of time in the course of two months by different experimenters. The results turned out to be the same, confirming the reproducibility of the kit.

## TROUBLESHOOTING REAL TIME PCR

| No. | Observation | Probable causes | Comments |
|---|---|---|---|
| 1 | Amplification signal in negative control | Cross contamination during handling | Check for contamination of kit's component |
| 2 | No amplification signal with positive controls | Incorrect PCR mixture | Check whether all components are added. |
| | | Missing control sample during DNA mixing | Be careful when pipetting |
| | | Changing DNA during DNA mixing | Write down sample number on the 1.5 ml micro centrifuge tube and the PCR tube |
| | | Leaving reagents at room temperature for a long time or incorrect storage condition | Please check the storage condition and the expiration date(see the kit label) of the reagents and use a new kit, if necessary |
| | | The PCR conditions do not comply with the protocol | Repeat the PCR with corrected settings |
| 3 | Weak or no signal of the Internal Control in Tex Red channel | Reagent has been thawed and frozen too often or exposed to inappropriate storage conditions | Please mind the storage conditions given in manual |
| | | The PCR was inhibited | DNA of Poor quality may interfere with the PCR reaction, use a recommended isolation method |

## TROUBLESHOOTING DNA EXTRACTION

| No. | Observation | Probable causes | Comments |
|---|---|---|---|
| 1 | No or poor DNA yield | Incomplete lysis | Sample not thoroughly homogenized and mixed with Buffer BT1/ Proteinase K. The mixture has to be vortexed vigorously immediately after the addition of Buffer BT1.<br>Decreased Proteinase K activity: Store dissolved Proteinase K at -20°C for 6 months. |
| | | Reagents not applied properly | Prepare Buffer BB3, Buffer BB5, and Proteinase K solution according to instructions. Add ethanol to the lysates before loading them onto the columns. |
| | | Suboptimal elution of DNA from the column | Preheat Buffer BBE to 70°C before elution. Apply Buffer BBE directly onto the center of the silica membrane.<br>Elution efficiencies decrease dramatically, if elution is done with buffers with a pH < 7.0. Use slightly alkaline elution buffers like Buffer BBE (pH 8.5).<br>Especially when expecting high yields from large amounts of material, we recommend elution with 200 µl Buffer BBE and incubation of the closed columns in an incubator at 70°C for 5 min before centrifugation. |
| 2 | Poor DNA quality | Incomplete lysis | Sample not thoroughly homogenized and mixed with Buffer BT1/ Proteinase K. The mixture has to be vortexed vigorously immediately after the addition of Buffer BT1.<br>Decreased Proteinase K activity: Store dissolved Proteinase K at -20°C for 6 months. |
| | | Reagents not applied properly | Prepare Buffer BB3, Buffer BB5, and Proteinase K solution according to instructions. Add ethanol to the lysates before loading them on the columns. |
| | | RNA in sample | If RNA-free DNA is desired, add 10 µl of RNase A solution (5 mg/ml; not supplied with the kit) before addition of Buffer BB3 and incubate at 37°C for 5 min. |
| 3 | Clogged columns | Too much sample material used | Do not use more sample material than recommended (25 mg for most tissue types). If insoluble material like bones or hair remains in the lysate, spin down the debris and transfer the clear supernatant to a new microcentrifuge tube before proceeding with addition of Buffer BB3 and ethanol. |
| | | Incomplete lysis | Sample not thoroughly homogenized and mixed with Buffer BT1/ Proteinase K. The mixture has to be vortexed vigorously immediately after the addition of Buffer BT1.<br>Decreased Proteinase K activity: Store dissolved Proteinase K at -20 °C for 6 months. |
| | | Reagents not applied properly | Prepare Buffer BB3, Buffer BB5, and Proteinase K solution according to instructions. Add ethanol to the lysates before loading them on the columns. |

## STORAGE AND HANDLING

All the components of 3B MTB/NTM KIT should be stored at -20˚C and stable until the date of expiry stated. The reagents can be aliquoted and stored at -20˚C in-order to maintain the stability and sensitivity.

All the components of MTB DNA EXTRACTION KIT should be stored at room temperature and stable until the date of expiry stated.

Note: Proteinase K should be stored at -20$^{o}$C, once dissolved.

## MATERIAL AND DEVICES REQUIRED BUT NOT PROVIDED

- Adjustable pipettes with sterile filter or positive displacement tips
- 2% NALC-NaOH
- Phosphate Buffer Saline (PBS)
- Disposable powder-free gloves
- Sterile bidistilled water
- Sterile 1.5 ml and 2 ml microcentrifuge tubes
- 50 ml conical tubes
- Vortex mixer
- Heating-block for incubation at 70$^{o}$C
- Water Bath
- 3B Termi-DNA-Tor or equivalent, in order to remove DNA from working surfaces
- Desktop centrifuge
- Real time PCR
- Laminar airflow cabinet
- PCR vials (0.2 ml, thin-walled)
- 96 – 100% ethanol
- Personal protection equipment (lab coat, gloves, goggles)

## GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, and protect eyes while samples and reagents handling. 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 samples and unused reagents in compliance with local authorities' requirements.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices. Infected material and disposable plasticware that was in contact with infected material must be treated with chlorine-containing solutions.
- Clean and disinfect all sample or reagent spills using a disinfectant, such as 0.5 % sodium hypochlorite or other suitable disinfectant.
- Avoid contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact, immediately flush with water, seek medical attention.
- 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 uni-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.

## REFERENCES

1. ICMR Bulletin. (2002). What is new in the diagnosis of Tuberculosis? Part I: Techniques for the diagnosis of Tuberculosis. Vol.32, No.8.
2. Kapur V, Li L-L, Hamrick MR, Plikaytis BB, Shinnick TM, Telenti A, et al. (1995). Rapid Mycobacterium species assignment and unambiguous identification of mutations associated with antimicrobial resistance in Mycobacterium tuberculosis by automated DNA sequencing. Arch Pathol Lab Med 119:131–8.
3. Kim H, Kim SH, Shim TS, Kim MN, Bai GH, et al. (2005). Differentiation of Mycobacterium species by analysis of the heat-shock protein 65 gene (hsp65). Int J Syst Evol Microbiol 55: 1649–1656
4. Tobler, N. E., M. Pfunder, K. Herzog, J. E. Frey, and M. Altwegg. (2006). Rapid detection and species identification of Mycobacterium spp. using real-time PCR and DNA-microarray. J. Microbiol. Methods 66:116-124.

## NOTICE

The user should always pay attention to the following:

This test is for use with **Sputum, Aseptic fluids (body fluid, blood, bone marrow aspiration, menstrual blood), Bronchial washing, Aseptic tissues (fresh tissues, paraffin- embedded tissue, endometrium tissue), Pus, Urine and Stool**.

**Store DNA samples at -20°C until ready for use and keep on ice during use.**

Avoid microbial contamination of reagents when removing aliquots from reagent tubes. The use of sterile disposable pipette tips is recommended.

Specimens should be handled as if infectious using safe laboratory procedures. Thoroughly clean and disinfect all work surfaces with 0.5% Sodium Hypochlorite in de-ionized or distilled water.