

Vertrieb:

LOXO GMBH, IMMUNBIOLOGIE - BIOCHEMIE POSTFACH 11 30

69215 DOSSENHEIM

Telefon 06221 868023 Fax 06221 8680255

E-Mail: info@loxo.de Internet: www.loxo.de



BIOTOOLS

BIOTOOLS B&M LABS.S.A.

Manufactured by:

BIOTOOLS B&M Labs, S.A.
Valle de Tobalina - 52 - Nave 39
28021 Madrid
Spain

Tel. (34) 91 710 00 74
Fax (34) 91 505 31 18
e-mail: diagnostics@biotools.eu
www.biotools.eu

BIOTUB-QT

*Kit for detection and quantification of DNA from
Mycobacterium tuberculosis in clinical samples*

Instructions for Use

STANDARD

Equipment: Applied Biosystems
(Cat. No. 90.572A)



**PLEASE READ THE INSTRUCTIONS FOR USE THOROUGHLY BEFORE USING THE KIT,
ESPECIALLY IF YOU ARE NOT FAMILIAR WITH THE PROTOCOL.**

BIOTUB-QT KIT

Some of the applications which may be performed with this product may be in certain countries under an applicable patent. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application. Biotools does not encourage the unlicensed use of patented applications.

PLEASE CHECK INTEGRITY OF THE KIT AND THE REAGENTS BEFORE USE. DETERIORATED KITS MAY CAUSE EQUIVOCAL RESULTS.

1. INTENDED USE

The BIOTUB-QT Kit is an *in vitro* diagnosis test for the quantitative determination of *Mycobacterium tuberculosis* in clinical samples by Real Time DNA amplification techniques, using specific hydrolysis primer-probes, LIONPROBES™ type. **BIOTOOLS has developed and patented LIONPROBES™ a new technology for detection of nucleic acids in Real Time.**

The BIOTUB-QT kit is to be used with the following samples:

- Lung and skin biopsies
For the use of the kit with biopsies do not treat the tissue with acetic acid or iodide. Paraffin-embedded tissues can be used provided the employed fixative reagents do not degrade or interact with DNA and deparaffination is performed.
- Sputum
The yield of the reaction will depend on the content and quality of DNA present in the sputum, and therefore, poor quality sputum samples may render equivocal results.
- Bacterial cultures

For purification of bacterial DNA, BIOTOOLS recommends the use of **Speedtools Mycobacteria DNA Extraction Kit** (Cat. No. 21.186/7), **Speedtools DNA Extraction Kit** (Cat. No. 21.131/2) or **Speedtools Tissue DNA Extraction Kit** (Cat. No. 21.136/7), though other methods guaranteeing $A_{260/280}=1.8 - 2.0$, 50-100 ng/μl concentration and the absence of amplification inhibitors can also be employed. When using purification methods based on silica matrix, it is most important to check for the complete absence of silica particles in the sample since this affects the performance of Real Time DNA amplification.

In the event that DNA concentration is not measurable by spectroscopy a serial dilution of the unknown sample (final volume of DNA sample is 10 μl) can be prepared so that results are within the range of the standard curve. It is recommended to check the quality and suitability of the purified DNA for amplification reactions, for example, by performing control amplifications in parallel.

For further information, please contact our Technical Dept. (diagnostics@biotools.eu).

2. FEATURES AND PRINCIPLES OF THE PROCEDURE

The BIOTUB-QT kit involves a DNA amplification technique, significantly shortening the time of diagnosis of tuberculosis from weeks, for bacterial culture techniques, to less than a day. Moreover, the quantification of *Mycobacterium tuberculosis* performed with the Kit allows therapy monitoring or study the course of the disease.

DNA from *Mycobacterium tuberculosis* present in positive samples is amplified and detected by using a consensus primer and a primer-probe (LIONPROBES™) complementary to bacterial conserved regions. The kit also includes an internal amplification control, which detects false negative results in each sample reaction due to amplification inhibition.

The LIONPROBES™ technique of the BIOTUB-QT Kit is based on the use of a DNA polymerase with 3'-5' exonuclease proofreading activity (Pfu) and a double labelled oligonucleotide (⁵TAMRA—FAM³) which presents a mismatch at 3' end of the hybridization area with the target DNA. During the amplification reaction the Pfu recognises the 3' mismatch and consequently excises the mismatched base pairs labelled one of them with the fluorophore (FAM). Once the 3'-end of the oligonucleotide is corrected amplification priming is enabled by the oligonucleotide. During each amplification cycle the fluorescence emerges as result of the release of the fluorophore.

The Kit consists of two amplification detection reactions which take place in the same amplification tube:

- Amplification reaction for the insert element IS6110¹ of the *Mycobacterium tuberculosis* complex—Samples positive for *Mycobacterium tuberculosis* in the presence of primer-probe (LIONPROBESTM 5'TAMRA—FAM³) and the consensus primer give an increase in the fluorescence in the reading channel for FAM.
- Amplification reaction for the Internal Control- Based on a sequence not related to *Mycobacterium tuberculosis*, which is amplified and detected by the consensus primer and primer-probe (LIONPROBESTM 5'VIC—TAMRA³). In this case the increase in fluorescence is observed in the reading channel for the fluorophore VIC (VIC channel) and it is due to the release of the quencher TAMRA. This reaction must always render a positive result. This control reaction reduces the possibility of false negative results due to amplification inhibition.

For the quantification of the number of *Mycobacterium tuberculosis* present in the clinical samples, a standard curve must be done using the positive control included in the kit (POS CTR vial). The amplification and detection of the standards should be performed in the same way as if it was a clinical sample.

3. CONTENTS OF THE KIT

The BIOTUB-QT Kit contains reagents for performance of 48 reactions (Cat. No. 90.572A). To minimize the risk of contamination and facilitate the use of the kit several times the Kit is presented in two set of 24 reactions. Sample Kit (Cat. No. 90.571A) contains one set of 24 reactions. The reagents are in liquid format, except the CONTROL vials (internal and positive) which are in gel format. **Store Kit vials at -15±8°C.**

CONTROL vials in gel format (without being regenerated with sterile bidistilled water) can be stored either at 2-8°C or -15±8°C, **once regenerated store the vials at -15±8°C.**

- **MTB MIXTURE:** Two vials: 2 x 110 µl
Amplification buffered solution containing a dNTPs mixture and the consensus primer at the appropriate concentrations.
- **MTB LIONPROBES:** Two vials: 2 x 50 µl
*A mixture of primer-probes (LIONPROBESTM) for the detection and amplification of *Mycobacterium tuberculosis* (5'TAMRA—FAM³) and the internal control (5'VIC—TAMRA³).
Avoid exposure to light.*
- **MgCl₂ (50 mM):** Two vials: 2 x 1.8 ml
Mix well before use.
- **Pfu DNA POLYMERASE (2.5 U/µl):** Two vials: 2 x 25 µl
Thermostable DNA Polymerase. Add to reaction mixtures shortly before introduction of vials in thermal cycler.
- **INTERNAL CONTROL:** Two vials: gel format
*Internal control of the amplification reaction in gel format, consisting of amplified DNA product, containing a sequence not related to *Mycobacterium tuberculosis* flanked by the consensus primer and the primer-probe (LIONPROBESTM) of the internal control. To be analysed simultaneously with all samples. **Regenerate each vial with 250 µl sterile bidistilled water.***
- **POS CTR (POSITIVE CONTROL):** Two vials: gel format
*Non-infective positive control of *Mycobacterium tuberculosis* in gel format. The positive control consists of amplified DNA product, containing a generic sequence from the target bacteria flanked by the consensus primer and the primer-probe (LIONPROBESTM) for *Mycobacterium tuberculosis*. **Regenerate with 500 µl sterile bidistilled water.** Final concentration is **5x 10⁴ copies/µl.***

NOTE

Thaw and handle reagents on ice. Do not freeze/thaw repeatedly. In case of frequent use, we recommend the aliquoting of the vial contents.

¹ The sequence of the insert element IS6110 is specific for the bacteria belonging to *Mycobacterium tuberculosis* complex, but its copy number varies among the different strains.

4. INSTRUCTIONS FOR USE

A. Master Mix Preparation

Proceed to Reagent Preparation Area in a laminar flow cabinet. Protect fluorescent probe vial from light at all times. Use of reaction mixtures and vials in non-refrigerated conditions may cause a drastic decrease in sensitivity and quality of the obtained fluorescence curves. Therefore, keep the amplification vials refrigerated until their introduction in the thermal cycler. Be careful not to wet the amplification vials.

1.- Regenerate INTERNAL CONTROL vial with 250 µl sterile bidistilled water.

2.- Final reaction volume is 20 µl (Master Mix + purified DNA). Prepare the **Master Mix** following the table bellow (Table 1) in a 1.5 ml vial, according to the number of reactions to be performed. For each round of analysis include at least one negative control and the necessary standards for the calibration curve. BIOTOOLS recommends to test each sample per duplicate, considering the mean value the final result. To ensure sufficient volume for all reactions, calculate the Master Mix for several additional reactions.

Table 1. Master Mix Preparation

Number of reactions = n° of samples + n° of standards + n° negatives controls + n° additional reactions

Reagent	1 Reaction
MTB MIXTURE	4 µl
MTB LIONPROBES	1.9 µl
MgCl ₂	1.6 µl
Pfu DNA POLYMERASE	0.8 µl
INTERNAL CONTROL	0.5 µl
Sterile bidistilled water	1.2 µl

Mix the required volume of all reagents

3.- Aliquot **10 µl of Master Mix** in each amplification vial.

B. Sample and Standard Curve Preparation

Proceed to DNA Purification Area separate from other sources of DNA. Never introduce DNA from samples or positive controls in the laminar flow cabinet at the reagent preparation area. Amplification must start in the next 10 minutes after adding purified DNA from samples and controls to the amplification reaction mix.

4.- Add **50 -100 ng of purified DNA** from samples to each amplification vial (maximum sample DNA volume is 10 µl). If necessary complete up to 20 µl final reaction volume with sterile bidistilled water.

5.- Regenerate POS CTR vial with 500 µl sterile bidistilled water. For the calibration curve **make four serial dilutions of the POS CTR** vial following the instructions of Table 2. The dilutions should be prepared just before performing the amplification and in a serial way.

Table 2. Preparation of Dilution Series of the Positive Control (POS CTR)

DILUTION	PREPARATION	CONCENTRATION
D5	POS CTR VIAL*	5x 10 ⁴ copies/µl
D4	3 µl of D5 + 27 µ sterile bidistilled H ₂ O	5x 10 ³ copies/µl
D3	3 µl of D4 + 27 µ sterile bidistilled H ₂ O	5x 10 ² copies/µl
D2	3 µl of D3 + 27 µ sterile bidistilled H ₂ O	5x 10 ¹ copies/µl
D1	3 µl of D2 + 27 µ sterile bidistilled H ₂ O	5 copies/µl

*D5 corresponds to POS CTR vial regenerated with 500 µl sterile bidistilled water

NOTE

Resuspend each dilution thoroughly by pipetting prior to use for the next one. Do not miss any of the dilutions in order to obtain a good calibration curve.

6.-The **standards** for the calibration curve are prepared adding **10 µl of the corresponding dilution** to an amplification vial containing 10 µl of Master Mix (see Table 3).

Table 3. Preparation of the Standards for the Calibration Curve

STÁNDAR	PREPARATION	MASTER MIX	CONCENTRATION
STD MTB 5	10 µl de D5	10 µl	5x 10 ⁵ copies/reaction
STD MTB 4	10 µl de D4	10 µl	5x 10 ⁴ copies/reaction
STD MTB 3	10 µl de D3	10 µl	5x 10 ³ copies/reaction
STD MTB 2	10 µl de D2	10 µl	5x 10 ² copies/reaction
STD MTB 1	10 µl de D1	10 µl	5x 10 ¹ copies/reaction

7.- **Negative Controls** should be prepared with **10 µl of sterile bidistilled water** instead.

8.- Close amplification vials. Be careful not to leave traces of powder, dust, etc.

Proceed to Amplification Area

9.- Place plate in the Applied Biosystems® 7500 thermal cycler. Store the remainder of the Kit reagents at -15±8°C.

C. Programming the Applied Biosystems® 7500

NOTE

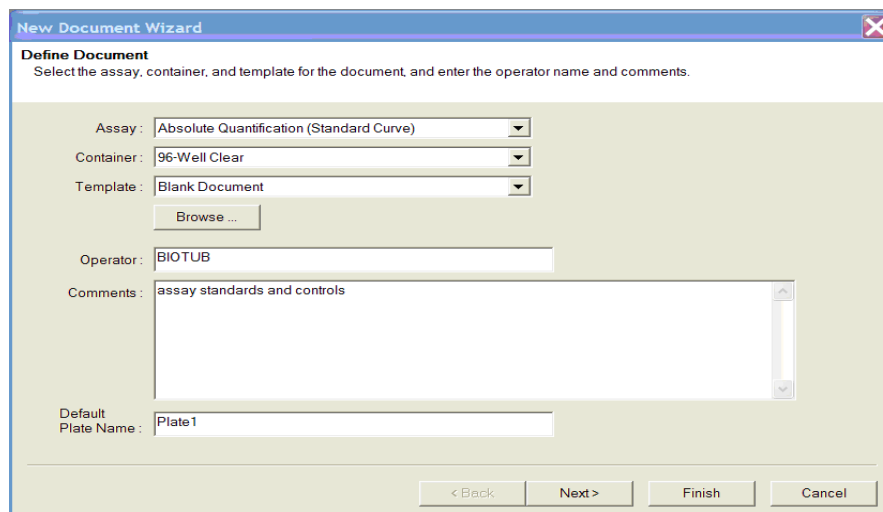
Fluorescence reading must be performed simultaneously with the following fluorescence channels:

FAM (*Mycobacterium tuberculosis*) **VIC** (Internal Control)

Use of this kit in other equipments has not been tested. For further information, contact our Technical Dept. (diagnostics@biotools.eu)

1.- Ensure that the Applied Biosystems® apparatus is switched on and is properly connected to the computer. For information on the installation of software and instrumentation setup, please consult the operator’s manual.

2.- Enter the Applied Systems 7300/7500 SDS Software and select File>New. In the “Assay” drop-down list of the “New Document Wizard” window, choose the **Absolute Quantitation (Standard Curve)** option. Accept the default settings for “Container” and “Template” (96-Well Clear and Blank Document respectively). Then click **Next**.



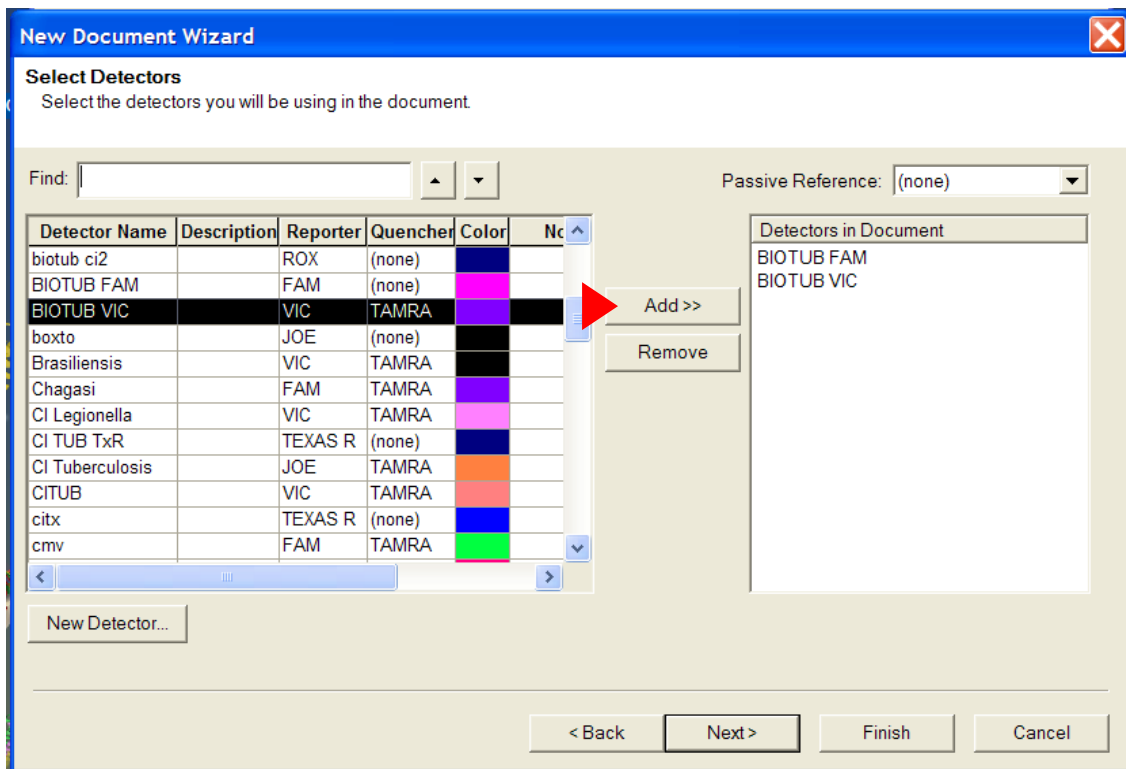
3.- Select the detectors that corresponds to the kit. If no detectors are listed in the “Detector Manager”, create detectors as explained:

- a) Select “New Detector”
- b) In the “New Detector” dialogue box enter a name for the detector, and select in the “Reporter Dye” the appropriate dye for each detector from the down list. The dyes that appear in the down list are those that have been previously entered in the “Detector Manager”. If the dyes used in this kit does not appear add the dye in the “Dye Manager” and then return to this step. See “Getting Started Guide: Absolute Quantification” for more information.

Detector Name	Reporter	Quencher
BIOTUB FAM	FAM	TAMRA
BIOTUB VIC	VIC	TAMRA

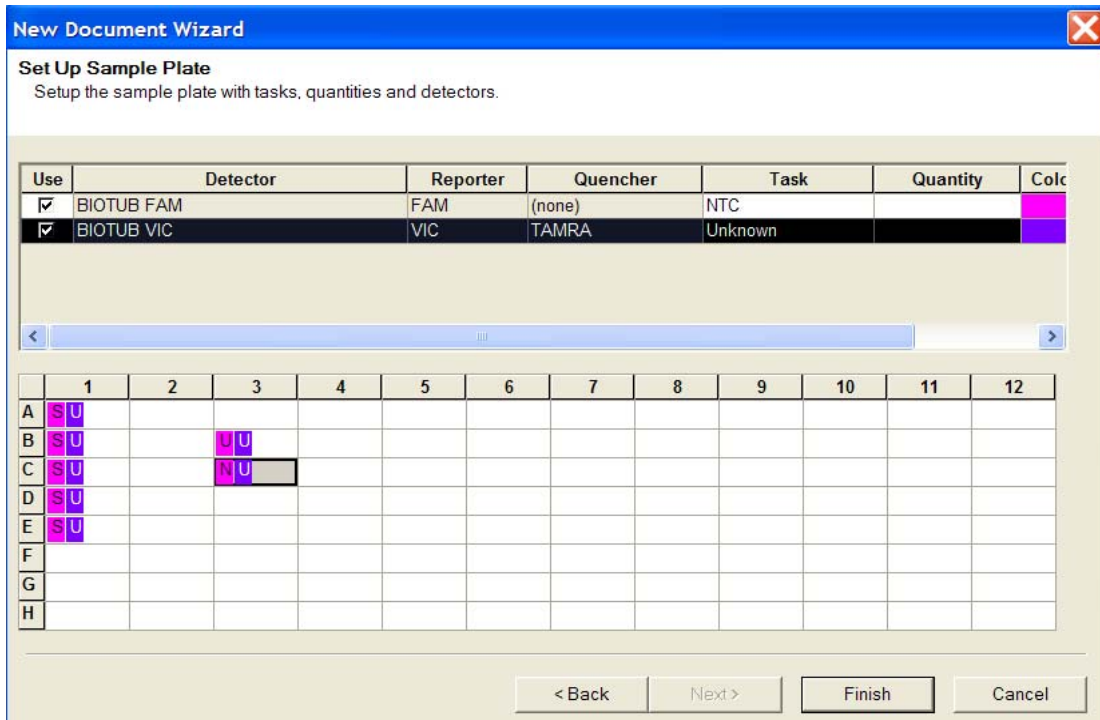
- c) Create the necessary detectors with “Create Another” option. Click OK to save the detector and return to the “New Document Wizard” window.

Once in the “New Document Wizard” window select the detectors to add to “Detectors in Document” dialogue box with the **Add** button. Deactivate detectors with the **Remove** button. Select **Next** to “Setup Sample Plate” window .

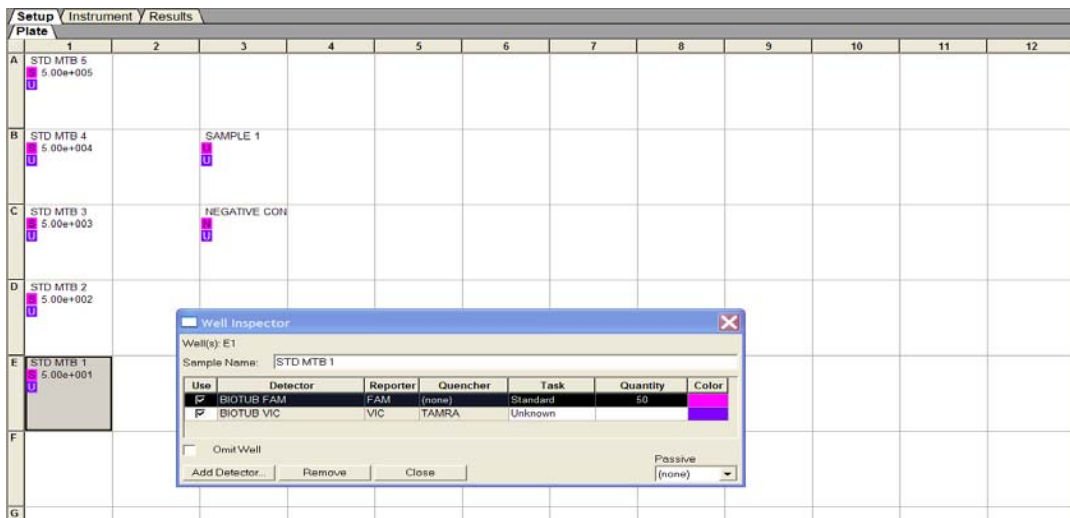


4.- Program the wells to be analysed according to the instructions and figure below:

- a) Select over a well (or group of wells, for replicates) with mouse.
- b) Under “Detector” select both BIOTUB VIC and BIOTUB FAM detectors for all analysed wells.
- c) Select “Task” to assign the detector task “Unknown”, “Standard” or “NTC” (blank) sample. Enter a quantity for wells that contain standards (see Table 3).
- d) Select **Finish** and proceed to the “Enter Sample Name” window.



5.- Next, the “Plate Setup” window will appear. Select a well, or group of wells, a “Well Inspector” dialogue box will appear. Enter the appropriate sample names for all the analysed wells.



6.- Program for according to the parameters in the table below. New values are entered in the Cycle Program Data Field using the instructions and sample screen below.

Table 4. Thermal Cycler Parameters

Stage	Temperature	Time (min:sec)	Repeat	Ramp Rate
1	95.0°C	4:00	1	100
2	97.0°C	0:05	10	100
	58.0°C	0:03		100
	68.0°C	0:40		100
3	97.0°C	0:05	35	100
	58.0°C	0:03		100
	68.0°C	0:34		100

- a) Double-Click on steps to Add or Delete step.
- b) Click over temperature and time parameters to change.
- c) Ensure that "Data Collection" is selected at the **stage 3 for the 68.0°C extension step**.
- d) Ensure "Sample Volume" is 20 µl.

Setup
Instrument
Results

Instrument Control

Start Estimated Time Remaining (hh:mm):

Stop

Disconnect Status:

Temperature

Sample: Heat Sink:

Cover: Block:

Cycle

Stage: Rep:

Time (mm:ss): Step:

State:

Thermal Cycler Protocol

Thermal Profile Auto Increment Ramp Rate

Stage 1

Reps:

95.0
4:00

Stage 2

Reps:

97.0
0:05

58.0
0:03

Stage 3

Reps:

97.0
0:05

68.0
0:40

58.0
0:03

68.0
0:34

Settings

Sample Volume (µL): 9600 Emulation

Data Collection: ▼

7.- Select "Start" to begin run.

5. INTERPRETATION OF RESULTS

The detection by Real Time of the amplified products is due to the hybridisation and hydrolysis of the primer-probes (LIONPROBES™). Thus, positive samples for *Mycobacterium tuberculosis* will render an increasing fluorescence curve in the reading channel for the fluorophore FAM. On the contrary, negative samples will not exhibit a fluorescence increase during the process, resulting in a basal fluorescence signal for the complete amplification run.

A) Fluorescence curves

Use of BIOTUB-QT kit generates different fluorescence curves depending on the reading channel:

- Reading in the **FAM Channel** represents amplification results for sequences from *Mycobacterium tuberculosis*. The cycle number at which an increase in the fluorescence rises above the background fluorescence (crossing point, Ct) is inversely proportional to the concentration of *Mycobacterium tuberculosis*.

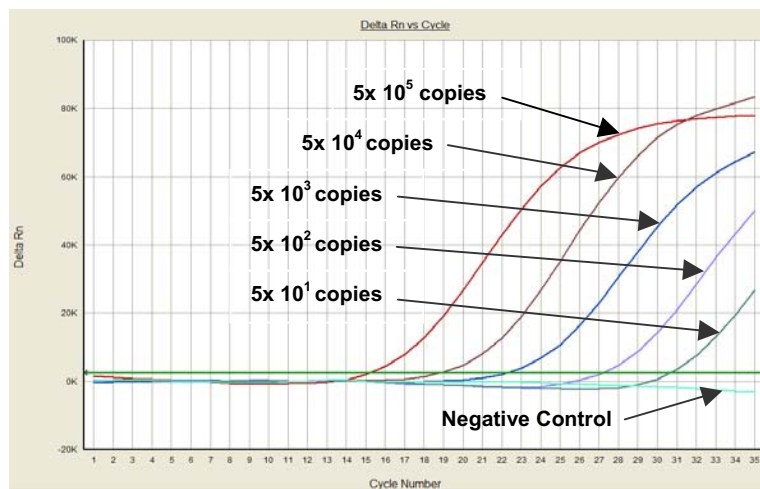


Figure 1. Fluorescence curves obtained in the FAM/ Channel using serial dilutions of the positive control provided by the Kit (STD MTB 5- 5×10^5 copies, STD MTB 4- 5×10^4 copies, STD MTB 3- 5×10^3 copies, STD MTB 2- 5×10^2 copies and STD MTB 1- 5×10^1 copies).

- Reading in the **VIC Channel** represents the results for the internal control amplification. As the concentration of internal control is the same in each amplification vial, Ct value should be similar for all samples (unknown samples, negative controls and standards). A negative result in the fluorescence curve in VIC Channel indicates an inhibition of the amplification reaction.

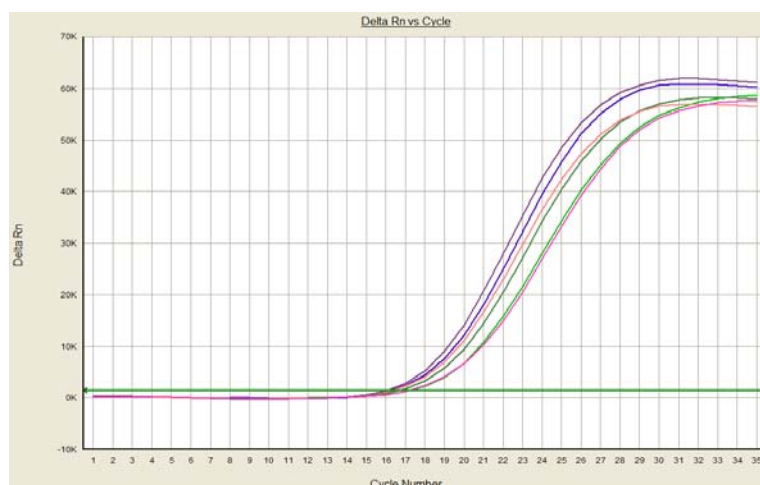


Figure 2. Fluorescent signal detected in the VIC Channel for the internal control amplification of the analysed samples shown in Figure 1.

In samples where an increase in the Ct value of the internal control is observed, some inhibition may have occurred. This will probably also affect detection of samples containing *Mycobacterium tuberculosis*. Therefore, it is recommended to purify the DNA from these samples, or even re-isolate it from the original sample.

Whereas the positive controls (standards) render a positive result in its reading channel, no fluorescence increase is observed in the negative control in any reading channel except for internal control channel. If the positive controls (standards) render a negative or no result then it is necessary to repeat the test.

NOTE

In samples with a high *Mycobacterium tuberculosis* concentration (Ct values may be low or very low in the FAM Channel), the amplification of internal control may be inhibited due to competitive effects. It is recommended, for these cases, to dilute the DNA sample so that concentration is in the acceptable range.

B) Standard Curve

The standard curve is made representing the logarithm of the concentration for each standard versus the crossing point (Ct). The graphical representation should render a straight line with a regression coefficient $r^2 \geq 0,9$. From this equation the initial concentration of a positive sample can be extrapolated. If the quality of the standard curve is poor, the results of the analysis must be discarded for quantification.

Fluorescence data used in quantification calculations correspond to the cycle where exponential phase is initiated. Fluorescence values obtained after the exponential phase, i.e. in the saturation phase (plateau) must not be used, as fluorescence data in this area are not proportional to the initial content of DNA.

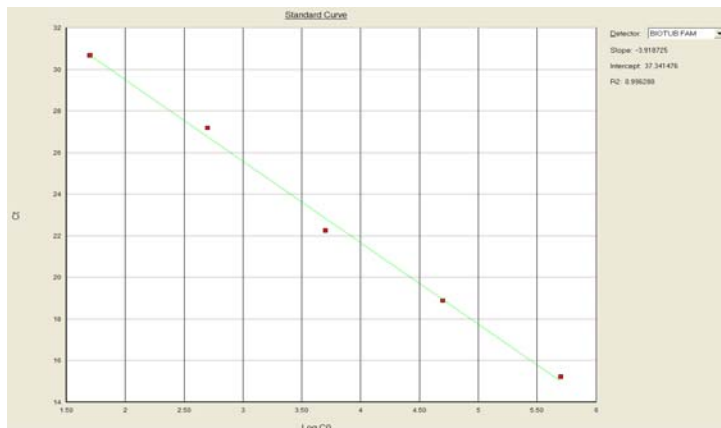


Figure 3. Standard curve obtained with the results of Figure 1.

A proper quantification of the analysed samples requires that the obtained crossing point is included in the standard curve. The extrapolation of the values above and under the ones belonging to the curve, results in purely theoretical values. The range of the standards covers a wide spectrum of concentrations, and therefore, this is unlikely to occur. However, if experimental results with these characteristics are found, it is recommended to repeat the experiment and dilute or concentrate, whatever necessary, the sample.

Determination of the Ct for each amplified sample can be performed following different methods depending on the software used. Usually, all the equipments have an automatic system that determines the Ct, following the second derivative method or modulating the fluorescence basal (threshold) level. Also, some equipment allow the manual modulation of fluorescence basal level. In this case, it must be taken into account that fluorescence data used in quantification calculations correspond to the cycle where exponential phase is initiated, or its inflexion point.

In order to determine the appropriate system for analysis of results read software instructions.

For laboratories requiring an external control, this must contain a defined number of copies of the target sequence of *Mycobacterium tuberculosis* to be within the range of the kit. The DNA must also be of sufficient quality for Real Time amplification analysis (see "Intended Use" section).

6. TROUBLESHOOTING

Problem	Cause	Recommendation
Standards give no results	Wrong channel has been chosen for detection	Check the channel chosen in the programming screen and change it
	Pipetting errors or omitted reagents	Check for missing reagents
	No fluorescence detection	Select acquiring at the stage 3 for the extension step at 68°C of Cycling 2
	Deteriorated positive control	Avoid repeated freezing and thawing. Follow "Storage Instructions"
Internal control gives no results in positive and negative controls	Wrong channel has been chosen for detection	Check the channel chosen in the programming screen and change it
	Pipetting errors or omitted reagents	Check for missing reagents
	No fluorescence detection	Select acquiring at the stage 3 for the extension step at 68°C of Cycling 2
	Deteriorated internal control	Avoid repeated freezing and thawing. Follow "Storage Instructions"
Fluorescence intensity of internal control varies in tested samples	Amplification inhibition due to DNA impurities	Re-purify samples Dilute the sample and re-do test
	Positive samples with high concentration of target bacteria inhibit detection of the internal control	Dilute the sample and re-do test
Negative control sample gives positive result	Contamination	Work in a clean bench with clean material. Exchange all reagents
Fluorescence intensity is too low	Low concentration or deterioration of dyes in the reaction mixture due to unsuitable storage conditions	Avoid repeated freezing and thawing Protect detection probes from light Follow "Storage Instructions"
Tested samples showed no Real Time amplification results whereas bands appeared in agarose gel	Fluorescence inhibition in tested samples. Storage and transport medium may contain inhibitors of Real Time detection e.g. NaN ₃	Follow "Storage Instructions"

7. MATERIALS REQUIRED BUT NOT PROVIDED

NOTE

For all equipment, regular maintenance and calibration is necessary. Follow manufacturer's instructions, and check working parameters regularly, especially thermal cyclers and pipettes. Maintenance and calibration of instruments allows their correct functioning, and helps to detect problems that may render an incorrect analysis result.

Pre-amplification Area (DNA Purification and Reagent Preparation Areas)

- Equipment, reagents and disposable material necessary for DNA purification (depending on the method, follow manufacturer's instructions)
- Laminar flow cabinet
- Timer
- Automatic pipettes² (10, 20 and 200 µl), filter or positive displacement tips, RNase-free³
- Disposable examination gloves, powder-free
- Sterile bidistilled water⁴ or equivalent

² Precision of automatic pipettes must be in the range of 3 % of the indicated volume. If necessary, calibrate and check regularly, following manufacturer's instructions. It is recommended to use RNase-free filter tips and positive displacement tips, in order to avoid cross contamination between samples and amplicons.

³ It is recommended to use different sets of pipettes for each reaction step (pre-amplification, amplification), in order to avoid contaminations that may render false positive results.

- Screw cap polypropylene tubes, 1.5 ml capacity, non siliconised, conical, sterile, RNase-free. It is recommended to use screw cap tubes, in order to avoid the potential contamination of samples and controls
- Racks for 1.5 ml vials
- Containers for disposal of potentially-infectious material
- Disposable filter paper for working surface, cleaning paper for accidental spills
- Termini-DNA-Tor⁵ or equivalent, in order to remove DNA from working surfaces

Amplification Area

- Real Time thermal cycler: AB 7500
- Laminar flow cabinet
- Racks for reaction vials
- Real Time amplification vials (as per manufacturer's requirements)
- Sterile bidistilled water or equivalent
- Automatic pipettes (10, 20 and 200 µl), filter or positive displacement tips, RNase-free
- Disposable examination gloves, powder-free
- Containers for disposal of potentially-infectious material
- Disposable filter paper for working surface, cleaning paper for accidental spills
- Termini-DNA-Tor or equivalent, in order to remove DNA from working surfaces

8. WARNINGS AND PRECAUTIONS

Following is a list of warning and precautions. For further information, please refer to the Material Safety Data Sheet (MSDS), available in our webpage (www.biotoools.eu), or by request to our Technical Dept. (diagnostics@biotoools.eu).

- In vitro* Diagnostic Medical Device.
- This test must be used with samples collected, handled and stored as indicated in the corresponding chapter. Efficiency of the test in other samples has not been tested.
- The kit detects DNA from *Mycobacterium tuberculosis*, and does not detect other bacteria in general, or belonging to the *Mycobacterium* genus in particular (except *M. tuberculosis* complex). The kit detects and quantifies the presence of a DNA region from the insert element IS6110 of *Mycobacterium tuberculosis* complex. The sequence of the insert element IS6110 is specific, but its copy number varies among the different strains. The kit does not differentiate between *Mycobacterium* species, and is not intended for resistance mutants detection.
- The kit detects the *Mycobacterium tuberculosis* complex (including *M. bovis*), and does not detect other *Mycobacterium* species (absence of cross-reactions with *M. avium-intracellulare* complex, *M. gordonae*, *M. xenopi*, *M. chelonae* complex, *M. kasasii* and *M. fortuitum* complex has been tested). No tests at experimental level have been performed with the following species: *M. africanum*, *M. canetti*, *M. microti*, *M. pinnipedii* and *M. pseudonotsii*, though efficiency of the reaction at theoretical level has been ascertained. Specificity tests have been performed with 500 ng of DNA from pure culture of the tested bacteria in absence of internal control, in order to avoid hypothetical inhibition effects due to competence between the amplification reactions. In all cases, it was observed that specificity is given by the primer sequence.
- The kit is based on the use of highly conserved regions (*IS6110*), where no mutations associated to resistance have been described up to date to our knowledge. However, the possibility remains that new mutants may appear in this region, and therefore, efficiency of the kit would be affected.
- Handle all samples and discarded material as infectious or potentially infectious.
- Use powder-free examination gloves while handling reagents or samples, as well as lab coat. Wash hands thoroughly after performing the test.
- Open and close reagent vials carefully. Observe temperature and light exposure instructions. After use, close vials and store at indicated temperatures.
- All materials used with the kit, including reagents and samples, must be discarded as to inactivate all possible infectious agents.
 - Solids:** autoclave.
 - Liquids:** add sodium hypochloride⁶ at a final concentration of 1 %, and incubate 30 minutes at room temperature before discarding any material.
- Spills: wash spills with a 5 % solution of sodium hypochloride. Cover surface with absorbent material, saturated with a 5 % solution of sodium hypochloride. Let at least for 10 minutes. In order to avoid

⁴ Available in Biotools' catalogue (Cat. No. 20.033).

⁵ Available in Biotools' catalogue (Cat. No. 22.001/2).

⁶ Commercial bleach usually contains sodium hypochloride at a concentration of 5 %. Bleach can be used, after performing the necessary calculations in order to achieve the indicated concentration.

- fume exposure, a plastic or glass cover can be used. All materials used for washing spills must be treated as infectious or potentially infectious material.
- K. Do not use product after expiry or best before date.
 - L. Kit components have been tested as a whole. **Do not interchange components** with other kits, or components from different lots.
 - M. Nucleic acids are very sensitive to degradation by nucleases. Nucleases are present in human skin and surfaces that have been in contact with humans. Use powder-free examination gloves throughout the whole process.
 - N. Extreme care must be taken when aliquoting the different volumes in each reaction step. Mix well after addition of each reagent, unless otherwise noted. Read instructions for use of automatic pipettes.
 - O. Do not pipette by mouth.
 - P. Packaging material included with the kit is resistant to the indicated storage conditions. Storage at different conditions can cause breakage of the material, and possible contamination of kit contents.
 - Q. Plastic material included with the kit is resistant in the normal conditions of use. Use of plastic material in extreme conditions may cause its breakage, and therefore, impossibility to use the kit.
 - R. Kit reagents, once used, must be discarded. Reagents cannot be reused once they have been used for the analysis of clinical samples, as this may cause false positive or false negative results.
 - S. Laboratory workflow must be unidirectional, from pre-amplification area to amplification area. Specific equipment for each working area must be used, in order to avoid cross contaminations. Equipment used for amplification must remain in this area at all times. Wash with Termi-DNA-Tor and cover working surfaces with suitable paper.

9. STORAGE AND HANDLING INSTRUCTIONS

1. After reception, store the different reagents at the indicated temperatures ($-15\pm 8^{\circ}\text{C}$). INTERNAL CONTROL and POS CTR vials (without being regenerated with sterile bidistilled water) can be stored either at $2-8^{\circ}\text{C}$ or $-15\pm 8^{\circ}\text{C}$, once regenerated store them at $-15\pm 8^{\circ}\text{C}$. Use non frost-free freezers. Also, for frequent use (more than 1 time a week), aliquot the contents of the reagent vials in different tubes, in order to avoid repeated freeze/thaw cycles.
2. Do not use the kit after expiry date. The closed kit is stable until the indicated date, if storage instructions are correctly followed. Do not mix reagents from other kits and/or other lots. If trace amounts of reagents remain, they must be discarded.

10. SAMPLE COLLECTION, TRANSPORT AND STORAGE

The different clinical samples recommended for use with the BIOTUB-QT kit are indicated at the 'Intended Use' chapter. Samples taken using other methods or transported by other means have not been tested for use with the kit.

Sputum samples

Samples should be stored at temperatures of $2-8^{\circ}\text{C}$, or freezing; and sent by express courier (maximum 24 hours) to the laboratory. These may be stored in the laboratory at $2-8^{\circ}\text{C}$ if analysed within one week. Prolonged storage (greater than one week) must be at $-15\pm 8^{\circ}\text{C}$. Samples must be kept away from heat sources protected from environmental humidity. Bacterial growth must be avoided and DNA integrity must be maintained. Results from sputum samples may vary depending on their quality and degree of conservation.

Lung and skin biopsies

Fresh biopsies of up to 5 mm diameter must be used. Biopsy must be immediately placed in a preservative medium and stored at $-15\pm 8^{\circ}\text{C}$. Biopsies can be shipped by express courier (24 hours maximum) at a maximum temperature of 25°C , and stored at $-15\pm 8^{\circ}\text{C}$ in the laboratory until performance of the test. Biopsies with a diameter under 2 mm must not be used.

Use of paraffin-embedded lung tissues is possible provided that tissue fixation method do not degrade DNA and purification of DNA is performed with methods specific for this kind of sample. For informative purposes, a protocol for purification of DNA from paraffin-embedded tissues is provided in our webpage (www.biotoools.eu/eng/products/paraffin.pdf).

In order to avoid accidents or accidental opening of the sample container, it is recommended to seal its closure with Parafilm® or equivalent before freezing.

Sample shipment must comply with local, national and international regulations for transport of etiological agents.

11. PROCEDURAL PRECAUTIONS

1. Laboratory workflow must be unidirectional, from pre-amplification area to amplification area. Pre-amplification tasks must be initiated with the preparation of the reagents and sample purification. Equipments, materials and reagents must be dedicated and they must not be used for other activities or be transferred from one to another area. Gloves must be worn in each area, and must be discarded before proceeding to the next area. Equipments and materials used for setting-up of reactions must not be used for other activities, or for pipetting or processing amplified DNA or other DNA sources.
2. As with any analytical procedure, it is fundamental to use a good laboratory practice to obtain good results with this technique. Due to the high analytical sensitivity of the test, extreme care must be taken in order to keep the purity of all kit reagents and all reaction mixes. All reagents must be carefully checked in order to ascertain their purity. Discard all suspect reagents.

12. PROCEDURAL LIMITATIONS

1. *In vitro* Diagnostic Medical Device.
2. *Instructions* must be followed in order to obtain correct results. Should the user have any questions, please contact our Technical Dept. (diagnostics@biotools.eu).
3. This test has been validated for use with the reagents provided by the kit. The use of other amplification methods, or the use of equipment not fulfilling the specifications, may render equivocal results. User is responsible for validating the modifications for this test, in any of the indicated parameters.
4. This test has been validated with samples collected and shipped as per the "Sample Collection" section. Any modification has not been validated, and therefore, obtained results may not be correct.
5. Detection of bacterial DNA depends on the number of bacteria present in the sample, and can be affected by sample collection methods, patient-related factors (e.g. age, symptoms), or for infection stage and sample size.
6. False negative results may be obtained due to polymerase inhibition. It is recommended to perform control reactions to distinguish between inhibition and true negatives. This is achieved by the Amplification Control included in the kit.
7. Cross contamination between samples and exogenous DNA can only be avoided by following good laboratory practice. Instructions in this document must be strictly followed.
8. Use of this product is limited to qualified professional personnel, experienced in DNA purification and DNA amplification techniques.
9. It is important to pipet the indicated quantities, and mix well after each reagent addition. Check pipettes regularly.

13. PERFORMANCE CHARACTERISTICS

Analytical specificity

At a theoretical level, the sequences of the primers used in the kit were compared to the databases (GenBank, EMBL), and no significant coincidences that could potentially result in amplification products were found. Study was mainly focused on the analysis of sequences of human origin, as well as sequences from pathogens that can be potentially present in the samples used for detection of tuberculosis.

Also, specificity of the reaction was empirically checked with the following bacterial species:

<i>M. avium-intracelulare</i> complex	<i>M. gordonae</i>	<i>M. xenopi</i>
<i>M. chelonae</i> complex	<i>M. kansasii</i>	<i>M. fortuitum</i> complex

In all cases, detection/amplification reaction using the primer-probes included in the kit rendered negative results, guaranteeing the specificity of the reaction in a wide variety of bacterial species.

Analytical sensitivity

Analytic sensitivity of the amplification-detection reaction for *Mycobacterium tuberculosis* was assessed using serial dilutions 1/10 of the Positive Control (POS CTR) included in the kit, containing a generic sequence from *Mycobacterium tuberculosis*. Also, tests were performed on a serial dilutions of a plasmid containing a cloned sequence from *Mycobacterium bovis*.









Amplification assays showed a consistent sensitivity of around 50 copies per reaction for both controls.

14. WARRANTY

Products are guaranteed to conform to the quality and content indicated on each vial and external labels during their shelf life. BIOTOOLS obligation and purchaser's rights under this warranty are limited to the replacement by BIOTOOLS of any product that is shown defective in fabrication, and that must be returned to BIOTOOLS, freight prepaid, or at BIOTOOLS' option, replacement of the purchasing price. Any complaint on damaged goods during transport must be directed to the handling or transport agent.

In vitro Diagnostic Medical Device, as per Directive 98/79/EC specifications. This product must be used by qualified professionals only. It is the responsibility of the user to ascertain that a given product is adequate for a given application. Any product not fulfilling the specifications included in the product sheet will be replaced. This warranty limits our responsibility to the replacement of the product. No other warranties, of any kind, express or implied, including, without limitation, implicit warranties of commercialisation ability or adequacy for a given purpose, are provided by BIOTOOLS. BIOTOOLS will not be held responsible for any direct, indirect, consequential or incidental damage resulting of the use, misuses, results of the use or inability to use any product.

Explanation of symbols:

Symbol	Meaning
	Batch code
	Use by
	Caution, consult accompanying documents
	Manufacturer
	Catalogue number
	Contains sufficient for <n> tests
	<i>In vitro</i> Diagnostic Medical Device
	Temperature Limitation

Manufactured by:

BIOTOOLS, Biotechnological & Medical Laboratories, S.A. have been evaluated and certified to accomplish ISO 9001:2000 requirements for the following activities: Research and development of biotechnology products and manufacture of biotechnology and *in vitro* products. Valle de Tobalina – 52 – Nave 39, 28021 Madrid – Spain

BIOTOOLS® is a registered mark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A.

BIOTUB-QT™ is a trademark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A.

SPEEDTOOLS MYCOBACTERIA/DNA/TISSUE DNA EXTRACTION KIT™ is a trademark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A.

Termi-DNA-Tor™ is a trademark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A.

LIONPROBES™ is a trademark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A.

Parafilm® is a registered trademark of Pechiney Plastic Packaging, Inc.

VIC, TAMRA™ and FAM™ are trademarks of Applera Corporation

Applied Biosystems® is a registered trademark of Applera Corp.



© 2008 BIOTOOLS, Biotechnological & Medical Laboratories, S.A. All rights reserved

Vertrieb:

LOXO GMBH, IMMUNBIOLOGIE - BIOCHEMIE POSTFACH 11 30
 69215 DOSSENHEIM
 Telefon 06221 868023 Fax 06221 8680255
 E-Mail: info@loxo.de Internet: www.loxo.de