MTB resistant OneStep PCR kit Multiplex - Real Time DNA

BioinGentech Biotechnology

50 / 100 /150 tests (Ready to use kit)

MTB resistant PCR kit Multiplex DNA Real Time PCR Kit is a screening assay for a rapid and accurate detection of MTB resistant OneStep PCR kit Multiplex.

Principles of the test:

One Step Bioingentech® PCR Kits provide components for "onestep" real time PCR detection in a convenient format that is compatible with both rapid and standard qPCR cycling conditions.
The One Universal qPCR DNA Master Mix include Bioingentech® all reagents for an optimized qPCR.
Specific primers and probe for MTB resistant OneStep PCR kit Multiplex are provided in the kit and can be detected through your Real Time thermal cycler by the 5' nuclease PCR detection method. During PCR amplification, forward and reverse primers hybridize to the MTB resistant OneStep PCR kit Multiplex target genomic DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5-reporter:
Rifampicin: rpoB gene in TAMRA and 3-quencher which can be detected through orange channel.
Isionazide: katG gene in CY5 and 3-quencher which can be detected through red channel.
Ethambutol: embB gene in ROX and 3-quencher which can be detected through orange channel.
Specific MTB gene in FAM and 3-quencher which can be detected through green channel.
To confirm extraction of a valid biological template, internal control primers and probe mix are included, which consist of a DNA probe labeled with a 5-reporter VIC and a 3-quencher which hybridize inside a specific housekeeping endogenous target gene. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. As a result, a fluorescence increase can be detected on a range of Real Time thermal cyclers through yellow channel.

Our kits also include Positive and Negative Controls which are detailed in the FAQ section.

Principle and use:

This amplification kit has been manufactured by Bioingentech Ltd. to detect MTB resistant OneStep PCR kit Multiplex via Real Time PCR. This allows absolute quantification or a qualitative assay.
Real Time PCR is based on fluorogenic dyes. Ct values between 12 - 36 should be taken as positive. Values between 36 - 40 Ct should be taken as marginal positive.
Ct values above 40 must be considered as negative (for more details see Table 5).

This kit needs DNA as a template, which can be isolated from blood, serum, faeces, respiratory fluid, cerebrospinal fluid, digestive system, tissues, egg yolk, milk, swabs, bacterial cultures, cell lines, among others.

We discarded the use of affinity columns due to reports of purification problems caused by lipids present in the biological samples, which quickly clog the column, decreasing its performance.

Table 1. Kit Components:

Reactions Tubes	50 test	100 test	150 test
Universal qPCR Master Mix	(1 vial)	(1 vial)	(1 vial)
Primer, Probes and Internal Control Universal Mix	(1 vial)	(1 vial)	(1 vial)
MTB resistant PCR kit Multiplex Positive Control	(1 vial)	(1 vial)	(1 vial)
MTB resistant PCR kit Multiplex Negative Control	(1 vial)	(1 vial)	(1 vial)
PCR grade Water	(1 vial)	(1 vial)	(1 vial)

Table 2. Instrument Compatibility:

* ABI 7300	* LightCycler 480
* ABI 7500FAST	* Mastercycler® ep realplex
* ABI 7900	* Mx3000P QPCR System
* AB Step One	* Mx3005P QPCR System
* AB Step One Plus	* RotorGene 3000
* Agilent Mx3005P	* RotorGene 6000
* CFX96 & CFX384	* RotorGeneQ
* ExiCyclerTM 96	* QuantGene 9600
* iQ5 & MyiQ Cycler	* QuantStudio™ 5
* Illumina Eco	* SLAN® Real-Time PCR
* LightCycler Nano	* Smartcycles II
* LightCycler 2.0	* Applied 7300 and 7500

Procedure:

Please read through the entire procedure before starting.

Before Starting

- Pulse-spin each tube in a centrifuge before opening.
- Homogenize the solutions for 5 seconds prior to pipetting
- Use different tips in order to avoid cross contamination.
- Use only sterile, RNAse, DNAase, and pyrogen-free tips.

Step 1

Prepare a Master mix according to the following reaction table.

Table 3. Reaction components for PCR

Reaction Tubes	Sample and Internal Control	Positive Control	Negative Control
Universal qPCR Master Mix	7,5 µL	7,5 µL	7,5 µL
Primer, Probes and Internal Control Universal Mix	0,6 µL	0,6 µL	0,6 µL
PCR grade Water	3,4 µL	3,4 µL	3,4 µL
DNA Sample	3,5 µL		
Positive Control		3,5 µL	
Negative Control			3,5 µL
Total Volume	15 µL	15 µL	15 µL

Step 2

Place the tubes in a thermal cycler and perform One Step qPCR according to the program outlined in Table 4.

Table 4. Recommended PCR Cycling table

Cycles	Steps	Time	Temp.(°C)
1 Cycle	Denaturation	2 min	95°C
40 Cycles	Denaturation	30 seg	95°C
	Annealing	30 seg	60°C
	Extension	30 seg	72°C
	Final Step	∞	4°C

Note: Measure the fluorescence at the end of the Extension Step.

Interpretation of the test

1) Qualitative analysis:

Ct (Threshold cycle) value of each sample can be interpreted as follows.

Table 5. Ct value result

Ct value	Result
0 - 11	**Negative**
12 - 36	Positive
36 - 40	Marginal Positive
> 40	**Negative**

* Ct values over 40 are considered Negative. If the Ct value is in the 12 - 36 range, it must be considered as Positive. This is depending of the sample initial concentration use d for each reaction. Note that the sample real concentration could be altered by the sample purity when it is quantified.
* For more technical information, request the quality control for each kit by writing to our email kitpcr@bioingentech.com

2) Quantitative analysis:

Table 6. Preparation of standard curve dilution series. MTB resistant PCR kit Multiplex positive control:

Average Positive Control Concentration	
MTB resistant PCR kit Multiplex	See Quality Control

Standard curve	Preparation series a fresh dilution	Concentration	Copy Number
Tube N°1:	2uL MTB resistant PCR kit Multiplex Positive Control (0,1 ng/µL) +18 µL de PCR grade Water	See quality control	See quality control
Tube N°2:	2uL Tube N°1 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°3:	2uL Tube N°2 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°4:	2uL Tube N°3 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°5:	2uL Tube N°4 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°6:	2uL Tube N°5 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°7:	2uL Tube N°6 + 18 µL de PCR grade Water	See quality control	See quality control

Important Note: Don't forget Homogenize the tubes.

* A Quality Control report will be sent for each purchase.
* For reaction mix you must use the Universal qPCR Master Mix included.
* If you wantto obtain less DNA copies, include a new dilution tube (Tube N° 8). Final DNA copy number will depend of the DNA concentration (check the Quality Control Report).

Table 7. Standard curve set up

	Tube A	Tube B	Tube C	Tube D	Tube E	Tube F	Tube G
Universal qPCR Master Mix	7,5 µL	7,5 µL	7,5 µL	7,5 µL	7,5 µL	7,5 µL	7,5 µL
Primer, Probes and Internal Control Mix	0,6 µL	0,6 µL	0,6 µL	0,6 µL	0,6 µL	0,6 µL	0,6 µL
PCR grade Water	3,4 µL	3,4 µL	3,4 µL	3,4 µL	3,4 µL	3,4 µL	3,4 µL
Tube N° 1 (Positive Control)	3,5 µL						
Tube N° 2		3,5 µL					
Tube N° 3			3,5 µL				
Tube N° 4				3,5 µL			
Tube N° 5					3,5 µL		
Tube N° 6						3,5 µL	
Tube N° 7							3,5 µL
Total Volume	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL

2.1.- Assess the Ct value when the amplification curve of Standard tubes 1, 2, 3, 4, 5, 6 passes the threshold line. Four tubes are sufficient for a standard curve (tubes 1 to 4).
2.2.- Calculate the quantitative value in order to compare the Ct value of the unknown samples with the standard curve.
2.3.- When visualizing the result for the Positive Control in the Real Time thermal cycler, there will be only one amplification curve for the target gene. It will not have an amplification curve for the Internal Control.

3) Test validation:

3.1.- Each Ct value standard should be as follows.
Standard 1 < Standard 2 < Standard 3 < Standard 4 < Standard 5 < Standard 6.

3.2.- R-value of standard curve should be 0.900 - 0.999.
R-value represent how well the experimental data fits the regression line. A significant difference in observed in Ct values between replicates will lower the R-value.

3.3.- The standard curve slope result should be negative.

3.4.- The desired amplification efficiencies vary from 90% to 110%. The theoretical maximum of 100% indicates that the polymerase enzyme is functioning at its maximum capacity. Low reaction efficiencies may be caused by poor primer design or by suboptimal reaction conditions. Reaction efficiencies >110 may indicate pipetting error in your serial dilutions or coamplification of nonspecific products, such as primer-dimers.

Visual explanation FAQ:

1.-Positive control:

The Positive control Rifampicin: rpoB gene assay uses a TAMRA dye and should be detected through the ORANGE channel of your Real Time thermal cycler (see Table 8).
The Positive control Isionazide: katG gene assay uses a CY5 dye and should be detected through the red channel of your Real Time thermal cycler (see Table 8).
The Positive control Ethambutol: embB gene assay uses a ROX dye and should be detected through the ORANGE channel of your Real Time thermal cycler (see Table 8).
The Positive control Specific MTB gene assay uses a FAM dye and should be detected through the GREEN channel of your Real Time thermal cycler (see Table 8).
For copy number determination and as a positive control for the PCR setup, the kit contains a plasmidial positive control template. This can be used to generate a standard curve of MTB resistant OneStep PCR kit Multiplex copy number / Ct value.
Alternatively, the positive control can be used at a single dilution if full quantitative analysis of the sample is not required. Each time the kit is used, at least one positive control reaction must be included in the run. We recommend running a positive control for each 12 samples.
A positive result indicates that the primer and probes for the target gene worked properly in that particular experimental scenario. If a negative result is obtained the test results should be invalid and must be repeated (see Table 10). Seal all other samples and negative controls before pipetting the positive control into its well.
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2.-Internal Control:

The internal control is included in the Primer, Probes and Internal Control Mix alongside the target pathogen detection. The internal control assay uses a VIC dye and should be detected through the Yellow channel of your Real Time PCR instrument and should result in a Ct value of 28 (+/-5), depending on the level of sample dilution and concentration. Therefore, a positive result through the yellow channel indicates that PCR conditions are suitable for detection of the target pathogen gene.
If a negative result is obtained through the green channel, the results should be analyzed following the Table 10 data.

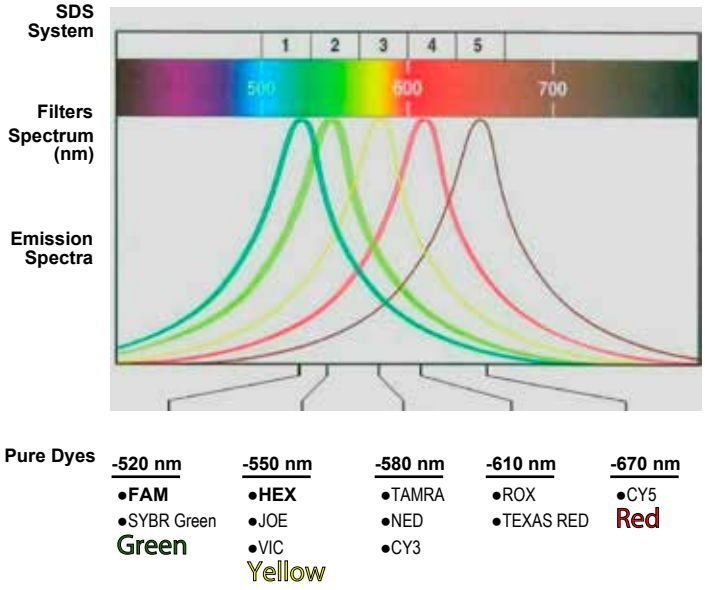
3.-Negative control:

To confirm absence of contamination, a Negative control reaction should be included every time the kit is used.
We recommend runing a Negative control for each 12 samples. In this instance, the PCR grade water should be used in place of template. A negative result indicates that the reagents are not contaminated.
If a positive result and a Ct value less than 36 is obtained, the results should be analyzed and checked if a correct amplification curve was obtained. If you obtain a clear amplification curve, you should consider repeating your assay due to sample contamination (see Table 10).

* Remember: We recomment running a Positive control and Negative control for each 12 samples. For reaction mix you must use Universal qPCR Master Mix.
* In order to setup your software to read the probes, see Table 8 and run the following channels:

Table 8. Fluorogenic probes, Channels and Dyes

Channel	Source	Detector	Dyes
Green	470 (nm)	520 (nm)	**FAM**, Sybr green1, Fluorescein, Eva green, Alerxa flour 488
Yellow	530 (nm)	550 (nm)	Joe, Vic, **HEX**, Tet, Cal Fluorgold 540, YaKima Yellow
Orange	585 (nm)	610 (nm)	Rox, Cal Fluor Red 610, Cy3.5, Texas Red, Alexa Fluor 568, TAMRA
Red	625 (nm)	660 (nm)	Cy5, Quasar 670, Lightcycler, Red 640, Alexa Fluor 633
Crimson	680 (nm)	710 (nm)	Quasar 705, Lightcycler Red 705, Alexa Fluor 680



Important Note:

Probes for the sample and controls mentioned in this manual are just referential and the purchased kits might have different ones. This information will be detailed in your Certificate of Analysis upon purchasing one of our PCR Kits. We can develop special requests for other pathogens or multiplex detection according to the client specifications.

We strongly recommend to not use or combine our products with reagents from another kits or unknown procedence. We cannot assure good results if incompatibility problems occur.

Temperature

All our reagents are made through protein engineering and are stable at room temperature, the label temperature is just a recommendation after the product is open.

Table 9. Store Temperature Kits

	Storage temperature	Label temperature
	Shipping temperature	At room temperature

Table 10. Interpretation of Results

Sample	I.C	N.C	P.C	Result
+	+	-	+	POSITIVE
+	-	-	+	POSITIVE
+	+	+	+	Check Ct and Consider repeat assay*
+	-	+	+	Check Ct and Consider repeat assay*
+	-	-	-	NEGATIVE
+	+	-	-	NEGATIVE
-	+/-	+/-	+/-	NEGATIVE

* Sometimes amplification curves for Negative or Internal control with Ct < 30 can be seen, however it might not be a Positive result. If the amplification curve is not sigmoid, you should consider it as a Negative result.

Table 11. Products Specifications

Technology	5' nuclease probe based real time PCR assay
Type of nucleic acid Kit	DNA
Kit storage	Shipped at room temperature, the label temperature is just a recommendation after the product is open.
Detection Limit	See Quality Control file. **Request it!**
Sensitivity & Specify	Ct value between 12 – 36 should be taken positive. Value between 36-40 Ct should be taken as marginal positive. Ct values above 40 must be considered as negative.
Controls included	Internal control, Positive control and Negative control included.
Channels	FAM Green channel ROX Orange chanel HEX Yellow channel CY5 Red channel TAMRA Orange chanel VIC Yellow channel detect Internal Control.