genetic PCR solutions™

30 years' experience, packed in microtubes

MTplex qPCR

F100 format

100 tests

Genetic detection of

***Mycobacterium tuberculosis* complex**

CE IVD

Sanitary License Number for the Operation of Sanitary Product Installations; 7722-PS

Edition E01 (01/2025)

NOTICES, DISCLAIMERS, AND TRADEMARKS

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DESCRIPTION

The **MTplex qPCR** comprises a series of specific targeted reagents, for human clinical diagnosis, designed for ***Mycobacterium tuberculosis* complex** detection by using qPCR. *Mycobacterium tuberculosis* complex refers to a set of variants belonging to *Mycobacterium tuberculosis* with the capacity to cause tuberculosis in humans and other animals. These are obligate pathogenic, aerobic bacteria that induce nodular lesions in the lungs, known as tubercles. Currently, this group includes the tuberculosis variants, *africanum*, *orygis*, *bovis*, *bovis* BCG, *caprae*, *microti*, *canettii*, *suricattae*, *pinnipedii*. Although the newly described species, *M. mungi*, has not yet been validated, it has been reported to contain the genetic element specific to this complex which has been used as target in the present test. Recently, all members of this complex have been considered part of the *M. tuberculosis* species and are being reclassified as variants of this species.

TARGET SPECIES AND NATURE OF THE SAMPLE

The assay is intended for use on human respiratory samples, specifically sputum and bronchial lavage samples.

PRINCIPLE OF THE METHOD

Polymerase chain reaction (PCR) allows the amplification of a target region from a DNA template by using specific oligonucleotides. In real-time PCR (qPCR), the accumulating amplified product can be detected at each cycle with fluorescent dyes. This increasing signal allows to achieve sensitive detection and quantification of pathogens.

KIT CONTENT

TargetSpecies qPCR-mix (AMBER TUBE), contains a dehydrated mixture of specific primers and probe. 100 reactions

Resuspension buffer (WHITE CAP), 130 µl

DNase/RNase free water (GREEN CAP), 1.5 ml

Internal Control qPCR-mix (AMBER TUBE), contains a dehydrated mixture of primers, probe and the DNA template to achieve a suitable internal control of PCR. 100 reactions

GPS™-mix (BLUE CAP), it is a 4X mastermix solution containing a polymerase, dNTPs, and buffer. 500 µl, 100 reactions

Positive Control (ORANGE CAP), dehydrated target copies for positive control.

Template buffer (BLACK CAP), exclusive for resuspension of the Positive Control, 150 µl

STORAGE CONDITIONS

All the components of **MTplex qPCR** are stable at room temperature for transport. Upon arrival, store at -15 to -25 °C if not used immediately. The product is stable for one year (see expiration date on the label).

For **Positive Control** (ORANGE CAP), once dissolved, we recommend storing **in a dedicated box** at -15 to -25 °C.

MATERIALS REQUIRED BUT NOT PROVIDED

- Nucleic acid isolation kit, appropriate purification method needs to be conducted to obtain pure DNA or RNA free of PCR inhibitors
- Certified DNase/RNase free water and low retention tubes to prepare the decimal dilution for quantification
- Micropipettes and sterile pipette tips with filters
- qPCR tubes, strips or plates
- Vortex mixer and spinner centrifuge
- Cooling block
- Real-time PCR device which must read on the FAM channel (target), and VIC/HEX/JOE channel (Internal Control)

ATTENTION NOTES

- For In Vitro Diagnostic (IVD).
- To avoid possible misuse, carefully read the handbook.
- A technologist with proper training in molecular biology is required for the correct operation of the kit.
- According to good laboratory practices, always wear a suitable lab coat, disposable gloves, and protective goggles.
- All the instruments used must been verified and calibrated according to the manufacturer's recommendations.
- Samples are potentially infectious materials, take appropriate measures for containment and follow the corresponding biosafety precautions.
- Any kind of sample can be analysed with this assay after appropriate nucleic acid extraction.

WARNINGS AND PRECAUTIONS

⚠ To prevent contamination of micropipettes, use sterile tips with filters.

⚠ Micropipettes used to dilute the Positive Control should not be used for other PCR reagents.

⚠ Extract, store and prepare positive materials (samples, positive controls and PCR products) in a separate laboratory environment.

⚠ To decrease the risk of contamination, we recommend that all pipetting be performed in a PCR clean environment. Ideally, this would be a designated PCR lab or PCR cabinet.

⚠ To avoid cross-contamination with the positive control, pipette it after closing reaction tubes with negative control and samples.

⚠ The workflow in the laboratory should be unidirectional, from clean preamplification area to the amplification area.

⚠ Keep components refrigerated in a cooling block.

⚠ Protect the primer/probe from prolonged exposure to light.

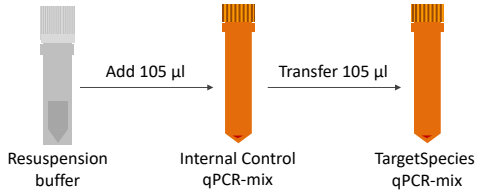
⚠ For dsRNA virus preincubate sample at 95 °C for 5 minutes.

⚠ Any incident related with the product, must be informed to GPS™ (see CONTACT INFORMATION).

RESUSPENSION PROTOCOLS

Resuspension of TargetSpecies qPCR-mix with Internal Control

For reconstitution of **Internal Control qPCR-mix** (AMBER TUBE) and **TargetSpecies qPCR-mix** (AMBER TUBE), pulse-spin tubes in a centrifuge to ensure that dehydrated product goes down before opening and will not be spilt. Add 105 µl of **Resuspension buffer** (WHITE CAP) to the **Internal Control qPCR-mix** (AMBER TUBE), vortex the tube thoroughly (or pipette the solution up and down on the sides of the tube), and harvest by pulse-spin in the centrifuge. Transfer the volume to the **TargetSpecies qPCR-mix** (AMBER TUBE), vortex the tube thoroughly and harvest by pulse-spin in the centrifuge. Use 1 µl of this suspension in PCR reactions with a final volume of 20 µl.



PREPARATION OF POSITIVE CONTROL

The sealed **Positive Control** (ORANGE CAP) contains a pure template and there is a significant contamination risk. To avoid contamination, please, follow recommendations of WARNINGS AND PRECAUTIONS section of this handbook. To prepare the **Positive Control** (ORANGE CAP) pulse-spin the tube, reconstitute with 120 µl of **Template buffer** (BLACK CAP), vortex thoroughly and pulse-spin.

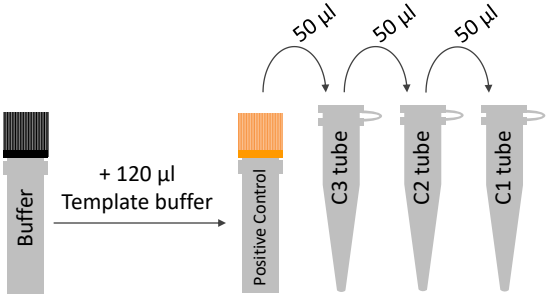


Pipette 5 µl of template into the well according to your plate set-up. The final volume in each qPCR reaction well is 20 µl.

PREPARATION OF A DECIMAL DILUTION

When quantification is required, the following dilutions can be prepared.

1) Pipette 450 µl of **DNAse/RNAse free water** (not provided) into three tubes and label as C3 to C1
2) Pulse-spin the **Positive Control** (ORANGE CAP), reconstitute with 120 µl of **Template buffer** (BLACK CAP), vortex thoroughly and pulse-spin
3) Pipette 50 µl of diluted **Positive Control** (ORANGE CAP), into tube C3
4) Vortex thoroughly and pulse-spin
5) Change tip and pipette 50 µl from tube C3 into tube C2
6) Vortex thoroughly and pulse-spin
7) Repeat steps 5 and 6 with the tubes C2 to C1 to complete serial dilution



Decimal dilution series	copies/µl	copies in 5 µl
Positive Control (ORANGE CAP)	2 x 10^3	10^4
C3 Tube	2 x 10^2	10^3
C2 Tube	2 x 10	10^2
C1 Tube	2	10

Pipette 5 µl of template into each well according to your plate set-up. The final volume in each qPCR reaction well is 20 µl.

PCR SET-UP PROTOCOL

It is advisable to prepare a reaction pre-mix in a nuclease-free tube on a cooling block, according to the following table. Prepare enough reaction mix for the desired number of reactions, for your samples and the positive/negative controls considered for your experiment.

Reagent	Volume
DNase/RNase free water (GREEN CAP)	9 µl
GPS™-mix (BLUE CAP)	5 µl
TargetSpecies qPCR-mix (AMBER TUBE)	1 µl
Reaction pre-mix volume	15 µl

Once prepared, pipette 15 µl of this reaction mix into each well or PCR tube according to your experimental platform.

Add 5 µl of the processed sample or, if the case of calibration curves/positive control, the corresponding diluted template to each PCR tube to reach a final PCR volume of 20 µl.

Reagent	Volume
Dispensed reaction pre-mix volume	15 µl
Processed sample (or DNase/RNase free water)[1]	5 µl
FINAL REACTION VOLUME	**20 µl**

1 In the case of negative control, add 5 µl of DNase/RNase free water (GREEN CAP) instead of sample (see Recommended Reaction Controls section).

AMPLIFICATION REGIME

Once all reactions mixes are ready and gently closed, vortex tubes thoroughly, harvest by pulse-spin in the centrifuge, and place plate or tubes in the block/rotor of the thermocycler programmed to run the cycling regime described on the table. Consider that the fluorogenic signal must be collected by using the FAM channel for the target and the HEX channel for the Internal Control. Probe includes a Dark Quencher which can be set up as any non-fluorescent quencher (NFQ) in the software. GPS™ reagents are compatible with all real-time PCR thermal cyclers. The use of a passive dye as ROX is not required.

Fast-Cycling	**Step**	Time	Temperature
	Activation	2 min	95 °C
40 Cycles	Denaturation	1 sec	95 °C
	Hybridization / Extension and data collection[1,2]	10 sec	**60 °C**

1 Fluorogenic signal must be collected during this step by using the appropriate channel.
2 For instruments with limitations on the time of the hybridization/extension step, the time can be increased up to 30 seconds.

RECOMMENDED REACTION CONTROLS

These qPCR reaction controls are recommended when considering the guidelines of UNE/EN ISO 17025. When setting-up your qPCR protocol, select the controls considered better suits your quality system.

Negative Control (Ctrl -): Add 5 µl of DNase/RNase free water (GREEN CAP) to 15 µl of reaction mix. Accordingly, this reaction should be negative. A positive result may be considered as a symptom of contamination in some reagents of reaction mix, making the test inconclusive. Reagents must be discarded.

Positive Control (Ctrl +): Prepare the **Positive Control** (ORANGE CAP) as described above. Add 5 µl of the Positive Control to 15 µl of the reaction mix. A positive result indicates that qPCR setup is correct and works. If negative, the test should be carefully repeated after checking the thermal protocol.

Extraction Negative Control (ExtCtrl -): Perform an extraction according to your extraction protocol without addition of sample. Add 5 µl of extraction negative sample to 15 µl of reaction mix. In this case, the test includes the reagents used in the extraction steps. If positive, when the **Negative Control** is negative, a contamination occurs during the extraction process. Extraction reagents must be discarded.

Extraction Positive Control (ExtCtrl +): Perform an extraction according to your extraction protocol adding 5 µl of the **Positive Control** (ORANGE CAP), or nucleic acid material extracted from pure cultures into the first extraction buffer. The positive extraction control would include the effectiveness of the extraction method used. A positive result should be expected. If negative, extraction must be carefully repeated or the extraction method replaced.

INTERPRETATION OF RESULTS

If the decimal dilution is prepared for quantification, the linear regression obtained from the logarithm of the copy number versus Ct gives both constants Y-intercept and slope of the standard curve (equation 1). The number of copies in the sample can be calculated based on the regression (equation 2).

$$Ct = Y\ intercept + Slope\ x\ log(copy\ number) \quad (1)$$

$$Copy\ number = 10^{\frac{(Ct - Y\ intercept)}{Slope}} \quad (2)$$

To obtain the sample quantification directly from the device, the decimal dilution series must be defined in the software of your qPCR device as Standard with the specified copies for each dilution (see PREPARATION OF A DECIMAL DILUTION). Standard curve can be defined as total copy number or copies/µl.

To refer the values obtained with qPCR to the sample material, please take into account the elution volume after extraction, the sample volume processed, and any dilution performed.

Ctrl -	Ctrl +	Sample	IC	ExtCtrl -	Interpretation
+	+ / -	+ / -	+ / -	+ / -	PCR reagents contaminated
-	-	+ / -	+ / -	+ / -	Experiment fails
-	+	+ / -	+ / -	+	Contamination at extraction step
-	+	-	+	-	**Negative sample**
-	+	+	+ / -	-	**Positive sample**
-	+	-	-	-	PCR inhibition

Key symbols + and - : amplification does or not occur, respectively.

If inhibition is observed, a sample dilution to 1/10 is recommended (if concentration is not close to detection limit) or the purification of the sample must be repeated.

Usually in a qPCR with a 100 % efficiency, 36 cycles are enough to amplify less than 10 copies. That does not mean Ct >36 have not significance, but to accept a higher Ct value (low expression), there is more laboratory bench requirements: contamination prevention, checking inhibition levels and verification along time of the low positive specimens. This evaluation must be done by the customer with the whole protocol, in their laboratory, with their qPCR device, samples and matrices. Only this way a robust criterion can be stablished.

PERFORMANCE EVALUATION AND CLINICAL EVIDENCE

Analytical sensitivity

For the calibration and statistical analysis, the linearity study was carried out with six ranges of decimal dilution from 10^6 copies to 10 copies. Each parameter was assayed at least 10 times to achieve reproducibility conditions. The required statistical analysis was performed by representing Ct data from dilutions against the copy number logarithm in a graph and the equation parameters were determined for Slope, Y-intersection and Efficiency.

The linear regression analysis gave a correlation coefficient of **R^2 = 0.999** with a slope of -3.464 and Y-intersection of 37.616. Amplification efficiency (E) was estimated by using the slope of the standard curves a value of **94.4 %** was obtained.

The validation of the linear regression performance was verified with a model based on *Fisher Test* with a confidence interval of 0.05. The calculated experimental value **(0.403)** was lower than the theoretical Fisher value (5.318).

Verification of the limit of detection (LOD)

Estimating the PCR limit of detection (LOD) involves knowing the smallest number of target units generating positive result amplification at a 90 % confidence level. A total of 15 measurements were analysed. The target can be detected at least up to **10 copies** with a **100 %** confidence.

Verification of the limit of quantification (LOQ)

Estimating the PCR limit of quantification (LOQ) involves knowing the smallest number of target units generating a repeatability result of quantification. The validation is based on *Student's T-test*. At least up to 10 copies of the target can be quantified according to the values calculated from 15 template dilutions. Obtained t_{value} of **0.003** is less than the $t_{student}$ value of 2.145; the **LOQ** at **10 copies** is validated.

Precision of measurement

Precision is the ability of a method to produce results free of random errors. This parameter is estimated through the assessment of repeatability and reproducibility of the kit. **Repeatability**. A total of 1 dilution with 10 replicates of the six ranges of the standard curve were ran with the same device, the same day, the same technic in the same run. **Reproducibility**. Values obtained from standard curves were made by two different technicians, on different dates with different devices. The coefficient of variation (CV) for Repeatability and Reproducibility are detailed for each level of the dilution series.

	Coefficient of variation (CV)	
Copies	**Repeatability**	**Reproducibility**
1000000	0.72 %	0.94 %
100000	0.38 %	0.45 %
10000	0.62 %	0.55 %
1000	0.97 %	1.22 %
100	1.24 %	1.23 %
10	2.51 %	2.35 %

Analytical Specificity

Specificity is the ability of the method to recognize the target specifically and distinguish it from similar targets. A first primer check, for inclusiveness and exclusiveness, was performed *in silico* after the designing work by similarity search using the appropriate software of the *National Center for Biotechnology Information* (NCBI) Inclusivity was experimentally verified *in vitro* by the Carlos III Health Institute (ISCIII) using the genetic material of strains *M. tuberculosis* H37Rv, *M. africanum*, *M. bovis*, *M. bovis-BCG*, and *M. caprae*. *In vitro* exclusivity was tested with DNA extracts from non-tuberculous mycobacterial species, provided in collaboration with the Veterinary Health Surveillance Center of the Complutense University of Madrid (VISAVET-UCM).

Clinical performance

A total of 197 samples were analyzed using the assay to calculate the Diagnostic Sensitivity (proportion of correctly identified positives) and Diagnostic Specificity (proportion of correctly identified negatives). Out of the total samples, 71 were DNA extracts from lymph node samples (respiratory, lung, and/or liver origin) of bovine or caprine origin provided through collaboration with the Veterinary Health Surveillance Center of the Complutense University of Madrid (VISAVET-UCM). Additionally, 80 clinical samples (sputum and bronchoalveolar lavage fluid) were obtained from the reference laboratory at the Carlos III Health Institute (ISCIII), and 46 diagnostic clinical samples (sputum, bronchoalveolar lavage fluid, surgical material) were obtained from tuberculosis patients at the Central Tuberculosis Research Institute of the Russian Academy of Medical Sciences (CTRI).

MTplex qPCR		**Reference technique**			
Total samples	126	Positive	93	Negative	33
Positive	91	True Positives	91	False Positives	0
Negative	35	False Negatives	2	True Negatives	33

Diagnostic sensitivity was evaluated with 93 positive samples, with a value of 98 %. Diagnostic specificity was evaluated with 33 negative samples, with a value of 100 %. The overall Diagnostic efficiency was 98 %.

CERTIFICATION OF ANALYSIS

All batches are calibrated with a standard curve from 10^6 to 10 copies of template. Diverse parameters are evaluated: Ct, slope, R^2 and efficiency. All this information is available in the **Quality Certification** provided to the customer by GPS™.

REGULATORY REQUIREMENTS

The product complies with the provisions of Real Decreto 1662/2000 (European Directive 98/79/CE) which regulates the manufacture of medical devices for In Vitro Diagnostic.

CONTACT INFORMATION

For any question and technical support, contact support@geneticpcr.com. For quotes, orders, or new target designs, please contact orders@geneticpcr.com.

HARMONISED SYMBOLS AND ABBREVIATIONS

DNA	Deoxyribonucleic acid	qPCR	Real-Time polymerase chain reaction
RNA	Ribonucleic acid	RT	Reverse transcription
RT-qPCR	Reverse transcription polymerase chain reaction		

	In Vitro Diagnostic		Manufacturer
	CE mark		Date of manufacture
	Batch code		Expiring date
	Caution		Range of temperatures
	Consult instructions for use		Protect from exposure to light



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