MIKROGEN
DIAGNOSTIK

Instruction for Use

*alpha*Cube MTB

For qualitive *in vitro* detection of Mycobacterium tuberculosis complex (MTB) DNA in clinical specimens.

REF	**830562**	**830561**
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1 Intended Use

The *alpha*Cube MTB real time PCR is an assay for the detection of the DNA of *MTB* in clinical specimens.

2 Pathogen Information

The *Mycobacterium tuberculosis* forms a complex with other higher related bacteria called the *M. tuberculosis* complex that consists of 6 members: *Mycobacterium tuberculosis* and *Mycobacterium africanum*, which infect humans; *Mycobacterium microti*, which infects vole; *Mycobacterium bovis*, which infects other mammalian species as well as humans; *M. bovis BCG*, a variant of *Mycobacterium bovis* and *Mycobacterium canettii*, a pathogen that infects humans. Tuberculosis (TB) is a disease caused by infection from the bacteria *M. tuberculosis*. If not treated properly, TB can be fatal. Currently, the World Health Organization estimates that over 13 million people have TB and about 1.5 million die each year from the disease. Tuberculosis most commonly affects the lungs (pulmonary TB). Patients with active pulmonary TB usually have a cough, an abnormal chest x-ray, and are infectious. TB can also occur outside of the lungs (extrapulmonary), most commonly in the central nervous, lymphatic, or genitourinary systems, or in the bones and joints. Tuberculosis which occurs scattered throughout the body is referred to as miliary TB. Extrapulmonary TB is more common in immunosuppressed persons and in young children. When a person with active pulmonary TB coughs, sneezes, or talks, the bacteria that cause TB may spread throughout the air. If another person breathes in these bacteria, there is a chance that they will become infected with tuberculosis. Repeated contact is usually required for infection (1). However, not everyone infected with TB bacteria becomes sick. Roughly 5 % of people infected with *M. tuberculosis* actually develop TB. People who are infected but not sick have latent TB infection. Those who have a latent infection are asymptomatic, do not feel sick, and are not contagious.

3 Principle of the Test

*alpha*Cube MTB contains specific primers and dual-labeled probes for the amplification and detection of MTB DNA in clinical specimens.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.

The fluorescence of the pathogen-specific probes is measured in the FAM channel.

Furthermore, *alpha*Cube MTB contains a Control DNA, which is added during DNA extraction and detected in the same reaction by a differently labeled probe.

The Control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimen.

The fluorescence of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel.

4 Package Contents

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of *alpha*Cube MTB.

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 512 µl	2 x 768 µl
Positive Control	red	1 x 50 µl	1 x 100 µl
Negative Control	green	1 x 50 µl	1 x 100 µl
Control DNA	colourless	1 x 160 µl	2 x 240 µl

5 Equipment and Reagents to be Supplied by User

- DNA isolation kit (e.g. *alpha*Clean Pure RNA/DNA)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation
- Optional: BLP-DNA (Bacterium-Like Particles, please look at chapter ‚Control DNA' for details)

6 Transport, Storage and Stability

*alpha*Cube MTB is shipped on dry ice or cool packs. All components must be stored at -18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package.
Up to 20 freeze and thaw cycles are possible.
For convenience, opened reagents can be stored at +2-8°C for up to 6 months.
Protect kit components from direct sunlight during the complete test run.

7 Important Notes

- *alpha*Cube MTB must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 General Precautions

- Stick to the protocol described in the Instruction for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.

- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine *alpha*Cube MTB components of different lot numbers.

9 Sample Material

Starting material for the *alpha*Cube MTB real time PCR is DNA isolated or released from clinical specimens (e.g. throat swabs, nasal swabs, bronchial lavage, sputum samples).

10 Sample Preparation

*alpha*Cube MTB is suitable for the detection of MTB DNA isolated from clinical specimens with appropriate isolation methods.
Commercial kits for DNA isolation such as the following are recommended:

- *alpha*Clean Pure RNA/DNA
- *alpha*Clean Mag RNA/DNA

Important:
In addition to the samples always run a ‚water control' in your extraction. Treat this water control analogous to a sample. Comparing the amplification of the Control DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

Please note the chapter 11 ‚Control DNA'.

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the manufacturer.

11 Control DNA

A Control DNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.
The Bacterium-Like Particles (BLP-DNA) are not supplied.

DNA isolation from clinical samples

a) Control DNA or BLP-DNA used as Extraction Control:
*alpha*Cube MTB Control DNA or BLP-DNA is added to the DNA extraction.
Add 5 µl Control DNA or BLP-DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions. Please follow protocol A.

The Control DNA must be added to the Lysis Buffer of the extraction kit.

b) Control DNA used as Internal Control of the real time PCR:
If only inhibition will be checked please follow protocol B.

12 Real time PCR

12.1 Important Points Before Starting:

- Please pay attention to the chapter 7 ‚Important Notes'.
- Before setting up the real time PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run one Positive Control and one Negative Control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed (do NOT vortex the Reaction Mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.

12.2 Procedure

If the Control DNA or BLP-DNA is used to control both, the real time PCR and the DNA isolation procedure, please follow protocol A. If the Control DNA is solely used to detect possible inhibition of the real time PCR, please follow protocol B.

Protocol A

The Control DNA or BLP-DNA was added during DNA extraction (see chapter 11 ‚Control DNA'). In this case, prepare the Master Mix according to Table 2.

The Master Mix contains all of the components needed for PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the Master Mix (Control DNA was added during DNA extraction)

Volume per Reaction	Volume Master Mix
16.0 µl Reaction Mix	16.0 µl x (N+1)

Protocol B

The Control DNA is used for the control of the real time PCR only (see chapter 11 ‚Control DNA'). In this case, prepare the Master Mix according to Table 3.

The Master Mix contains all of the components needed for real time PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the Master Mix (Control DNA is added directly to the Master Mix)

Volume per Reaction	Volume Master Mix
16.0 µl Reaction Mix	16.0 µl x (N+1)
0.5 µl Control DNA*	0.5 µl x (N+1)*

*The increase in volume caused by adding the Control DNA is not taken into account when preparing the PCR assay.

Protocol A and B: real time PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube.
- Add **4 µl** of the eluates from the DNA isolation (including the eluate of the water control), the Positive Control, and the Negative Control to the corresponding optical PCR reaction tube (Table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

12.3 Instrument Settings

For the real time PCR use the thermal profile shown in Table 5.

Table 5: real time PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of DNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec	60°C	
	Aquisition at the end of this step		

Dependent on the real time PCR instrument used, further instrument settings have to be adjusted according to Table 6.

Table 6: Overview of the instrument settings required for *alpha*Cube MTB.

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler 480I	MTB	483-533	pre-installed universal Color Compensation FAM (510) – VIC (580)
	Control DNA	523-568	
LightCycler 480II	MTB	FAM (465-510)	
	Control DNA	HEX (533-580)	
Stratagene Mx3000P / Mx3005P	MTB	FAM	Gain 8 Reference Dye: None
	Control DNA	HEX	Gain 1
ABI 7500	MTB	FAM	Option Reference Dye ROX: NO
	Control DNA	JOE	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	MTB	Green	Gain 5
	Control DNA	Yellow	Gain 5

13 Data Analysis

The MTB specific amplification is measured in the FAM channel. The amplification of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel.

Following results can occur:

- **A signal in the FAM channel is detected:**
  The result is positive, the sample contains MTB DNA.
  In this case, detection of a signal of the Control DNA in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the Control DNA.

- **No signal in the FAM channel, but a signal in the VIC®/HEX/JOE™/TET channel is detected:**
  The result is negative, the sample does not contain MTB DNA.
  The signal of the Control DNA excludes the possibilities of DNA isolation failure (in case the Control DNA is being used as an Extraction Control) and/or real time PCR inhibition. If the C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see ‚Troubleshooting').

- **Neither in the FAM nor in the VIC®/HEX/JOE™/TET channel a signal is detected:**
  A diagnostic statement cannot be made.
  The DNA isolation was not successful or an inhibition of the PCR has occurred. In case the Control DNA was added during DNA isolation and not directly to the PCR Master Mix, the Negative Control is negative in both channels.

Figure 1 and **Figure 2** show examples for positive and negative real time PCR results.

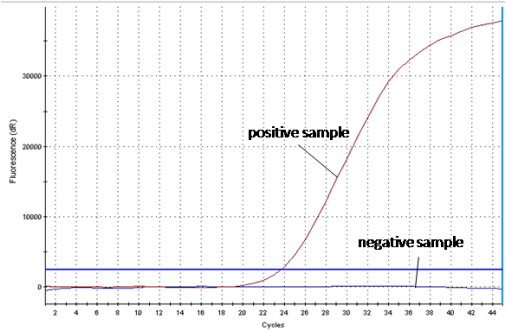

Figure 1: The positive sample shows bacteria specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

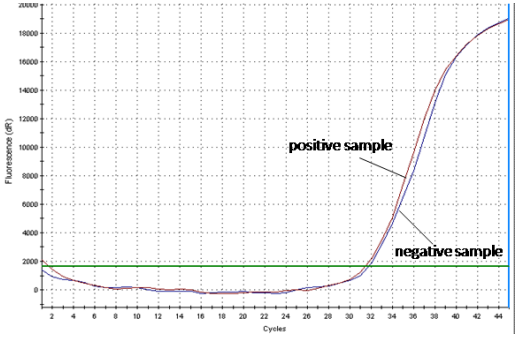

Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the bacteria specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

14 Assay Validation

Set a threshold as follows:

Negative Controls
All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive Controls
All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal Controls
All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a C_T of 33.

15 Limitations of the Method

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.
A negative test result does not exclude a MTB infection.

16 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR.

No fluorescence signal in the FAM channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the MTB specific amplification and the VIC®/HEX/JOE™/TET channel for the amplification of the Control DNA.
Incorrect configuration of the real time PCR	Check your work steps and compare with ‚Procedure' on page 6.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 7).
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport, Storage and Stability', page 4.

Weak or no signal of the Control DNA and simultaneous absence of a signal in the bacteria specific FAM channel.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 6).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.
DNA loss during isolation process	In case the Control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport, Storage and Stability', page 4.
Detection of a fluorescence signal in the FAM channel of the Negative Control	
Contamination during preparation of the PCR	Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

17 Kit Performance

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the *alpha*Cube MTB real time PCR 11 positive and 13 negative samples were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 %

Table 7: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	**negative samples**
*alpha*Cube MTB positive	11	0
*alpha*Cube MTB negative	0	13
Sensitivity	100 %	
Specificity	100 %	

During the validation study of the *alpha*Cube MTB real time PCR ring trial samples were tested. The table below shows the outcome of the ring trial.

Table 8: Results of MTB ring trial.

Sample	Sample Content	Expected Result	Result *alpha*Cube MTB	Sample Type
MTBDNA14-01	M. tuberculosis Complex	positive	positive	core
MTBDNA14-02	Mycobacterium negative	negative	negative	core
MTBDNA14-03	M. tuberculosis Complex	positive	positive	core
MTBDNA14-04	M. tuberculosis Complex	positive	positive	educational
MTBDNA14-05	M. xenopi	negative	negative	educational
MTBDNA14-06	M. tuberculosis Complex	positive	positive	core
MTBDNA14-07	M. tuberculosis Complex	positive	positive	educational
MTBDNA14-08	M. tuberculosis Complex	positive	positive	core
MTBDNA14-09	Mycobacterium negative	negative	negative	core
MTBDNA14-10	M. tuberculosis Complex	positive	positive	core

17.2 Analytical Sensitivity

The limit of detection (LoD) of *alpha*Cube MTB real time PCR was determined using serial dilutions of synthetic target DNA-sequences in a Stratagene Mx3000 real time PCR instrument. The LoD of *alpha*Cube MTB real time PCR for *MTB* is ≥ 1 target copie per reaction each.

17.3 Analytical Specificity

The specificity of the *alpha*Cube MTB real time PCR was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:

The *alpha*Cube MTB real time PCR showed a positive result for the sample containing *MTB,* whereas samples containing other pathogens were reliably tested negative. The results are shown in Table 9.

Table 9: Bacterial and viral pathogens tested for the determination of the analytical specifcity of the *alpha*Cube MTB real time PCR Kit.

Strain	**Expected Result**	**Result**
Influenza Virus A A/ Brisbane H1N1 59/2007 E40/08	negative	negative
Influenza Virus A Indonesia H5N1 05/2005	negative	negative
Influenza Virus A New Caledonia 20/99 H1N1	negative	negative
Influenza Virus A Panama H3N2 2007/99	negative	negative
Influenza Virus B B/ Brisbane 60/2008 E09/09	negative	negative
Influenza Virus B Jiangsu 10/2003	negative	negative
RSV Strain A2 ATCC-VR-1540	negative	negative
RSV Strain B WV/14617/85 ATCC-VR-1400	negative	negative
Parainfluenzavirus Typ 3 Str. C243 VR93	negative	negative
Mycoplasma pneumoniae ATCC 15531	negative	negative
Chlamydophila pneumoniae Str. CM-1, ATCC-VR-1360	negative	negative
Adenovirus	negative	negative
Legionella pneumophila Serogroup 2	negative	negative
Rhinovirus Typ 3 FEBVR483	negative	negative
Streptococcus agalactiae	negative	negative
Coxsackievirus B5	negative	negative
MRSA	negative	negative
M. tuberculosis complex	positive	positive

18 Abbreviations and Symbols

DNA	Deoxyribonucleid Acid
PCR	Polymerase Chain Reaction
MTB	Mycobacterium tuberculosis
REACTION MIX	Reaction Mix
CONTROL +	Positive Control
CONTROL −	Negative Control
CONTROL DNA IC	Control DNA
REF	Catalog number
Σ	Contains sufficient for <n> test

- 18°C	Upper limit of temperature
	Manufacturer
	Use by YYYY-MM
LOT	Batch code
CONT	Content
	Consult instructions for use
IVD	*In vitro* diagnostic medical device
CE	European Conformity

gerbion gmbH & Co. KG
Remsstr. 1
70806 Kornwestheim
Germany
phone: +49 7154 806 20 0
fax: +49 7154 806 20 29
e-mail: info@gerbion.com
www.gerbion.com

Distributor
Mikrogen GmbH
Floriansbogen 2-4
D-82061 Neuried
Germany
phone: +49 (0) 89-54801 0
fax: +49 (0) 89-54801 100
e-mail: mikrogen@mikrogen.de
www.mikrogen.de

19 Literature

[1] Centers for Disease Control and Prevention (2016) "Division of Tuberculosis Elimination (DTBE)", <HTTP: default.htm tb www.cdc.gov>