Instruction for Use

diarellaMTB/NTM/MAC

real time PCR Kit

For qualitive *in vitro* detection of purified DNA of Mycobacterium tuberculosis complex (MTB), Nontuberculous Mycobacteria (NTM) and Mycobacterium avium complex (MAC) extracted from biological samples.

REF	G01114-32	G01114-96
Σ	32	96
- 18°C		

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1 Intended Use

The diarellaMTB/NTB/MAC real time PCR is an assay for the amplification of purified DNA of Mycobacterium tuberculosis complex (MTB), Non Tuberculous Mycobacteria (NTM) and Mycobacterium avium complex (MAC), extracted from biological samples.

2 Background Information

Mycobacterium tuberculosis forms a complex with other higher related bacteria called the **M. tuberculosis complex (MTB)** 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.

Nontuberculous mycobacteria (NTM), also known as environmental mycobacteria, atypical mycobacteria and mycobacteria other than tuberculosis (MOTT), are mycobacteria which do not cause tuberculosis or leprosy. NTM do cause pulmonary diseases that resemble tuberculosis. The most common clinical manifestation of NTM disease is lung disease, but lymphatic, skin/soft tissue, and disseminated disease are also important.

Organisms of the **Mycobacterium avium complex (MAC)** are ubiquitous in the environment. M. avium is the etiologic agent in >95 % of patients with AIDS who acquire disseminated MAC disease. An estimated 7 % to 12 % of adults have been previously infected with MAC, although rates of disease vary in different geographic locations. Although epidemiologic associations have been identified, no environmental exposure or behavior has been consistently linked to subsequent risk of developing MAC disease.

3 Principle of the Test

The diarellaMTB/NTM/MAC real time PCR contains specific primers and dual-labeled probes for the amplification and detection of the DNA of MTB, NTM and MAC. The presence of nucleic acids is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The fluorescence signals of the specific probes are measured in the FAM (MTB), ROX (MAC) and Cy5 (NTM) channels.

Furthermore, diarellaMTB/NTM/MAC real time PCR 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 the diarellaMTB/NTM/MAC real time PCR Kit

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 purification kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- DNase/RNase-free disposable pipette tips with aerosol barriers
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes or optical PCR reaction plates
- Optional: Liquid handling system for automation

6 Transport, Storage and Stability

The diarellaMTB/NTM/MAC real time PCR Kit is shipped on dry ice or cool packs. All components must be stored at maximum -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 Warnings and Precautions

Read the Instructions for Use carefully before using the product.
Before first use check the product and its components for:

- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.

8 Sample Preparation

Purified DNA is suitable for downstream processing in real time PCR. For the extraction and purification of DNA from various biological materials, commercial kits are available. The operator needs to evaluate the suitability of respective DNA extraction kit.

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 ‚Control DNA'.

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

9 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.

a) Control DNA used as Extraction Control:
diarellaMTB/NTM/MAC real time PCR Control DNA is added to the DNA extraction.
Add 5 µl Control 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.

10 Real time PCR

10.1 Important Points Before Starting:

- 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.

10.2 Procedure

If the Control 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 was added during DNA extraction (chapter 9 ‚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 9 ‚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

10.3 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		

The real time PCR thermal profile mentioned in table 5 represents the universal settings for gerbion real time PCR and real time RT-PCR kits. Therefore, different kits can be used in the same run. For gerbion real time PCR kits used for amplification of DNA, the reverse transcription can be omitted. 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 the diarellaMTB/NTM/MAC real time PCR.

Real time PCR Instrument	Parameter Reaction Mix	Detection Channel	Notes		
			Colour Compensation required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
LightCycler 480II	MTB	465-510	1	10	1
	MAC	533-610	1	10	2
	Control DNA	533-580	1	10	2
	NTM	618-660	1	10	3
Stratagene Mx3000P / Mx3005P	MTB	FAM	Gain 8		
	MAC	ROX	Gain 1		Reference Dye: None
	Control DNA	HEX	Gain 1		
	NTM	Cy5	Gain 4		
Agilent Aria Mx BioRad CFX 96	MTB	FAM			
	MAC	ROX			Reference Dye: None
	Control DNA	HEX			
	NTM	Cy5			
ABI 7500	MTB	FAM	Option Reference Dye ROX: NO		
	MAC	ROX			
	Control DNA	JOE			
	NTM	Cy5			
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	MTB	Green	Gain 5		
	MAC	Orange	Gain 5		
	Control DNA	Yellow	Gain 5		
	NTM	Red	Gain 5		
mic qPCR Cycler	MTB	Green	Gain 8		
	MAC	Orange	Gain 10		
	Control DNA	Yellow	Gain 10		
	NTM	Red	Gain 10		

11 Data Analysis

The specific amplifications are measured in the FAM, ROX, and Cy5 channel. The amplification of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel. The Positive Control contains nucleic acid target sequences of MTB, NTM and MAC. For the Positive Control, signals in the FAM, ROX, and Cy5 channels must be detected. The interpretation of the test results is described in table 7.

Table 7: Interpretation of results

Signal/Ct Values				Interpretation
FAM MTB	**ROX** MAC	**Cy5** NTM	**HEX** Control DNA	
positive	negative	positive or negative	positive or negative*	Positive result, the eluate contains MTB DNA. NTM sequences can be present in MTB DNA, therefore a signal in the Cy5 channel can be observed
negative	negative	**positive**	positive or negative*	Positive result, the eluate contains NTM DNA.
negative	**positive**	positive or negative	positive or negative*	Positive result, the eluate contains MAC DNA. NTM sequences can be present in MTB DNA, therefore a signal in the Cy5 channel can be observed
negative	negative	negative	≤ 34**	Negative result, the eluate contains no MTB, NTM and MAC DNA.
negative	negative	negative	negative or > 34**	No diagnostic statement can be made. The real time PCR is either inhibited or errors occurred while DNA extraction.

* A strong positive signal in the FAM, Cy5 and/or ROX can inhibit the IC. In such cases the result for the Control DNA can be neglegted.

** Depending on the PCR instrument and/or the chosen extraction method, the Ct values might be shifted. The water control can be used as reference. If the HEX Ct value of a sample differs a lot from the water control, partial inhibition has occurred, leading to false negative results in case of weak positive samples.

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

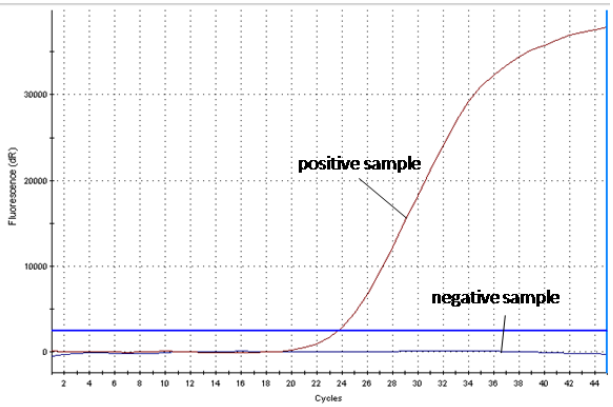


Figure 1: The positive eluate shows specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative eluate.

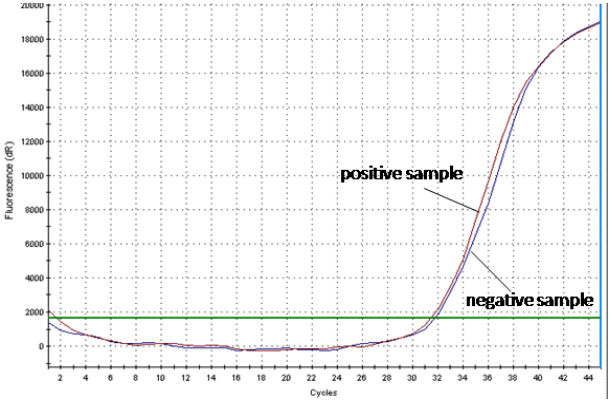


Figure 2: The positive eluate as well as the negative eluate show a signal in the Control DNA specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA in the negative eluate shows, that the missing signal in the specific FAM channel is not due to PCR inhibition or failure of DNA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

12 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 $\leq$34. If the internal control is above C_T 34, this points to a purification problem in DNA-extraction or a strong positive eluate 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 $\leq$34.

13 Limitations

- Strict compliance with the Instructions for Use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and in vitro diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors may cause false negative or invalid results.
- Potential mutations within the target regions of the MTB, NTM or MAC genome covered by the primers and/or probes used in the kit may result in failure to detect the respective DNA.
- As with any diagnostic test, results of the diarellaMTB/NTM/MAC real time PCR Kit need to be interpreted in consideration of all clinical and laboratory findings.

14 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the bacteria specific channels of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the channel according to Table 6.
Incorrect configuration of the real time PCR	Check your work steps and compare with ‚Procedure' on page 7.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 8).
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 channels.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 7).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see chapter ‚Sample Preapartion') 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'.
Detection of a fluorescence signal in the bacteria specific 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.

15 Kit Performance

15.1 Analytical Sensitivity

The limit of detection (LoD) of diarellaMTB/NTM/MAC real time PCR was determined using serial dilutions of synthetic target DNA-sequences in a Stratagene Mx3005 real time PCR instrument. The LoD of diarellaMTB/NTM/MAC real time PCR for MTB is ≤ 1 target copies per reaction each for NTM ≤ 10 target copies per reaction each and for MAC ≤ 1 target copies per reaction each.

During the validation study of the diarellaMTB/NTM/MAC real time PCR ring trial samples were tested. The table below shows the outcome of testing.

Table 8: Results of MTB ring trial.

Sample	Sample Content	Expected Result	Result FAM MTB	Result ROX MAC	Result Cy5 NTM	Sample Type
MTBDNA14-01	M. tuberculosis Complex	positive	positive	negative	positive	core
MTBDNA14-02	Mycobacterium negative	negative	negative	negative	negative	core
MTBDNA14-03	M. tuberculosis Complex	positive	positive	negative	positive	core
MTBDNA14-04	M. tuberculosis Complex	positive	positive	negative	positive	educational
MTBDNA14-05	M. xenopi	negative	negative	negative	positive	educational
MTBDNA14-06	M. tuberculosis Complex	positive	positive	negative	positive	core
MTBDNA14-07	M. tuberculosis Complex	positive	positive	negative	positive	educational
MTBDNA14-08	M. tuberculosis Complex	positive	positive	negative	positive	core
MTBDNA14-09	Mycobacterium negative	negative	negative	negative	negative	core
MTBDNA14-10	M. tuberculosis Complex	positive	positive	negative	positive	core

15.2 Analytical Specificity

The specificity of the diarellaMTB/NTM/MAC real time PCR was evaluated by testing a panel of genomic RNA/DNA extracted from viruses and bacteria.

The diarellaMTB/NTM/MAC real time PCR kit did not cross-react with the DNA and RNA from the following viruses and bacteria.

Table 9: Cross-reactivity of the diarellaMTB/NTM/MAC real time PCR Kit.

Strain	Expected Result	Result FAM MTB	Result ROX MAC	Result Cy5 NTM
Influenza Virus A	negative	negative	negative	negative
Influenza Virus B	negative	negative	negative	negative
RSV Strain A2 ATCC-VR-1540	negative	negative	negative	negative
RSV Strain B WV/14617/85 ATCC-VR-1400	negative	negative	negative	negative
Parainfluenzavirus Typ 3 Str. C243 VR93	negative	negative	negative	negative
Mycoplasma pneumoniae ATCC 15531	negative	negative	negative	negative
Chlamydophila pneumoniae Str. CM-1, ATCC-VR-1360	negative	negative	negative	negative
Adenovirus	negative	negative	negative	negative
Legionella pneumophila Serogroup 2	negative	negative	negative	negative
MRSA	negative	negative	negative	negative

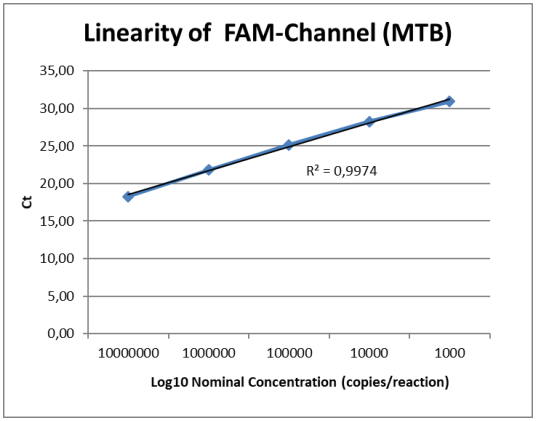
The diarellaMTB/NTM/MAC real time PCR kit did amplify the DNA and RNA from the following viruses and bacteria.

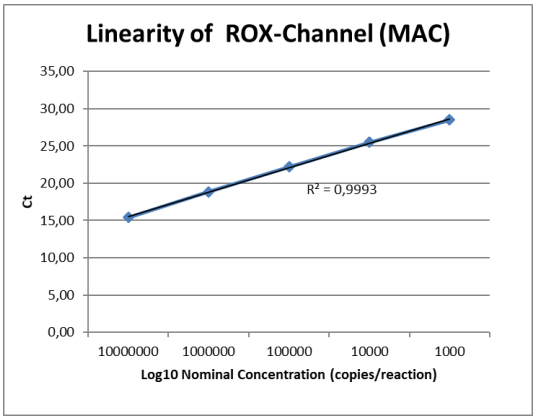
Table 10: Specificity of the diarellaMTB/NTM/MAC real time PCR Kit.

Strain	Expected Result	Result FAM MTB	Result Cy5 NTM	Result ROX MAC
M. tuberculosis complex	positive	positive	positive	negative
M. avium ssp. paratuberculosis (DSM 44135)	positive	negative	positive	positive
M. avium ssp. avium (DSM 44156)	positive	negative	positive	positive
M. phlei (DSM 43473)	positive	negative	positive	negative
M. avium ssp. hominissuis (FLI)	positive	negative	positive	positive
M. fortuitum ssp. fortuitum (DSM 43478)	positive	negative	positive	negative
M. kansasii (DSM 43502)	positive	negative	positive	negative
M. diernhoferi (DSM 43523)	positive	negative	positive	negative
M. terrae (DSM 43540)	positive	negative	positive	negative
M. smegmatis (DSM 44201)	positive	negative	positive	negative
M. intracellulare (DSM 44365)	positive	negative	positive	positive
M. palustre (DSM 44571)	positive	negative	positive	negative
M. chelonae ssp. chelonae (DSM 45527)	positive	negative	positive	negative
M. rhodesiae (DSM 44223)	positive	negative	positive	negative

15.3 Linear Range

The linear range of the diarellaMTB/NTM/MAC real time PCR was evaluated by analyzing logarithmic dilution series of synthetic DNA fragments (Figure 3).





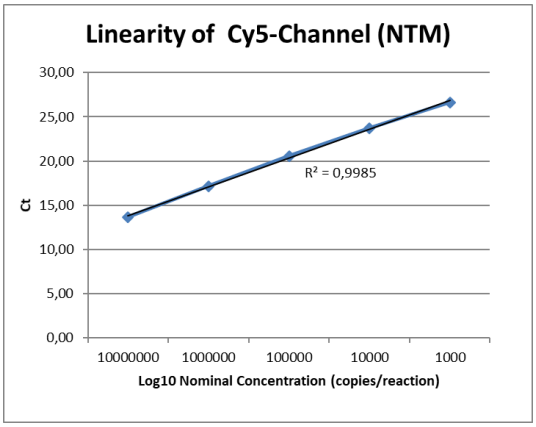


Figure 3: The linear range of the diarellaMTB/NTM/MAC real time PCR Kit was determined to be 10^3 copies/reaction to 10^7 copies/reaction.

15.4 Precision

The precision of the diarellaMTB/NTM/MAC real time PCR Kit was determined as intra-assay variability, inter-assay variabilitity and inter-lot variability.
Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of MTB, NTM and MAP specific DNA and on the threshold cycle of the Control-DNA.

MTB	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.12	0.37
Inter-Assay-Variability	25	0.22	0.67
Inter-Lot Variability	25	0.05	0.15

MAC	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.11	0.36
Inter-Assay-Variability	25	0.13	0.43
Inter-Lot Variability	25	0.02	0.06

NTM	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.03	0.11
Inter-Assay-Variability	25	0.02	0.05
Inter-Lot Variability	25	0.00	0.01

Control DNA	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.05	0.18
Inter-Assay-Variability	25	0.25	0.81
Inter-Lot Variability	25	0.06	0.21

16 Abbreviations and Symbols

DNA	Deoxyribonucleid Acid
PCR	Polymerase Chain Reaction
MTB	Mycobacterium tuberculosis
NTM	Nontuberculous Mycobacteria
MAC	Mycobacterium avium complex
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-DD
LOT	Batch code
CONT	Content
	Consult instructions for use
IVD	*In vitro* diagnostic medical device
CE	European Conformity

17 Literature

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

[2] Yon Ju Ryu, Won-Jung Koh, and Charles L. Daley, Diagnosis and Treatment of Nontuberculous Mycobacterial Lung Disease: Clinicians' Perspectives. Tuberc Respir Dis (Seoul). 2016 Apr; 79(2): 74–84.

[3] Porvaznik I, Solovič I, and Mokrý J. Non-Tuberculous Mycobacteria: Classification, Diagnostics, and Therapy. Adv Exp Med Biol. 2017; 944:19-25.