MIKROGEN
DIAGNOSTIK

Instruction for Use

*alpha*Cube Rickettsia

For qualitive *in vitro* detection of purified DNA of Rickettsia species, extracted from biological specimens.

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Σ	32	96

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1 Intended Use

The *alpha*Cube Rickettsia real time PCR is an assay for the amplification of purified DNA of Rickettsia species, extracted from biological specimens.

2 Background Information

Rickettsia is a genus of bacteria of the tribe Rickettsiae, made up of small, gram-negative, rod-shaped to coccoid, often pleomorphic microorganisms, which multiply only in host cells. Organisms occur in the cytoplasm of tissue cells or free in the gut lumen of lice, fleas, ticks, and mites and are transmitted by their bites.

R. conorii is the etiologic agent of Boutonneuse Fever (a tickborne disease endemic in the Mediterranean area, Crimea, Africa, and India with chills, fever, primary skin lesion (tache noire), and rash appearing on the second to fourth day).

R. prowazekii is transmitted between humans by the human body louse and from flying squirrels to humans by fleas and lice. R. prowazekii is the agent of epidemic typhus and Brill-Zinsser disease. Epidemic typhus is a form of typhus so named because the disease often causes epidemics following wars and natural disasters. The Brill-Zinsser disease is characterized by a delayed relapse of epidemic typhus. After a patient contracts epidemic typhus from the fecal matter of an infected louse (Pediculus humanus), the rickettsia can remain latent and reactivate months or years later, with symptoms similar to or even identical to the original attack of typhus, including a maculopapular rash.

R. typhi is the cause of murine typhus, which is transmitted to humans chiefly by rat fleas. Murine typhus is a mild, acute, endemic form of typhus characterized by fever, headache, and muscular pain. Rickettsial diseases are not common in communities with good sanitary standards, since prevention depends on controlling the rodent and insect populations. Major epidemics have occurred, especially in times of war when standards of sanitation drop.

3 Principle of the Test

The *alpha*Cube Rickettsia real time PCR contains specific primers and dual-labeled probes for the amplification and detection of the DNA Rickettsia species. 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 channel.

Furthermore, *alpha*Cube Rickettsia 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 HEX channel.

4 Package Contents

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

Table 1: Components of *alpha*Cube Rickettsia.

Label	Lid Colour	Content 32	96
Reaction Mix	yellow	1 x 512 µl	1 x 1536 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	1 x 480 µl

5 Equipment and Reagents to be Supplied by User

- DNA isolation kit (e.g. *alpha*Clean Pure RNA/DNA or *alpha*Clean Mag 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

6 Transport, Storage and Stability

*alpha*Cube Rickettsia 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 Important Notes

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. 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 ‚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.

DNA isolation from biological specimens
a) Control DNA used as Extraction Control:
*alpha*Cube Rickettsia 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:

- Please pay attention to the chapter ‚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 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 (see chapter ‚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 in the table shown below.

Table 5: real time PCR thermal profile

Description	**Time**	**Temperature**	**Number of Cycles**
Initial Denaturation	10 min	95°C	1
Amplification			45
Denaturation	10 sec	95°C	
Annealing	20 sec	60°C	
	Aquisition at the end of this step		
Extension	10 sec	72°C	

If in the same run samples should be tested for pathogens with RNA genome, e.g. with the *alpha*CubeTBE real time RT-PCR Kit, use the thermal profile shown in

Table 6: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	20 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of DNA			
Denaturation	10 sec	95°C	45
Annealing	20 sec Aquisition at the end of this step	60°C	
Extension	10 sec	72°C	

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

Table 7: Overview of the instrument settings required for *alpha*Cube Rickettsia.

Real time PCR Instrument	Parameter Reaction Mix	Detection Channel	Notes	
LightCycler 480II	Rickettsia Control DNA	465-510 533-580	use pre-installed Colour Compensation	
Stratagene Mx3000P / Mx3005P	Rickettsia Control DNA	FAM HEX	Gain 8 Gain 1	Reference Dye: None
Agilent Aria Mx BioRad CFX 96	Rickettsia Control DNA	FAM HEX		Reference Dye: None
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Rickettsia Control DNA	Green Yellow	Gain 5 Gain 5	
mic qPCR Cycler	Rickettsia Control DNA	Green Yellow	Gain 8 Gain 10	

11 Data Analysis

The specific amplification is measured in the FAM channel. The amplification of the Control DNA is measured in the HEX channel. The Positive Control contains nucleic acid target sequences of Rickettsia species. For the Positive Control, a signal in the FAM channel must be detected.

Table 8: Interpretation of results

Signal/Ct Values		**Interpretation**
FAM Rickettsia	**HEX** Control DNA	
positive	positive or negative*	Positive result, the eluate contains Rickettsia DNA.
negative	≤ 34**	Negative result, the eluate contains no Rickettsia DNA.
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-channel can inhibit the amplification of the Control DNA. 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.

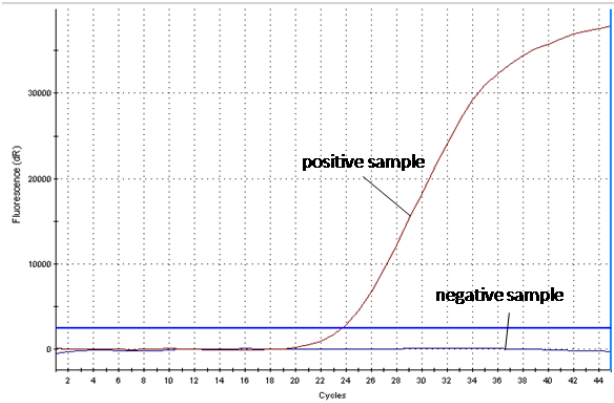


Figure 1: The positive eluate shows specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative eluate.

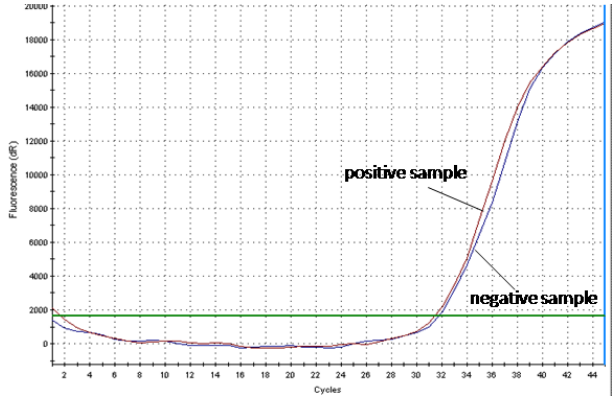


Figure 2: The positive eluate as well as the negative eluate show a signal in the Control DNA specific HEX channel. The amplification signal of the Control DNA in the negative eluate shows, that the missing signal in the specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but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

- 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.
- As with any diagnostic test, results of the *alpha*Cube Rickettsia 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.

No fluorescence signal in the specific channels of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the channel according to chapter ‘Data Analysis’.
Incorrect configuration of the real time PCR	Check your work steps and compare with chapter ‚Procedure‘.
The programming of the thermal profile is incorrect	Compare the thermal profile with chapter ‚Instrument Settings‘.

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 chapter ‚Transport, Storage and Stability‘.
Weak or no signal of the Control DNA and simultaneous absence of a signal in the specific channels	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (chapter ‚Real time PCR‘).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see chapter ‚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 chapter ‚Transport, Storage and Stability‘.
Detection of a fluorescence signal in the specific channels 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

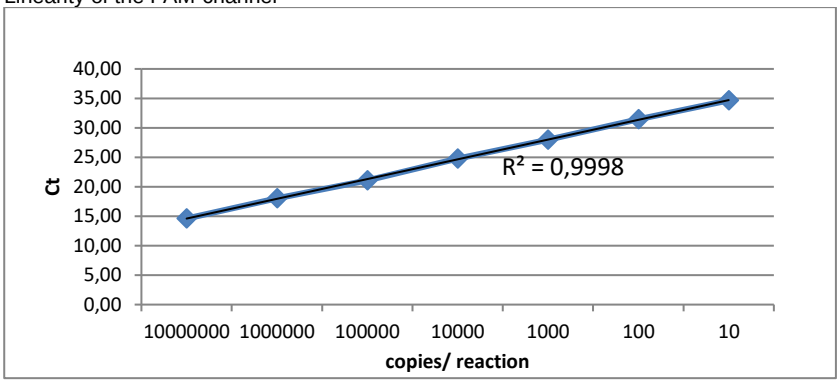
15.1 Analytical Sensitivity

The limit of detection (LoD) of *alpha*Cube Rickettsia real time PCR was determined using serial dilutions of synthetic target DNA-sequences in a Stratagene Mx3005 real time PCR instrument. The LoD of *alpha*Cube Rickettsia real time PCR for Rickettsia is at least 10 copies per reaction each.

15.2 Linear Range

The linear range of the *alpha*Cube Rickettsia real time PCR was evaluated by analyzing logarithmic dilution series of synthetic DNA fragments.

Linearity of the FAM-channel



15.3 Analytical Specificity

The specificity of the *alpha*Cube Rickettsia real time PCR was evaluated by testing a panel of DNA extracted from bacteria.
The *alpha*Cube Rickettsia real time PCR kit did not cross-react with the DNA from the following bacteria.

Table 9: Determination of the analytical specificity of *alpha*Cube Rickettsia real time PCR.

Strain	**Expected Result**	**Result *alpha*Cube Rickettsia**
Borrelia burgdorferi 4681	negative	negative
Borrelia miyamotoi	negative	negative
Borrelia spielmanii	negative	negative
Borrelia afzelii	negative	negative
Babesia microti	negative	negative
Babesia divergens	negative	negative
Anaplasma phagocytophilum	negative	negative
Ehrlichia canis ebony	negative	negative
Coxiella burnetii	negative	negative
Leptospira	negative	negative
Treponema phagedenis	negative	negative

15.4 Precision

The precision of the *alpha*Cube Rickettsia real time PCR 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 Rickettsia specific DNA and on the threshold cycle of the Control-DNA.

Table 10: Precision of *alpha*Cube Rickettsia real time PCR.

Rickettsia	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.12	0.49
Inter-Assay-Variability	25	0.36	1.47
Inter-Lot Variability	25	0.15	0.61

Control DNA	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.15	0.49
Inter-Assay-Variability	25	0.50	1.62
Inter-Lot Variability	25	0.29	0.93

16 Abbreviations and Symbols

DNA	Deoxyribonucleid Acid
PCR	Polymerase Chain Reaction
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

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17 Literature

[1] www.health.nsw.gov.au/Infectious/factsheets/Factsheets/typhus.PDF
[2] cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/rickettsial-including-spotted-fever-and-typhus-fever-rickettsioses-scrub-typhus-anaplasmosis-and-ehrlichiosis