MIKROGEN
DIAGNOSTIK

Instruction for Use

*alpha*Cube Babesia

For qualitative *in vitro* detection of *Babesia* DNA in clinical specimens and in ticks.

REF	830526	830525
Σ	32	96

- 18°C

IVD

CE

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1 Intended Use

*alpha*Cube Babesia is a real-time PCR assay for the detection of DNA of Babesia divergens, Babesia microti and Babesia sp. EU1 (Babesia venatorum) in clinical specimens and in ticks.

2 Pathogen Information

Babesiosis is a malaria-like parasitic disease caused by infection with *Babesia*, a genus of protozoal piroplasm.
The parasites are transmitted by ticks such as *dermacentor reticulatus*, a hard-bodied tick nowadays common throughout Central Europe.
Dependent on the species, the incubation time varies between 7 to 20 days. Rupture of the erythrocytes causes anaemia. Infections can remain without symptoms or with mild fever and diarrhea. However, in more severe cases, high fever with chills can occur.
In Europe infections are mainly caused by *B. divergens* and B. sp. EU1, while in the US *B. microti* (East Coast and Mid-West) and *B. duncani* (North-West Coast) are the causative agents for human babesiosis.

3 Principle of the Test

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

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 or *alpha*Clean Mag RNA/DNA, *alpha*Clean TS (tissue shred))
- 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 Babesia 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

- *alphaCube* Babesia 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 Babesia components of different lot numbers.

9 Sample Material

Starting material for the *alpha*Cube Babesia real time PCR is DNA isolated or released from clinical specimens (e.g. EDTA-blood, plasma, serum, cerebrospinal fluid and tissue samples) or from ticks.

10 Sample Preparation

*alpha*Cube Babesia is suitable for the detection of *Babesia* DNA isolated from clinical specimens or ticks 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

It is recommended to use mechanical disruption of ticks before DNA extraction. Please follow the instructions for use of the respective 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 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.

DNA isolation from EDTA-blood, plasma, serum, cerebrospinal fluid, tissue samples and ticks

a) Control DNA used as Extraction Control:
*alpha*Cube Babesia 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.

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 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 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 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
Initial Denaturation	10 min	95°C	1
Amplification			
Denaturation	10 sec	95°C	45
Annealing	20 sec Aquisition at the end of this step	60°C	
Extension	10 sec	72°C	

If in the same run samples should be tested for pathogens with RNA genome, e.g. with *alpha*Cube TBE, use the thermal profile shown in Table 6.

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			
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 instrument used, further instrument settings have to be adjusted according to Table 7.

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

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler 480I	Babesia	483-533	pre-installed universal Color Compensation FAM (510) – VIC (580)
	Control DNA	523-568	
LightCycler 480II	Babesia	FAM (465-510)	
	Control DNA	HEX (533-580)	
Stratagene Mx3000P / Mx3005P	Babesia	FAM	Gain 8 Reference Dye: None
	Control DNA	HEX	Gain 1
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Babesia	Green	Gain 5
	Control DNA	Yellow	Gain 5
Mic qPCR Cycler	Babesia	Green	Gain 8
	Control DNA	Yellow	Gain 10

13 Data Analysis

The *Babesia* 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 *Babesia* 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 parasitic 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 *Babesia* 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.

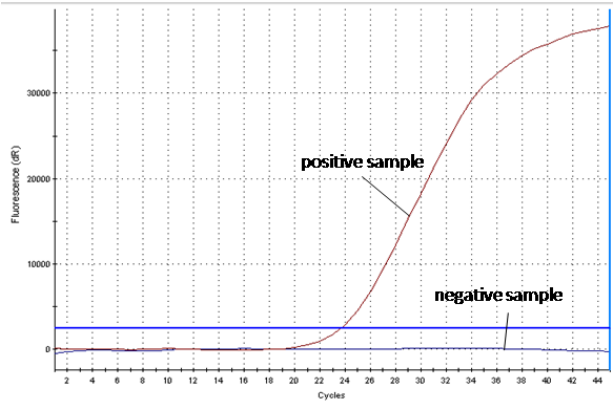

Figure 1: The positive sample shows parasite specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

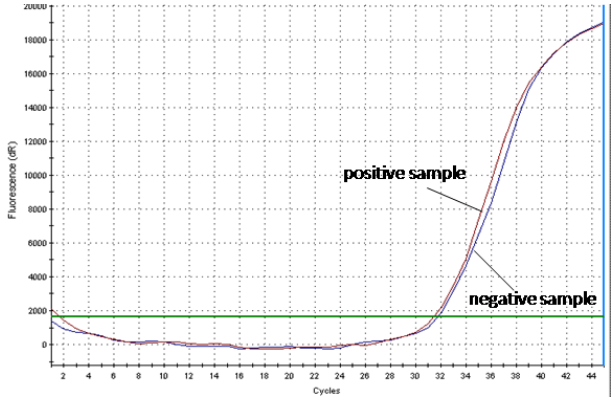

Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific VIC®/HEX/JOE/TET channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the parasite specific FAM channel is not due to PCR inhibition or failure 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 *Babesia* 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 *Babesia* 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 5.
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 parasite specific FAM channel.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 5).
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 Analytical Sensitivity

The limit of detection (LoD) of *alpha*Cube Babesia was determined using serial dilutions of synthetic DNA-fragments containing the Babesia microti and Babesia divergens target sequences in a Stratagene Mx3000 real time PCR instrument. The results of the determinations in triplicates for the Babesia microti system and the Babesia divergens system within the *alpha*Cube Babesia Kit are shown in Table 8 and Table 9.
The LoD of *alpha*Cube Babesia for *Babesia* is ≥10 genome copies per reaction each.

Table 8: Determination of the analytical sensitivity of the Babesia microti system within *alpha*Cube Babesia.

copies per reaction	**C_T-value FAM**	**mean C_T FAM**
1.000.000	19,07 19,86 18,69	19,21
100.000	22,20 22,52 22,37	22,36
10.000	26,35 26,02 26,00	26,12
1.000	29,74 29,64 29,39	29,59
100	33,54 32,32 32,51	32,79
10	37,40 36,79 38,14	37,44
1	no C_T no C_T no C_T	no C_T

Table 9: Determination of the analytical sensitivity of the Babesia divergens system within *alpha*Cube Babesia.

copies per reaction	C_T-value FAM	mean C_T FAM
1.000.000	19,94 20,41 20,12	20,16
100.000	22,14 24,35 23,35	23,28
10.000	27,24 27,51 26,97	27,24
1.000	30,53 31,32 31,05	30,97
100	34,20 34,97 35,08	34,75
10	36,36 38,22 42,99	39,19
1	no C_T no C_T no C_T	no C_T

17.2 Analytical Specificity

The specificity of *alpha*Cube Babesia was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:

The *alpha*Cube Babesia Kit showed a positiv result for the samples containing *Babesia microti, Babesia divergens and Babesia sp EU1,* whereas samples containing other pathogens were reliably tested negative. The results are shown in Table 10.

Table 10: Parasite, bacterial and viral pathogens tested for the determination of the analytical specificity of *alpha*Cube Babesia.

Strain	Expected Result	result
Enterovirus 68	negative	negative
Coxsackievirus B3	negative	negative
Coxsackievirus A16	negative	negative
Coxsackievirus A9	negative	negative
Coxsackievirus B5	negative	negative
Influenza Virus A A/ Brisbane H1N1 59/2007 E40/08	negative	negative
Influenza Virus A Indonesia H5N1 05/2005	negative	negative
Influenza Virus A Panama H3N2 2007/99	negative	negative
Influenza Virus B B/ Brisbane 60/2008 E09/09	negative	negative
TBEV (Tick Borne Encephalitis Virus)	negative	negative
Ehrlichia chaffeensis	negative	negative
Ehrlichia ewingii	negative	negative
Ehrlichia canis	negative	negative
Ehrlichia phagocytophilum	negative	negative
Anaplasma platy	negative	negative
Coxiella burnetii	negative	negative
West Nile Virus	negative	negative
Borrelia burgdorferi Strain 4681	negative	negative
Borrelia burgdorferi sensu stricto	negative	negative
Borrelia afzelii	negative	negative
Borrelia garinii	negative	negative
Borrelia spielmanii	negative	negative
Borrelia bavariensis	negative	negative
Borrelia bisettii	negative	negative
Borrelia lustianae	negative	negative
Borrelia valaisiana	negative	negative
Borrelia kurtenbachii	negative	negative
Borrelia japonica	negative	negative
Borrelia miyamotoi	negative	negative
Treponema phagedenis	negative	negative
Leptospira	negative	negative
Babesia caballi	negative	negative
Babesia canis canis	negative	negative
Babesia canis vogeli	negative	negative
Babesia duncani	negative	negative
Babesia equi	negative	negative
Babesia gibsoni	negative	negative
Babesia divergens	positive	positive
Babesia microti	positive	positive
Babesia sp. EU1	positive	positive

18 Abbreviations and Symbols

DNA	Deoxyribonucleid Acid	REF	Catalog number
PCR	Polymerase Chain Reaction	Σ	Contains sufficient for <n> test
REACTION MIX	Reaction Mix	-18°C	Upper limit of temperature
CONTROL +	Positive Control		Manufacturer
CONTROL −	Negative Control		Use by YYYY-MM-DD
CONTROL DNA IC	Control DNA	LOT	Batch code
	Consult instructions for use	CONT	Content
CE	European Conformity	IVD	*In vitro* diagnostic medical device

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19 Literature

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