Instruction for Use

diarella Q-Fever

real time PCR Kit

For qualitative *in vitro* detection of *Coxiella burnetii* (Q-Fever) DNA, extracted from clinical specimens and ticks.

REF	G01106-32	G01106-96
Σ	32	96

- 18°C

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

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1 Intended Use

diarella Q-Fever is a real-time PCR assay for the detection of *Coxiella burnetii* DNA, extracted from clinical specimens and ticks.

2 Pathogen Information

The pathogen *Coxiella burnetii* (Q-Fever) is a zoonotic, gramnegative bacterium and belongs to the family Rickettsiaceae. It is the causative agent of Q-Fever, an acute rickettsial disease.

It can be considered the most infectious disease in the world, as a human being can be infected by a single bacterium. It can be found worldwide, including tropical countries, with the exception of New Zealand. In Europe it appears as hepatitis rather than pneumonia as in the United States.

The most common manifestation is flu-like symptoms. The fever lasts approximately 7 to 14 days. The disease can progress to an atypical pneumonia, which can result in a life threatening acute respiratory distress syndrome (ARDS). Occasionally, Q-Fever causes hepatitis, which may be asymptomatic or becomes symptomatic with malaise, fever, liver enlargement and pain in the right upper quadrant of the abdomen.

The chronic form of Q-Fever is very similar to inflammation of the inner lining of the heart, which can occur months or decades following the infection. It is fatal if left untreated, however with the correct treatment the mortality rate falls under 10%.

3 Principle of the Test

The diarella Q-Fever real time PCR Kit contains specific primers and dual-labeled probes for the amplification and detection of *Coxiella burnetii* DNA, extracted from 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, the diarella Q-Fever real time PCR Kit 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 diarella Q-Fever 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 isolation kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012), NukEx TS (tissue shred, gerbion Cat. No. G06007).
- 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

The diarella Q-Fever real time PCR Kit 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

- The diarella Q-Fever real time PCR Kit 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 diarella Q-Fever real time PCR Kit components of different lot numbers.

9 Sample Material

Starting material for the diarella Q-Fever real time PCR is DNA, extracted from clinical specimens (e.g. EDTA-blood, plasma, serum, cerebrospinal fluid and tissue samples) or from ticks.

10 Sample Preparation

The diarella Q-Fever real time PCR is suitable for the detection of *Coxiella burnetii* DNA isolated from clinical specimens or ticks with appropriate isolation methods.

Commercial kits for DNA isolation such as the following are recommended:

- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

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 ‚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:
diarella Q-Fever 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.

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 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 (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.

Important: Dilute the Control DNA 1:10 in PCR-grade water (e.g. 1 µl Control DNA + 9 µl dH_2O) before adding in to the Master Mix.

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* (diluted 1:10)	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 of DNA			
Denaturation	10 sec	95°C	
Annealing	20 sec	60°C	45
	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 virellaTBE real time RT-PCR Kit, 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 of DNA			
Denaturation	10 sec	95°C	
Annealing	20 sec	60°C	45
	Aquisition at the end of this step		
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 the diarella Q-Fever real time PCR.

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler 480I	*Coxiella burnetii*	483-533	pre-installed universal CC FAM (510) – VIC (580)
	Control DNA	523-568	
LightCycler 480II	*Coxiella burnetii*	FAM (465-510)	
	Control DNA	HEX (533-580)	
Stratagene Mx3000P / Mx3005P	*Coxiella burnetii*	FAM	Gain 8 Reference Dye: None
	Control DNA	HEX	Gain 1
ABI 7500	*Coxiella burnetii*	FAM	Option Reference Dye ROX: NO
	Control DNA	JOE	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	*Coxiella burnetii*	Green	Gain 5
	Control DNA	Yellow	Gain 5
Mic qPCR Cycler	*Coxiella burnetii*	Green	Gain 8
	Control DNA	Yellow	Gain 10

13 Data Analysis

The *Coxiella burnetii* 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 *Coxiella burnetii* 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 *Coxiella burnetii* 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 chapter „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.

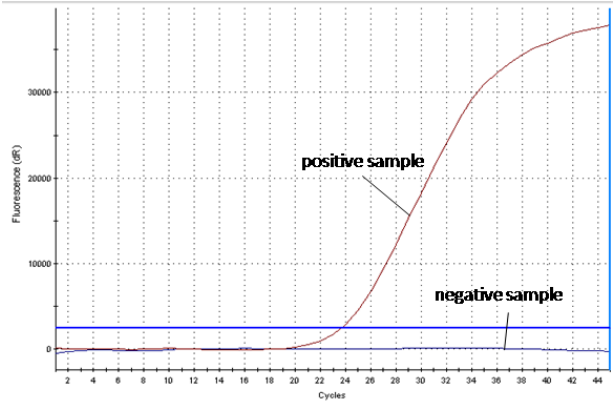

Figure 1: The positive sample shows bacteria specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

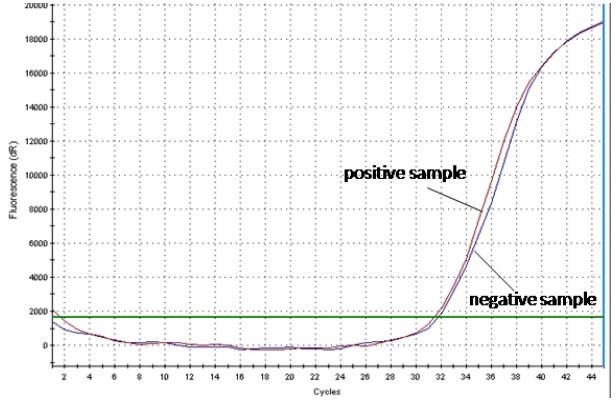

Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the bacteria specific FAM channel is not due to PCR inhibition or failure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

16 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 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 *Coxiella burnetii* specific amplification and the VIC®/HEX/JOETM/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 7.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 9).

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 7).
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 ‚Transport, Storage and Stability
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 diarella Q-Fever real time PCR was determined using serial dilutions of a plasmid containing the *Coxiella burnetii* target sequence using a Stratagene Mx3000 real time PCR instrument. Total nucleic acids were extracted using NukEx Pure RNA/DNA according to the manufacturer´s instructions. Each sample was supplemented with 5 µl Control-DNA prior to extraction. Total nucleic acids were eluted with 50 µl and 4 µl of the eluates were applied to the subsequent real time PCR.
The LoD of diarella Q-Fever real time PCR for *Coxiella burnetii DNA* is ≥10 genome copies per reaction each.

17.2 Analytical Specificity

The specificity of diarella Q-Fever real time PCR was evaluated additionally with different other relevant viruses and bacteria found in clinical samples by in vitro testing (see table 9) and in silico analysis.

Results:
The diarella Q-Fever real time PCR showed a positive result for the sample containing *Coxiella burnetii,* whereas samples containing other pathogens were reliably tested negative. The results are shown in Table 8.

Table 8: Bacterial and viral pathogens tested for the determination of the analytical sensitivity of the diarella Q-Fever real time PCR Kit.

Strain	Expected Result	Result
Enterovirus 68	negative	negative
Coxsackievirus B3	negative	negative
Coxsackievirus A16	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
TBE-Virus	negative	negative
Ehrlichia chaffeensis	negative	negative
Ehrlichia ewingii	negative	negative

Ehrlichia canis	negative	negative
Ehrlichia phagocytophilum	negative	negative
Anaplasma platy	negative	negative
Babesia divergens	negative	negative
Babesia microti	negative	negative
Babesia sp. EU1	negative	negative
Borrelia burgdorferi Strain 4681	negative	negative
Zikavirus	negative	negative
Coxiella burnetti	positive	positive

In silico analysis of the primers and probes used in diarella Q-Fever real time PCR showed no cross-reaction of the complete system with any other known genome sequences.

17.3 Diagnostic Sensitivity and Specificity

During the validation study of the diarella Q-Fever real time PCR 34 positive and 62 negative samples were tested. The diagnostic sensitivity was found to be 100% and the diagnostic specificity 100 %.
The positive predictive value was found to be 100%, the negative predictive value showed to be 100 %.

Table 9: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	negative samples
diarella Q-Fever positive	34	0
diarella Q-Fever negative	0	62

Sensitivity	100 %
Specificity	100 %

Furthermore, the German Ring Trial samples (RV 542) from 2011 – 2016 (10 Ring Trials) were tested retrospectively with 100% correct results.

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

19 Literature

[1] Bundesgesundheitsblatt 2013, 56: 1178 - 1190 Coxiella burnetii – Erreger des Q (query) – Fiebers.

[2] Arricau-Bouvery, N., Rodolakis, A., 2005. Is Q Fever an emerging or re-emerging zoonosis? Vet. Res. 36, 327-349.

[3] Fretz, R., Schaeren, W., Tanner, M., Baumgartner, A., 2007. Screening of various foodstuffs for occurrence of Coxiella burnetii in Switzerland. Int. J. Food Microbiol. 116, 414-418.