Instruction for Use

virellaTBE

real time RT-PCR Kit LC

For the *in-vitro* detection of TBE Virus RNA in clinical specimens and in ticks.

REF	G01065-32	G01065-96
Σ	32	96
- 18°C		

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1 Intended Use

The virellaTBE real time RT-PCR LC is an assay for the detection of TBE Virus RNA in clinical specimens and in ticks by real time RT-PCR using real time RT-PCR in the capillary system of the LightCycler® 1.5 or 2.0 (Roche Diagnostics).

2 Pathogen Information

Tick-borne encephalitis (TBE) is a disease caused by the tick-borne encephalitis virus. The disease pattern includes flu-like symptoms and fever. TBE most often manifests as meningitis, encephalitis or meningoencephalitis. However, most patients show no symptoms after infection. The disease is transmitted by the sting of an infected tick, mainly *Ixodes ricinus*.

A causative treatment against TBE is not possible. Beside common precautions like scanning the body for ticks, active vaccination is the most effective method for preventing TBE. Vaccination is recommended for all persons in high-risk areas.

Reliable diagnosis can be made on the basis of symptoms, course of disease, anamnesis and serological findings.

To better evaluate the risk of infection after the sting of a tick, the tick can be tested by real time RT-PCR for the presence of TBE Virus RNA.

There is no curative therapy for TBE. In severe cases interferons are administered. Alltogether the therapy is restricted to symptomatic measures. Bed rest and dimout of the sick room can help to avoid complications.

3 Principle of the Test

The virellaTBE real time RT-PCR LC Kit contains specific primers and dual-labeled probes for the detection of TBE Virus RNA in clinical specimens and in ticks.

The reverse transcription (RT) of viral RNA to cDNA and the subsequent amplification of virus specific fragments are performed in an one-step RT-PCR. The amplification can be detected when specific probes are hydrolysed by the polymerase. The emitted fluorescence is measured in the 530 nm channel (F1 of LightCycler®).

Furthermore, the virellaTBE real time RT-PCR LC Kit contains a Control RNA, which is added during RNA extraction and detected in the same reaction by a differently labeled probe. The Control RNA allows the detection of RT-PCR inhibition and acts as control, that the nucleic acid was isolated from the

clinical specimen. The amplification of the Control RNA is measured in the 705 nm channel (F3 of LightCycler® 1.5, F6 of LightCycler® 2.0).

4 Package Contents

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

Table 1: Components of the virellaTBE real time RT-PCR Kit LC.

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 506 µl	2 x 759 µl
Enzyme	blue	1 x 6,4 µl	1 x 19,2 µl
Positive Control	red	1 x 50 µl	1 x 100 µl
Negative Control	green	1 x 50 µl	1 x 100 µl
Control RNA	colourless	1 x 160 µl	2 x 240 µl

5 Equipment and Reagents to be Supplied by User

- RNA isolation kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004) or NukEx Nucleic Acid Release Reagent Kit (gerbion Cat. No. G01013)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument LightCycler® 1.5 or 2.0
- LightCycler® Capillaries
- Optional: Liquid handling system for automation
- Optional: VLP-RNA for tick analysis (Virus-Like Particles, please look at chapter ‚Control RNA' for details)

6 Transport, Storage and Stability

The virellaTBE real time RT-PCR Kit LC 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 virellaTBE real time RT-PCR Kit LC 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 RT-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 virellaTBE real time RT-PCR Kit LC components of different lot numbers.

9 Sample Material

Starting material for the virellaTBE real time RT-PCR LC is viral RNA isolated or released from clinical specimens (e.g. EDTA-blood, plasma, serum, cerebrospinal fluid and tissue samples) or from ticks.

10 Sample Preparation

The virellaTBE real time RT-PCR LC is suitable for the detection of TBE Virus RNA isolated from clinical specimens or ticks with appropriate isolation methods.

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

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

We recommend the release of RNA from ticks with the gerbion NukEx Nucleic Acid Release Reagent (Cat. No. G01013). This is the fastest and most convenient method for the release of nucleic acid from ticks, because column based purification of the RNA can be omitted.

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 RNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time RT-PCR. Furthermore, possible contaminations during RNA extraction will be detectable.

Please note the chapter 11 ‚Control RNA'.

If the real time RT-PCR is not performed immediately, store extracted RNA and NukEx Nucleic Acid Release Reagent lysates according to the instructions given by the RNA extraction kit's manufacturer.

11 Control RNA

A Control RNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.

Control RNA or VLP-RNA used as Extraction Control:
virellaTBE real time RT-PCR Kit LC Control RNA or VLP-RNA is added to the RNA extraction.
Add 5 µl Control RNA or VLP-RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer's instructions. Please follow protocol A.

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

Control RNA used as Internal Control of the real time RT-PCR:
If only inhibition will be checked please follow protocol B.

RNA isolation from ticks
The use of VLP-RNA is recommended if NukEx Nucleic Acid Release Reagent is used for sample preparation (e.g. for nucleic acid release from ticks), because in this case Control RNA cannot be used as extraction control. Please follow protocol A.

12 Real time RT-PCR

12.1 Important Points Before Starting::

- Please pay attention to the chapter 7 ‚Important Notes'.
- Before setting up the real time RT-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 at least one Positive Control and one Negative Control should be included.
- Before each use, all reagents - except the Enzyme - should be thawed completely at room temperature, thouroughly 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 RNA or VLP-RNA is used to control both the real time RT-PCR and the RNA isolation procedure, please follow protocol A. If the Control RNA is solely used to detect possible inhibition/failure of the real time RT-PCR, please follow protocol B.

Protocol A

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

The Master Mix contains all of the components needed for RT-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 RNA was added during RNA extraction)

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

Protocol B

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

The Master Mix contains all of the components needed for real RT-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 RNA **1:10** in PCR grade Water (e.g. 1 µl Control RNA + 9 µl PCR grade Water) before adding to the Master Mix.

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

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Control RNA * **(diluted 1:10)**	0.2 µl x (N+1)*
0.2 µl Enzyme	0.2 µl x (N+1)

*The increase in volume caused by adding the Control RNA is not taken into account when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Protocol A and B: real time RT-PCR set up

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

Table 4: Preparation of the real time RT-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 RT-PCR use the thermal profile shown in Table 5.

Table 5: real time RT-PCR thermal profile

Step	Time	Temperature	Number of Cycles
Reverse Transcription	20 min	45 °C	1
Initial Denaturation	5 min	95°C	1
Amplification of cDNA			
Denaturation	10 sec	95°C	45
Annealing	20 sec Aquisition mode SINGLE	57°C	
Extension	15 sec	72°C	
Cooling	5 sec	40°C	

Ramping time for all steps 20°C/sec.

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

Table 6: Overview of the instrument settings required for the virellaTBE real time RT-PCR LC.

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler® 1.5	TBE Virus	F1 (530 nm)	Color Compensation Kit needed, e.g. LightCycler® Color Compensation Set
	Control RNA	F3 (705 nm)	
LightCycler® 2.0	TBE Virus	F1 (530 nm)	
	Control RNA	F6 (705 nm)	

13 Data Analysis

The virus specific amplification is measured in the 530 nm channel (F1 of LightCycler® 1.5 and 2.0). The amplification of the Control RNA is measured in the 705 nm channel (channel F3 of LightCycler® 1.5; channel F6 of LightCycler® 2.0).

Following results can occur:

- A signal in the F1 is detected:
  The result is positive, the sample contains TBE Virus RNA.
  In this case, detection of a signal of the Control RNA in F3 (LightCycler® 1.5) or F6 (LightCycler® 2.0) is inessential, as high concentrations of cDNA may reduce or completely inhibit amplification of the Control RNA.

- No signal in F1, but a signal in F3 (LightCycler® 1.5) or F6 (LightCycler® 2.0) is detected:
  The result is negative, the sample does not contain TBE Virus RNA.
  The signal in channel F3 (LightCycler® 1.5) or F6 (LightCycler® 2.0) excludes the possibilities of RNA isolation failure (in case the Control RNA is being used as an extraction control) and/or real time RT-PCR inhibition. If the C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occurred, which can lead to negative results in weak positive samples (see chapter ‚Troubleshooting').

- Neither in F1 nor in F3 (LightCycler® 1.5) or F6 (LightCycler® 2.0) a signal is detected:
  A diagnostic statement cannot be made.
  The RNA isolation was not successful or an inhibition of the RT-PCR has occurred. In case the Control RNA was added during RNA 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 RT-PCR results.

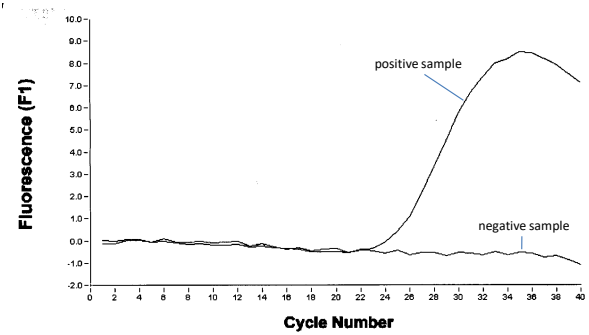

Figure 1: The positive sample shows virus-specific amplification in the F1 channel, whereas no fluorescence signal is detected in the negative sample.

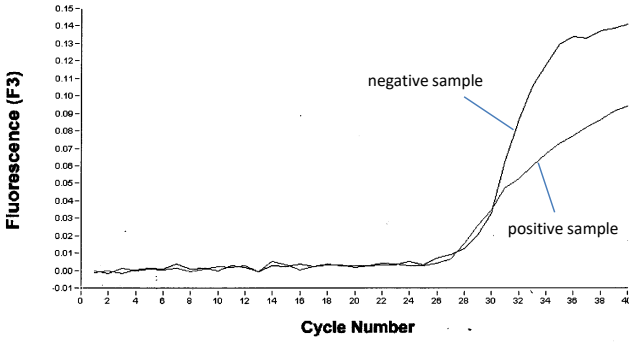

Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA-specific channel (F3 or F6). The amplification signal of the Control RNA in the negative sample shows, that the missing signal in the virus-specific channel (F1) is not due to real time RT-PCR inhibition or failure of RNA 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 TBE Virus infection.

16 Troubleshooting

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

No fluorescence signal in the F1 channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the F1 channel for analysis of the virus specific amplification and either the 640 nm or the 705 nm channel (LightCycler® 1.5: F2 or F3; LightCycler® 2.0: F4 or F6) for the amplification of the Control RNA.
Incorrect configuration of the real time RT-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 RNA and simultaneous absence of a signal in the virus specific F1 channel	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 9).
real time RT-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 wash buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the RNA.
RNA loss during isolation process	In case the Control RNA was added before extraction, the lack of an amplification signal can indicate that the RNA 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 F1 channel of the Negative Control	
Contamination during preparation of the RT-PCR	Repeat the real time RT-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 RT-PCR.

17 Kit Performance

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the virellaTBE real time RT-PCR Kit LC 32 positive and 98 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 7: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	negative samples
virellaTBE positive	32	0
virellaTBE negative	0	98
Sensitivity	100%	
Specificity	100%	

17.2 Analytical Sensitivity

The limit of detection (LoD) of virellaTBE real time RT-PCR Kit LC was determined using serial dilutions of TBE-Virus cell culture supernatant in culture medium. Total nucleic acids were extracted using NukEx Pure RNA/DNA according to the manufacturer´s instructions. Each sample (200 µl of diluted supernatant) was supplemented with 5 µl Control-RNA prior to extraction. Total nucleic acids were eluted with 50 µl and 4 µl of the eluates were applied to the subsequent real time RT-PCR.

The LoD of virellaTBE real time RT-PCR Kit LC for *TBE-Virus* is $\leq$0.02 TCID 50 per reaction each.

Table 8: Samples tested for the validation of the sensitivity of the virellaTBE real time RT-PCR Kit LC.

Sample	TCID50/ml	Expected Result	CT-value *virellaTBE LC*	Mean CT
K617 10-2	100.000	positive	23.14 23.27	23.21
K617 10-3	10.000	positive	26.75 25.86	26.30
K617 10-4	1.000	positive	30.51 29.64	30.08
K617 10-5	100	positive	33.64 34.37	34.01
K617 10-6	10	positive	37.22 36.45	36.84
K617 10-7	1	positive	39.57 40.29	39.93

17.3 Analytical Specificity

The specificity of virellaTBE real time RT-PCR Kit LC was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:

The virellaTBE real time RT-PCR Kit LC showed a positive result for the sample containing *TBE-Virus,* 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 sensitivity of virellaTBE real time RT-PCR Kit LC.

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
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
Borrelia afzelii	negative	negative
Treponema phagedenis	negative	negative
Borrelia miyamotoi	negative	negative
Borrelia bavariensis	negative	negative
Borrelia garinii Ospa Typ 8	negative	negative
Borrelia kurtenbachii	negative	negative
TBE-Virus	positive	positive

18 Abbreviations and Symbols

cDNA	complementary Deoxyribonucleid Acid
RNA	Ribonucleid Acid
PCR	Polymerase Chain Reaction
RT	Reverse Transcription
TBE	Tick-borne encephalitis
TCID50	Tissue Culture Infective Dose 50%
REACTION MIX	Reaction Mix
ENZYME	Enzyme
CONTROL +	Positive Control
CONTROL −	Negative Control
CONTROL RNA IC	Control RNA

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

19 Literature

[1] Fischer, M. et al. Tickborne Encephalitis. CDC 24/7, 2016.

[2] Robert-Koch-Institut, RKI. FSME - Fruehsommermeningoencephalitis. http://www.rki.de/DE/Content/Infekt/EpidBull/Merkblaetter/Ratgeber_FSME.html