


Instruction for Use

virellaTBE

real time RT-PCR Kit TM

For qualitative *in vitro* detection of TBE Virus RNA, extracted from clinical specimens and ticks.

REF	G01066-32	G01066-96
	32	96



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1 Intended Use

The virellaTBE real time RT-PCR TM is an assay for the detection of TBE Virus-RNA, extracted from clinical specimens and ticks.

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. Altogether 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 Kit TM contains specific primers and dual-labeled probes for the amplification and detection of TBE Virus RNA, extracted from clinical specimens.

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, virellaTBE real time RT-PCR Kit TM 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 fluorescence of the Control RNA 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 virellaTBE real time RT-PCR Kit TM.

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 506 μ l	2 x 759 μ l
Enzyme	blue	1 x 6.4 μ l	1 x 19.2 μ l
Positive Control	red	1 x 50 μ l	1 x 100 μ l
Negative Control	green	1 x 50 μ l	1 x 100 μ l
Control RNA	colourless	1 x 160 μ l	2 x 240 μ l

5 Equipment and Reagents to be Supplied by User

- RNA 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 virellaTBE real time RT-PCR Kit TM 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 $+2-8^{\circ}\text{C}$ 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 TM 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.
- Regularly decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine virellaTBE real time RT-PCR Kit TM components of different lot numbers.

9 Sample Material

Starting material for the virellaTBE real time RT-PCR TM is viral RNA extracted 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 TM 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

It is recommended to use mechanical disruption of ticks before RNA 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 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 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.

RNA isolation from clinical specimens

a) Control RNA used as Extraction Control:

virellaTBE Control RNA is added to the RNA extraction.

Add 5 µl Control 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.

b) Control RNA used as Internal Control of the real time RT-PCR:

If only inhibition will be checked please follow protocol B.

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 RT-PCR set up.
- In every RT-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 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 of the real time RT-PCR, please follow protocol B.

Protocol A

The Control 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 it 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 Enzyme	0.2 μl x (N+1)
0.2 μl Control RNA* (diluted 1:10)	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 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 RNA isolation (including the eluate of the water control) or NukEx Nucleic Acid Release Reagent lysates, 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 RT-PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

For the real time RT-PCR use the thermal profile shown in Table 5.

Table 5: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
<i>Reverse Transcription</i>	20 min	45°C	1
<i>Initial Denaturation</i>	5 min	95°C	1
<i>Amplification</i>			
Denaturation	10 sec	95°C	45
Annealing	20 sec	60°C	
	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 6.

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

Real time RT-PCR Instrument	Parameter	Detection Channel	Notes
LightCycler 480I	TBE	483-533	Color Compensation Kit needed, e.g. pre-installed universal CC FAM (510) – VIC (580)
	Control RNA	523-568	
LightCycler 480II	TBE	FAM (465-510)	VIC (580)
	Control RNA	HEX (533-580)	
Stratagene Mx3000P / Mx3005P	TBE	FAM	Gain 8 Reference Dye:
	Control RNA	HEX	Gain 1 None
ABI 7500	TBE	FAM	Option Reference Dye ROX: NO
	Control RNA	JOE	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	TBE	Green	Gain 5
	Control RNA	Yellow	Gain 5
Mic qPCR Cyclcr	TBE	Green	Gain 8
	Control RNA	Yellow	Gain 10

13 Data Analysis

The virus specific amplification is measured in the FAM channel. The amplification of the Control RNA 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 TBE Virus RNA.
In this case, detection of a signal of the Control RNA in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of cDNA may reduce or completely inhibit amplification of the Control RNA.
- **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 TBE Virus RNA.
The signal of the Control RNA 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 „Troubleshooting“).
- **Neither in the FAM nor in the VIC®/HEX/JOE™/TET channel 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.

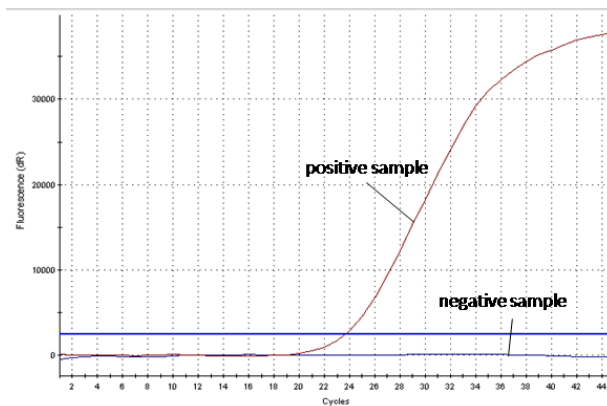


Figure 1: The positive sample shows virus-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

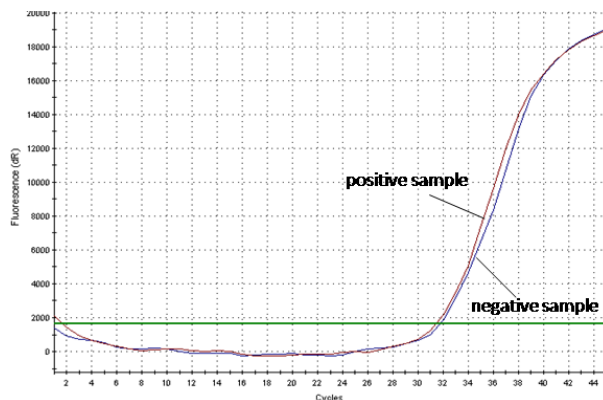


Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA-specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control RNA in the negative sample shows, that the missing signal in the virus-specific FAM channel is not due to RT-PCR inhibition or failure 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 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 virus specific amplification and the VIC®/HEX/JOETM/TET channel 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 FAM channel.

real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 7).
real time RT-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 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 FAM 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 occurred 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 TM 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 TM was determined using serial dilutions of a cell culture supernatant containing TBE virus K617 in a Stratagene Mx3000 real time PCR instrument. The results of the determinations in duplicates are shown in Table 8.

The LoD of virellaTBE real time RT-PCR Kit TM is $\geq 0,016$ TCID₅₀ per reaction each.

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

TCID50 per reaction	C _T -value FAM	mean C _T FAM
16	27,19 27,55	27,37
1,6	30,28 30,21	30,25
0,16	33,04 32,83	32,94
0,016	36,56 36,51	36,54
0,0016	45,00 40,78	42,89
0,00016	38,09 45,00	42,70

17.3 Analytical Specificity

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
















Results:

The virellaTBE real time RT-PCR Kit TM 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 specificity of virellaTBE real time RT-PCR Kit TM.

Strain	Expected Result	Result
<i>Enterovirus 68</i>	negative	negative
<i>Coxsackievirus B3</i>	negative	negative
<i>Coxsackievirus A16</i>	negative	negative
<i>Coxsackievirus B5</i>	negative	negative
<i>Influenza Virus A A/ Brisbane H1N1 59/2007 E40/08</i>	negative	negative
<i>Influenza Virus A Indonesia H5N1 05/2005</i>	negative	negative
<i>Influenza Virus A Panama H3N2 2007/99</i>	negative	negative
<i>Influenza Virus B B/ Brisbane 60/2008 E09/09</i>	negative	negative
<i>Ehrlichia chaffeensis</i>	negative	negative
<i>Ehrlichia ewingii</i>	negative	negative
<i>Ehrlichia canis</i>	negative	negative
<i>Anaplasma phagocytophilum</i>	negative	negative
<i>Anaplasma platy</i>	negative	negative
<i>Babesia divergens</i>	negative	negative
<i>Babesia microti</i>	negative	negative
<i>Babesia sp. EU1</i>	negative	negative
<i>Borrelia burgdorferi Strain 4681</i>	negative	negative
<i>Borrelia afzelii</i>	negative	negative
<i>Treponema phagedenis</i>	negative	negative
<i>Borrelia miyamotoi</i>	negative	negative
<i>Borrelia bavariensis</i>	negative	negative
<i>Borrelia garinii Ospa Typ 8</i>	negative	negative
<i>Borrelia kurtenbachii</i>	negative	negative
<i>Coxiella burnetii</i>	negative	negative
<i>TBE-Virus</i>	positive	positive
West Nile Virus	negative	negative
Zika Virus MR766	negative	negative
Chikungunya Virus S27 Afrika	negative	negative
Yellow Fever Virus 17D	negative	negative
Dengue Virus 1	negative	negative
Dengue Virus 2	negative	negative
Dengue Virus 3	negative	negative
Dengue Virus 4	negative	negative

18 Abbreviations and Symbols

cDNA	complementary Deoxyribonucleid Acid		Catalog number
RNA	Ribonucleid Acid		Contains sufficient for <n> test
PCR	Polymerase Chain Reaction		Upper limit of temperature
RT	Reverse Transcription		Manufacturer
TBE	Tick-borne encephalitis		Use by YYYY-MM-DD
TCID50	Tissue Culture Infective Dose 50%		Batch code
	Reaction Mix		Content
	Enzyme		Consult Instructions for Use
	Positive Control		<i>In vitro</i> diagnostic medical device
	Negative Control		European Conformity
	Control RNA		

19 Literature

- [1] Fischer, M. et al. Tickborne Encephalitis. CDC 24/7, 2016.
- [2] Robert-Koch-Institut, RKI. FSME - Frühsommermeningoencephalitis.
http://www.rki.de/DE/Content/Infekt/EpidBull/Merkblaetter/Ratgeber_FSME.html