Instructions for Use

RealLine TBEV
Fla-Format

KIT FOR THE QUALITATIVE DETECTION OF RNA FROM TICK-BORNE ENCEPHALITIS VIRUS BY REVERSE TRANSCRIPTION AND REAL TIME PCR

In vitro Diagnostics

CE

RealLine TBEV (Fla-Format)	VBD1199	50 Tests
valid from	September 2019	

RealLine TBEV
Fla-Format

Explanation of symbols used in labeling

IVD	*In vitro* diagnostic medical device
LOT	Batch code
REF	Catalogue number
Σ	Contains sufficient for <n> tests
	Use-by-date
	Temperature limit
	Consult instructions for use
	Keep away from sunlight
	Manufacturer

BIORON Diagnostics GmbH

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Trademarks:
Rotor-Gene® is a registered trademark of Qiagen Group, Germany.

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1. INTENDED USE

Clinical information:

TBEV is endemic in an area ranging from northern China and Japan, through far-eastern Russia to Europe. TBEV is transmitted by the bite of infected ticks (Ixodes scapularis, Ixodes ricinus and Ixodes persulcatus), or (rarely) through the non-pasteurized milk of infected cows, sheeps and goats. TBE is a viral infectious disease involving the central nervous system (CNS). The disease is incurable once manifested, so there is no specific drug therapy for TBE.

RealLine TBEV assay kit is intended for the detection of the tick-borne encephalitis virus (TBE) RNA using reverse transcription of viral RNA and real-time polymerase chain reaction (RT-PCR) method with fluorescent detection of amplified product.

The kit is designed for the analysis of RNA extracted from clinical specimens (whole blood, serum (plasma), leukocyte blood fraction, cerebrospinal fluid, biopsy materials and tick suspensions). The extraction of RNA from clinical specimens can be performed using the **RealLine Extraction 100** kit.

The results of PCR analysis are taken into account in complex diagnostics of disease.

The **Fla-format** Kit contains 5 vials with the lyophilized Mastermix, each vial with 10 reactions, for volume of 50 µl per reaction. The sample kit contains reagents required for 50 tests, including the positive control samples.

The kit is intended for use with block cyclers iQ™ iCycler, iQ5™ iCycler, CFX96™ (*Bio-Rad, USA*), RealLine Cycler (*BIORON Diagnostics GmbH*), DT96 (*DNA-Technology, Russia*); and rotor type cyclers Rotor-Gene® 3000, 6000 and Q (*Qiagen, Germany*).

The use of:

- ! **Extraction Kits for nucleic acids from clinical specimen from other supplier**
- ! **other real-time PCR devices**
- ! **appropriate reaction volumes, other than 50 µl**

has to be validated in the lab by the user. The special notes regarding the internal control IC have to be strongly followed.

2. KIT CONTENTS

Positive Control sample (PC)	1 vial, 1 ml
Master Mix (MM), lyophilized	5 tubes (10 tests each)
Recovery Solution (RS)	1 vial, 2 ml
Solution for Sample Preparation (SSP)	4 vials, 4 ml each

3. PRINCIPLE OF THE METHOD

The assay is based on reverse transcription of a selected region of tick-borne encephalitis virus RNA in combination with amplification of the cDNA being formed. The amplification of the selected cDNA fragment (consisting of repeated cycles: thermal denaturation, hybridization of primers with complementary sequences, extension of polynucleotide sequences from these primers with Taq DNA polymerase) is recorded in real time mode.

Real-time PCR is based on the detection of the fluorescence produced by a reporter molecule, which increases as the reaction proceeds. Reporter molecule is a dual-labeled DNA-probe that specifically binds to the target region of pathogens cDNA. Fluorescence signal increases due to the separation of fluorescence dye and quencher by Taq DNA-polymerase exonuclease activity during amplification. PCR consists of repeated cycles: temperature denaturation of cDNA, primer annealing and complementary chain synthesis.
Threshold cycle value (Ct) is a cycle number at which the fluorescence generated within a reaction crosses the threshold and the fluorescence signal rises significantly above the background. Increased signal is due to the use of a DNA hybridization probe that is specific for the given cDNA sequence: it binds to the cDNA in the course of reaction and provides additional specificity of the method. A DNA probe consists of a fluorescence dye at the 5'-end and a fluorescence quencher at the 3'-end that significantly reduces the fluorescence intensity. During the polymerase synthesis of the complementary strand, the probe is cleaved from the 5'-end due to the 5'-3' nuclease activity of Taq DNA polymerase, the quencher and the dye become separated, thus increasing the fluorescence signal due to accumulation of the reaction product. The detected fluorescence intensity depends on initial quantity of pathogens cDNA template in the specimen.

The use of **Internal Control sample (IC)** prevents generation of false negative results associated with possible loss of cDNA template during specimen preparation. IC indicates if PCR inhibitors occur in the reaction mixture. IC template should be added in each single specimen (including control samples) prior to nucleic acids (NA) extraction procedure. The amplification and detection of IC does not influence the sensitivity or specificity of the target cDNA PCR.

Note: Internal Control sample is added to the specimen during NA extraction step and is used throughout the whole process of NA extraction, amplification, detection.

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4. SPECIFICATIONS

4.1. Sensitivity:

Sensitivity control was performed on five samples containing 100 TBE RNA copies per sample, prepared from CRS (Standard Reference Sample containing TBE RNA, certified by QC Department).
The sensitivity equals 100% (detection of 100 TBE RNA copies in five TBE RNA SRS samples).

4.2. Specificity:

Specificity of TBE RNA detection was determined using 4 negative samples of Standard Serum Panel. Specificity equals 100%.

4.3. Diagnostic sensitivity:

Diagnostic sensitivity of TBE RNA detection: clinical trials conducted on 63 positive samples showed 100 % sensitivity (interval 95.4% -100%, with a confidence level of 90%).

4.4. Diagnostic specificity:

Diagnostic specificity of TBE RNA detection: clinical trials conducted on 127 negative samples showed 100 % specificity (97.7% -100% interval, with a 90% confidence level).

Analysis by the CE-marked reference kit showed full match of results.

5. PRODUCT USE LIMITATIONS

- This assay must not be used on the clinical specimen directly. Appropriate nucleic acids extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors (e.g. heparin) may cause false negative or invalid results.
- When monitoring a patient the same extraction method must be used in all determinations. Otherwise, results may not be comparable.
- The kit is designed for use in patients with a clinical history and/or symptoms consistent with *TBEV.* infections. The kit may be used for screening purposes.
- Diagnostic sensitivity of the kit may vary depending on the pathogen prevalence and characteristics of the enrolled cohort.
- Reliable results depend on adequate specimen sampling.
- Positive results indicate active or asymptomatic infection; clinical history and symptoms should be taken into account.
- Negative results indicate lack of detectable RNA but do not exclude the infection or disease.
- Potential mutations within the target regions of the TBEV genome covered by the primers and/or probes used in the kit may result in failure to detect the presence of the pathogens.
- The kit is not intended to replace culture and other methods (e.g., cervical exam) for diagnosis of infections.

6. WARNING AND PRECAUTIONS

- For in vitro use only.
- The kits must be used by skilled personnel only.
- When handling the kit, follow the national safety requirements for working with pathogens.
- To prevent contamination, the stages of RNA isolation and PCR test run must be spatially separated.
- Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents.
- Every workplace must be provided with its own set of variable-volume pipettes, necessary auxiliary materials and equipment. It is prohibited to relocate them to other workplaces.
- To conduct real-time amplification reaction with PCR products detection, use only disposable tips with filters.
- Never use the same tips for different samples.
- Do not pool reagents from different lots or from different vials of the same lot.
- Dispose unused reagents and waste in accordance with country, federal, state and local regulations.
- Do not use the kit after the expiration date at the side label of the box.

7. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- Real time PCR system, like described in paragraph 1
- NA-Extraction Kit: **RealLine Extraction 100**;
- Internal Control reagent (VBC8881), if the kit is used with the extraction kits of other supplier;
- Negative Control Sample, if the kit is used with the extraction kits of other supplier;
- Plates or Tubes suitable for the used device with caps or a sealing foil for PCR
- Laminar safety box;
- Refrigerator;
- Microcentrifuge for 1.5-2 ml tubes;
- Vortex mixer with adjustable rotation speed;
- Half-automatic variable-volume single-channel pipettes with disposable tips;
- Disposable medical non-sterile powder-free gloves;
- Disposable pipette tips with filters;
- Biohazard waste container.

8. PREPARATION OF SPECIMENS

*Each group of samples undergoing the procedure of RNA isolation must include a **Positive Control sample (PC)** from this kit and a **Negative Control sample (NC)** that is a component of the NA extraction kit.*

The Internal Control IC, the Negative Control NC and Positive Control PC samples must be implemented to the extraction procedure.

When using a kit of another supplier for the extraction of nucleic acids as recommended in p1., add **20 µl** of **IC (VBC8881)** to each tube:

- For the Negative Control NC use **100 µl** of the Negative Control Sample
- For the PC use **70 µl** of Negative Control Sample and **30 µl** of Positive Control to the tube marked PC.

The assay is performed on extracted RNA specimens obtained from the clinical material using one of the NA extraction kits listed in p.1, according to the Instruction Manual to the kit. To prepare tick suspension, SSP is used (see Annex I).

Extracted RNA cannot be stored! Perform RNA extraction from clinical material immediately before running the RT-PCR*

9. PROCEDURE

9.1. Preparation of the reagents.

Open the package, take the necessary number of tubes with the **Master Mix for RT-PCR (MM)**, taking into account prepared specimens and control samples. 1 NC and 1 PC for each test is required. Keep the tubes at (18 – 25) °C for at least 30 min.

Put the remaining tubes immediately back into the foil pouch, squeeze the air out and tightly close with the clip.
Attention! Each tube is intended for 10 tests.

After initial opening, store PC at (2 – 8) °C for no more than 1 month, or in 50 µl aliquots at minus (18 – 60) °C for no more than 3 months.

After initial opening, store MM at (2 – 8) °C for no more than 3 months.

To prepare diluted Master Mix, add **300 µl** of RS to each tube with MM. Mix gently, keep at (18 - 25) °C for 15 min, and mix thoroughly. Spin the tubes to collect any drops.

Do not store, use the recovered MM on the day of the assay.
After initial opening, store RS at (2 – 8) °C for no more than 3 months.

9.2. Prepare an appropriate number of 0.2 ml tubes or a plate. (including necessary controls).
Attention! *Labels should be placed on the caps of tubes for rotor-type cyclers. For block-cyclers labels should be placed on the lateral side of the tubes.*

9.3. Add **25 µl** of recovered MM to all test tubes.

9.4. Add **25 µl** of corresponding isolated RNA solution to each tube using a separate pipette tip with filter. Add **25 µl** of NC and PC to the corresponding tubes. Tightly close the tubes with caps.

9.5. Place the tubes into the real-time PCR system

9.6. Program real time PCR system as follows:

For Rotor-Gene® 3000 (6000, Q):

Stage 1:	45 °C	30 min	
Stage 2:	95°C	2 min	
Stage 3:	94°C	10 sec	50 cycles
	60°C*	40 sec	

* Measure the fluorescence at 60°C

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For iQ™ iCycler, iQ™5 iCycler, CFX™96, DT-96:

Stage 1:	45 °C	30 min	
Stage 2:	95°C	2 min	
Stage 3:	94°C	10 sec	50 cycles
	60°C*	20 sec	

* Measure the fluorescence at 60°C

9.7. Select the amplification detection channels:

- Collect data through **FAM** channel (iQ5 iCycler, RealLine Cycler, CFX96, DT-96, Rotor-Gene 3000) and ***Green*** channel (Rotor-Gene 6000, Rotor-Gene Q) for the detection of amplification signal of IC cDNA;

- Collect data through **ROX** channel (iQ5 iCycler, CFX96, DT-96, Rotor-Gene 3000), and ***Orange*** channel (Rotor-Gene 6000, Rotor-Gene Q) for the detection of amplification signal of TBE cDNA.

9.8. Program the positions of test tubes with samples, positive and negative controls according to the instruction manual for the real time PCR system in use.

9.9. Run the program.

10. DATA ANALYSIS AND INTERPRETATION

10.1 For **NC** the program should detect the increase of the amplification signal of IC cDNA in channel **FAM** (*Green*) and determine the **Ct** IC. The program must not detect the signal rise of the TBEV cDNA amplification via **ROX** (*Orange*) channel.

10.2 For **PC** the program should detect:

- increase of the amplification signal of IC cDNA in channel **FAM (*Green*)** and determine the threshold cycle, **Ct** IC;
- increase of the signal of a specific amplification product of TBEV cDNA via channel **ROX (*Orange*)** and determine the threshold cycle, **Ct** PC.

10.3 For each specimen the program should detect the increase of the amplification signal of IC cDNA and determine **Ct** IC.

10.4 Calculate (**Ct** IC)$_{av}$ as an average **Ct** IC of all analyzed samples (including PC and NC). **Ct** IC values that differ by more than 2 from the (**Ct** IC)$_{av}$ should be ignored. Recalculate the (**Ct** IC)$_{av}$ for the remaining values after the screening.

10.5 The sample is considered **negative** (not containing **TBEV cDNA**), if **Ct** value via **ROX** (*Orange*) channel for this sample is **above 40** or is not determined.
When **Ct** IC value for such sample differs from the (**Ct** IC)$_{av}$ value by more than 2 cycles, the result is not regarded as negative. A repeated analysis of the sample, starting with the isolation step is necessary.

10.6 The sample is considered **positive** (containing **TBEV cDNA**) when **Ct** value via **ROX** (*Orange*) channel for this sample is **less than or equal to 40.**

10.7 When **Ct** NC value via the **ROX** (*Orange*) channel **is less than or equal to 40,** this indicates the presence of contamination. In this case all positive results of the individual PCR run are considered unreliable. Actions are required to identify and eliminate the source of contamination, and repeat the analysis of all samples of this run that were identified as positive. Samples that showed negative results in this run should be considered as negative.

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11. STORAGE AND TRANSPORTATION

- Store the assay kit at (2 - 8) °C in the manufacturer's packing.
- Transport at (2 - 8) °C. Transportation at up to 25 °C for up to 10 days is allowed.
- Do not freeze the kit!
- Do not pool reagents from different lots or from different vials of the same lot.
- Strictly follow the Instruction manual for reliable results.
- Do not use kits with damaged inner packages and get in contact with BIORON Diagnostics GmbH.

- **Storage and shelf life of solutions and components of the kit after initial opening:**
  Positive Control sample: 1 month at (2 – 8) °C *or in 50 µl aliquots at minus (18 – 60) °C for no more than 3 months*.
  Master Mix (MM): unused MM at (2 – 8) °C up to 3 month.
  Recovery Solution: at (2 – 8) °C for 3 months.

Technical Support: techsupport@bioron.de

ANNEX I: PREPARATION OF TICK SUSPENSIONS

Equipment required:

- Laminar box;
- 1.5 ml tube rack;
- IsoFreeze double-sided refrigerator SSI-5610-43 or floating stand SSI-5100-43 ("SSI", USA);
- Cooling element or a container with ice;
- Metal pestles for grinding ticks.

Place ticks into the numbered 1.5 ml Eppendorf type tubes.
To clean the ticks from contamination by substances used for the removal of attached insects, wash them before preparation of suspension (see p. a). In case of analysis of free ticks, the suspension can be prepared immediately, (see p. b or c).

a) Preliminary washing of ticks:

Add 300 µl of 96% ethanol to each tube with a tick, vortex the tubes, and then centrifuge briefly. Remove ethanol using a pipette or an aspirator without touching the tick, using separate tips for each specimen. Add 500 µl of 0.15 M sodium chloride to tubes, vortex the tubes and spin for 5 sec at 5000 rpm to collect any drops; discard the supernatant using a pipette or an aspirator with a new tip for each specimen.

b) Preparation of tick suspensions:

Attention! *Ticks that died recently as well as dried or crushed ticks are suitable for PCR analysis.*
To prevent degradation of nucleic acids (NA) extracted from the ticks, avoid heating of the sample to a temperature above 8 °C at all stages of ticks suspension preparation.
Before analysis, full ticks, which diameter is comparable to that of the tube, should be pierced with a disposable needle or a pipette tip.
When preparing the ticks for the analysis use SSP (Solution for Sample Preparation (SSP) – is a component of some of the kits) pre-cooled to a temperature of (2 - 8) °C.

Method 1. Freeze the tubes with ticks in liquid nitrogen (for at least 5 min). Take one frozen tube and immediately carefully grind the tick with a separate sterile pestle combining rotational movements and pressing. Without removing the pestle, put the tube with the crushed tick into the rack, placed on ice. Add 250 µl of pre-chilled SSP to the tube. Gently rinse the pestle in the tube and discard it into disinfectant solution.
Vortex the tube for 5-10 sec. Centrifuge briefly to collect the drops from the inside tube walls. Perform the grinding procedure with other specimens. Without touching the pellet, take 100 µl of the specimen for nucleic acids extraction and further assay.

Method 2. Add 30 µl of SSP to the tubes with ticks (for full or large ticks add 50 µl of SSP). Place the tubes into liquid nitrogen or pre-chilled thermal rack and keep until completely frozen in a freezer at minus (20-30) °C. Take one tube with the tick frozen in the SSP, and crush the tick thoroughly with a sterile pestle before the solution thaws. Without removing the pestle, put the tube with the crushed tick into the rack, placed on ice. Add 200 µl of pre-chilled SSP to the tube. Gently rinse the pestle in the tube and discard it into disinfectant solution.
Vortex the tube for 5-10 sec. Centrifuge briefly to collect any drops from the inside tube walls. Without touching the pellet, take 100 µl of the specimen for nucleic acids extraction and further assay. Perform the grinding procedure with other specimens.

c) Tick suspension preparation using MagNA Lyser (Roche Diagnostics, Switzerland) or similar homogenizer:

Prepare a tube with ceramic beads for each specimen; place the tubes into pre-chilled thermal rack. Add a tick and 300 µl of SSP to each tube and close the tubes tightly. Place the tubes into the homogenizer's rotor (up

to 16 tubes) and fix the rotor according to the Instruction manual. Homogenize specimens for 90 sec at 7000 rpm.

Attention! To ensure biological safety and prevent contamination, before opening the homogenizer, check through the window in the lid that no leakage of material has occurred.

If droplets or aerosols appear in the working chamber, the homogenizer should be moved to the laminar box and kept with closed lid for 30-40 min. Open the homogenizer and process the device in accordance with the instructions for handling potentially infectious material and the disinfection instruction given in the device manual.

After disinfection, remove the tubes from the device and place into pre-chilled thermal rack. In case of insufficient grinding of individual specimens, repeat the homogenization procedure.

Discard the drops of the suspension from the walls of the tubes by brief centrifugation. To extract nucleic acids using the **RealLine Extraction 100** kit, take 100 µl of the supernatant.

Attention! Add additional 300 µl SSP after homogenization when working with large ticks (i.e. ticks larger than 5 mm in diameter, representatives of the genera *Dermacentor* and *Hyalomma*, full ticks).

Transportation and storage of ticks and tick suspension specimens:

- At (2 - 8) °C – for no more than 24 hours;
- At minus (18 - 60) °C – for no more than 2 weeks.
- At minus 70 °C and below – for no more than 1 year.

Do not freeze - thaw specimens repeatedly!

ANNEX II: Settings for RealLine Cycler and DT96:

for these cyclers the measurement exposure must be adjusted. Choose the **Operation with the device** mode in the **Settings** menu, select the item **Measurement exposition:**

- **FAM** to **250**
- **ROX** to **1000**

Confirm that the current exposure value is saved by pressing **YES**

Attention! The specified exposure values are applicable only for RealLine kits and, if necessary, must be changed for other purposes.

ANNEX III PROGRAMMING AND ANALYSIS USING ROTOR-TYPE CYCLERS:

Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q (Qiagen, Germany)

Hereinafter, detection channels and terms corresponding to different versions of PCR cyclers and software are listed in the following order: Rotor-Gene 3000 (Rotor-Gene 6000, Rotor-Gene Q).

Program real-time PCR cycler.

1) Click **"New"** button.
2) Select **"Advanced"** template from the tab of the New Run wizard. Click "**New"** button.
3) Select **36-Well Rotor** type, check that No Domed 0.2 ml Tubes are used. Click **"Next"** button.
4) In the new window determine Reaction volume as **50 µl**. Click **"Next"** button.
5) The temperature profile of real-time PCR should be set. Click **"Edit Profile"** button.

Stage 1:	45 °C	30 min	
Stage 2:	95°C	2 min	
Stage 3:	94°C	10 sec	50 cycles
	60°C*	40 sec	

* Measure the fluorescence at 60°C

6) Then temperature profile is set, click **"OK"** button.

7) In the **"New Run Wizard"** window, click **"Calibrate"** (**"Gain optimization"**) button. The window **"Auto Gain Calibration Setup"** opens. In the line **"Channel Settings"** choose **"ROX"** (Orange), click **"Add"**. Set **"Tube Position"** 1, **"Min Reading"** 5, **"Max Reading"** 10, click **"OK"**. In the line **"Channel Settings"** choose **"FAM"** (Green), click **"Add"**. Set **"Tube Position"** 1, **"Min Reading"** 5, **"Max Reading"** 10, click **"OK"**.
8) Tick off **"Perform Calibration Before 1st Acquisition"**. Click **"Close"** button.
9) Click **"Next"** button, start the amplification process by clicking **"Start Run"** button.
10) Save a file in the Rotor-Gene/templates folder, named RealLine with *.ret extension. In subsequent work RealLine template would be presented in New run wizard.
11) Save reaction result file with Rotor-Gene Run File *.rex extension.
12) Record the positions of the control samples and specimens according to the instruction manual of the PCR cycler in use. Click **"Start run"** button.

Results of Internal Control cDNA amplification

1) Click **"Analysis"** button, choose **"Quantitation"** from the list, choose **"Cycling A. FAM"** ("Cycling A. Green"**)**, click **"Show"** button.
2) Click **"OK"** button, and cancel automatic **Threshold** determination.
3) Click **"Linear scale"** button. Settings should change to **"Log. scale"**.
4) In the **Quantitation analysis** menu buttons **"Dynamic tube"** and **"Slope Correct"** should be pressed.
5) Click **"More Settings"** (Outlier Removal) **button,** determine **NTC threshold** value as **5 %**.
6) In the column **"CT Calculation"** (right part of the window) determine **"Threshold"** value as **0,04**.
7) In the result table (**"Quant. Results"** window) **Ct** will be displayed.

Results of TBE cDNA amplification

1) Click **"Analysis"** button, choose **"Quantitation"** from the list, choose **"Cycling A. ROX"** ("Cycling A. Orange"**)** click, **"Show"** button.
2) Click **"OK"** button, and cancel automatic **Threshold** determination.
3) Click **"Linear scale"** button. Settings should change to **"Log. scale"**.
4) In the **Quantitation analysis** menu buttons **"Dynamic tube"** and **"Slope Correct"** should be pressed.
5) Click **"More Settings"** (Outlier Removal) **button,** determine **NTC threshold** value as **5 %**.
6) In the column **"CT Calculation"** (right part of the window) determine **"Threshold"** value as **0,04**.
7) In the result table (**"Quant. Results"** window) Ct will be displayed.

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