Instruction for Use

virella arbo10

real time RT-PCR Kit

For the simultaneous *in vitro* detection of the RNA of Toscana Virus (Genotype A and B) and West Nile Virus (Lineage 1 and 2).

REF **G01149-96**

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1 Intended Purpose

The Kit is designed for the simultaneous qualitative detection of RNA of Toscana Virus (Genotype A and B) and West Nile Virus (Lineage 1 and 2).

2 Pathogen Information

Toscana Virus (TOSV) belongs to the genus of Phlebovirus and is an arthropod-borne segmented, enveloped, negative stranded RNA virus. The virus can be transmitted to humans by the bite of an infected sandfly. While most infections with TOSV are symptomless, nonspecific symptoms such as fever, headache and myalgia are reported. In most cases, illnesses caused by Toscana virus mimics a flulike syndrome with fever, photophobia, headache, red eyes, and stiff neck [1]. In rare cases, meningoencephalitis in patients with unusual symptoms and life-threatening complications were described [2]. The Virus is endemic in Italy and Spain and commonly found among all states around the Mediterranean Sea. Coinfections of TOSV with West Nile Virus have been reported in a few cases [3].

West Nile Virus (WNV) infection is an arthropod-borne zoonosis that is endemo-epidemic in Europe. The disease affects countries in Southern, Eastern and Western Europe. WNV is transmitted among birds via the bite of infected mosquitoes and ticks and incidentally humans and other mammals may become infected. About 80 % of WNV infections in humans are asymptomatic. West Nile fever (WNF) is the most common clinical manifestation [4]. The elderly and immunocompromised persons are at higher risk of developing West Nile neuroinvasive disease (WNND). No specific prophylaxis or treatment exist against the disease in humans. WNF is characterised by a sudden onset of symptoms that may include headache, malaise, fever, myalgia, vomiting, rash, fatigue and eye pain. Symptom severity ranges from a mild self-limiting illness from which patients recover within one week to a protracted debilitating disease that can last for months. WNND involves symptoms that affect the central nervous system. These can be categorised clinically as meningitis, encephalitis and acute flaccid paralysis or a combination of the three. Risk factors include advanced age, malignancies disrupting the blood–brain barrier, hypertension, hematologic disorders, diabetes mellitus, renal disease, alcohol abuse and genetic factors. The case fatality ratio among patients with WNND can be up to 17 %.

3 Principle of the Test

The virella arbo10 real time RT-PCR Kit contains specific primers and dual-labelled probes for the amplification of RNA (cDNA) of Toscana Virus A and B (nucleocapsid protein) and West Nile Virus 1 and 2 (3' untranslated region).
The presence of RNA is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The fluorescence of the Toscana Virus specific probe (Genotype A and B) is measured in the Cy5 channel, the fluorescence of the West Nile Virus specific probe (Lineage 1 and 2) is measured in the FAM channel.
Furthermore, the virella arbo10 real time RT-PCR Kit contains a Control RNA (Internal Process Control, IPC), which is added during RNA extraction and detected in the same reaction by a HEX-labelled probe.
The Control RNA allows the detection of RT-PCR inhibition and acts as control, that the nucleic acid was isolated from the biological specimen.

4 Package Contents

The reagents supplied are sufficient for 96 reactions.

Table 1: Components of the virella arbo10 real time RT-PCR Kit

Label	**Lid Colour**	**Content 96**
Reaction Mix	yellow	1 x 1325 µl
Enzyme	blue	1 x 19.2 µl
Positive Control	red	1 x 150 µl
Negative Control	green	1 x 150 µl
Control RNA	colourless	1 x 480 µl

5 Equipment and Reagents to be Supplied by User

- RNA isolation kit (e.g. NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- 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 or optical PCR reaction plate with optical foil
- Optional: Liquid handling system for automation

6 Transport, Storage and Stability

The virella arbo10 real time RT-PCR Kit 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.
Protect kit components from direct sunlight during the complete test run.

7 Warnings and Precautions

Read the Instruction for Use carefully before using the product.
Before first use check the product and its components for:

- Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organisations.

- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.
- Do not mix components from different lots.

8 Sample Material

Starting material for virella arbo10 real time RT-PCR is RNA isolated from biological specimens.

9 Sample Preparation

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

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

Please follow the Instruction 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 ‚Control RNA'.

If the real time RT-PCR is not performed immediately, store extracted RNA according to the instructions given by the manufacturer of the respective nucleic acid extraction kit.

10 Control RNA

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

Add 5 µl Control RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer's instructions.

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

11 Real time RT-PCR

11.1 Important Points Before Starting

- Please pay attention to chapter 7 ‚Warnings and Precautions'.
- 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 each 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 (except the Enzyme) and centrifuged very briefly.
- Due to the high viscosity of the Enzyme (blue lid), prewarming at room temperature for 15 min is recommended.

11.2 Procedure

The Master Mix contains all of the components needed for the real time RT-PCR except the sample. Prepare the Master Mix according to Table 2. 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

Volume per Reaction	Volume Master Mix
13.8 µl Reaction Mix	13.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

11.3 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 / take an optical PCR reaction plate.
- Pipet **14 µl** of the Master Mix into each optical PCR reaction tube / the optical PCR reaction plate.
- Add **6 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 optical PCR reaction tube / the optical PCR reaction plate (Table 3).
- Close the optical PCR reaction tubes / the optical PCR reaction plate immediately after filling in order to reduce the risk of contamination.

Table 3: Preparation of the real time RT-PCR

Component	Volume
Master Mix	14.0 µl
Sample	6.0 µl
Total Volume	20.0 µl

11.4 Instrument Settings

To ensure the compatibility with other gerbion real time RT-PCR, one of the thermal profiles shown in Table 4 and Table 5 can be used.

Table 4: real time RT-PCR thermal profile 1

Description	Time	Temperature	Number of Cycles	Acquisition
Reverse Transcription	10 min	45°C	1	no
Initial Denaturation	5 min	95°C	1	no
Denaturation	10 sec	95°C	45	no
Annealing and Extension	40 sec	60°C		end of step

Table 5: real time RT-PCR thermal profile 2

Description	Time	Temperature	Number of Cycles	Acquisition
Reverse Transcription	20 min	45°C	1	no
Initial Denaturation	5 min	95°C	1	no
Denaturation	10 sec	95°C	45	no
Annealing	20 sec	60°C		end of step
Extension	10 sec	72°C		no

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

Table 6: Overview of the instrument settings required for the virella arbo10 real time RT-PCR

Real time PCR Instrument	**Parameter**	**Detection Channel**	**Notes**		
LightCycler 480II			Color Compensation Kit not required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	West Nile Virus	FAM (465-510)	1	10	1
	Control RNA	HEX (533-580)	1	10	2
	Toscana Virus	CY5 (618-660)	1	10	3
Stratagene Mx3000P / Mx3005P	West Nile Virus	FAM	Gain 8	Reference Dye: None	
	Control RNA	HEX	Gain 1		
	Toscana Virus	Cy5	Gain 4		
QuantStudio 5 CFX96 CFX Opus 96 NEOS qPCR 96 Aria Mx qTower³ G	West Nile Virus	FAM	Option Reference Dye ROX: NO		
	Control RNA	HEX			
	Toscana Virus	Cy5			
Mic qPCR Cycler	West Nile Virus	Green	Gain 8		
	Control RNA	Yellow	Gain 10		
	Toscana Virus	Red	Gain 10		

12 Data Analysis

Following results can occur:

Signal/C_T Values			
Cy5 Channel Toscana Virus A and B	**FAM Channel** West Nile Virus 1 and 2	**HEX Channel** IPC	**Interpretation**
positive	negative	positive or negative[1]	**Positive result.** The sample contains Toscana Virus RNA
negative	**positive**	positive or negative[1]	**Positive result.** The sample contains West Nile Virus RNA
positive	**positive**	positive or negative[1]	**Positive result.** The sample contains Toscana Virus RNA and West Nile Virus RNA.
negative	negative	≤ 34 [2]	**Negative result.** The sample contains no Toscana Virus RNA and West Nile Virus RNA**.**
negative	negative	negative or > 34 [2]	**No statement can be made.** The real time RT-PCR is either inhibited or errors occurred during RNA extraction.

[1] A strong positive signal in the FAM or Cy5 channel can inhibit the IPC. In such cases the result for the Control RNA can be neglected.

[2] In case of high C_T values, the IPC should be compared to the water extraction control as described in the chapter 'Assay validation'.

13 Assay Validation

Negative Control
The Negative Control must show no C_T in the Cy5, FAM and HEX channel.

Positive Control
The Positive Control must show a positive (i.e. exponential) amplification curve in the Cy5 and the FAM channel. The Positive Control must be below C_T 30.

Internal Control
The following values for the amplification of the Internal Control are valid using gerbion nucleic acid extraction kit NukEx Mag RNA/DNA. The Control RNA (IPC) must show a positive (i.e. exponential) amplification curve.
The IPC must be below a C_T of 34. If the IPC is above C_T 34 this points to a purification problem or a strong positive sample that can inhibit the IPC. In the latter case, the assay is valid. It is recommended to perform the extraction of a water control in each run. The IPC in the water control must be below a C_T of 34.
If other nucleic acid extraction kits are used, the customer must define own cut offs. In this case the C_T value of the Control RNA (IPC) in an eluate from a sample should not be delayed for more than 4 C_T in comparison to an eluate from an extracted water control.

14 Limitations of the Method

- Strict compliance with the Instruction for Use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of RT-PCR inhibitors may cause false negative or invalid results.

15 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 Cy5 and FAM channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the Cy5 channel for analysis of the Toscana Virus specific amplification, the FAM channel for analysis of the West Nile Virus specific amplification and the HEX channel for the amplification of the Control RNA (IPC).
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with chapter 'Procedure'.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol in chapter 'Instrument Settings'.
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 chapter 'Transport, Storage and Stability'.
Weak or no signal of the Control RNA (IPC) and simultaneous absence of a signal in the specific Cy5 and/ or FAM channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time PCR conditions (chapter 'Real time RT-PCR').
real time RT-PCR inhibited	Make sure 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.
sample material not sufficient	Make sure that enough sample material has been applied to the extraction. Use an appropriate isolation method (see chapter 'Sample Preparation') and follow the manufacturer 's instructions.
RNA loss during isolation process	The lack of an amplification signal of the Control RNA 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 chapter 'Transport, Storage and Stability'.
Detection of a fluorescence signal in the Cy5 and/ or 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.

16 Kit Performance

16.1 Analytical Sensitivity

The limit of detection (LoD) of the virella arbo10 real time RT-PCR Kit was determined using serial dilutions of synthetic RNA-fragments containing the specific gene target sequence on the following real time PCR instruments:

Instrument	Limit of Detection [copies/µl]			
	Toscana Virus A	Toscana Virus B	West Nile Virus 1	West Nile Virus 2
CFX Opus 96	≤0.05	0.025	≤1	0.25
CFX 96 C1000 Touch	≤0.05	≤0.25	≤0.1	0.25
NEOS qPCR 96	≤0.5	≤0.25	1	0.25
Mic qPCR Cycler	0.05	≤0.025	≤1	≤0.25
LightCycler 480 II	≤0.05	≤0.025	≤1	0.25
QuantStudio 5	≤0.05	0.025	≤1	0.25

The limit of detection was reached with both temperature profiles for all targets.

16.2 Analytical Specificity

The specificity of the virella arbo10 real time RT-PCR Kit was evaluated with different ring trial samples of known status and different other relevant viruses and bacteria found in biological samples and basing on in silico analyses. The results for the sample analysis are shown in Table 7.

Table 7: Eluted DNA/RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of virella arbo10 real time RT-PCR Kit, Cy5 channel.

Eluates with known status	**virella arbo10** Toscana Virus **Cy5 channel**	**virella arbo10** West Nile Virus **FAM channel**
Japanese Encephalitis Virus	negative	negative
Tickborne Encephalitis Virus	negative	negative
Rift Valley Fever Virus	negative	negative
Echovirus 7	negative	negative
Coxsackievirus B3	negative	negative
Enterovirus 68	negative	negative
Influenzavirus A	negative	negative
Influenzavirus B	negative	negative
Yellow Fever Virus	negative	negative
Zika Virus	negative	negative
Chikungunya Virus	negative	negative
Dengue 1 Virus	negative	negative
Dengue 2 Virus	negative	negative
Dengue 3 Virus	negative	negative
Dengue 4 Virus	negative	negative
Babesia microti	negative	negative
Coxiella burnetii	negative	negative
Borrelia persica	negative	negative
Borrelia burgdorferi	negative	negative
Ehrlichia Canis Ebony	negative	negative
Anaplasma phagocytophilum	negative	negative
Amplirun® Toscana Virus RNA Control	**positive**	negative
West Nile Virus Lineage 1	negative	**positive**
West Nile Virus Lineage 2	negative	**positive**

16.3 Linear Range

The linear range of the virella arbo10 real time RT-PCR Kit was evaluated by analysing logarithmic dilution series of synthetic RNA-fragments containing the specific gene target sequence on a CFX Opus 96 real time PCR instrument.

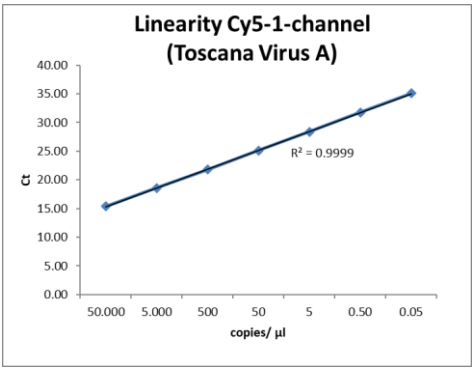

Figure 1: Determination of the linear range of the virella arbo10 real time RT-PCR Kit for Toscana Virus A in the Cy5 channel.

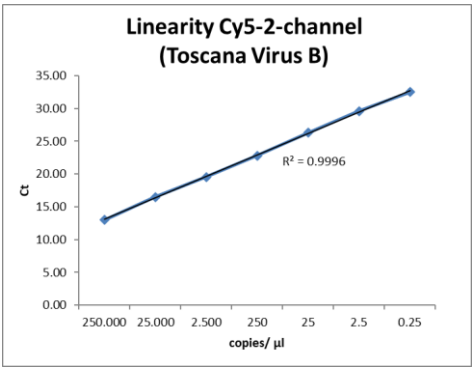

Figure 2: Determination of the linear range of the virella arbo10 real time RT-PCR Kit for Toscana Virus B in the Cy5 channel.

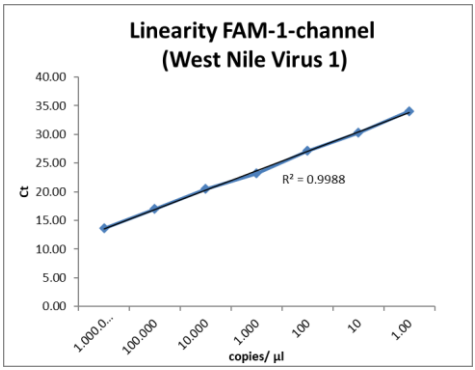

Figure 3: Determination of the linear range of the virella arbo10 real time RT-PCR Kit for West Nile Virus 1 in the FAM channel.

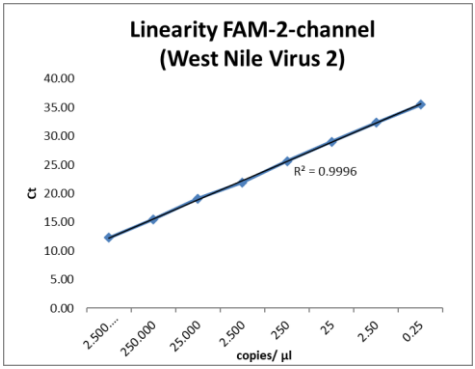

Figure 4: Determination of the linear range of the virella arbo10 real time RT-PCR Kit for West Nile Virus 2 in the FAM channel.

16.4 Precision

The precision of the virella arbo10 real time RT-PCR Kit was determined as intra-assay variability, inter-assay variability and inter-lot variability.

Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of in vitro transcripts of Toscana Virus A and B (nucleocapsid gene), West Nile Virus 1 and 2 (3' UTR) and on the threshold cycle of the Control RNA (IPC). The results are shown in Table 8.

Table 8: Precision of the virella arbo10 real time RT-PCR Kit

Toscana Virus A (Cy5)	copies/µl	Standard Deviation	Coefficient of Variation
Intra-Assay Variability	0.5	0.29	0.91
Inter-Assay Variability	0.5	0.15	0.49
Inter-Lot Variability	0.5	0.11	0.35
Toscana Virus B (Cy5)	copies/µl	Standard Deviation	Coefficient of Variation
Intra-Assay Variability	0.25	0.29	0.87
Inter-Assay Variability	0.25	0.03	0.09
Inter-Lot Variability	0.25	0.20	0.60
West Nile Virus 1 (FAM)	copies/µl	Standard Deviation	Coefficient of Variation
Intra-Assay Variability	1	0.48	1.43
Inter-Assay Variability	1	0.43	1.28
Inter-Lot Variability	1	0.12	0.36
West Nile Virus 2 (FAM)	copies/µl	Standard Deviation	Coefficient of Variation
Intra-Assay Variability	2.5	0.16	0.49
Inter-Assay Variability	2.5	0.20	0.61
Inter-Lot Variability	2.5	0.10	0.31
IPC (HEX)	copies/µl	Standard Deviation	Coefficient of Variation
Intra-Assay Variability	4.000	0.10	0.38
Inter-Assay Variability	4.000	0.13	0.49
Inter-Lot Variability	4.000	0.06	0.23

17 Abbreviations and Symbols

RNA	Ribonucleic Acid	REF	Catalog number
cDNA	complementary Deoxyribonucleic Acid	Σ	Contains sufficient for <n> test
RT-PCR	Reverse Transcription Polymerase Chain Reaction	-18°C	Upper limit of temperature
REACTION MIX	Reaction Mix		Manufacturer
ENZYME	Enzyme		Use by YYYY-MM-DD
CONTROL +	Positive Control	LOT	Batch code
CONTROL −	Negative Control	RUO	Research use only
CONTROL RNA IPC	Control RNA (IPC)		Consult Instruction for Use
CONT	Content		

18 Literature

[1] Peyrefitte C., Devetakov I., Pastorino B., et al. (2005). Toscana Virus and Acute Meningitis, France. Emerg. Infect Dis. 11(5): 778 – 780

[2] Baldelli F., Ciufolini M., Francisci D., et al. (2004) Unusual presentation of life-threatening Toscana virus meningoencephalitis.Clin. Infect Dis. 5: 515-520

[3] Erdem H., Ergunay K., Yilmaz A., et al. (2014) Emergence and co-infections of West Nile virus and Toscana virus in Eastern Thrace, Turkey. Clin. Microbiol. Infect 20: 319-325

[4] Kramer L., Li J., Shi P.-Y. (2007) West Nile virus. The Lancet 6(2): 171-181