Instruction for Use

virellaWNV
real time RT-PCR Kit

For the in vitro detection of RNA of West Nile Virus (Lineage 1 and 2), extracted from biological specimens.

REF **G01135-96**

96

- 18°C

gerbion GmbH & Co. KG
Remsstr. 1
70806 Kornwestheim
Germany
phone: +49 7154 806 20 0
fax: +49 7154 806 20 29
e-mail: info@gerbion.com
www.gerbion.com

IVD

CE

Index

1 Intended Use

The virellaWNV real time RT-PCR Kit is an assay for the detection of West Nile Virus (Lineage 1 and 2), extracted from biological specimens.

2 Pathogen Information

West Nile virus 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. 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 virellaWNV real time RT-PCR Kit contains specific primers and dual-labelled probes for the amplification of RNA (cDNA) of West Nile Virus (Lineage 1 and 2) extracted from biological specimen.

Furthermore, virellaWNV 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 virellaWNV 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 Pure RNA/DNA, gerbion Cat. No. G05004, NukEx Mag RNA/DNA, gerbion Cat. No. G05012).
- 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 virellaWNV 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. 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 Warnings and Precautions

Read the Instructions 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 pipet 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 organizations.
- Do not autoclave PCR reaction tubes or the optical PCR reaction plate 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.

8 Sample Material

Starting material for virellaWNV real time RT-PCR Kit is RNA isolated from biological specimens.

9 Sample Preparation

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

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 ‚Control RNA'.

If the real time RT-PCR is not performed immediately, store extracted RNA according to the instructions given by the manufacturer.

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 not be added to the sample material.

11 Real time RT-PCR

11.1 Important Points Before Starting:

- Please pay attention to the 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 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 (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 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)

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

11.3 Instrument Settings

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

Table 4: real time RT-PCR thermal profile 1

Description	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of cDNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec Acquisition at the end of this step	60°C	

Table 5: real time RT-PCR thermal profile 2

Description	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 Acquisition at the end of this step	60°C	
Extension	10 sec	72°C	

Further instrument settings have to be adjusted according to the table below.

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

Real time PCR Instrument	**Parameter**	**Detection Channel**	**Notes**		
LightCycler 480II			Pre-installed universal Color Compensation FAM (510) – VIC (580)		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	WNV	465-510	1	10	1
	Control RNA (IPC)	533-580	1	10	2
Mx3000P / Mx3005P	WNV	FAM	Gain 8	Reference Dye: None	
	Control RNA (IPC)	HEX	Gain 1		
AriaMx CFX96 NEOS-48 qPCR NEOS-96 qPCR	WNV	FAM		Reference Dye: None	
	Control RNA (IPC)	HEX			
ABI 7500 QuantStudio 5	WNV	FAM		Reference Dye: None	
	Control RNA (IPC)	JOE			
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	WNV	Green	Gain 5		
	Control RNA (IPC)	Yellow	Gain 5		
Mic qPCR Cycler	WNV	Green	Gain 8		
	Control RNA (IPC)	Yellow	Gain 10		

12 Data Analysis

Following results can occur:

Signal/C_T Values		**Interpretation**
FAM WNV	**HEX** Control RNA (IPC)	
positive	positive or negative [1]	**Positive result.** **The sample contains RNA of WNV.**
negative	≤ 34	**Negative result.** **The sample contains no RNA of WNV.**
negative	negative or > 34 [2]	**Caution!** The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction.

1 A strong positive signal in the FAM 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'.

Figure 1 and Figure 2 show examples for positive and negative real time RT-PCR results.

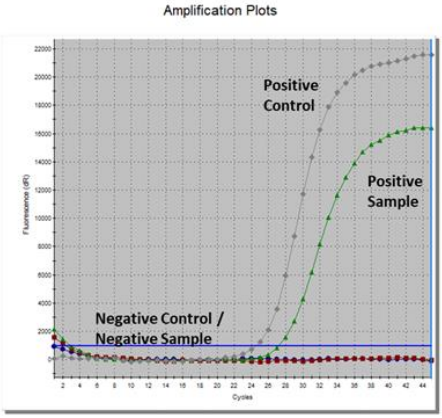

Figure 1: The positive sample shows pathogen specific amplification in the FAM channel (positive WNV sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (Mx3005P qPCR System).

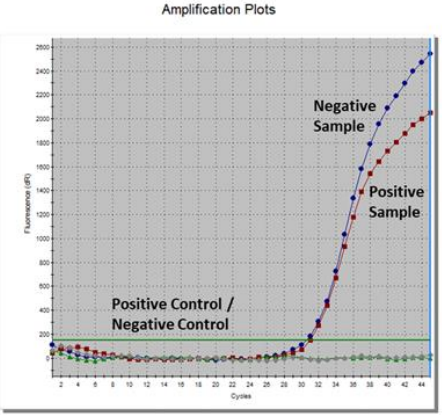

Figure 2: The positive sample and the negative sample show an amplification curve, whereas the Positive Control and the Negative Control don't show a signal in the Control RNA specific HEX channel (Mx3005P qPCR System). The amplification signal of the Control RNA in the negative eluate shows that the missing signal in the specific channels is not due to PCR inhibition or failure of RNA isolation, but that the eluate is a true negative.

13 Assay Validation

Negative Control
The Negative Control must show no C_T in the channels FAM and HEX.

Positive Control
The Positive Control must show no C_T in the HEX channel.
The Positive Control must show a positive (i.e. exponential) amplification curve in the FAM channel. The Positive Control must fall below a C_T of 30.

Internal Controls
The following values for the amplification of the internal controls are valid using gerbion nucleic acid extraction kits NukEx Mag RNA/DNA or NukEx Pure RNA/DNA. The Control RNA (IPC) must show a positive (i.e. exponential) amplification curve and fall below a C_T of 34. If the Control RNA 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 fall 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 and in vitro diagnostic procedures.
- 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.
- As with any diagnostic test, results of the virellaWNV real time RT-PCR Kit need to be interpreted in consideration of all clinical and laboratory findings.

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 for the Positive Control in the FAM channel	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the WNV (Lineage 1 and 2) specific amplification and the HEX channel for the amplification of the Control RNA.
Incorrect preparation of the Master Mix	Make sure that the Enzyme is added to the Master Mix (chapter 11).
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with 'Procedure' on page 6.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol 'Instrument Settings' in Table 4 or Table 5.
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'.

Weak or no signal of the Control RNA (IPC) in the HEX channel and simultaneous absence of a signal in the FAM channel	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions in Table 4, Table 5 and Table 6.
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 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	Lack of an amplification signal in the HEX channel 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'.
Detection of a fluorescence for the Negative Control signal in the FAM channel	
Contamination during preparation of the real time 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. Make sure to pipet the Positive Control last. If the same result occurs, one or more of the kit components might be contaminated. Make sure that workspace 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 virellaWNV real time RT-PCR Kit was determined testing serial dilutions of synthetic RNA-fragments containing the specific gene target sequence on a QuantStudio 5 real time PCR instrument. The estimated LoD of virellaWNV real time RT-PCR Kit is ≤ 10 genome copies per reaction for WNV Lineage 1 and ≤ 5 genome copies per reaction for WNV Lineage 2.

The LoD is independent of the thermal profile used for the PCR.

16.2 Analytical Specificity

The specificity of the virellaWNV real time RT-PCR Kit was evaluated with ring trial samples of known status and different other relevant viruses and bacteria found in clinical samples and basing on in silico analyses.
All ring trial samples were detected correctly. Results are shown in Table 7.
The results for the sample analysis are shown in Table 8, the results for the in silico analysis are shown in Table 9.
No difference in the specificity was observed between the two possible thermal profiles.

Table 7: Ring trial samples tested for the validation of the sensitivity of the virellaWNV real time RT-PCR Kit.

sample	ring trial	expected result WNV-1 and WNV-2	result virellaWNV
14-01	QCMD 2014	positive	positive
14-02	QCMD 2014	positive	positive
14-03	QCMD 2014	positive	positive
14-04	QCMD 2014	positive	positive
14-05	QCMD 2014	positive	positive
14-06	QCMD 2014	negative	negative
14-07	QCMD 2014	negative	negative
14-08	QCMD 2014	positive	positive
14-09	QCMD 2014	positive	positive
14-10	QCMD 2014	positive	positive
14-11	QCMD 2014	negative	negative
14-12	QCMD 2014	positive	positive
391029	Instand 2016	positive	positive
391030	Instand 2016	positive	positive
391031	Instand 2016	positive	positive
391032	Instand 2016	negative	negative
391033	Instand 2016	positive	positive
391034	Instand 2016	positive	positive

sample	ring trial	expected result WNV-1 and WNV-2	result virellaWNV
391041	Instand 2017	positive	positive
391042	Instand 2017	negative	negative
391043	Instand 2017	positive	positive
391044	Instand 2017	positive	positive
391045	Instand 2017	positive	positive
391046	Instand 2017	positive	positive
391047	Instand 2018	negative	negative
391048	Instand 2018	positive	positive
391049	Instand 2018	positive	positive
391050	Instand 2018	positive	positive
391051	Instand 2018	negative	negative
391052	Instand 2018	positive	positive
391083	Instand 2020	positive	positive
391084	Instand 2020	positive	positive
391085	Instand 2020	negative	negative
391086	Instand 2020	positive	positive
391087	Instand 2020	positive	positive
391088	Instand 2020	positive	positive

Table 8: Eluted RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of virellaWNV real time RT-PCR Kit.

sample	**expected result WNV-1 and WNV-2**	**result virellaWNV**
West Nile Virus Lineage 1	**positive**	**positive**
West Nile Virus Lineage 2	**positive**	**positive**
Influenzavirus A	negative	negative
Influenzavirus B	negative	negative
Coxsackievirus A16	negative	negative
Coxsackievirus B3	negative	negative
Enterovirus 68	negative	negative
Ehrlichia canis ebony	negative	negative
Anaplasma phagocytophilum	negative	negative
Borrelia burgdorferi	negative	negative
TBE-Virus	negative	negative
Babesia microti	negative	negative
Coxiella burnetti	negative	negative
Zika Virus	negative	negative
Chikungunya Virus	negative	negative
Yellow Fever Virus	negative	negative
Dengue Virus 1	negative	negative
Dengue Virus 2	negative	negative
Dengue Virus 3	negative	negative
Dengue Virus 4	negative	negative

Table 9: Inclusivity of the virellaWNV real time RT-PCR Kit Primers and Probes (in silico analysis).

	Target	Sequences	Mismatches		
			0	1	2
Probe	WNV 1 WNV 2	1000	1221	0	0
Forward Primer	WNV 1	1000	999	1	0
Reverse Primer		1000	1000	0	0
Forward Primer	WNV 2	221	168	53	0
Reverse Primer		221	167	46	8

None of the observed mismatches shows an crucial impact on the PCR efficiency of the specificity of the test.

16.3 Sample Testing

Positive (30) and negative (26) ring trial samples of known status and different other relevant viruses and bacteria found in clinical samples were tested with both thermal profiles.

The RNA was extracted by using the NukEx Mag RNA/DNA (gerbion Cat. No. G05012) extraction kit on a KingFisher™ Flex 96 Purification System.

The testing of the confirmed samples with virellaWNV real time RT-PCR Kit showed a sensitivity of 100% and a specificity of 100%.

	WNV positive samples	WNV negative samples
virellaWNV positive WNV-1 / WNV-2	30	0
virellaWNV negative WNV-1 / WNV-2	0	26
	Sensitivity (%)	Specificity (%)
	100	100

16.4 Linear Range

The linear range of the virellaWNV real time RT-PCR Kit was evaluated by analysing logarithmic dilution series of in vitro transcripts of the target sequences with both thermal profiles.

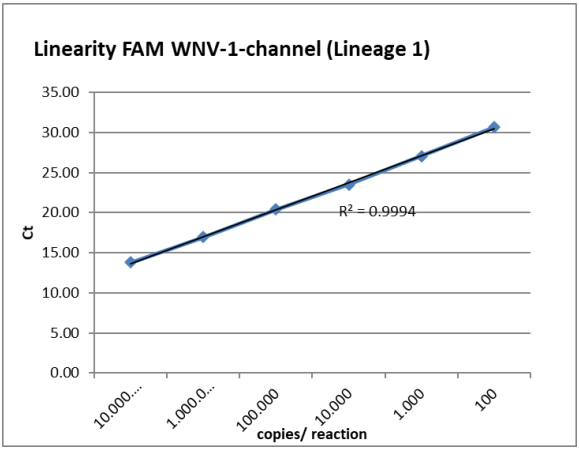

Figure 3: Determination of the linear range of virellaWNV real time RT-PCR Kit for WNV-1 in the FAM channel with thermal profile 1.

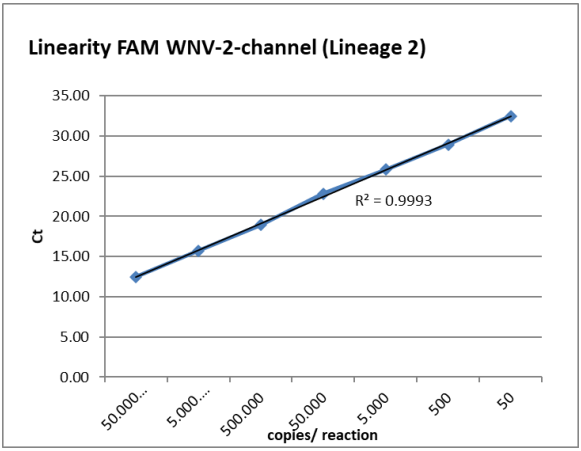

Figure 4: Determination of the linear range of virellaWNV real time RT-PCR Kit for WNV-2 in the FAM channel with thermal profile 1.

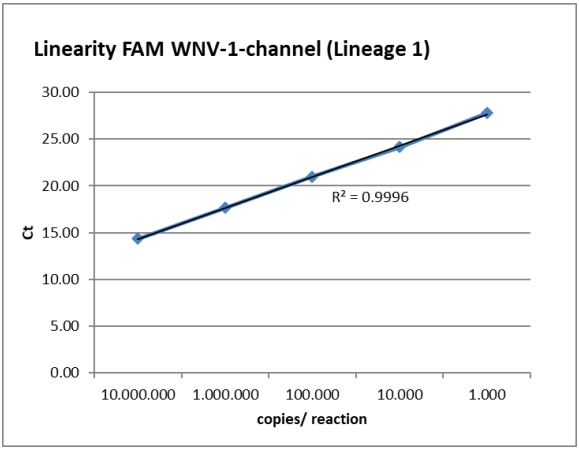

Figure 5: Determination of the linear range of virellaWNV real time RT-PCR Kit for WNV-1 in the FAM channel with thermal profile 2.

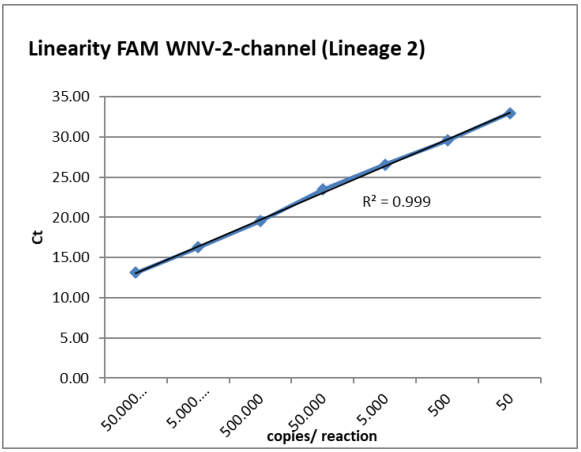

Figure 6: Determination of the linear range of virellaWNV real time RT-PCR Kit for WNV-2 in the FAM channel with thermal profile 2.

16.5 Precision

The precision of the virellaWNV 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 WNV Lineage 1 and Lineage 2 in vitro transcripts and on the threshold cycle of the Control RNA (IPC). The results are shown in Table 10 and Table 11.

Table 10: Precision of the virellaWNV real time RT-PCR Kit with thermal profile 1.

WNV Lineage 1 (FAM)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	100	0.23	0.75
Inter-Assay-Variability	100	0.39	1.25
Inter-Lot-Variability	100	0.46	1.48
WNV Lineage 2 (FAM)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	50	0.18	0.55
Inter-Assay-Variability	50	0.27	0.84
Inter-Lot-Variability	50	0.36	1.10
IPC (HEX)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	1000	0.37	1.29
Inter-Assay-Variability	1000	1.45	5.23
Inter-Lot-Variability	1000	0.37	1.28

Table 11: Precision of the virellaWNV real time RT-PCR Kit with thermal profile 2.

WNV Lineage 1 (FAM)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	100	0.27	0.86
Inter-Assay-Variability	100	0.69	2.24
Inter-Lot-Variability	100	0.45	1.43
WNV Lineage 2 (FAM)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	50	0.24	0.73
Inter-Assay-Variability	50	0.17	0.52
Inter-Lot-Variability	50	0.47	1.43
IPC (HEX)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	1000	0.25	0.92
Inter-Assay-Variability	1000	0.45	1.66
Inter-Lot-Variability	1000	0.55	2.05

16.6 Diagnostic Sensitivity

The diagnostic sensitivity of real time (RT-)PCR assays is mainly dependent on the DNA/RNA extraction method used to isolate DNA and RNA from various biological specimens. DNA/RNA extraction reagents are not part of the gerbion real time (RT-)PCR kits. gerbion real time (RT-)PCR kits include an extraction control and guidelines for the validation criteria of the extraction control in each reaction. The extraction control indicates inhibition of the real time (RT-)PCR and/or inefficient nucleic acid extraction. It cannot be used as a calibrator.

Therefore, gerbion guarantees the analytical sensitivities and specificities of the real time (RT-)PCR kits, performed with eluted DNA and RNA from reference materials and ring trial samples and with synthetic nucleic acid fragments. gerbion does not guarantee diagnostic sensitivities. If diagnostic sensitivities are mentioned in manuals of gerbion real time (RT-)PCR kits, the data are strictly correlated to a specific nucleic acid extraction method that has been used during the validation of the respective kits and cannot be transferred to other extraction methods. It is the responsibility of the user to qualify the extraction methods used for DNA/RNA isolation from biological samples.

17 Abbreviations and Symbols

RNA	Ribonucleic Acid		Upper limit of temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction		Manufacturer
REACTION MIX	Reaction Mix		Use by YYYY-MM-DD
ENZYME	Enzyme	LOT	Batch code
CONTROL +	Positive Control	CONT	Content
CONTROL −	Negative Control		Consult instruction for use
CONTROL RNA IPC	Control RNA (IPC)	IVD	*In vitro* diagnostic medical device
REF	Catalog number	CE	European Conformity
	Content sufficient for <n> tests		

18 Literature

[1] Pérez-Ramírez et al. (2017) Pathogenicity evaluation of twelve West Nile virus strains belonging to four lineages from five continents in a mouse model: discrimination between three pathogenicity categories

[2] Zehender et al. (2017) Reconstructing the recent West Nile virus lineage 2 epidemic in Europe and Italy using discrete and continuous phylogeography

[3] Barzon et al. (2013) New endemic West Nile virus lineage 1a in northern Italy, July 2012