MIKROGEN
DIAGNOSTIK

Instruction for Use

*alpha*Cube TBEV/WNV

For simultaneous qualitive in vitro detection of Tick Borne Encephalitis Virus RNA and West Nile Virus (Lineage 1 and 2) RNA, extracted from biological specimens.

REF	830569	830568
Σ	32	96

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1 Intended Use

*alpha*Cube TBEV/WNV is an assay for the simultaneous qualitive in vitro detection of Tick Borne Encephalitis Virus (TBEV) RNA and West Nile Virus (WNV) RNA, extracted from biological specimens.

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

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

*alpha*Cube TBEV/WNV contains specific primers and dual-labeled probes for the amplification and detection of TBEV and WNV RNA.

The presence of nucleic acids is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.

The fluorescence of the TBEV specific probes is measured in the FAM channel. The fluorescence of the WNV specific probes (Lineage 1 and 2) is measured in the ROX channel.

Furthermore, *alpha*Cube TBEV/WNV 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 biological 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 *alpha*Cube TBEV/WNV.

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

5 Equipment and Reagents to be Supplied by User

- RNA isolation kit (e.g. *alpha*Clean Pure RNA/DNA or *alpha*Clean Mag RNA/DNA, *alpha*Clean TS (tissue shred))
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- If using LightCycler® 480 (Roche) alphaCube Colour Compensation (831009) is required.
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation

6 Transport, Storage and Stability

*alpha*Cube TBEV/WNV 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 Important Notes

- *alpha*Cube TBEV/WNV must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Biological samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

- 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 PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine *alpha*Cube TBEV/WNV components of different lot numbers.

8 Sample Preparation

*alpha*Cube TBEV/WNV is suitable for the detection of TBEV RNA or WNV RNA isolated from biological specimens with appropriate isolation methods.
Commercial kits for RNA isolation such as the following are recommended:

- *alpha*Clean Pure RNA/DNA
- *alpha*Clean Mag RNA/DNA

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 9 ‚Control RNA'.

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

9 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 biological specimens
a) Control RNA used as Extraction Control:
*alpha*Cube TBEV/WNV 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 PCR:
If only inhibition will be checked please follow protocol B.

10 Real time RT-PCR

10.1 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 - except the Enzyme - should be thawed completely at room temperature, thoroughly mixed (do NOT vortex the Reaction Mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.

10.2 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 9 ‚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 9 ‚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.

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), 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 PCR

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

10.3 Instrument Settings

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

Table 5: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	20 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification			
Denaturation	10 sec	95°C	45
Annealing	20 sec Aquisition at the end of this step	60°C	
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 *alpha*Cube TBEV/WNV.

Real time PCR Instrument	**Parameter Reaction Mix**	**Detection Channel**	**Notes**		
LightCycler 480II			*alpha*Cube 480 Colour Compensation (831009) required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	TBEV	FAM (465-510)	1	10	1
	Control RNA	HEX (533-580)	1	10	2
	WNV	ROX (533-610)	1	10	2
Stratagene Mx3000 / Mx3005	TBEV	FAM	Gain 8		
	Control RNA	HEX	Gain 1	Reference Dye: None	
	WNV	ROX	Gain 1		
AriaMx BioRad CFX 96	TBEV	FAM			
	Control RNA	HEX		Reference Dye: None	
	WNV	ROX			
RotorGene Q, RotorGene 3000 RotorGene 6000	TBEV	Green	Gain 5		
	Control RNA	Yellow	Gain 5		
	WNV	Orange	Gain 5		
Mic qPCR Cycler	TBEV	Green	Gain 8		
	Control RNA	Yellow	Gain 10		
	WNV	Orange	Gain 10		

11 Data Analysis

The TBEV Virus specific amplification is measured in the FAM channel. The WNV specific amplification is measured in the ROX channel. The amplification of the Control RNA is measured in the VIC®/HEX/JOE/TET channel.

Table 7: Interpretation of results

Signal/Ct Values			**Interpretation**
FAM TBEV	**ROX** WNV	**HEX** Control RNA	
positive	negative	positive or negative*	Positive result, the eluate contains TBEV RNA.
negative	**positive**	positive or negative*	Positive result, the eluate contains WNV RNA.
negative	negative	≤ 34**	Negative result, the eluate contains neither TBEV RNA nor WNV RNA.
negative	negative	negative or > 34**	No diagnostic statement can be made. The real time PCR is either inhibited or errors occurred while DNA extraction.

* A strong positive signal in the FAM and/or ROX can inhibit the amplification of the Control RNA. In such cases the result for the Control RNA can be neglegted.

** Depending on the PCR instrument and/or the chosen extraction method, the Ct values might be shifted. The water control can be used as reference. If the HEX Ct value of a sample differs a lot from the water control, partial inhibition has occurred, leading to false negative results in case of weak positive samples.

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

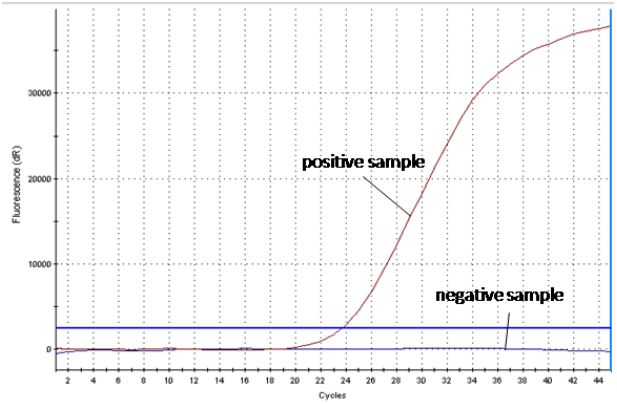


Figure 1: The positive sample shows virus-specific RNA amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

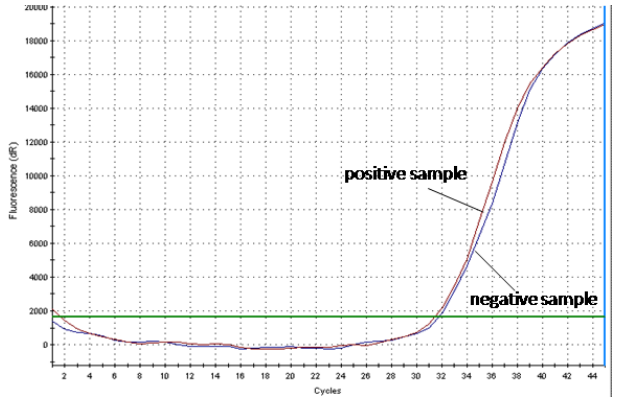


Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA specific VIC®/HEX/JOE/TET channel. The amplification signal of the Control RNA in the negative sample shows, that the missing signal in the virus specific FAM channel is not due to RT-PCR inhibition or failure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

12 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.

13 Limitations of the Method

*alpha*Cube TBEV/WNV is designated for the detection of TBEV and WNV RNA in watery solutions (eluates) from biological samples. The extraction of the RNA from biological samples is not part of *alpha*Cube TBEV/WNV. The Control RNA added during extraction does not reflect the quality of the sample but gives a hind for the quality of the extraction and/or remaining inhibitors in the eluate. The LoD of the reaction is $\geq$10 TBEV target sequences and $\geq$10 WNV target sequences per reaction, respectively. Therefore, a negative result in the *alpha*Cube TBEV/WNV real time RT-PCR does not exclude the complete absence of TBEV or WNV RNA in an eluate.

14 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time RT-PCR.

No fluorescence signal in the FAM and ROX channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the TBEV specific amplification, the ROX channel for analysis of the WNV 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 6.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 8).

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 and/or ROX channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 6).
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 buffers have 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 and/or ROX channel of the Negative Control	
Contamination during preparation of the RT-PCR	Repeat the real time RT-PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time RT-PCR.

15 Kit Performance

15.1 Analytical Sensitivity

The limit of detection (LoD) of *alpha*Cube TBEV/WNV was determined using serial dilutions of TBEV and WNV specific target sequences. The LoD of *alpha*Cube TBEV/WNV real time RT-PCR Kit is ≥10 genome copies per reaction each.

The sensitivity of *alpha*Cube TBEV/WNV was also analysed by testing ring trial samples and samples of Inter-Laboratory comparisons of known status. All samples were detected correctly. Results are shown in Table 8.

Table 8: Ring trial samples tested for the validation of the sensitivity of the alphaCube TBEV/WNV.

Sample	Ring Trial number	Expected Result	Result *alpha*Cube TBEV/WNV ROX Channel	Result *alpha*Cube TBEV/WNV FAM Channel
391029 WNV	Instand 198/16	positive	positive	negative
391030 WNV	Instand 198/16	positive	positive	negative
391031 WNV	Instand 198/16	positive	positive	negative
391033 WNV	Instand 198/16	positive	positive	negative
391034 WNV	Instand 198/16	positive	positive	negative
391941 WNV	Instand 195/17	positive	positive	negative
391042 WNV	Instand 195/17	negative	negative	negative
391043 WNV	Instand 195/17	positive	positive	negative
391044 WNV	Instand 195/17	positive	positive	negative
391045 WNV	Instand 195/17	positive	positive	negative
391046 WNV	Instand 195/17	positive	positive	negative
391047 WNV	Instand 208/18	positive	positive	negative
391048 WNV	Instand 208/18	positive	positive	negative
391049 WNV	Instand 208/18	negative	negative	negative
391050 WNV	Instand 208/18	positive	positive	negative
391051 WNV	Instand 208/18	negative	negative	negative
391052 WNV	Instand 208/18	positive	positive	negative
14-01 WNV	QCMD 112/14	positive	positive	negative
14-02 WNV	QCMD 112/14	positive	positive	negative
14-03 WNV	QCMD 112/14	positive	positive	negative

14-04 WNV	QCMD 112/14	positive	positive	negative
14-05 WNV	QCMD 112/14	positive	positive	negative
14-06 WNV	QCMD 112/14	negative	negative	negative
TBEV-1	Inter-Lab comparison	negative	negative	negative
TBEV-2	Inter-Lab comparison	positive	negative	positive
TBEV-3	Inter-Lab comparison	positive	negative	positive

15.2 Analytical Specificity

The specificity of *alpha*Cube TBEV/WNV was evaluated additionally with different other relevant viruses and bacteria found in biological samples.

Results:

*alpha*Cube TBEV/WNV showed a positive result for the sample containing TBEV and for the sample containing WNV, 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 *alpha*Cube TBEV/WNV.

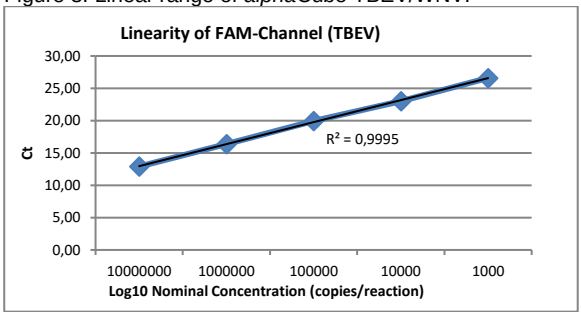
Strain	**Expected Result**	**Result *alpha*Cube TBEV/WNV ROX Channel**	**Result *alpha*Cube TBEV/WNV FAM Channel**
Enterovirus 68	negative	negative	negative
Coxsackievirus B3	negative	negative	negative
Coxsackievirus A16	negative	negative	negative
Coxsackievirus B5	negative	negative	negative
Influenza Virus A A/ Brisbane H1N1 59/2007 E40/08	negative	negative	negative
Influenza Virus A Indonesia H5N1 05/2005	negative	negative	negative
Influenza Virus A Panama H3N2 2007/99	negative	negative	negative
Influenza Virus B B/ Brisbane 60/2008 E09/09	negative	negative	negative
Zikavirus	negative	negative	negative
Chikungunya Virus	negative	negative	negative
Yellow Fever Virus	negative	negative	negative
Dengue Virus 1	negative	negative	negative
Dengue Virus 2	negative	negative	negative
Dengue Virus 3	negative	negative	negative

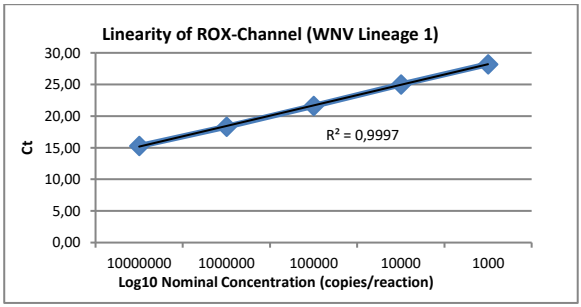
Dengue Virus 4	negative	negative	negative
Coxiella burnetii	negative	negative	negative
Ehrlichia chaffeensis	negative	negative	negative
Ehrlichia ewingii	negative	negative	negative
Ehrlichia canis	negative	negative	negative
Ehrlichia phagocytophilum	negative	negative	negative
Anaplasma platy	negative	negative	negative
Babesia divergens	negative	negative	negative
Babesia microti	negative	negative	negative
Babesia sp. EU1	negative	negative	negative
Borrelia burgdorferi Strain 4681	negative	negative	negative
Borrelia burgdorferi sensu stricto	negative	negative	negative
Borrelia afzelii	negative	negative	negative
Borrelia garinii	negative	negative	negative
Borrelia spielmanii	negative	negative	negative
Borrelia bavariensis	negative	negative	negative
Borrelia bisettii	negative	negative	negative
Borrelia lustianae	negative	negative	negative
Borrelia valaisiana	negative	negative	negative
Borrelia kurtenbachii	negative	negative	negative
Borrelia japonica	negative	negative	negative
Borrelia miyamotoi	negative	negative	negative
T. phagedenis	negative	negative	negative
Leptospira	negative	negative	negative
TBEV	positive	negative	positive
WNV Lineage 1	positive	positive	negative
WNV Lineage 2	positive	positive	negative

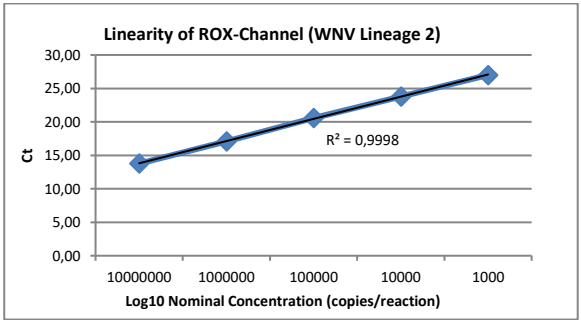
15.3 Linear Range

The linear range of *alpha*Cube TBEV/WNV was evaluated by analyzing logarithmic dilution series of in vitro transcripts (Figure 3).

Figure 3: Linear range of *alpha*Cube TBEV/WNV.







15.4 Precision

The precision of *alpha*Cube TBEV/WNV was determined as intra-assay variability, inter-assay variabilitity 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 TBEV, WNV 1 and WNV 2 specific RNA and on the threshold cycle of the Control-RNA. The data are shown in Table 10.

Table 10: Precision of *alpha*Cube TBEV/WNV.

TBEV	copies/µl	Standard Deviation	Variation Coefficient [%]
Intra-Assay Variability	25	0.31	0.95
Inter-Assay-Variability	25	0.21	0.64
Inter-Lot Variability	25	0.00	0.01

WNV 1	copies/µl	Standard Deviation	Variation Coefficient [%]
Intra-Assay Variability	25	0.07	0.21
Inter-Assay-Variability	25	0.05	0.15
Inter-Lot Variability	25	0.32	1.03

WNV 2	copies/µl	Standard Deviation	Variation Coefficient [%]
Intra-Assay Variability	25	0.19	0.57
Inter-Assay-Variability	25	0.58	1.73
Inter-Lot Variability	25	0.33	0.98

Control RNA	copies/µl	Standard Deviation	Variation Coefficient [%]
Intra-Assay Variability	250	0.19	0.68
Inter-Assay-Variability	250	0.11	0.37
Inter-Lot Variability	250	0.29	1.03

16 Abbreviations and Symbols

RNA	Ribonucleid Acid		Upper limit of temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction		Manufacturer
	Reaction Mix		Use by YYYY-MM-DD
	Enzyme		Batch code
	Positive Control		Content
	Negative Control		Consult instructions for use
	Control RNA		*In vitro* diagnostic medical device
	Catalog number		European Conformity
	Contains sufficient for <n> tests		

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17 Literature

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