IVD For *in Vitro* Diagnostic Use

CE

West Nile Virus Real-TM

Handbook

Real Time PCR Kit for the qualitative detection of West Nile Virus (WNV) RNA

REF V53-50FRT

REF TV53-50FRT

Σ 50

NAME

West Nile Virus (WNV) Real-TM

INTRODUCTION

The West Nile virus is one of the many members of the genus Flavivirus that are known to cause human disease. The life cycle of the West Nile virus involves the microbe's transmission from nonhuman animals to humans by way of Aedes, Culex, or Anopheles mosquitoes. The West Nile virus can infect horses, birds dogs, and other mammals. However, wild birds are apparently the optimal hosts for harboring and replicating the virus. The West Nile virus causes serious manifestations in approximately 1% of person who are infected, with increased morbidity and mortality in individuals older than 50 years. In hospitalized patients, neurologic sequelae of the West Nile virus included severe muscle weakness, with approximately 10% of patients developing a complete flaccid paralysis. One in 150 West Nile viruses infections results in encephalitis or meningitis, and the mortality rate from severe illness is 3-15%.

INTENDED USE

WNV Real-TM is Real-Time amplification test for the qualitative detection of West Nile Virus (WNV) RNA in clinical specimens (plasma, serum; white blood cells; cerebrospinal fluid), autopsy material of human and animals (brain tissue), biological material (mosquitoes) and water.

PRINCIPLE OF ASSAY

WNV Real-TM Test is based on three major processes: isolation of WNV RNA from specimens, one-step reverse transcription of the RNA and Real Time amplification of the cDNA. *WNV* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific primers and detection via fluorescent dyes. These dyes are linked with probes of oligonucleotides which bind specifically to the amplified product. The real-time PCR monitoring of fluorescence intensities allows the accumulating product detection without reopening of reaction tubes after the PCR run. **WNV Real-TM** PCR kit is a qualitative test which contains the Internal Control (IC). It must be used in the isolation procedure in order to control the process of each individual sample extraction and serves also to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V53-50FRT)

Part N°2 – **"Controls":** Controls kit

- **Negative Control (C-)***, 1,6 ml ;
- **Pos *WNV*-RNA-rec****, 5 x 0,03 ml ;
- **Internal Control (IC) RNA*****, 5 x 0,12 ml ;
- **RNA-buffer**, 2 x 0,6 ml;
- **Pos Control cDNA WNV/IC (C+)**, 0,1 ml;

Part N°3–**"WNV Real-TM":** Real Time amplification kit

- **RT-G-mix-2**, 0,015 ml;
- **RT-PCR-mix-1-TM WNV**, 0,6 ml;
- **RT-PCR-mix-2-TM**, 0,3 ml;
- **Hot Start Taq Polymerase**, 0,03 ml;
- **M-MLV Revertase**, 0,015 ml;

Contains reagents for 55 tests.

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *must be used in the isolation procedure as Positive Control of Extraction.*
*** *add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture*

Module No.2: Complete Real Time PCR test with RNA purification kit (TV53-50FRT)

Part N°1 – **Ribo-Sorb:** Sample preparation kit

- **Lysis Solution**, 22,5 ml;
- **Washing Solution**, 20,0 ml;
- **Sorbent**, 1,25 ml;
- **RNA-eluent**, 5 x 0,5 ml;

Contains reagents for 50 tests.

Part N°2 – **"Controls":** Controls kit

- **Negative Control (C-)***, 1,6 ml ;
- **Pos *WNV*-RNA-rec****, 5 x 0,03 ml ;
- **Internal Control (IC) RNA*****, 5 x 0,12 ml ;
- **RNA-buffer**, 2 x 0,6 ml;
- **Pos Control cDNA WNV/IC (C+)**, 0,1 ml;

Part N°3–**"WNV Real-TM":** Real Time amplification kit

- **RT-G-mix-2**, 0,015 ml;
- **RT-PCR-mix-1-TM WNV**, 0,6 ml;
- **RT-PCR-mix-2-TM**, 0,3 ml;
- **Hot Start Taq Polymerase**, 0,03 ml;
- **M-MLV Revertase**, 0,015 ml;

Contains reagents for 55 tests.

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *must be used in the isolation procedure as Positive Control of Extraction.*
*** *add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- RNA extraction kit (Module No. 1)
- Biosafety cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- 60°C ± 2°C dry heat block
- Vortex mixer
- Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Tube racks
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone
- Refrigerator
- Freezer

Zone 2: RT and amplification:

- Real Time Thermalcycler
- Workstation
- Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

STORAGE INSTRUCTIONS

Ribo-Sorb and **Controls** must be stored at 2-8°C. **WNV Real-TM** must be stored at -20°C. The kits can be shipped at 2-8°C for 3-4 days but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

WNV Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

WNV Real-TM can analyze RNA extracted from:

- ***Plasma:***
  - EDTA tubes may be used. Follow sample tube manufacturer's instructions.
  - Whole blood collected in EDTA should be separated into plasma and cellular components by centrifugation at 800-1600 x g for 20 min within six hours. The isolated plasma has to be transferred into a sterile polypropylene tube. Plasma may be stored at 2-8°C for an additional 3 days. Alternatively, plasma may be stored at -18°C for up to one month or 1 year when stored at -70°C.
  - Do not freeze whole blood.
  - Specimens anti-coagulated with heparin are unsuitable for this test.
  - Thaw frozen specimens at room temperature before using.
  - Whole blood must be transported at 2-25°C and processed within 6 hours of collection. Plasma may be transported at 2-8°C or frozen.
  - Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.
- ***Tissue:***
  - Homogenize internal organs of animals and autopsy material by means of a porcelain mortar and pestle and prepare 10% suspension with sterile saline solution or phosphate buffer. Use 30 µl of the suspension for RNA extraction.
- ***Mosquetoes:***
  - Prepare mosquitoes pools (up to 50 mosquitoes). Homogenize gnats in sterile saline solution or phosphate buffer calculating 30 µl of the solution per 1 mosquito. Centrifuge the samples at 13,000 rpm for 1 minute. Collect 100 µl of the supernatant for RNA extraction.
- ***Water:***
  - Centrifuge 10-20 ml for 10 min at maximum speed. Discard the supernatant and leave about 100 µl of solution for DNA extraction

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.

Sacace Biotechnologies recommends to use the following kit:

- ⇨ **Ribo-Sorb** (Sacace, REF K-2-1);
- ⇨ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003) for plasma.

Please carry out RNA extraction according to the manufacture's instruction. Add 10 µl of Internal Control during RNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the module no.2)

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including controls.
2. Add to each tube **450 µl Lysis Solution** and **10 µl** of **IC RNA**.
3. Add **100 µl** of samples to the appropriate tube containing Lysis Solution and IC. Mix by pipetting and incubate 5 min at room temperature.
4. Prepare Controls as follows:
   - add **100 µl** of **C– Negative Control** to the tube labeled *Cneg*.
   - add **90 µl** of **C– Negative Control** and **10 µl** of **Pos *WNV*-RNA-rec** to the tube labels *Cpos*.
5. Vortex the tubes and centrifuge for 5 sec at 5000g. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 1 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for RNA/DNA extraction
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
8. Centrifuge all tubes for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
9. Add **400 µl** of **Washing Solution** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g. and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **500 µl** of **Etanolo al 70%** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g. and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
11. Repeat step 10.

12. Add **400 µl** of **Acetone** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
13. Incubate all tubes with open cap for 10 min at 60°C.
14. Resuspend the pellet in **50 µl** of **RNA-eluent.** Incubate for 10 min at 60°C and vortex periodically. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g).

RT AND AMPLIFICATION

Total reaction volume is **25 µl**, the volume of RNA sample is **10 µl**.

1 Prepare the reaction mix for required number of samples.

2 For N reactions mix in a new tube:

10*(N+1) µl of RT-PCR-mix-1-TM WNV,

5.0*(N+1) µl of RT-PCR-mix-2

0.5*(N+1) µl of Taq Polymerase

0.25*(N+1) µl of RT-G-mix-2

0.25*(N+1) µl of MMlv

3 Vortex the tube, then centrifuge shortly. Add **15 µl** of prepared reaction mix into each tube.

4 Using tips with aerosol filter add **10 µl** of RNA samples obtained at the stage of RNA isolation and mix carefully by pipetting**.**

N.B. If the Ribo-Sorb isolation kit is used as a RNA extraction kit, re-centrifuge all the tubes with extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction

5 Prepare for each panel 2 controls:

- add **10 µl** of **RNA-buffer** to the tube labeled PCR Negative Control;
- add **10 µl** of **Pos Control cDNA WNV(C+)** to the tube labeled C_{pos};

Create a temperature profile on your Real-time instrument as follows:

	***Rotor type instruments*[1]**				***Plate type or modular instruments*[2]**			
Stage	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***
Hold	50	30 min	–	1	50	30 min	–	1
Hold	95	15 min	–	1	95	15 min	–	1
Cycling	95	5 s	–	5	95	5 s	–	5
	56	25 s	–		56	30 s	–	
	72	15 s	–		72	15 s	–	
Cycling 2	95	5 s	–	40	95	5 s	–	40
	56	25 s	FAM(Green), JOE(Yellow)		56	30 s	FAM, JOE/HEX/Cy3	
	72	15 s	–		72	15 s	–	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)

[2] For example, *SaCycler-96™ (Sacace),* iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500/StepOne Real Time PCR (Applera), SmartCycler® (Cepheid), Line Gene K® (Bioer), Eco PCR Real Time System® (Illumina).

INSTRUMENT SETTINGS

Settings for rotor-type instruments (Rotor-Gene 3000/6000, Rotor-Gene Q)

Channel	*Threshold*	*More Settings/Outlier Removal*	*Slope Correct*	*Eliminate Cycles before*	*Calibrate/Gain Optimisation…*
FAM/Green	0.03	5 %	on	5	from 5Fl to 10Fl
JOE/Yellow	0.03	5 %	on	5	from 5Fl to 10Fl

Note: *if the fluorescence curves in FAM and JOE channels do not correspond to exponential growth, set the threshold level of negative samples (NTC threshold) equal to 10 %.*

Settings for plate-type instruments (iCycler iQ, iQ5 and Mx3000P)

iCycler iQ, iQ5	When analyzing amplification results, set the following parameters: in ***Base Line cycles*** menu, select ***User Defined***, ***Select all***, ***Edit Range*** and set ***Start Cycle* = 2**, ***Ending Cycle* = 10**. In ***Crossing Threshold*** menu, select ***User Defined***, set ***Threshold Position*** for **FAM** channel equal to **50** and for **JOE** channel equal to **200**.
Mx3000P	1. In ***Plate Setup*** menu, set fluorescence detection parameters. To do this, select all cells with test tubes with **PCR-mix-1-FRT *WNV***, then select fluorophores FAM and JOE in ***Collect fluorescence data*** menu. 2. In ***Threshold fluorescence*** menu, set the threshold line at a level where the fluorescence curves are linear. It is recommended to select the threshold line level equal to **150** for **FAM** channel and **500** for **JOE** channel. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross the baseline; otherwise, the threshold level should be raised.

RESULTS ANALYSIS

1. The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line.

   cDNA of WNV is detected on the JOE (Yellow)/HEX/Cy3 channel and *IC* on the FAM (Green) channel.

Results are accepted as relevant if positive and negative controls of amplification and extraction are passed (see table 1).

Table. *Ct* boundary values

Sample	**Instrument**			
	Rotor-Gene 3000/6000/Q		**SaCycler, Smart Cycler, AB 7300/7500/StepOne, iCycler iQ, iQ5, Mx3000P**	
	FAM/Green	JOE/Yellow	FAM	HEX/JOE
	IC detection	*WNV* detection	IC detection	*WNV* detection
Neg Control C-	< 28	-	< 31	-
Positive Control *WNV*-rec RNA	< 28	< 30	< 31	< 33
cDNA WNV/IC(C+)	< 28	< 28	< 31	< 31
Clinical samples	< 28 (for blood serum, CSF), < 31 (for homogenized mosquitoes, ticks, and internal organs and for urine)	< 38	< 31 (for blood serum, CSF), < 33 (for homogenized mosquitoes, ticks, and internal organs and for urine)	< 39

PERFORMANCE CHARACTERISTICS

The kit **WNV Real-TM** allows to detect WNV in 100% of the tests with a sensitivity of not less than 500 copies/ml.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam (Green) channel): retesting of the sample is required.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended RNA extraction method and follow the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting the extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the RNA extraction procedure.
2. Weak (Ct > 37) signal on the Joe (Yellow)/Cy3/HEX channel: retesting of the sample is required.
3. Joe (Yellow)/Cy3/HEX signal with Negative Control of extraction.
    - Contamination during RNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips among tubes.
        - ⇒ Repeat the RNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive controls at the end.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**C–**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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