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NOTICE of CHANGE dated 13/10/2020

IMPORTANT COMMUNICATION FOR THE USERS OF PRODUCT:

«WNV ELITe MGB Kit»
Ref. RTS100PLD

This new revision of the Instruction for Use (IFU) contains the following changes:

- *Minor changes in the Intended Use description. The Intended Use of the product remains unchanged.*

Composition, use and performance of the product remain unchanged.

PLEASE NOTE

	LA REVISIONE DI QUESTO IFU E' COMPATIBILE ANCHE CON LA VERSIONE PRECEDENTE DEL KIT
	THE REVIEW OF THIS IFU IS ALSO COMPATIBLE WITH THE PREVIOUS VERSION OF THE KIT
	CET IFU MIS A JOUR ANNULE ET REMPLACE ET EST PARFAITEMENT COMPATIBLE AVEC LA VERSION PRECEDENTE DU KIT
	LA REVISIÓN DE ESTE IFU ES COMPATIBLE TAMBIÉN CON LA VERSIÓN ANTERIOR DEL KIT
	A REVISÃO DO ESTE IFU ÉTAMBÉM COMPATÍVEL COM A VERSÃO ANTERIOR DO KIT
	DIESE FASSUNG DER GEBRAUCHSANLEITUNG IST KOMPATIBEL MIT DER VORHERIGEN VERSION DES TESTKITS

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WNV ELITe MGB® Kit

reagents for RNA reverse transcription and cDNA Real Time amplification

REF RTS100PLD

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-20°C

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INTENDED USE

The **«WNV ELITe MGB® Kit»** product is a quantitative reverse transcription and nucleic acids amplification assay for the **detection and quantification of the RNA of the human Flavivirus West Nile Virus (WNV, lineage 1a**, Mediterranean strains included, and **lineage 2)** in total RNA samples extracted from Whole Blood samples collected in EDTA, Cerebrospinal Fluid (CSF), Urine collected without preservatives.

The product is intended for use, alongside clinical data and other laboratory tests, in the diagnosis and monitoring of WNV infections. No validation data are available for use of the product in association with biological matrices or for intended uses different from those reported in this instruction for use manual.

ASSAY PRINCIPLE

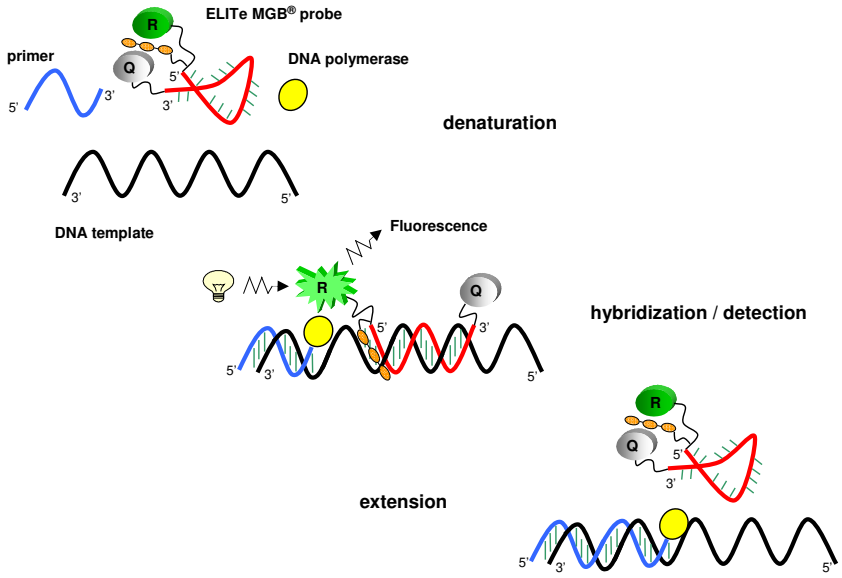
The assay consists of a reverse transcription and a real time amplification reaction (one-step method) in a microplate with a programmable thermostat provided with a fluorescence detection optical system (real time amplification thermal cycler).

In each well a reverse transcription and amplification reaction is carried out. The reaction is specific for a region of **NS5** gene of **WNV**, codifying a non structural protein, and for a region of the genomic RNA of **MS2 phage** (Internal Control of inhibition). The reaction starts directly from the RNA extracted from the samples in analysis. The WNV specific probe with ELITe MGB® technology, labelled with FAM fluorophore, is activated when hybridized with the specific product of the WNV amplification reaction. The Internal Control specific probe with ELITe MGB® technology, labelled with AP525 fluorophore (similar to VIC), is activated when hybridized with the specific product of Internal Control amplification reaction. As the specific product of the amplification reaction increases, the fluorescence emission increases and is measured and recorded by the instrument. The processing of the data determines the presence and the titre of WNV RNA in the starting sample.

At the end of the session it is possible to perform a melting curve analysis and to determine the melting temperature in order to confirm the identity of the target or detect mutated target.

The validation of the assay was carried out with 7300 Real Time PCR System and 7500 Fast Dx Real-Time PCR Instrument

The following picture shows the mechanism of activation and fluorescence emission of ELITe MGB® technology probe. Note that the probe is not hydrolyzed during the amplification cycle so it can be used for the dissociation curve analysis.



PRODUCT DESCRIPTION

The **«WNV ELITe MGB® Kit»** product provides the following components:

- **WNV PreMix**

A mixture of primer oligonucleotides for reverse transcription and real time amplification, in a stabilizing solution, **aliquoted into two test tubes** (NEUTRAL cap). Each tube contains **270 µL** of solution, sufficient for **50 reactions**.

Primers and the probe for WNV (stabilized by MGB® group, labelled by FAM fluorophore and quenched by a non fluorescent molecule) are specific for a region of the **WNV NS5 gene**.

Primers and the probe for Internal Control (stabilized by MGB® group, labelled by AP525 fluorophore, similar to VIC, and quenched by a non fluorescent molecule) are specific for a region of the **phage MS2** genomic RNA.

The reaction mixture also provides AP593 fluorophore, used instead ROX or CY5 as passive reference for fluorescence normalisation.

- **PCR MasterMix**

An optimized and stabilized mixture of reagents for reverse transcription and real time amplification **aliquoted into two test tubes** (NEUTRAL cap). Each tube contains **820 µL** of solution, sufficient for **50 reactions**.

The reaction mixture provides the buffer, magnesium chloride, the nucleotide triphosphates and the DNA Polymerase enzyme with hot start capability.

- **RT EnzymeMix**

An optimized and stabilized mixture of enzymes for reverse transcription, **pre-aliquoted into two test tubes** (BLACK cap). Each tube contains **20 µL** of solution, sufficient for **50 reactions**.

The reaction mixture provides enzymes for reverse transcription.

The product enables **100 determinations**, including standards and controls.

MATERIALS PROVIDED IN THE PRODUCT

Component	Description	Quantity	Hazard classification
WNV PreMix	Primer/probe oligonucleotides mixture NEUTRAL cap	**2 x 270 µL**	-
PCR MasterMix	mixture of reagents for reverse transcription and real time amplification NEUTRAL cap	**2 x 820 µL**	-
RT EnzymeMix	Reverse transcriptase BLACK cap	**2 x 20 µL**	-

MATERIALS REQUIRED BUT NOT PROVIDED IN THE PRODUCT

- Laminar airflow hood.
- Disposable nitrile powder-free gloves or similar material.
- Vortex mixer.
- Bench microcentrifuge (12,000 - 14,000 RPM).
- Sterile micropipettes and tips with aerosol filter or positive displacement (2-20 µL, 5-50 µL, 50-200 µL, 200-1000 µL).
- Polypropylene micro tube for molecular biology, 1.5 mL.
- Molecular biology grade water.
- Programmable thermostat with optical fluorescence detection system 7300 Real Time PCR System or 7500 Fast Dx Real-Time PCR Instrument calibrated following manufacturer's instructions.

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OTHER PRODUCTS REQUIRED

The reagents for the extraction of RNA from samples, the Internal Control of extraction and inhibition, the amplification microplates, the amplification Positive Control and known-quantity DNA standards **are not** included in this kit.

For automatic RNA extraction from samples, it is necessary to use the generic product **«ELITe STAR 200 Extraction Kit»** (ELITechGroup S.p.A., code INT011EX), kit for nucleic acid extraction from biological samples with the instrument **«ELITe STAR»** (ELITechGroup S.p.A., code INT010).

For extraction control and inhibition control, it is necessary to use the generic product «**CPE - Internal Control**» (ELITechGroup S.p.A., code CTRCPE), plasmid DNA and phage RNA template from Internal Control reactions.

When a 7300 Real-Time PCR System is used, it is recommended to use the generic product **«Q - PCR Microplates»** (ELITechGroup S.p.A., code RTSACC01), microplates with 0.2 mL wells and adhesive sealing sheets for real time amplification.

When a 7500 Fast Dx Real-Time PCR Instrument is used, it is recommended to use the generic product: **«Q - PCR Microplates Fast»** (ELITechGroup S.p.A., code RTSACC02), microplates with 0.1 mL wells and adhesive sealing sheets for real time amplification.

If a qualitative result of the analysis is required, it is necessary the use of the specific product **«WNV - Positive Control»** (ELITechGroup S.p.A., code CTR100PLD).

If a quantitative result of the analysis is required, to use the specific product **«WNV ELITe Standard»** (ELITechGroup S.p.A., code STD100PLD), four dilutions of known-quantity plasmid DNA to calculate the standard curve.

SAMPLES AND CONTROLS

Samples

This product must be used with **RNA** extracted from the following clinical samples: Whole Blood collected in EDTA, Cerebrospinal Fluid (CSF) and Urine collected without preservatives.

This product must be used by directly adding the **extracted RNA** (up to 300 ng) to reverse transcription and real time amplification reaction (one step procedure).

Whole Blood collected in EDTA

The Whole Blood samples, used for nucleic acid extraction, must be collected in EDTA according to laboratory guidelines, carried at +2 / +8 °C and stored at +2 / +8 °C for a maximum of four hours. Otherwise they must be frozen and stored at -20 °C for a maximum of two days or at -70 for longer period.

We suggest to split the samples in aliquots in order not to freeze / thaw them several times.

Cerebrospinal Fluid

The Cerebrospinal Fluid samples, used for nucleic acid extraction, must be collected according to laboratory guidelines, carried at +2 / +8 °C and stored at +2 / +8 °C for a maximum of four hours. Otherwise they must be frozen and stored at -20 °C for a maximum of two days or at -70 for longer period.

We suggest to split the samples in aliquots in order not to freeze / thaw them several times.

Urine

The Urine samples, used for nucleic acid extraction, must be collected without preservatives according to laboratory guidelines, carried at room temperature (+18 / +25 °C) and stored at room temperature (+18 / +25 °C) for a maximum of four hours. Otherwise they must be frozen and stored at -20 °C for a maximum of two days or at -70 for longer period. We suggest to split the samples in aliquots in order not to freeze / thaw them several times.

Urine sample freezing frequently causes the formation of precipitates that can interfere with downstream steps of the method: extract only the supernatant.

N.B.: when you carry out the RNA extraction from Whole Blood, Cerebrospinal Fluid and Urine with the kit **«ELITe STAR 200 Extraction Kit»** and the instrument **«ELITe STAR»** with **software version 3.4.13** (or higher), use the extraction protocol **UUNI_E100S200_ELI** that processes 200 µL of sample and elutes the extracted RNA in 100 µL. Samples in primary tubes can be directly loaded on the instrument **«ELITe STAR»**. A minimum sample volume of 600 µL is always requested. Add **200 µL** of **CPE** Internal Control in the Proteinase-Carrier solution tube as indicated in the instruction for use manual of the extraction kit. For the extraction procedure, follow the indications of the user manual in the instruction for use manual of the extraction kit.

Interfering substances

The RNA extracted from samples must not contain heparin, haemoglobin, ethanol or 2-propanol in order to prevent inhibition problems and the possibility of frequent not valid results.

Quantity of extracted RNA higher than 300 ng could inhibit the reverse transcription reaction and the real time amplification.

Large quantities of human genomic DNA in the RNA extracted from the sample could inhibit the reverse transcription reaction and the real time amplification.

There are no data available concerning inhibition caused by antibiotics, antiviral drugs, chemotherapeutic or immunosuppressant drugs.

Amplification controls

It is absolutely mandatory to validate each amplification session with a negative control reaction and a positive control reaction.

As negative control, use molecular biology grade water (not supplied with the product) added to the reaction in place of the RNA obtained from the sample.

As positive control use the **«WNV ELITe Standard»** or the **«WNT - ELITe Positive Control»** product.

Quality controls

It is recommended to validate the whole analysis procedure of each session, extraction, reverse transcription and amplification, by processing a negative sample and a positive sample that have already been tested or a calibrated reference material.

WARNINGS AND PRECAUTIONS

This product is exclusively designed for *in-vitro* use.

General warnings and precautions

Handle and dispose of all biological samples as if they were able to transmit infective agents. Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with the biological samples must be treated for at least 30 minutes with 3% sodium hypochlorite or autoclaved for one hour at 121°C before disposal.

Handle and dispose of all reagents and all materials used to carry out the assay as if they were able to transmit infective agents. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be handled and disposed of in compliance with adequate safety standards. Disposable combustible material must be incinerated. Liquid waste containing acids or bases must be neutralised before disposal.

Wear suitable protective clothes and gloves and protect eyes and face.
Never pipette solutions by mouth.
Do not eat, drink, smoke or apply cosmetic products in the work areas.
Carefully wash hands after handling samples and reagents.
Dispose of leftover reagents and waste in compliance with the regulations in force.
Carefully read all the instructions provided in the product before running the assay.
While running the assay, follow the instructions provided in the product.
Do not use the product after the indicated expiry date.
Only use the reagents provided in the product and those recommended by the manufacturer.
Do not use reagents from different batches.
Do not use reagents from other manufacturers.

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Warnings and precautions for molecular biology

Molecular biology procedures, such as nucleic acids extraction, reverse transcription, amplification and detection, require qualified and trained staff to avoid the risk of erroneous results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.

It is necessary to have available separate areas for the extraction / preparation of amplification reactions and for the amplification / detection of amplification products. Never introduce an amplification product in the area designated for extraction / preparation of amplification reactions.

It is necessary to have available lab coats, gloves and tools which are exclusively used for the extraction / preparation of the amplification reactions and for the amplification / detection of amplification products. Never transfer lab coats, gloves or tools from the area designated for the amplification / detection of amplification products to the area designated for the extraction / preparation of the amplification reactions.

The samples must be exclusively used for this type of analysis. Samples must be handled under a laminar airflow hood. Tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively used for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips used must be sterile, free from DNases and RNases, free from DNA and RNA.

The reagents must be handled under a laminar airflow hood. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes used to handle the reagents must be exclusively used for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips used must be sterile, free from DNAses and RNAses, free from DNA and RNA.

Amplification products must be handled in such a way as to reduce as much as possible dispersion into the environment in order to avoid the possibility of contamination. The pipettes used to handle amplification products must be exclusively used for this purpose.

Warnings and precautions specific for the components

WNV PreMix

The **WNV PreMix** must be stored at -20 °C in the dark.

The **WNV PreMix** can be frozen and thawed for no more than **five times**: further freezing / thawing cycles may cause a loss of product performances.

PCR MasterMix

The **PCR MasterMix** must be stored at -20 °C.

The **PCR MasterMix** can be frozen and thawed for no more than **five times**: further freezing / thawing cycles may cause a loss of product performances.

RT EnzymeMix

The **RT EnzymeMix** must be stored at -20°C.

The **RT EnzymeMix** must not be exposed to temperatures higher than -20 °C for more than 10 minutes.

PROCEDURE

Setting of the real time amplification session
(To perform in the amplification / detection area)

When **7300 Real-Time PCR System** instrument is used.

Before starting the session, referring to instrument documentation, it is important to do the following:
- switch on the real time thermal cycler, switch on the control computer, launch the software and open an "absolute quantification" session;
- set the "detector" for the WNV probe with the “reporter” = "FAM" and the "quencher" = “none " (non fluorescent) " and name it "WNV";
- set the "detector" for the Internal Control probe with the “reporter” = "VIC" and the "quencher" = “none” (non fluorescent) and name it "IC";
- for each well in use in the microplate, set (Well Inspector) the "detector" (type of fluorescence to be measured), the "passive reference" = "ROX" (AP593 is used instead of ROX, for normalisation of the measured fluorescence) and the type of reaction (sample, negative amplification control or known quantity standard). Add this information to the **Work Sheet** enclosed at the end of this manual or print the microplate set up. The **Work Sheet** must be followed carefully when transferring the reaction mixture and samples into the wells.

N.B.: in order to determine the RNA titre in the starting sample, set up a series of reactions with the **Q - PCR standard** (10^5 copies, 10^4 copies, 10^3 copies, 10^2 copies) to obtain the **standard curve**.

Referring to the instrument documentation, set on the dedicated software (Instrument > Thermal Cycler Protocol > Thermal Profile) the parameters of the **thermal cycle**:
- add to amplification stage the step (Add Step) of **extension at 72 °C**,

N.B.: the fluorescence acquisition (Instrument > Thermal Cycler Protocol > Settings > Data Collected) must be set during the step of **hybridization at 60 °C**.

- modify timing as indicated in the table **"Thermal cycle"**,
- set the cycles number to **45**,
- set the the reaction volume to **30 µL**,
- optional: add the melting curve analysis (Add Dissociation Stage) and set the temperature from **40 °C** to **80 °C**.

Thermal cycle		
Phase	**Temperature**	**Time**
Reverse-transcription	50 °C	20 min.
Initial denaturation	94 °C	5 min.
Amplification and detection (45 cycles)	94 °C	10 sec.
	60 °C (fluorescence acquisition)	30 sec.
	72 °C	20 sec.
Dissociation (optional)	95 °C	15 sec.
	40 °C	30 sec.
	80 °C	15 sec.

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When **7500 Fast Dx Real-Time PCR Instrument** is used.

Before starting the session, referring to instrument documentation, it is important to do the following:
- switch on the real time thermal cycler, switch on the control computer, launch the software and open an "absolute quantification" session;
- set the "detector" for the WNV probe with the “reporter” = "FAM" and the "quencher" = “none " (non fluorescent) " and name it "WNV";
- set the "detector" for the Internal Control probe with the “reporter” = "VIC" and the "quencher" = “none” (non fluorescent) and name it "IC";
- for each well in use in the microplate, set (Well Inspector) the "detector" (type of fluorescence that is to be measured), the "passive reference" = "CY5" (AP593 is used instead of CY5, for the normalisation of the measured fluorescence) and the type of reaction (sample, negative amplification control, positive amplification control or known quantity standard). Add this information to the **Work Sheet** enclosed at the end of this manual or print the microplate set up. The **Work Sheet** must be followed carefully during the transfer of the reaction mixture and samples into the wells.

N.B.: in order to determine the RNA titre in the starting sample, set up a series of reactions with the **WNV Q - PCR standard** (10^5 copies, 10^4 copies, 10^3 copies, 10^2 copies) to obtain the **standard curve**.

Referring to the instrument documentation, set on the dedicated software (Instrument > Thermal Cycler Protocol > Thermal Profile) the parameters of the **thermal cycle**:
- add to amplification stage the step (Add Step) of **extension at 72 °C**,

N.B.: the fluorescence acquisition (Instrument > Thermal Cycler Protocol > Settings > Data Collected) must be set during the step of **hybridization at 60 °C**.

- modify time as indicated in the table **"Thermal cycle"**,
- set the cycles number to **45**,
- set the volume for the software emulation of thermal transfer to reaction ("Sample volume") to **30 µL**,
- optional: add the melting curve analysis (Add Dissociation Stage) and set the temperature from **40 °C** to **80 °C**.

Thermal cycle		
Phase	**Temperature**	**Time**
Reverse-transcription	50 °C	20 min.
Initial denaturation	94 °C	5 min.
Amplification and detection (45 cycles)	94 °C	10 sec.
	60 °C (fluorescence acquisition)	30 sec.
	72 °C	20 sec.
Dissociation (optional)	95 °C	15 sec.
	40 °C	1 min.
	80 °C	15 sec.
	60 °C	15 sec.

Amplification set-up
(To be performed in the extraction / preparation area)

Before starting the session it is important to do the following:
- remove and thaw at room temperature (+18 / 25 °C) the test tubes containing the samples to be analysed. Mix by vortexing for 10 seconds, centrifuge the tubes for 5 seconds to bring the contents to the bottom and keep in ice,
- remove and thaw for 30 minutes at room temperature (+18 / 25 °C) the **WNV PreMix** (NEUTRAL cap) test tubes needed for the session, remembering that the content of each test tube is enough for **50 reactions.** Mix by vortexing for 10 seconds three times and centrifuge the tubes for 5 seconds to bring the content to the bottom and keep in ice,

- remove and thaw for 30 minutes at room temperature (+18 / 25 °C) the **PCR MasterMix** (NEUTRAL cap) tubes necessary for the session, remembering that the content of each tube is sufficient to set up **50 reactions**. Mix by vortexing for 10 seconds three times and centrifuge the tubes for 5 seconds to bring the content to the bottom and keep in ice,
- when needed, remove the **RT EnzymeMix** (BLACK cap) tubes necessary for the session remembering that the content of each tube is sufficient to set up **50 reactions**. Gently shake the tubes, centrifuge for 5 seconds to bring the contents to the bottom and keep in ice,

N.B.: The **RT EnzymeMix** should not be exposed to temperatures above -20 °C for more than 10 minutes.

- remove and thaw for 30 minutes at room temperature (+18 / 25 °C) the **WNV Q - PCR Standard** tubes or the **WNV - Positive Control** tube. Mix by vortexing for 10 seconds, centrifuge the tubes for 5 seconds to bring the content to the bottom and keep in ice,
- take the **Amplification microplate** that will be used during the session, being careful to handle it with powderless gloves and not to damage the wells,
- take the **Amplification Sealing Sheet** that will be used during the session, being careful to handle it with powderless gloves and not to damage,
- prepare one 1.5 mL molecular biology grade polypropylene tubes (not provided with this product) for the complete reaction mixture **WNV Q - PCR Mix** and mark them in a recognizable manner with a permanent marker,
- calculate the volumes of the three components provided by kit that are needed for preparing the complete reaction mixture **WNV Q - PCR Mix** on the basis of the number of samples to be analyzed, as described in the following table.

N.B.: In order to calculate the volumes of the three components it is necessary to define the number of reactions (N) of the session by counting the number of the samples to be tested, a negative control, four Q - PCR Standard plus one reaction as safety margin.

Reaction Number	WNV PreMix	PCR MasterMix	RT EnzymeMix
1	5 µL	15 µL	0.3 µL
N	N x 5 µL	N x 15 µL	N x 0.3 µL

- prepare the complete reaction mixture **WNV Q - PCR Mix** by adding to the dedicated tube the calculated volumes of the three components.
- mix by **vortexing at low speed** for 10 seconds three times, centrifuge the tube for 5 seconds to bring the content to the bottom and keep in ice;

N.B.: The complete reaction mixture should be used within 30 minutes. The complete reaction mixture **cannot** be stored

Set up the reactions as described below:

1. Accurately pipet **20 µL** of complete reaction mixture **WNV Q - PCR Mix** on the bottom of the **Amplification microplate** wells, as previously established in the **Work Sheet**. Avoid creating bubbles.
2. Accurately pipet, by placing into the reaction mixture, **10 µL** of **RNA extract** from the first sample in the corresponding well of the **Amplification microplate**, as previously established in the **Work Sheet**. Mix thoroughly the sample by pipetting the **extracted RNA** three times into the reaction mixture. Avoid creating bubbles. Proceed in the same way with all the other **extracted RNA**.
3. Accurately pipet, by placing into the reaction mixture, **10 µL** of **Molecular Biology grade water** (not provided with this product) in the corresponding well of **Amplification microplate**, as previously established in the **Work Sheet**. Mix thoroughly the negative control by pipetting the **Molecular Biology grade water** three times into the reaction mixture. Avoid creating bubbles.

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4. On the basis of the result required (qualitative or quantitative), one of these two options must be followed:

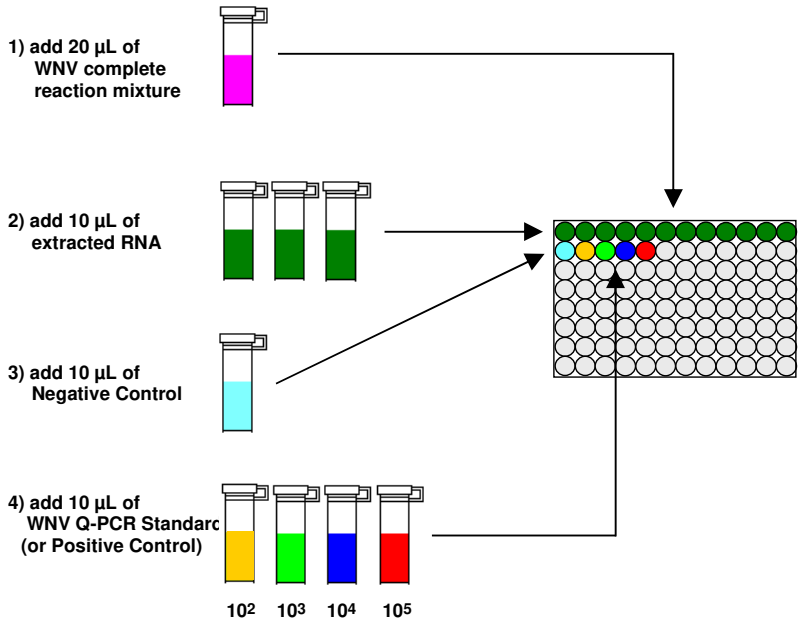
   - When a **qualitative** result is required (detection of WNV RNA): accurately pipet, by placing into the reaction mixture, **10 µL** of **WNV - Positive Control** in the corresponding well of **Amplification microplate**, as previously established in the **Work Sheet**. Mix the positive control thoroughly by pipetting the **WNV - Positive Control** three times into the reaction mixture. Avoid creating bubbles.

   - When a **quantitative** result is required (quantification of WNV RNA): accurately pipet, by placing into the reaction mixture, **10 µL** of **WNV Q - PCR Standard 10^2** in the corresponding well of **Amplification microplate**, as previously established in the **Work Sheet**. Mix the standard thoroughly by pipetting the **WNV Q - PCR Standard** three times into the reaction mixture. Avoid creating bubbles. Proceed in the same way with the other **WNV Q - PCR Standards** (**10^3, 10^4, 10^5**).

5. Accurately seal the **Amplification microplate** with the **Amplification Sealing Sheet**.
6. Transfer the **Amplification microplate** into the real time thermal cycler in the amplification / detection area and start the thermal cycle for the amplification saving the session setting with an unambiguous and recognizable file name (e.g. "year-month-day-WNV-EGSpA").

N.B.: At the end of the thermal cycle the **Amplification microplate** with the reaction products must be removed from the instrument and eliminated without producing environmental contaminations. In order to avoid the spilling of the reaction products, the **Amplification Sealing Sheet must not be removed from the Amplification microplate**.

The following figure shows an example of the preparation of the amplification reactions for quantitative analysis of 12 samples.



Qualitative analysis of the results

The recorded values of the fluorescence emitted by the WNV specific probe (FAM detector "WNV") and by the Internal Control specific probe (VIC detector "IC") in the amplification reactions must be analysed by the instrument software.

Before starting the analysis, referring to the instrument documentation, it is necessary to:
- manually set (Results > Amplification plot > delta Rn vs Cycle) the calculation range for the **Baseline** (fluorescence background level) from cycle 6 to cycle 15;

N.B.: In the case of a positive sample with a high titre of WNV RNA, the FAM fluorescence of the WNV specific probe may begin to increase before the cycle 15. In this case the calculation range for the **Baseline** must be adapted from cycle 6 to the cycle in which the FAM fluorescence of the sample begins to increase, as detected by the instrument software (Results > Component).

When a **7300 Real-Time PCR System** instrument is used:

- manually set the **Threshold** for the FAM detector "WNV" to **0.1**;
- manually set the **Threshold** for the VIC detector "IC" to **0.05**.

When a **7500 Fast Dx Real-Time PCR Instrument** is used:

- manually set the **Threshold** for the FAM detector "WNV" to **0.1**;
- manually set the **Threshold** for the VIC detector "IC" to **0.1**.

The values of fluorescence emitted by the specific probes in the amplification reaction and the **Threshold** value of fluorescence allow determination of the **Threshold cycle (Ct)**, the cycle in which the fluorescence reached the **Threshold** value.

Positive Control or Q-PCR Standard

In the **Positive Control** or **Q - PCR Standard 10^5** amplification reaction, the **Ct** value of WNV (Results > Report) is used to validate the amplification and the detection as described in the following table:

Positive Control reaction (or Q-PCR Standard 10^5) detector FAM "WNV"	Assay result	Amplification / Detection
Ct ≤ 25	POSITIVE	CORRECT

If the result of the **Positive control** or **Q - PCR Standard 10^5** amplification reaction is **Ct > 25** or **Ct Undetermined** for WNV, the target DNA was not correctly detected. This means that problems occurred during the amplification or detection step (incorrect dispensation of the reaction mix or of the positive control, degradation of the reaction mix or of the positive control, incorrect setting of the positive control position, incorrect setting of the thermal cycle, see "Troubleshooting" section) which may lead to incorrect results. The session is not valid and needs to be repeated starting from the amplification step.

Negative Control

In the **Negative control** amplification reaction, the **Ct** value of WNV (Results > Report) is used to validate the amplification and the detection as described in the following table:

Negative control reaction detector FAM "WNV"	Assay result	Amplification / Detection
Ct Undetermined	NEGATIVE	CORRECT

If the result of the amplification reaction for the **Negative control** is different from **Ct Undetermined (Undetermined)** for WNV, the target DNA has been detected. This means that problems have occurred during the amplification step (contamination, see "Troubleshooting" section), which may lead to incorrect results and false positives. The session is not valid and needs to be repeated starting from the amplification step.

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Samples

In the amplification reaction of each **sample**, the **Ct** value of WNV is used to detect the target RNA while the **Ct** value of Internal Control is used to validate extraction, reverse transcription, amplification and detection.

N.B: Verify with the instrument software (Results > Amplification plot > delta Rn vs Cycle) that the **Ct** was determined by a fast and regular increase of the fluorescence values and not by peaks or an increase of the background (irregular or high background).

This product is able to detect a minimal quantity from about 450 to 200 copies of WNV RNA per mL, corresponding to the genome Equivalents per mL (detection limit of the product, see "Performance Characteristics" section).

The results as **Ct** of the amplification reactions of each **sample** (Results > Report) are used as described in the following table:

Sample reaction		Sample suitability	Assay result	WNV RNA
detector FAM "WNV"	detector VIC "IC"			
Ct Undetermined	Ct > 35 or Ct Undetermined	unsuitable	not valid	-
	Ct ≤ 35	suitable	valid, negative	NOT DETECTED
Ct Determined	Ct > 35 or Ct Undetermined	suitable	valid, positive	DETECTED
	Ct ≤ 35	suitable	valid, positive	DETECTED

If the result of the amplification reaction of a sample is **Ct Undetermined** for WNV and **Ct > 35** or **Ct Undetermined** for the Internal Control, it means that it was impossible to detect efficiently the RNA for the Internal Control. In this case problems have occurred during the reverse transcription and amplification step or during the extraction step (degradation of Internal Control RNA, loss of RNA during extraction or presence of inhibitors in the extracted RNA, see "Troubleshooting" section) which may lead to incorrect results and false negatives. The sample is not suitable, the assay, is not valid and it needs to be repeated starting from the extraction of a new sample.

If the result of the amplification reaction of a sample is **Ct Undetermined** for WNV and **Ct ≤ 35** for the Internal Control, it means that the WNV RNA is not detected in the RNA extracted from the sample; but it cannot be excluded that the WNV RNA has a lower titre than the detection limit of the product (see "Performance Characteristics" section). In this case the result could be a false negative.

The results obtained with this assay must be interpreted taking into consideration all the clinical data and the other laboratory test outcomes concerning the patient.

N.B.: When in the amplification reaction of a sample the WNV RNA is detected, the Internal Control may result with Ct > 35 or Ct Undetermined. In fact, the low efficiency amplification reaction for the Internal Control may be displaced by competition with the high efficiency amplification reaction for WNV RNA. In this case the sample is nevertheless suitable and the positive result of the assay is valid.

Quantitative analysis of the results

After carrying out the procedure for qualitative analysis of the results it is possible to perform the quantitative analysis of the results of the positive samples.

Standard Curve

In the amplification reactions of the four **Q - PCR standards**, the **Ct** values are used to calculate the **Standard Curve** (Results > Standard Curve) for the amplification session, as described in the following table:

Standard Curve detector FAM "WNV"	Acceptability range	Amplification / Detection
Correlation coefficient (R2)	0.990 ≤ R2 ≤ 1.000	CORRECT

If the **Correlation coefficient (R2)** value does not fall within the limits, this means that it was impossible to efficiently detect the target DNA. Problems have occurred during the amplification or detection step (incorrect preparation of the reaction mixture, incorrect dispensation of the reaction mix or of the standards, degradation of the reaction mixture or of the standards, incorrect setting of the position of the standards, incorrect setting of the thermal cycle, see "Troubleshooting" section) which may lead to incorrect results. The session is not valid and needs to be repeated starting from the amplification step.

Samples

The **Ct** values of WNV in the amplification reaction of each **sample** and the **Standard Curve** (Results > Standard Curve) of the amplification session are used to calculate the **Quantity** of target RNA present in the amplification reactions of the samples.

This was tested with samples with a concentration range from 1,000,000 to 316 copies of WNV RNA per mL, corresponding to the genome Equivalents per mL (linear measuring range, see "Performance Characteristics" section).

The results (**Quantity**) of each **sample** (Results > Report) are used to calculate the genome Equivalents (**gEq**) of WNV present in the sample used in the extraction (**Nc**) according to this formula:

$$\text{Nc (gEq / mL)} = \frac{\text{Ve x Quantity}}{\text{Vc x Va x Ep}}$$

Where:
Vc is the quantity of the sample used in the extraction **expressed in mL**;
Ep is the efficiency of the procedure (extraction, reverse transcription and amplification) **expressed in decimal**;
Ve is the total volume of the extraction product **expressed in µL**;
Va is the volume of the reverse transcription and amplification extraction product used in the amplification reaction **expressed in µL**;
Quantity is the result of the amplification reaction of the sample **expressed in gEq per reaction**.

When «**ELITe STAR**» extraction system is used with samples from Whole Blood and the result is required **in gEq / mL**, the formula becomes:

Simplified formula for «ELITe STAR» and Whole Blood samples
Nc (gEq / mL) = 66.7 x Quantity

When «**ELITe STAR**» extraction system is used with samples of Cerebrospinal Fluid or Urine and the result is required **in gEq / mL**, the formula becomes:

Simplified formula for «ELITe STAR» and Cerebrospinal Fluid or Urine samples
Nc (gEq / mL) = 50 x Quantity

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PROCEDURE LIMITATIONS

Use this product only with RNA extracted from the following clinical samples: Whole Blood collected in EDTA, Cerebrospinal Fluid (CSF) and Urine.

Do not use RNA extracted from heparinized samples with this product: heparin inhibits the amplification reaction of nucleic acids and causes not valid results.

Do not use extracted RNA that is contaminated with haemoglobin, ethanol or 2-propanol with this product: these substances inhibit the reverse transcription reaction and the amplification of nucleic acids and may cause not valid results.

Quantity of extracted RNA higher than 300 ng per reaction may inhibit the reverse transcription reaction and the amplification of nucleic acids.

Do not use with this product extracted RNA containing high quantity of human genomic DNA that may inhibit the reverse transcription reaction and the amplification of nucleic acids.

There are no data available concerning product performances with RNA extracted from the following clinical samples: Plasma collected in EDTA.

There are no data available concerning inhibition caused by antiviral, antibiotic, chemotherapeutic or immunosuppressant drugs.

The results obtained with this product depend on an adequate identification, collected, transport, storage and processing of the samples. To avoid incorrect results, it is therefore necessary to take care during these steps and to carefully follow the instructions for use provided with the products for nucleic acids extraction.

Owing to its high analytical sensitivity, the real time amplification method used in this product is sensitive to cross-contaminations from the WNV positive clinical samples, the positive controls and the same amplification products. Cross-contaminations cause false positive results. The product format is able to limit cross-contaminations. However, the cross-contaminations can be avoided only by good laboratory practices and following these instructions for use.

This product must be handled by qualified personnel trained in the processing of potentially infective biological samples and chemical preparations classified as dangerous to prevent accidents with potentially serious consequences for the user and other persons.

This product requires the use of work clothes and areas that are suitable for the processing of potentially infective biological samples and chemical preparations classified as dangerous to prevent accidents with potentially serious consequences for the user and other persons.

This product must be handled by qualified personnel trained in molecular biology techniques, such as extraction, reverse transcription, amplification and detection of nucleic acids, to avoid incorrect results.

It is necessary to have separate areas for the extraction / preparation of amplification reactions and for the amplification / detection of amplification products to prevent false positive results.

This product requires the use of special clothing and instruments for extraction / preparation of amplification reactions and for amplification / detection of amplification products to avoid false positive results.

Due to inherent differences between technologies, it is recommended that users perform method correlation studies to estimate technology differences prior to switching to a new technology.

A negative result obtained with this product means that the WNV RNA is not detected in the RNA extracted from the sample; but it cannot be excluded that the WNV RNA has a lower titre than the product detection limit (see "Performance Characteristics" section). In this case the result could be a false negative.

Results obtained with this product may sometimes be not valid due to failed internal control and require retesting, starting from extraction, that can lead to a delay in obtaining final results.

Possible polymorphisms within the region of the viral genome covered by the product primers and probes may impair detection and quantification of WNV RNA.

As with any other diagnostic medical device, the results obtained with this product must be interpreted taking into consideration all the clinical data and other laboratory tests done on the patient.

As with any other diagnostic medical device, there is a residual risk of invalid, false positive and false negative results obtained with this product. This residual risk cannot be eliminated or further reduced. In some cases, as the prenatal or emergency diagnosis, this residual risk could contribute to wrong decisions with potentially dangerous effects for the patient.

PERFORMANCE CHARACTERISTICS

Limit of Detection

The detection limit of this assay used in association to Whole Blood samples was verified with a dilutions panel of WNV. The panel was prepared using WNV DNA negative Whole Blood samples spiked with calibrated and certified reference material QCMD 2012 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, U.K.) of WNV lineage 1a (strain NY99). The panel ranged from 562 copies / mL a 10 copies / mL. Each sample of the panel was tested in 24 replicates carrying out the whole analysis procedure, extraction, reverse transcription and amplification, with ELITechGroup S.p.A. products. The statistical analysis was performed by the Probit regression. The limit of detection was calculated for the concentrations at which the probability of a positive result is the 95%.

The final results are reported in the following tables.

Limit of Detection for WNV lineage 1a in Whole Blood samples and «ELITe STAR»			
		95% confidence range	
		lower limit	upper limit
LoD (95% positivity)	**447 copies / mL**	310 copies / mL	798 copies / mL

The detection limit of this assay used in association to Urine collected without preservatives samples was verified with two dilutions panels of WNV. The panels were prepared using WNV DNA negative Urine samples spiked with calibrated and certified reference material QCMD 2012 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, U.K.) of WNV lineage 1a (strain NY99) and lineage 2a (strain Heja). The panels ranged from 316 copies / mL a 10 copies / mL. Each sample of the panel was tested in 24 replicates carrying out the whole analysis procedure, extraction, reverse transcription and amplification, with ELITechGroup S.p.A. products. The statistical analysis was performed by the Probit regression. The limit of detection was calculated for the concentrations at which the probability of a positive result is the 95%.

The final results are reported in the following tables.

Limit of Detection for WNV lineage 1a in Urine samples and «ELITe STAR»			
		95% confidence range	
		lower limit	upper limit
LoD (95% positivity)	**318 copies / mL**	228 copies / mL	554 copies / mL

Limit of Detection for WNV lineage 2 in Urine samples and « ELITe STAR »			
		95% confidence range	
		lower limit	upper limit
LoD (95% positivity)	**282 copies / mL**	188 copies / mL	545 copies / mL

The detection limit of this assay used in association to Cerebrospinal Fluid (CSF) samples was verified with a dilutions panel of WNV. The panel was prepared using WNV DNA negative CSF samples spiked with calibrated and certified reference material QCMD 2012 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, U.K.) of WNV lineage 1a (strain NY99) . The panel ranged from 316 copies / mL a 10 copies / mL. Each sample of the panel was tested in 24 replicates carrying out the whole analysis procedure, extraction, reverse transcription and amplification, with ELITechGroup S.p.A. products. The statistical analysis was performed by the Probit regression. The limit of detection was calculated for the concentrations at which the probability of a positive result is the 95%.

The final results are reported in the following tables.

Limit of Detection for WNV lineage 1a in CSF samples and «ELITe STAR»			
		95% confidence range	
		lower limit	upper limit
LoD (95% positivity)	**201 copies / mL**	145 copies / mL	345 copies / mL

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Linear measuring range

The linear measuring range of this assay was determined using three panels of dilutions of WNV in Whole Blood, Urine and CSF. The panels were prepared with negative matrixes for WNV RNA spiked with calibrated and certified reference material QCMD 2012 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, U.K.) of WNV lineage 1a (strain NY99) and lineage 2 (strain Heja). The panels ranged from 1,000,000 copies / mL to 316 copies / mL. Each sample of the panel was tested in 9 replicates carrying out the whole analysis procedure, extraction, reverse transcription and amplification, with ELITechGroup S.p.A. products. The analysis of the obtained data, performed by linear regression, demonstrated that the assay shows a linear response for all the dilutions (square correlation coefficient greater than 0.99).

The final results are reported in the following tables.

Linear measuring range WNV lineage 1a in Whole Blood with «ELITe STAR»	
Lower limit	Upper limit (tested)
447 copies / mL	**1,000,000 copies / mL**

Linear measuring range WNV lineage 1a in Urine with «ELITe STAR»	
Lower limit	Upper limit (tested)
318 copies / mL	**1,000,000 copies / mL**

Linear measuring range WNV lineage 2 in Urine with «ELITe STAR»	
Lower limit	Upper limit (tested)
316 copies / mL	**1,000,000 copies / mL**

Linear measuring range WNV lineage 1a in CSF with «ELITe STAR»	
Lower limit	Upper limit (tested)
316 copies / mL	**1,000,000 copies / mL**

N.B.: In the case of Whole Blood collected in EDTA and Urine spiked with WNV lineage 1a the Limit of Detection value has been imposed as lower limit of linear measuring range.

Precision & Accuracy

The precision of the assay, as the variability of results obtained with 9 replicates of a sample tested within the same session, was evaluated as a percentage coefficient of variation (CV%) values of Ct and as the standard deviation (SD) of the results expressed in Log copies / mL for the concentrations of WNV within the linear measuring range from 1,000,000 copies / mL to 316 copies / mL. The results are reported in the following tables.

Precision: CV% of Ct				
Samples	**WNV lineage 1a in Whole Blood**	**WNV lineage 1a in Urine**	**WNV lineage 2 in Urine**	**WNV lineage 1a in CSF**
6.0 Log copies / mL	0.43	1.16	0.81	0.98
5.0 Log copies / mL	0.30	1.25	0.73	0.50
4.0 Log copies / mL	0.68	1.09	1.02	0.71
3.0 Log copies / mL	0.76	2.18	1.23	0.56
2.5 Log copies / mL	0.97	2.82	2.71	1.01

Precision: SD of Log copies / mL				
Samples	**WNV lineage 1a in Whole Blood**	**WNV lineage 1a in Urine**	**WNV lineage 2 in Urine**	**WNV lineage 1a in CSF**
6.0 Log copies / mL	0.15	0.09	0.06	0.07
5.0 Log copies / mL	0.10	0.11	0.06	0.04
4.0 Log copies / mL	0.23	0.11	0.10	0.07
3.0 Log copies / mL	0.26	0.24	0.13	0.06
2.5 Log copies / mL	0.33	0.32	0.31	0.11

The accuracy of the assay, as the variability of results obtained with 9 replicates of a sample tested within the same session, was evaluated as a deviation from the theoretical value of the average value measured expressed in Log copies / mL for the concentrations of WNV within the linear measuring range from 1,000,000 copies / mL to 316 copies / mL. The results are reported in the following tables.

Accuracy: Quantification deviation (Log copies / mL) of technical value				
Samples	**WNV lineage 1a in Whole Blood**	**WNV lineage 1a in Urine**	**WNV lineage 2 in Urine**	**WNV lineage 1a in CSF**
6.0 Log copies / mL	0.30	0.43	0.08	0.50
5.0 Log copies / mL	0.47	0.41	0.04	0.42
4.0 Log copies / mL	0.28	0.36	0.02	0.37
3.0 Log copies / mL	0.48	0.32	0.06	0.49
2.5 Log copies / mL	0.29	0.08	0.03	0.58

The precision and the accuracy were calculated using data obtained for the study of the linear measuring range.

Reproducibility with panel for proficiency test

The reproducibility of the assay results compared with results obtained using other methods in different laboratories has been verified by testing the proficiency testing panel for QCMD 2013 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, UK). Each sample of the panel was used to perform the whole procedure for analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products. The results are reported in the following table.

Panel for proficiency test and « ELITe STAR »				
Samples	**Contents**	**Sample Status**	**Positive / Replicates**	**Mean results Log_{10} copies / mL**
WNV13-01	WNV NY99, lineage 1a	Frequently detected	2 / 2	8.28
WNV13-02	WNV NY99, lineage 1a	Frequently detected	2 / 2	7.17
WNV13-03	WNV NY99, lineage 1a	Frequently detected	2 / 2	5.92
WNV13-04	WNV NY99, lineage 1a	Frequently detected	2 / 2	5.99
WNV13-05	WNV NY99, lineage 1a	Detected	2 / 2	4.97
WNV13-06	WNV NY99, lineage 1a	Detected	2 / 2	3.95
WNV13-07	WNV Heja, lineage 2	Frequently detected	2 / 2	8.12
WNV13-08	WNV Heja, lineage 2	Frequently detected	2 / 2	7.10
WNV13-09	WNV Ug37, lineage 2	Frequently detected	2 / 2	7.60
WNV13-10	flavivirus not-WNV	Negative	0 / 2	-
WNV13-11	flavivirus not-WNV	Negative	0 / 2	-
WNV13-12	VTM Negative	Negative	0 / 2	-

All samples were correctly detected. The sample WNV13-10 containing Japanese Encephalitis virus, Dengue 1 virus, Dengue 2 virus, Dengue 4 virus, and the sample containing WNV13-11 Yellow Fever 17D virus, Dengue 3 virus, dengue 4 virus, Tick borne Encephalitis virus resulted negative.

Detection and quantification efficiency with different genotypes

The efficiency of detection and quantification of the different genotypes was evaluated by comparison of sequences with nucleotide databases. The analysis of the regions chosen for the hybridization of primer and fluorescent probe on the alignment of the sequences available in the database of the NS5 gene of WNV, Lineage Lineage 1a and 2, showed conservation and the absence of significant mutations.

The detection and quantification efficiency of the different genotypes was evaluated using a plasmid construct containing the amplified region of WNV lineage 1a of Mediterranean strain Ita09.

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A plasmid construct containing the amplified region of the first WNV lineage 1a strain Ita09 (ENA accession number GU011992) was quantified by spectrophotometer reading and diluted to concentrations of 100,000, 10,000, 1,000, 100 copies per reaction. Each sample was tested in triplicate with ELITechGroup S.p.A. products. The results are reported in the following table.

Detection and quantification efficiency on WNV lineage 1a strain Ita09 sequences		
Theoretical concentration copies / reaction	**Positives / Replicates**	**Mean results copies / reaction**
5.00	3 / 3	5.11
4.00	3 / 3	3.98
3.00	3 / 3	2.94
2.00	3 / 3	2.06

The detection and quantification efficiency of different genotypes was evaluated by analyzing certified reference material positive for WNV lineage 2 lineage 1a and Institute of Health (ISS, Italy). Each sample was used to perform the whole procedure of analysis, extraction, reverse transcription and amplification, with ELITechGroup S.p.A. products. The results are reported in the following table.

Detection and quantification efficiency on WNV lineage 1a strain Ita09 sequences			
Samples	**Theoretical quantity Log copies / mL**	**Positivity**	**Measured quantity Log copies / mL**
WNV RNA ISS0213 (lineage 1a)	3.176	Yes	3.371
WNV RNA ISS0411 (lineage 2)	3.000 - 3.698	Yes	3.764

Potential interfering markers

The absence of cross-reactivity with other markers potentially interfering, was evaluated by comparison of sequences with nucleotide databases.

The analysis of the alignment of the sequences of the oligonucleotide primer and fluorescent probe with the sequences available in the database of different organisms from WNV, including other flavivirus of the Japanese encephalitis antigenic complex including the Usutu virus, showed their specificity and the lack of significant homologies.

The absence of cross-reactivity with other potentially interfering markers has been verified by testing the proficiency testing panel for QCMD 2013 West Nile Virus (RNA) EQA Programme.

The results obtained with the panel are presented in "Reproducibility with proficiency test panel". In particular, the sample WNV13-10 containing Japanese Encephalitis virus, Dengue 1 virus, Dengue 2 virus, Dengue 4 virus, and the sample WNV13-11 containing Yellow Fever 17D virus, Dengue 3 virus, Dengue 4 virus, Tick Borne Encephalitis virus, resulted negative.

The absence of cross-reactivity with other markers potentially interfering, was verified using reference materials from proficiency panel (Qnostics Ltd, UK) for CMV (QCMD 2012 Human Cytomegalovirus (DNA) EQA Programme), EBV (Epstein 2008 QCMD Barr virus (DNA) EQA Programme), for HSV1 and HSV2 (Herpes Simplex virus 2008 QCMD (DNA) EQA Programme) and VZV (Varicella Zoster virus QCMD 2012 (DNA) EQA Programme).

Each sample panel was used to perform the whole procedure for analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products.

All samples were confirmed negative.

Interfering substances

The possible effect on the rate of interfering substances was evaluated by analyzing the panel "AcroMetrix® Inhibition Panel" (Life Technologies Inc.) with samples containing potential interfering endogenous substances, resulting from hemolysis, jaundice and lipemia, and exogenous, anticoagulants EDTA and heparin. Samples of the panel were spiked with reference material QCMD 2012 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, UK) at a concentration of 3 x LoD. Each sample was used to perform the whole procedure of analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products.

All samples were positive, but the sample with heparin is inhibited (Ct of WNV and Internal Control significantly delayed).

Absence of cross-reactivity

The absence of cross-contamination, was verified by analyzing the results of three sessions in which samples positive for WNV RNA were alternated with samples negative for WNV RNA. No sample negative for WNV RNA was positive.

A sample of whole blood negative for WNV RNA was used as a negative sample and, after addition of reference material QCMD 2012 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, UK) to a title of 10,000 copies / mL, as a positive sample. Three series of 6 positive samples alternating with 6 negative samples were used to perform the whole procedure of analysis, extraction, reverse transcription and amplification, with ELITechGroup S.p.A. products. The results are reported in the following table.

Samples	N	positive	negative
WNV RNA positive Whole Blood	18	18	0
WNV RNA negative Whole Blood	18	0	18

Whole system failure rate

The overall failure rate of the system leading to false negative results was verified by analyzing a panel of samples spiked for WNV RNA of low titre. No sample spiked for WNV RNA was negative.

A sample of whole blood negative for WNV RNA was spiked with reference material QCMD 2012 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, UK) to a title of 1,500 copies / mL. The sample was used in 60 replicates to perform the whole procedure of analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products. The results are summarized in the following table.

Samples	N	positive	negative
WNV RNA spiked Whole Blood	60	60	0

Inter-lot reproducibility

The inter-lot reproducibility assay was verified by analyzing the correlation and the variability of the results obtained with reference material and three different lots of product. The essay presented a CV% of Ct values lower than 4%.

A sample of whole blood collected in EDTA negative for WNV RNA and reference material QCMD 2012 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, UK) were used to prepare the following panel of 12 samples:

- 3 samples spiked to 1,350 copies / mL (3x LoD);
- 3 samples spiked to 450 copies / mL (1x LoD);
- 3 samples spiked to 225 copies / mL (0.5x LoD);
- 3 samples negative for WNV.

The panel has been used by three different operators in association to the extraction system **«ELITe STAR»**. The extracted samples were reverse transcribed and amplified, using 3 lots of ELITechGroup S.p.A. products, on different days and using different real time amplification instruments. The results are reported in the following table.

Samples	Positive / replicates	Mean of Ct WNV	SD	CV%	Mean of Ct CI	SD	CV%
3x LoD	9 / 9	35.25	0.81	2.29	31.28	0.87	2.78
1x LoD	9 / 9	36.22	1.43	3.95	31.13	0.55	1.78
0,5x LoD	9 / 7	37.79	1.15	3.04	30.91	0.27	0.88
Negative	0 / 9	-	-	-	30.88	0.39	1.27

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Diagnostic Sensitivity: confirmation of positive samples

The diagnostic sensitivity of the assay, as confirmation of positive clinical samples, was evaluated by analyzing some clinical samples of Whole Blood, CSF and Urine spiked for WNV RNA, given the difficulty of finding a significant number of positive clinical samples. The overall diagnostic sensitivity was equal to 96.5%.

The test with whole blood collected in EDTA was performed on 30 samples from different subjects. The samples were spiked in a first series with the RNA of WNV lineage 1a and in a second series with that of the lineage 2 QCMD the 2010 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, UK) to a title of 500 copies / mL. Test with WNV lineage 2 were then added with another 10 samples of whole blood collected in EDTA spiked to a title of 500 copies / mL. Each sample was used to perform the whole procedure of analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products. The results are summarized in the following table.

Samples	N	positive	negative
WNV RNA Lineage 1a spiked Whole Blood	30	30	0
WNV RNA Lineage 2 spiked Whole Blood	39	34	5
Total	69	64	5

Five samples were negative with ELITechGroup S.p.A. products, probably due to the concentration near the LOD value (447 copies / mL).

A sample result is not valid due to an unidentified inhibitor and was not included in the calculation of diagnostic sensitivity.

The diagnostic sensitivity of the assay in this test with Whole Blood was equal to 92.7%.

The test with urine collected without preservatives was performed on 30 samples from different subjects. The samples were spiked in a first series with the RNA of WNV lineage 1a and in a second series with that of the lineage 2 QCMD the 2010 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, UK) to a titre/total of 500 copies / mL. Each sample was used to perform the whole procedure of analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products. The results are summarized in the following table.

Samples	N	positive	negative
WNV RNA Lineage 1a spiked Urine	30	29	1
WNV RNA Lineage 2 spiked Urine	30	30	0
Total	60	59	1

A sample was negative with ELITechGroup S.p.A. products, probably due to the concentration near the LoD (318 copies / mL).

The diagnostic sensitivity of the assay in this test with Urine was equal to 98.3%.

The CSF test was performed on 20 samples from different subjects. The samples were spiked in a first series with the RNA of WNV lineage 1a and in a second series with that of the lineage 2 QCMD the 2010 West Nile Virus (RNA) EQA Programme (Qnostics Ltd, UK) to a titre/total of 500 copies / mL. Each sample was used to perform the whole procedure of analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products. The results are summarized in the following table.

Samples	N	positive	negative
WNV RNA Lineage 1a spiked CSF	20	20	0
WNV RNA Lineage 2 spiked CSF	20	20	0
Total	40	40	0

The diagnostic sensitivity of the assay in this test with CSF was equal to 100%.

Diagnostic Specificity: confirmation of negative samples

The diagnostic specificity of the assay, as confirmation of negative clinical samples, was evaluated by analyzing some clinical samples of whole blood, CSF and urine tested negative for WNV RNA. The overall diagnostic specificity was equal to 100%.

The test with whole blood collected in EDTA was performed on 30 samples from different subjects. The samples were tested negative for WNV RNA with a "home-made" real time amplification product. Each sample was used to perform the whole procedure of analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products. The results are summarized in the following table.

Samples	N	positive	negative
Whole Blood negative for WNV RNA	24	0	24

Six samples were "not valid" because of an unidentified inhibitor and were not included in the calculation of diagnostic specificity.
The diagnostic specificity of the assay in this test with Whole Blood was equal to 100%.

The test with Urine collected without preservatives was performed on 30 samples from different subjects. The samples were tested negative for WNV RNA with a "home-made" real time amplification product. Each sample was used to perform the whole procedure of analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products. The results are summarized in the following table.

Samples	N	positive	negative
Urine negative for WNV RNA	27	0	27

Three samples were "not valid" because of an unidentified inhibitor and were not included in the calculation of diagnostic specificity.
The diagnostic specificity of the assay in this test with Urine was equal to 100%.

The CSF test was performed on 20 samples from different subjects. The samples were tested negative for WNV RNA with a "home-made" real time amplification product. Each sample was used to perform the whole procedure of analysis, extraction, reverse transcription and amplification with ELITechGroup S.p.A. products. The results are summarized in the following table.

Samples	N	positive	negative
CSF negative for WNV RNA	20	0	20

The diagnostic specificity of the assay in this test with CSF was equal to 100%.

N.B.: The complete data and results of the tests carried out to evaluate the performance characteristics of the product are recorded in Section 7 of the Product Technical File "WNV ELITe MGB® Kit", FTP RTS100PLD.

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TROUBLESHOOTING

Target DNA not detected in the Q - PCR Standard reactions or not valid correlation coefficient of the Standard curve	
Possible Causes	**Solutions**
Error in preparation of complete reaction mix.	Check the volumes of dispensed reagents during the preparation of the complete reaction mix.
Incorrect dispensing into the microplate wells.	Take care when dispensing reactions into the microplate wells and comply with the work sheet. Check the volumes of reaction mixture dispensed. Check the volumes of standard dispensed.
Probe degradation.	Use a new aliquot of PreMix.
PCR MasterMix degradation.	Use a new aliquot of PCR MasterMix.
Standard degradation.	Use a new aliquot of standard.
Instrument setting error.	Check the position settings for the standard reactions on the instrument. Check the thermal cycle settings on the instrument.

Target RNA / DNA detected in the Negative control reaction	
Possible Causes	**Solutions**
Incorrect dispensing into the microplate wells.	Avoid spilling the contents of the sample test tube. Always change tips between one sample and another. Take care when dispensing samples, negative controls, standards into the microplate wells and comply with the work sheet.
Error while setting the instrument	Check the position settings of the samples, negative controls, standards on the instrument
Microplate badly sealed.	Take care when sealing the microplate.
Contamination of the Molecular Biology grade water.	Use a new aliquot of Molecular Biology grade water.
Contamination of the complete reaction mixture.	Prepare a new aliquot of complete reaction mixture.
Contamination of the extraction / preparation area for amplification reactions.	Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use.

Target and Internal Control RNA not detected in the sample reactions	
Possible Causes	**Solutions**
Incorrect complete reaction mix preparation.	Check the reagent volumes dispensed during complete reaction mix preparation. Verify that RT EnzymeMix was added to complete reaction mix.
Degradation of RT EnzymeMix.	Use a new aliquot of RT EnzymeMix.
Incorrect reagent storage.	Verify that RT EnzymeMix was not exposed to temperature higher than -20 °C for more than 10 minutes. Verify that complete reaction mix was not exposed to room temperature for more than 30 minutes.
Problems during extraction	Verify quality and concentration of extracted RNA.

Irregular or high background fluorescence in the reactions	
Possible causes	**Solutions**
Incorrect dispensing of sample.	Take care, by pipetting thoroughly three times, when mixing samples, negative controls and standards into the reaction mixture. Avoid creating bubbles.
Baseline setting error.	Set the baseline calculation range within cycles where the background fluorescence has already stabilized (check the "Results", "Component" data) and the signal fluorescence has not yet started to increase, e.g. from cycle 6 to cycle 15. Use the automatic baseline calculation by setting the "Auto Baseline" option.

Anomalous dissociation curve	
Possible causes	**Solutions**
Absence of a defined peak. Defined peak but different from that of the other samples and of the standards or positive control.	Check for detector FAM Ct lower than 30. High quantity of amplification product at the end of the reaction may interfere with the melting curve analysis. Repeat the sample amplification to confirm the presence of target RNA with a possible mutation. The target RNA of the sample should be sequenced to confirm mutation.

WNV ELITe MGB® Kit
reagents for RNA reverse transcription and cDNA Real Time amplification

REF RTS100PLD

SYMBOLS

REF	Catalogue Number.
	Upper limit of temperature.
LOT	Batch code.
	Use by (last day of month).
IVD	*in vitro* diagnostic medical device.
CE	Fulfilling the requirements of the European Directive 98\79\EC for *in vitro* diagnostic medical device.
	Contains sufficient for "N" tests.
	Attention, consult instructions for use.
CONT	Contents.
	Keep away from sunlight.
	Manufacturer.

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ELITe® MGB detection reagents are covered by one or more of U.S. Patent numbers 6,127,121, 6,485,906, 6,660,845, 6,699,975, 6,727,356, 6,790,945, 6,949,367, 6,972,328, 7,045,610, 7,319,022, 7,368,549, 7,381,818, 7,662,942, 7,671,218, 7,715,989, 7,723,038, 7,759,126, 7,767,834, 7,897,736, 8,008,522, 8,067,177, 8,163,910, 8,389,745, 8,969,003, 8,980,855, 9,056,887, 9,085,800, 9,169,256 and EP patent numbers 1068358, 1144429, 1232157, 1261616, 1430147, 1781675, 1789587, 1975256, 2714939 as well as applications that are currently pending.

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WORK SHEET

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