West Nile Virus Quantification Kit v2

USER MANUAL

For *in vitro* Diagnostic Use

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IVD CE

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1. PRODUCT DESCRIPTION

Bosphore® West Nile Virus Quantification Kit v2 detects and quantitates West Nile Virus RNA in human biological samples such as serum, plasma, urine and CSF. The linear range of quantitation is $1x10^2$ -$1x10^8$copies/ml and the analytic sensitivity is 50 copies/ml. A region within the 3' UTR region is amplified and fluorescence detection is accomplished using the FAM filter.

An internal control has been integrated into the kit in order to check PCR inhibition. The amplification data of the internal control is detected with the HEX filter.

2. CONTENT

Bosphore® West Nile Virus RNA Quantification Kit v2 is composed of following components:

Component	REAGENT	100 Tests	50 Tests	25 Tests
1	dH_2O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Master Mix	(1660 µl)	(830 µl)	(415 µl)
3	Internal Control	(15 µl)	(15 µl)	(15 µl)
4	Positive Control	(44 µl)	(22 µl)	(15 µl)
5	Standard 1 (1000 copies/µl)*	(88 µl)	(44 µl)	(22 µl)
6	Standard 2 (100 copies/µl)*	(88 µl)	(44 µl)	(22 µl)
7	Standard 3 (10 copies /µl)*	(88 µl)	(44 µl)	(22 µl)
8	Standard 4 (2 copies/µl)*	(88 µl)	(44 µl)	(22 µl)

*****See section "Analysis" Section for RNA quantitation

3. STORAGE

Bosphore® West Nile Virus Quantification Kit v2 PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min. and the detection mix components should not be exposed to light or air more than necessary, vials must be kept closed except during pipetting. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

4. REQUIRED MATERIALS AND DEVICES

- Montania® 483, Montania® 484 or Montania® 4896 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM and HEX filters (such as iCycler, iQ5, CFX–BioRad, LightCycler 2.0, 480-Roche, 7500 Real-Time PCR System-ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, LineGene 9600-Bioer, Rotorgene 2000, 3000, 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes, PCR plates or strips
- Magnesia® 16 Nucleic Acid Extraction System or Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot / Magnesia® Viral Nucleic Acid Extraction Kit or Magnesia® 2448 Viral DNA/RNA Extraction Kit/ Magrev™24 stand and Magrev™ Viral DNA/RNA Extraction Kit/ Bosphore® Viral RNA Extraction Spin Kit (Anatolia Geneworks), or other high quality viral RNA extraction kits and systems
- Deep freezer (-20°C)

- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20ºC.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- Pathogen information should be reviewed to be aware of the health related risks.
- Serum/plasma samples should be handled with extreme caution: Physical contact with pathogens should be avoided by; wearing lab coats and gloves, no allowance for eating or drinking within the workspace, prevention of unauthorized individuals' access to the working area.
- All the pathogenic wastes produced during the nucleic acid isolation step; including the serum/plasma samples and material contacted with them, should be discarded into medical waste and disposed safely.

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual.
- This product is to be used by personnel specially trained to perform in vitro diagnostic procedures.

7. PATHOGEN

Causative agents

West Nile Virus (WNV) is a flavivirus belonging to the Flaviviridae family that was first isolated in 1937 in "West Nile" province of Uganda/Africa and was named after this region. WNV virions are approximately 50 nm in diameter and are composed of a single-stranded RNA genome of length between 11kb and 12 kb. [1]

Epidemiology

WNV was originally isolated in the West Nile region of Uganda in 1937. It was then identified in Congo, Sudan, India, Egypt and Israel. Later on, infection spread outside Africa and was eventually found to be widespread in Africa,

West Asia, Middle East, Southern Europe, Australia and US. Several WNV outbreaks has occurred in Israel (1957, 2000), US (1999, 2003-2007), Romania (1996-1997) and Canada (2002-3003). Recent WNV outbreak in Greece in 2010 with more than 10 deaths has been reported. WNV infection has been identified to be the cause of 3 deaths between August and September of 2010 in Turkey. Although WNV was first emerged as a distinct virus, currently it is classified into two lineages where lineage 1 is associated with clinical human encephalitis and lineage 2 with several outbreaks in horses. [2], [3]

Modes of Transmission

Most of the WNV infections are asymptomatic without any symptoms in people. Second type of WNV infections result in mild fever (West Nile Fever) with recovery from the illness, but however, third type of WNV infections with the virus crossing the blood-brain barrier cause severe outcomes such as inflammation of the brain (encephalitis) and the tissue surrounding the brain and spinal cord (meningitis) that all resulting in long-term effects like memory loss, depression, etc. The common route of WNV transmission is shown to be the mosquitos as vectors carrying the virus across vertebrate hosts. Mosquitos receive the virus upon feeding on infected birds, and then virus is transmitted to other hosts via mosquito bites. Humans, horses and most other mammals are widely accepted as incidental hosts as they don't transmit virus to others. However, with increasing research after WNV outbreaks, new WNV transmission routes has been shown to be transmitted via blood transfusion, organ transplantation, intrauterine exposure and breast feeding. [3]

8. METHOD

Bosphore® West Nile Virus Quantification Kit v2 is based on the Real Time RT PCR method. West Nile genetic material is amplified by Reverse transcription technique because it is composed of RNA. RT-PCR which is also referred as RNA PCR is a two-step reaction. Firstly complementary DNA is synthesized from RNA by reverse transcription and then complementary DNA is amplified by standard PCR. The primer binds to the target RNA region in RT-PCR and RNA-DNA double strand is synthesized by reverse transcriptase enzyme using the RNA template for complementary DNA. Afterwards, standard PCR continues.

Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. As a brief explanation, primers are small synthetic DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis. In addition to these components, in RT PCR reverse transcriptase is added to the reaction and cDNA synthesis from the RNA template is acquired.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity.

The assay utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent probe during the extension phase of PCR. The probe is labeled at the 5′ end with a fluorescent 'reporter' molecule, and at the 3′end with another fluorescent molecule that acts as a 'quencher' for the 'reporter'. When the two fluorophores are in close proximity, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step

of PCR, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, fluorescence signal can be detected.

The fluorescence generated by the reporter increases as the PCR product is accumulated; the point at which the signal rises above background level and becomes distinguishable, is called the threshold cycle (C_T). There is a linear relationship between the log of the starting amount of a template and its threshold cycle, thus starting amount of unknown templates can be determined using standard curves constructed using C_T values of the known starting amounts of target templates.

Bosphore West Nile Virus Quantification Kit v2 employs multiplex PCR, and an internal control is incorporated into the system in order to check for possible PCR inhibition. West Nile Virus RNA (cDNA) and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the West Nile Virus amplification is detected by a probe labeled at the 5′ end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification, is detected by a second probe (labeled at the 5′ end with a different reporter molecule, HEX) through the HEX channel.

9. PROCEDURE

9.1. Sample Preparation, Storage and Transport

To isolate serum from the clinical specimen, the blood sample should be collected into sterile vacutainers without any anticoagulant. For venipuncture, only sterile material should be used.

The serum should be separated from blood within 6 hours after blood collection. To separate the serum, the blood container should be centrifuged at 800-1600 x g for 20 minutes. The separated serum should be transferred to polypropylene tubes and stored at -20ºC or lower, until use.

The samples should be transported in containers with capacity to resist pressure. Transportation should be done according to local and national regulations for pathogen material transport.

9.2. RNA Isolation

We recommend that the Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot or Magnesia® 16 Nucleic Acid Extraction System / Magnesia® 2448 Viral DNA/RNA Extraction Kit or Magnesia® Viral Nucleic Acid Extraction Kit/Magrev™24 stand and Magrev™ Viral DNA/RNA Extraction Kit/ Bosphore® Viral RNA Extraction Spin Kit (Anatolia Geneworks) isolation system is used with Bosphore West Nile Virus Quantification Kit v2. The RNA isolation should be performed according to the manufacturers' instructions.

9.3. Kit Components

9.3.1. PCR Master Mix

PCR mix contains; HotStarTaq DNA Polymerase, Probe RT-PCR Buffer, ROX passive reference dye, RT Mix, Detection Mix 1 and Detection Mix 2.

HotStarTaq DNA Polymerase: HotStarTaq DNA Polymerase is a modified form of Taq DNA Polymerase, and is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme remains completely inactive during the reverse-transcription reaction and does not interfere with it. This prevents formation of misprimed RT-PCR products and primer–dimers during reaction setup, reverse transcription, and the first denaturation step. The enzyme is activated after the reverse-transcription step by a 15-minute, 95°C incubation step.

The hot start also inactivates the reverse-transcription enzymes, ensuring temporal separation of reverse transcription and PCR, and allowing both steps to be performed sequentially in a single tube.

Probe RT-PCR Buffer: It is a unique OneStep RT-PCR buffer system, and has been specifically adapted for realtime RT-PCR using sequence-specific probes. The buffer contains a balanced combination of KCl and $(NH_4)2SO_4$.

ROX passive reference dye: For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR–related variations in fluorescence detection such as differences in fluorescence detection between wells due to slight variations in reaction volume or to differences in well position. The use of ROX dye is necessary only for some instruments. For the other instruments which do not require ROX dye, like Montania 483, the presence of ROX dye in the master mix does not interfere with real-time PCR on any instrument, since the dye is not involved in the reaction and has an emission spectrum completely different from fluorescent dyes commonly used for probes.

RT Mix contains a unique Omniscript and Sensiscript blend. Both enzymes exhibit a high affinity for RNA, facilitating transcription through secondary structures that may inhibit other reverse transcriptases. Omniscript is designed for reverse transcription of RNA amounts greater than 50 ng, and Sensiscript is optimized for use with very small amounts of RNA (<50 ng). This enzyme combination provides highly efficient and sensitive reverse transcription over a wide range of RNA template amounts.

Detection Mix 1 contains West Nile Virus -specific forward and reverse primers and a dual-labeled probe.

Detection Mix 2 contains internal control-specific forward and reverse primers and a dual-labeled probe.

9.3.2. Internal Control

The internal control is a synthetic DNA molecule. Internal control is added directly into the PCR master mix to control the RT-PCR inhibition exclusively. For this purpose, 0.1 µl of internal control should be added for each reaction into the master mix. Lack of internal control amplification in the FAM negative samples, may indicate a problem in isolation or PCR inhibition. In this case, isolation and PCR should be repeated. In samples that contain a high viral load, internal control can be suppressed and no increase of the signal is detected. Please use the table below for the interpretation of internal control data:

West Nile Virus (FAM)	Internal Control (HEX)	Interpretation
+	+	Sample positive
-	+	Sample negative
+	-	Sample positive
-	-	Repeat the test!

9.3.3. Quantitation Standards

The quantitation standards are calibrated standards of 1×10^3, 1×10^2, 1×10^1, and 2 copies/µl.

9.4. Preparing the RT-PCR

All four external quantitation standards should be added into the RT-PCR reaction together with the samples and the negative control (PCR-grade water). Make sure that all the kit components are thawed before use. Refer to the table below for preparing the RT-PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 5 samples, an extra 10% should be added to the total sample number.

PCR Master Mix	14.9µl
Internal Control	0.1 µl
Sample RNA **Standard** **Negative/Positive Control**	10 µl
Total Volume	25 µl

Pipette 15 µl of the master mix into the PCR tubes or strips, and add 10 µl of RNA (sample/ standard/positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

9.5. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore® West Nile Virus Quantification Kit v2 is composed of two-steps; firstly a reverse transcription step and secondly Real Time PCR steps; an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

Reverse Transcription	50°C	30:00 min.	
Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	50 cycles
Annealing and Synthesis (Data Collection)	55.5°C	01:10 min.	
Hold	22°C	05:00 min.	

Before starting a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM and HEX),
- Identify unknown samples, standards, positive and negative controls, assign quantitative values to the standards,
- Select the correct thermal protocol,
- Start the protocol

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold.

Example of an amplification curve is given in Fig. 1.

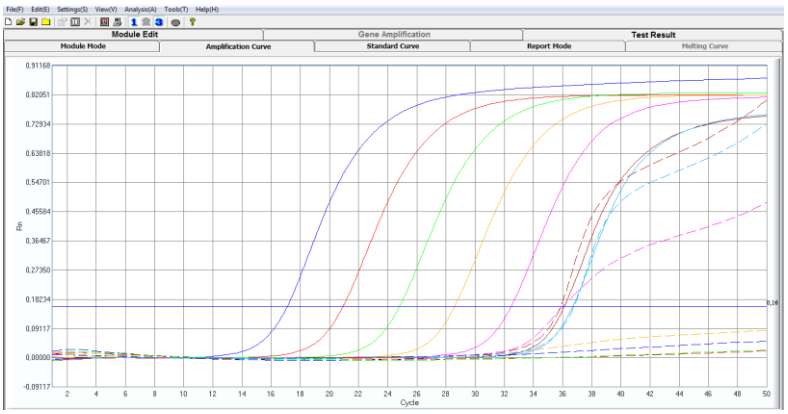

Fig. 1: Amplification Curve of a Bosphore® WNV v2 test

The standard curve is plotted using the data obtained from the defined standards, with the axes Ct-Threshold Cycle and Log Starting Quantity. Example of a standard curve is given in Fig. 2.

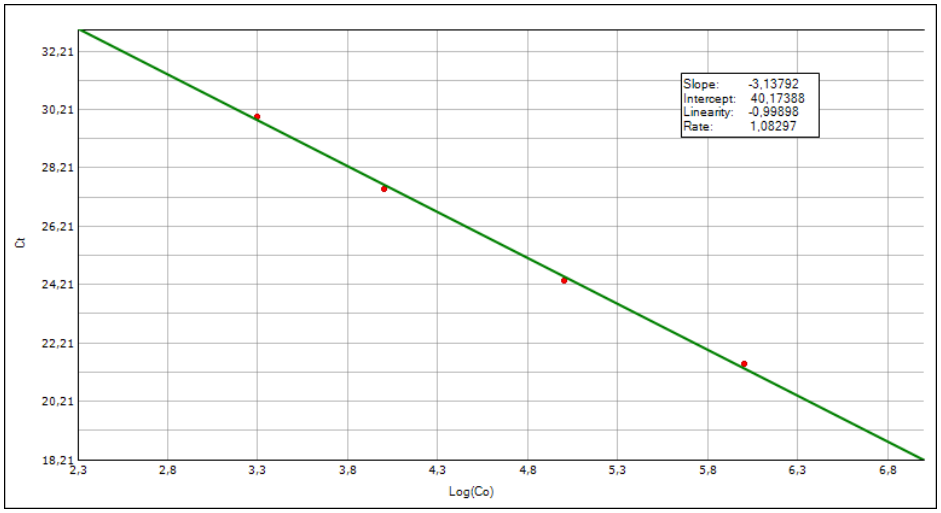

Fig.2: Standard Curve of a Bosphore™ West Nile Virus v2 test

Caution!: Since the quantitation standards are provided as plasmid samples and as copies/µl, the following formula should be applied to the resulting copies/µl values obtained for the samples, to assess the quantitation values of the samples in copies/ml. This mathematical factor takes the starting volume of RNA extraction and the elution volume into consideration, to ensure correct quantitation of the samples:

$$\frac{\text{(Result in copies/µl) x (Elution Volume in µl)}}{\text{(Starting Extraction Volume in ml)}} = \text{Result in copies/ml}$$

For example, if a sample's result from the automated Standard Curve was calculated as 1000 copies/µl, considering that the starting extraction volume is 400 µl and the elution volume is 60 µl, applying the formula; 1000 x 60 ÷ 0.4 = 150 000 copies/ml is calculated as the West Nile Virus RNA that the sample material contains.

Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, and if the system's software allows pulling down the threshold as much as possible in order to detect low positive samples, attention should be paid to keep the threshold line above the background and to keep the correlation coefficient at the maximum possible value (and within its acceptance criteria.

The table below displays the acceptance criteria for Bosphore® West Nile Virus v2.

Component/Parameter	Cycle Threshold (C_T)
Standard 1	21±2
Standard 2	24±2
Standard 3	27.5±2
Standard 4	30±2

Correlation Coefficient	27±4
PCR Efficiency	>0.970

Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if an impairment in the product's performance is observed (See the last page for contact information).

The quantitative results of the test are displayed on the "Report Mode" screen. A spread sheet containing the calculated starting quantities of the unknown samples in each tube is shown. The samples that cross the threshold in Channel 1 (FAM) are displayed with a calculated starting quantity, samples that do not cut the threshold are displayed as "No Ct". These samples are regarded as negative or having a viral load below the detection limit of the assay. For these undetectable samples, the HEX data of the internal control should also be checked to avoid false negative results.

The following table shows the possible results and their interpretation:

Signal detected in FAM filter pair	The sample contains West Nile Virus RNA, the result is positive	No need to check the internal control since the sample is positive (high positive samples may suppress the signal from the internal control)
No signal in FAM, signal in HEX	The West Nile Virus RNA in the sample is not detectable	Signal from HEX filter pair rules out the possibility of PCR inhibition
No signal in FAM and HEX	The diagnosis is inconclusive	No signal in HEX points out to PCR inhibition or to a problem in RNA isolation

11. SPECIFICATIONS

11.1. Sensitivity

Analytical sensitivity may be expressed as the limit of detection: i.e. the smallest amount of the target marker that can be precisely detected. The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value. The analytical sensitivity or detection limit for NAT assays is expressed by the 95% positive cut-off value.

The analytical detection limit for Bosphore® West Nile Virus v2 was found to be 50 copies/ml. The sensitivity was determined using serial dilutions of RNA calibrated with the plasmid positive control. The dilutions were tested in different runs in replicates. The results were analyzed by probit method.

11.2. Linear Range

The linear range of Bosphore® West Nile Virus Quantification Kit v2 was determined to be $1x10^2$ -$1x10^8$ copies/ml. The standard curve correlation coefficient was found to be 0.999 for the linearity run with a dilution series of the West Nile Virus Positive Control (Figure 3 and 4).

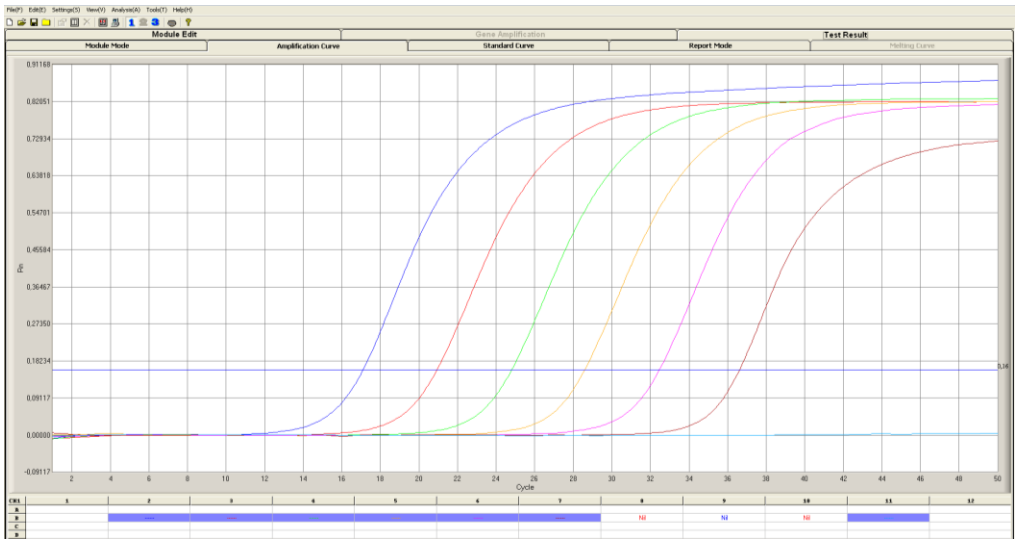

Fig. 3: Linear Range Amplification Curve

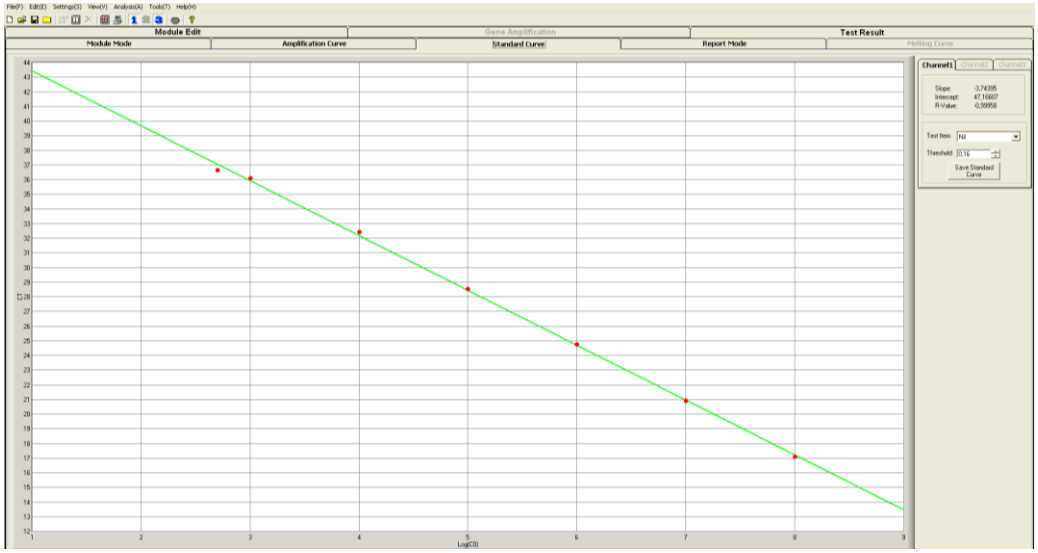

Fig. 4: Linear Range Standard Curve

11.3. Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were employed. Primer and probe sequences were checked for possible homology to other known pathogen sequences by sequence comparison analysis using database alignment. Samples of HCV, HIV, HDV with known high positivity were tested, and found negative.

11.4. Reproducibility

Reproducibility data (on C_T value basis) were obtained by the analysis of one of the quantitation standards of the Bosphore® West Nile Virus Quantification Kit v2. Test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given in Table 1:

Table 1: Reproducibility Data.

West Nile Virus (10^5 Copies/ml)	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay Variability N=4	0,089	0,008	0,27
Inter-lot Variability N=3	0,05	0,002	0,14
Inter-operator Variability N=3	0,10	0,010	0,30
Total Inter-assay Variability N=5	0,11	0,013	0,36

12. REFERENCES

1. Brinton MA: The molecular biology of West Nile Virus: a new invader of the western hemisphere. Ann Rev Microbiol 2002;56:371-402
2. 2008 Final West Nile Activity Map, Centers for Disease Control and Prevention
3. Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL (2005). "Epidemiology and transmission dynamics of West Nile virus disease". Emerging Infect. Dis. 11 (8): 1167–73.

13. SYMBOLS


Use by

Lot/Batch

Catalog number


Temperature limitation


Caution, consult accompanying documents


Manufacturer

In Vitro Diagnostic Medical Device

14. CONTACT INFORMATION

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