West Nile Virus (WNV) detection & quantitation by qRT-PCR (Eva green).

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This protocol describes the real-time polymerase chain reaction with reverse transcription (qRT-PCR) approach developed for the detection and quantitation of viral titres in biological specimens using either a two-step (separate RT and PCR reactions) or one-step EVA-Green modality (but easily TaqMan adaptable). WNV is a group IV (+)ssRNA species of an unassigned order, Flaviviridae family, Flavivirus genus related to the dengue, yellow fever, Japanese encephalitis, and Zika viruses. WNV is a member of the Japanese encephalitis antigenic serocomplex of viruses, exhibits a similar virion morphology as DENV of 45‑50 nm. Its genome is 11 to 12 kb in length encodes seven nonstructural proteins and three structural proteins. The RNA strand is held within a nucleocapsid formed from 12-kDa protein blocks; the capsid is contained within a host-derived membrane altered by two viral glycoproteins.

Oligonucleotide primers

Name	Sequence[1]	bp	%GC	Tm	Hair	HomD	HetD	Amplicon
WNV-F	5′-CAg-ACC-ACg-CTA-Cgg-Cg-3′	17	70.6	59	-3.1	-6.75	-13.12	101
WNV -R	5′-CTA-ggg-CCg-CgT-ggg-3′	15	80	59.4	-0.59	-10.36		

Note 1: Reverse oligonucleotide primer sequences given in this table are the reverse-complement of sequence present in alignments and as they should be ordered for synthesis; Hairpin (Hair), homodimer (homD) and heterodimer (HetD) ΔQ's given in kcal/mol.

Oligonucleotide primer map in artificial gene[2] (underlined primer sites)

AAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCA
AATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGTCTATAATATGGA
CTACCCGCCCTTTGGCGCAGGAAGACCAGGACAATTTGG|CAGACCACGCTACGGCGTGCTACTCTGCGGAGAGTGCAGTCTGCG
ATAGTGCCCCAGGAGGACTGGGTTAACAAAGGCAAACCAACGCCCCACGCGGCCCTAG|AAGGACTAGAGGTTAGAGGAGACCAT
GTCCATGTCACCCACGGTCATCATCCTGGCATGTCTTGGGTTCTATCCTGCTGTCTCTACAGCATCATTCCAGGCACAGAACGCC
|AAGGACTAGAGGTTAGAGGAGACCCCCCCAACACAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGC
AACATCAATCCAGGCACAGAGCGCC|AAATACACATACCAAAACAAAGTGGTGAAGGTTCTCAGACCAGCTGAAGGAGGGAAAAC
AGTCATGGACATCATCTCAAGACAAGACCAGAGAGGGAGTGGA|ATGTATGTGAGTGCTGATGCCACGAAATGGTCACCAGGAGA
TAATTCGGCAAAGTTTAAGAGATTCACACAGGCATTATATGATGGCTTGTCAGATGAGAAGTTAAAATGTTGCGTTGTTGATGC|
ATGTAACACCTCTACAATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTCTCTTTGAAGCCTGAGATTATCGTG
GATCAATATGAGTACAAGTACCCTGC|GCATAGCTGTAGGAAGGACTCTCAAGGCATTTTTCTCCTGGTCGCTTACTGACCCTTT
AGGGAACGAAGCCCCTGGGGGATACTGTCTTGAAAAGTGGATGCTT|GCAACGCGCGATTCAGTTCCTCTTCACATAATCGCCCC
GAGCTCGCTTATCGTTTAAGCAGCTCTGCGCTACTATGGGTCCCGTGTAGAGGC|TGATGATGCCGTCGTGTGCTACAACAGTAA
CTATGCGGCTCAAGGTTTAGTAGCTAGCATTAAGAACTTTAAGGCAGTTCTGTATTATCAAGATAATGTGTTCATGTCTGAGGCA
AAATGTTGGACTGAGACTGACCTTACTAAAGGACCTCACGAATTTTGCTCACA

Note 2: Primer sites for different pathogen sequences shown in red, TaqMan probe sites shown in blue,

WNV specific primer binding sites shown in grey highlight. WNV artificial nucleocápside 3' UTR gene sequence derived from Bethesda reference strain partial sequence as available from GenBank accession number AY646354.

Two-step (separate RT and PCR) components and conditions

Two-step (separate RT and PCR) uses M-MULV RT Enzyme (Vivantis Cat. No.: ME2305) and iTaq qPCR Master Mix (Biorad Cat. No.: 1725150).

First strand synthesis (RT)

	cf	1x
dH_2O	---	2.5 µL
10 µM Forward oligo	1.125 µM	2.3 µL
10 µM Reverse oligo	1.125 µM	2.3 µL
10 mM dNTPs 10 mM	250 µM	0.5 µL
RNA	---	10 µL
		vf: 17.6 µl

↓

Run RT-1 program in Axygen TC-1 →

Total time: 6 min		
95 ºC	2 min	1 cycle
4 ºC	2 min	

↓

RT Buffer	1x cf	2 µL
RT Enzyme 250 IU/µl	5 IU/µL	0.4 µL
		vf: 20 µl

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Run RT-2 program in Axygen TC-1 →

Total time: 1:12 hrs		
38 ºC	60 min	1 cycle
95 ºC	5 min	
4 ºC	5 min	

↓

Polymerase Chain Reaction (PCR)

	cf	1x
dH_2O	---	3.7 µL
2x iTaq SYBR Green	1 x	5 µL
10 µM Forward oligo	150 nM	0.15 µL
10 µM Reverse oligo	150 nM	0.15 µL
Template	10 ng	1 µL
		vf: 10 µl

↓

Run generic program in Applied Biosystems 7500 →

Total time: 2:10 hrs		
95 ºC	3 min	
94 ºC	15 sec	40 cycles
60 ºC[3]	60 sec	
95 ºC	15 sec	
60 ºC	20 sec	
Ramp[3]	60 min	
95 ºC	15 sec	

Note 3: Data acquisition.

One-step qRT-PCR components and conditions

The One-Step qRT-PCR procedure uses SCRIPT One-Step RT-qPCR EvaGreenMaster (Jena Bioscience Cat No. PCR-514S).

qRT-PCR		1x
dH_2O	---	1.2 µL
10 µM Forward oligo	400 nM	0.4 µL
10 µM Reverse oligo	200 nM	0.2 µL
ROX	0.5 x	0.2 µL
SCRIPT 2x	1 x	5 µL
Template	< 1 µg	3 µL
		vf: 10 µl

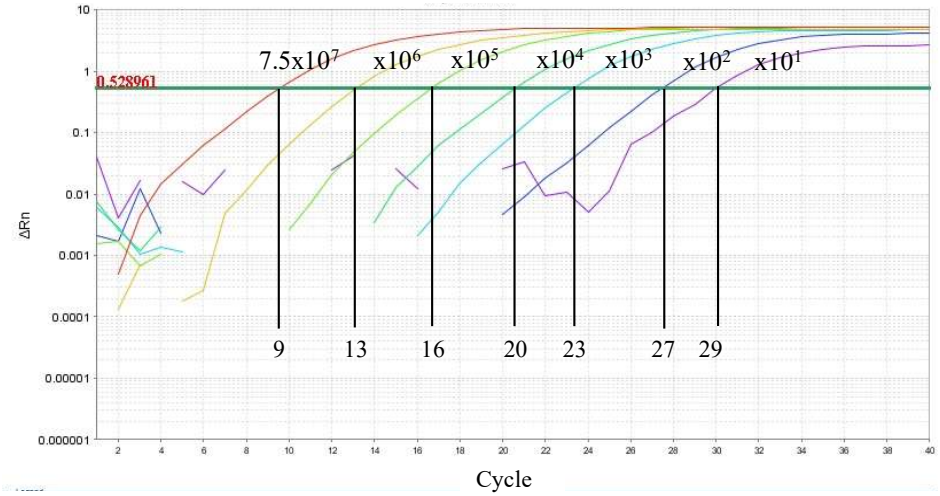
↓

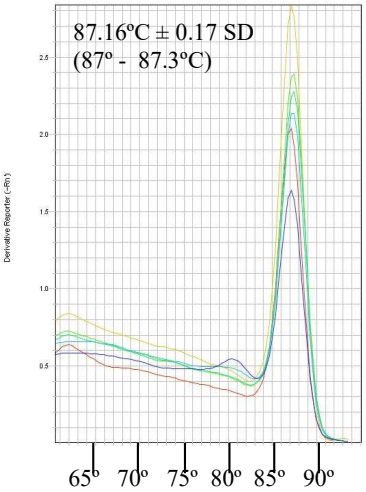
Run Green Master program in Applied 7500 ➔

50 ºC	10 min	
95 ºC	5 min	
94 ºC	15 sec	40 cycles
60 ºC[3]	60 sec	
95 ºC	15 sec	
60 ºC	20 sec	
Ramp[3]	60 min	
95 ºC	15 sec	

Note 3: Data acquisition.

Performance summary





Standard curve: ***m* = -3.41, Y = 36.708, R^2 = 0.995**
Target amplicon Tm: **87.16 ºC ± 0.17 SD (86.99 to 87.32ºC)**
Limit of detection (LODet): **$7.51x10^{-1}$ cp/µL**
Limit of discrimination (LODis): **$7.51x10^{2}$ cp/µL**

Titration curve preparation for quantitative analysis of viral titres

Using Artificial Gene (AG) sequence stock having all viral references (CHIKV, WNV, DENV1-3, DENV2-4, ZIKV, SNV, RABV, TCRV, MERS and SARS) prepare the following log titres (stock currently at $7.51x10^7$ cp/µL, working dilution of AG prepared at $7.51x10^6$ cp/µL).

		AG stock	dH_20 vol	Ct
Working	$7.5x10^7$ cp/µL	10 µL	90 µL	9.20 (±0.07)
1^{st}	$7.5x10^6$ cp/µL	10 µL	90 µL	12.78 (±0.02)
2^{nd}	$7.5x10^5$ cp/µL	10 µL	90 µL	16.37 (±0.29)
3^{rd}	$7.5x10^4$ cp/µL	10 µL	90 µL	20.25 (±0.20)
4^{th}	$7.5x10^3$ cp/µL	10 µL	90 µL	22.85 (±0.63)
5^{th}	$7.5x10^2$ cp/µL	10 µL	90 µL	27.15 (±0.30)
6^{th}	$7.5x10^1$ cp/µL	10 µL	90 µL	29.32 (±0.24)

Add 90 µL to each of the 6 PCR 0.2 mL tubes. Take 10 µL of initial working stock (at $7.51x10^7$ cp/µL) and dispense into 1^{st} PCR tube, wash tip 30 times, cap, vortex for 10 seconds and spin down for 10 seconds. Retrieve 10 µL from volumetric centre of PCR tube and dispense into 2^{nd} PCR tube repeating the same procedure for further dilutions.

References

1. Naze, F., Le Roux, K., Schuffenecker, I., Zeller, H., Staikowsky, F., & Grivard, P. et al. (2009). Simultaneous detection and quantitation of Chikungunya, Dengue and West Nile viruses by multiplex RT-PCR assays and Dengue virus typing using High Resolution Melting. Journal Of Virological Methods, 162(1-2), 1-7.

Revision history

1.0 Original document.

2.0 Optimized protocol.

3.0 Changes to document format only.

4.0 Changes to reagent volumes for optimized yield.