

Detection, Validation and Quantitation of West Nile Virus RNA by the Alternative NAT WNV Assay

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INTRODUCTION

West Nile Virus is an RNA virus of the family *Flaviviridae*, genus *Flavivirus*. WNV was first isolated in the West Nile District of Uganda in 1937 and is commonly found in humans and other vertebrates in Africa, Eastern Europe, West Asia and the Middle East.

It wasn't until 1999 that the first documented cases of WNV was reported in the northeastern US. Since then, WNV has recurred and spread to more geographical regions in the North America. West Nile Virus circulates in natural transmission cycles via mosquitoes and birds. Humans are incidental hosts when infected mosquitoes transmit the virus while taking a blood meal. Most WNV infections are mild and clinically unapparent, while 20% of those infected develop a mild illness with symptoms of malaise, headache, anorexia, myalgia, nausea, rash, etc. Symptoms usually last 3-6 days. 1 in 150 infections results in severe neurological disease and can include fever, muscle weakness, flaccid paralysis cranial nerve abnormalities, optic neuritis, seizures, etc. Advanced age is the primary risk factor for severe neurological disease and death.

The virus is an enveloped, spherical virion of approximately 40-50nm consisting of two envelope proteins, an icosahedral capsid and a positive stranded 11kb genome. The single long open reading frame encodes for polyproteins that are co- and post-translationally processed by viral and cellular proteases into 3 structural and seven nonstructural proteins. Both the 5' and 3' UTR's form highly conserved secondary and tertiary structures.

METHODS and RESULTS

Efficiency of Amplification

Amplification PRIMERS AND PROBES

- 3 Regions of the gene targeted initially.
- 5' UTR performed the best.

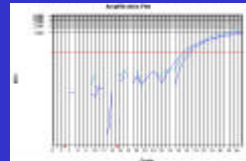


Internal Control (IC) RNA

An IC RNA of 960 nt amplified with 5'UTR primers, with altered probe binding sequence is used to identify false negatives. Detection Limit of IC RNA

Region	Ct	Average	Std Dev	%CV
Capsid	32.43	32.56	0.11	0.34
NS1/NS2	33.90	33.31	0.52	1.56
3'UTR	32.97	33.43	0.41	1.22

Amplification efficiency of Capsid, NS1 and 3'UTR. Of the three regions amplification of Capsid was the most robust.



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Alternative NAT WNV assay by Bead Capture-Taqman method.

- Oligo dT magnetic capture beads.
- Four capture primers.
- Two amplification primers.
- Three Probes, one each for Lineage 1, 2, and IC.
- IC probe detected with TET, Target probes with FAM.
- 96 samples analyzed in 3 hours.

Magnetic Bead based Target Capture

- Large Sample volume 0.5 ml, 1.0 ml for increased sensitivity.
- One tube isolation, no transfers.
- Magnetic beads, no centrifugations.
- Semi-automated operation.
- High-through put, 40 min. 100 samples, 60 min. 200 samples.
- Generic reagents.

Sensitivity of Alternative NAT WNV Assay

Detection of BBI WNV RNA Qualification Panel-QWN702 and 701

Target Cps/ml	BBI 702 Lineage 1-US Target Ct	BBI 702 Lineage 1-US IC Ct	BBI 701 Lineage 2-Ugandan Target Ct	BBI 701 Lineage 2-Ugandan IC Ct
2500	34.5	43.1	34	44.6
1250	35.4	43.4	35.2	42.4
625	36.9	41.3	35.6	40.3
312	37.3	40.8	36.7	40.8
156	38.2	40.7	37.4	40.2
78	40.2	40	39.5	39.5
39	45	40	41.8	39.6
19	45.6	40	45.7	39.6
9	47.8	39	45	40.5
Negative	50	39	50	39.5

- 10,000 Cps/ml BBI panel member serially diluted and tested in triplicates.
- >45 Ct is considered Negative.
- 100% positivity at 39 cps/ml for both Lineages.

RESULTS, continued

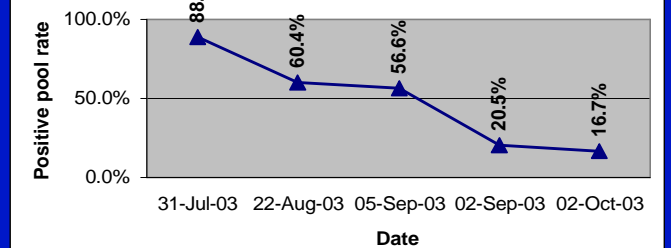
Validation Parameters for Alternative NAT WNV Assay

Test Condition	Copy Levels Tested	Number of Replicates	Results % positive
Analytical Sensitivity Using dilutions of Vero cell cultured WNV	900,300, 100, 30, 10, 3 Cps/mL	n=80	100% @ 30cps/mL
Analytical Sensitivity Study for Lineages 1 and 2	300, 100 Cps/mL	n=5	100% @ 300cps/mL and 100cps/mL
Specificity of random donor specimens using 2 lots of reagents	negative	500 individual specimens per lot	0/1000
Specificity for other blood borne pathogens	Positive for HCV, HTLV, HAV, CMV, EBV, HSV, HIV-1	Number of specimens per pathogen: HCV(5), HTLV(5), HAV(5), CMV(5), EBV(2), HSV(5), HIV-1(2) n=5 for each specimen	0/145
Anticoagulant Study including K2EDTA, K3EDTA, CPD, CPDA-1, CP2D, ACD, PPT, Serum and Na Heparin	300, 100, and 0 Cps/mL	n=5	100% @ 300cps/mL
Potentially interfering substances including hemolyzed, icteric, and lipemic samples as well as specimens spiked with <i>Propionibacterium</i> spp., <i>Corynebacterium</i> spp., <i>Micrococcus</i> spp., <i>Staphylococcus aureus</i> , and <i>Staphylococcus epidermidis</i> . Also elevated lev	300, 100, and 0 Cps/mL	n=5	100% @ 300cps/mL
Freeze-thaw study required samples undergo 1,3, and 5 freeze-cycles prior to testing	300, 100, and 0 Cps/mL	n=5	100% @ 300cps/mL
Reproducibility Study included testing 2 lots of reagents using 2 operators on 2 days	300 and 0 Cps/mL	n=480 @ 300 Cps/mL and n=96 @ 0 Cps/mL	100% @ 300cps/mL 0% @ 0cps/mL

Alternative NAT WNV Assay Testing at Bayer Reference Testing Laboratory of Procleix[®] WNV Assay Positive Samples from July 14th to October 10th 2003.

Blood Banks	#Pos/Total	%
ARC	12/30	40
Assoc. of Indep. Blood	0/2	0
Blood Center SE Wisconsin	263	3.2
Blood Systems (Tempe, AZ)	178/623	28.6
Blood Systems (Bedford, TX)	35/55	63.6
Bonfils Blood Center	194/380	51.1
Camp Memorial Blood Center	2/79	2.5
Florida Blood Service	11/22	50.0
Gen-Probe	0/1	0
Indiana Blood Center	3/30	10
Life South Community	3/4	75
Michigan Community	0/8	0
Miller Memorial	2/4	50
NIH TTVL	0/4	0
Oklahoma Blood Institute	15/233	6.4
Robertson Blood Center	2/157	1.3
South Texas Blood/Tissue	4/22	18.2
The Blood Center (New Orleans)	7/84	8.3
Tripler Army Medical Center	0/1	0
U of T MD Anderson Center	7/135	5.2
Total	500/1944	25.7

Positive Pool Rate July-October Bonfils Blood Center (Denver, CO)

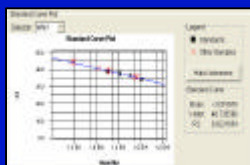


Improved Alternative NAT WNV Assay % Positivity N=10

Cps/mL	ACD	Heparin	K3EDTA
30	100	100	100
15	70	80	90
7.5	80	60	80
0	0	0	0

Quantitative Assay for West Nile Virus RNA

Standard graph generated with BBI panel members 100-10,000 Cps/mL tested in



Sample	Advised viral load	Replicate	IC Ct	Target Ct	Viral Load	Ave Viral Load	Ave Target Ct	STD of Target Ct	%CV
BBI (+) A385-5227	300 cp/mL	1	43.96	32.27	305	277	38.53	0.78	2%
		2	44.31	39.92	338				
		3	43.80	40.41	128				
Impath++ "ProcessCheck"	10,000 cp/mL	1	50.00	30.05	341800	504139	29.63	0.38	2%
		2	50.00	29.67	392494				
		3	50.00	28.97	778192				
Impath+ "ProcessCheck"	300 cp/mL	1	50.00	35.15	7015	5992	35.37	0.24	1%
		2	45.42	35.82	4895				
		3	50.00	35.34	6066				

QUALITATIVE WNV NAT ASSAY SUMMARY

1. Confirmatory assay for TMA positives detects 30 cp/mL, and meets the required sensitivity of 300 cp/mL of 0.5 log of TMA assay detection.
2. Validation studies begin Mid April and finish Mid May.
3. Validation Report end of May.
4. June 1st IND submission.

QUANTITATIVE WNV NAT ASSAY SUMMARY

1. A standard panel of 12 members ranging from 10⁶-100 copies of RNA.
2. The standards and unknown run in triplicates for quantitation.
3. Correlate Plaque forming units with copy numbers.
4. Correlate Infectivity with copy numbers.
5. Confirm Run controls for Procleix[®]- WNV assay.