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# Performance Evaluation of the PROCLEIX® West Nile Virus Assay on Semi-Automated and Automated Systems

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The PROCLEIX® West Nile virus assay (WNV assay) is a qualitative nucleic acid test based on transcription-mediated amplification (TMA). The assay was used under an investigational protocol in the United States to screen blood donations for West Nile virus (WNV) RNA starting in the summer of 2003, and was licensed by the FDA in December 2005 for use on the PROCLEIX System, also known as the enhanced semi-automated system (eSAS). Performance characteristics for the assay were determined on both eSAS and the fully automated PROCLEIX® TIGRIS® (TIGRIS) System. Detection of both lineage 1 and lineage 2 WNV was demonstrated on both systems. For lineage 1, the 95% detection limit was 8.2 copies/ml for eSAS and 9.8 copies/ml for the TIGRIS system. For lineage 2, $\geq$95% detection was seen at $\geq$30 copies/ml on both systems. The overall specificity of the assay was $>$99.9% in fresh and frozen plasma specimens. Reproducibility studies on the TIGRIS system yielded $\geq$99.1% agreement with expected results for the 3-member panel tested (0, 30, and 100 copies/ml). The WNV assay exhibited robust performance in cadaveric specimens and specimens representing various donor and donation conditions, including specimens from different plasma collection tubes that were subjected to multiple freeze/thaw cycles; specimens with elevated levels of endogenous substances; specimens containing other viruses and microorganisms; and specimens from patients with autoimmune and other diseases. Overall, these studies demonstrate high sensitivity, specificity, and reproducibility of the WNV assay on both the semi-automated and automated systems. ***J. Med. Virol. 79:1422–1430, 2007.***


**KEY WORDS:** donor blood screening; NAT; TMA

## INTRODUCTION

West Nile virus (WNV) is a pathogenic flavivirus that in its most severe form of infection can cause neurological disease and death [Campbell et al., 2002]. Since 1999, the U.S. Centers for Disease Control and Prevention (CDC) have received 23,758 reports of WNV related human disease, including 931 patient deaths [CDC, 2006]. Despite the potential severity of the disease, the majority of infected healthy adults are asymptomatic, making exclusion of WNV infected blood donors difficult without laboratory testing [Orton et al., 2006]. WNV is a mosquito-borne virus that is spread primarily through a number of different *Culex* mosquito species, with birds as the primary reservoir [Hayes et al., 2005]. In contrast to this normal route of infection, in 2002 there were 23 documented cases of WNV infection resulting from the transfusion of infected donor blood [Pealer et al., 2003], as well as reports of WNV infection resulting from organ donation [Iwamoto et al., 2003]. These were the first reported cases of human-to-human WNV transmission. In September 2002, the U.S. Food and Drug Administration (FDA) strongly urged test manufacturers to develop WNV assays for blood screening in response to this new risk to blood safety. WNV infection can be detected in blood donations using either nucleic acid testing (NAT) or antibody based technologies. As antibodies are not detectable at the earliest stages of infection, and can persist long after the virus is cleared, NAT has been determined to be the best method for primary

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WNV blood screening [Busch et al., 2005; Stramer et al., 2005].

To address the need for NAT based WNV blood donor screening, an investigational use assay was developed and implemented at U.S. blood centers by the summer of 2003, 9 months after the FDA's request. The PROCLEIX® WNV Assay (WNV assay) was first developed and implemented on the PROCLEIX® system, also known as the enhanced semi-automated system (eSAS), which is the same platform already in place in blood screening laboratories for use with the licensed PROCLEIX HIV-1/HCV Assay [Giachetti et al., 2002]. Testing under an Investigational New Drug (IND) protocol started in June 2003, and in December 2005 the FDA approved the Biologics License Application (BLA) for the WNV assay on the eSAS platform. Including data from 2006, blood screening using NAT has intercepted 1,793 presumptively positive WNV blood donations [CDC, 2006].

Reported here is a comprehensive evaluation of the assay performance of the WNV assay using a large number of samples in a non-clinical setting. The assay was evaluated on two systems: eSAS and the fully automated PROCLEIX TIGRIS® system. Both systems use the same assay chemistry but differ in throughput and level of automation. The results demonstrate the effectiveness of the assay for specific detection of low copy levels of WNV and the comparability of assay performance on both systems.

## MATERIALS AND METHODS

### PROCLEIX WNV Assay

The PROCLEIX WNV assay is based on the same technology as the FDA-licensed PROCLEIX HIV-1/HCV Assay. These qualitative in vitro tests involve three main steps: (1) sample preparation with magnetic based target capture; (2) viral RNA target amplification by transcription-mediated amplification (TMA); and (3) detection of the amplification products with chemiluminescent probes using the hybridization protection assay (HPA) [Giachetti et al., 2002]. These steps are performed manually in the eSAS system, or are automated by the TIGRIS system.

To detect the amplification products, chemiluminescent acridinium ester-labeled probes complementary to both the viral (analyte) and internal control (IC) RNAs are added to the reaction tubes. The analyte and IC probes have different light emission kinetics, and are distinguishable from one another. Results are expressed as relative light units (RLU) values and as signal-to-cutoff (S/CO) values. The cutoff value is a floating cutoff based on the RLU values obtained with calibrator samples included in each run. Cutoff values for the WNV Assay IC and analyte signals are determined as follows:

$$\text{IC Cutoff} = (0.5)[\text{NC}_\text{X}(\text{Internal Control})]$$

$$\text{Analyte Cutoff} = \text{NC}_\text{X}(\text{Analyte}) + [(0.03)\text{PC}_\text{X}(\text{Analyte})]$$

where $\text{NC}_\text{X}$ (Internal Control) is the mean of the IC RLU values of the negative calibrators, $\text{NC}_\text{X}$ (Analyte) is the mean of the analyte RLU values of the negative calibrators, and $\text{PC}_\text{X}$ (Analyte) is the mean of the analyte RLU values of the positive calibrators. The constants for the IC and analyte cutoff formulas were derived by statistically determining the optimal IC and analyte cutoff values. The optimal analyte cutoff value was determined by using receiver operating characteristic (ROC) analysis to identify the range of analyte RLU values where both the sensitivity and specificity of the assay are greater than or equal to 99.5%. The optimal IC cutoff value was determined by testing negative and WNV positive samples under both normal and known inhibitory conditions, and identifying the range of IC RLU values that maintain the sensitivity claim of the assay while minimizing false negative results. In both cases, the constants chosen result in cutoff values that are within the determined optimal range.

A sample is considered "reactive" if the analyte S/CO is $\geq$1.0. A sample is considered "non-reactive" (negative) if the analyte S/CO is $<$1.0 and the internal control signal is greater than the internal control cutoff. For any samples where the internal control signal is greater than the set maximum, or the analyte S/CO value is $<$1.0 and the internal control signal is below the internal control cutoff, the sample is considered "invalid." The "set maximum" is the maximum allowable IC RLU value. Any sample that exceeds this value is considered invalid and must be retested. The purpose of this is to control for errors either in the assay chemistry or in the processing of the assay results.

### eSAS and the Tigris System

The semi-automated platform, eSAS, is comprised of the instruments and software needed to run a PROCLEIX assay with the appropriate reagent kit. For increased automation, the TECAN® Genesis™ automated pipettor (Tecan Trading AG, Zurich, Switzerland) can be used to pipette TCR and specimens. Alternatively, specimen pipetting can be performed manually.

The TIGRIS system is an integrated NAT system that automates all the steps necessary to perform a PROCLEIX assay. The two main components of the TIGRIS system are the analyzer and the computer workstation. The assay software in the computer workstation directs the analyzer modules to perform each assay step. The analyzer unit contains the automation robotics, and is designed to hold all required fluids, reagents, and consumables. Using a single TIGRIS instrument, one technician can complete up to 1,000 tests in 14 hr.

### Dilution Panels for Determination of Analytical Sensitivity

Two panels were used to assess the analytical sensitivity of the WNV assay for lineage 1 WNV. The first lineage 1 sensitivity panel was prepared from the Health Canada WNV reference standard [Saldanha

et al., 2005], by serial dilution of a 1,000 copies/ml (c/ml) heat-treated tissue culture-derived stock. The panel consisted of six members at the following concentrations: 0, 1, 3, 10, 30, and 100 c/ml. A second lineage 1 WNV panel, the FDA WNV Reference Panel (distributed by BBI Diagnostics, West Bridgewater, MA), consisted of the following concentrations: 0, 5, 10, 50, 100, 500, and 1,000 c/ml.

To assess detection of lineage 2 WNV, 2 panel members from WNV RNA Qualification Panel QWN701 (BBI Diagnostics) with WNV RNA copy levels of 10,000 c/ml were pooled and diluted to create 6 panel members at the following concentrations: 0, 1, 3, 10, 30, and 100 c/ml.

### Specimens for Determination of Analytical Specificity

Frozen specimens from normal negative donors were obtained from the Community Blood Center of Greater Kansas City (Kansas City, MO). Fresh specimens were obtained from the San Diego Blood Bank and American Red Cross National Testing Laboratory in San Diego, CA. Fresh specimens, collected in $K_3$EDTA and plasma preparation tubes (PPT™; Becton-Dickinson, Franklin Lakes, NJ), were tested within 8 days of being drawn and were never frozen. A total of 3,116 normal blood donor specimens were tested on eSAS, including 1,591 frozen specimens and 1,525 fresh specimens. A total of 3,000 normal blood donor specimens were tested on the TIGRIS system, including 1,500 frozen specimens and 1,500 fresh specimens. For every condition, testing was conducted using three different reagent kit lots.

### Specimens Containing Potentially Interfering Substances

The effects of donor and donation factors on assay performance were evaluated using various types of clinical and analytical specimens. Conditions, factors, and sample characteristics that were evaluated included the following: (1) viral infections other than WNV (herpes simplex virus types 1 and 2, human T-cell lymphotrophic virus types I and II, hepatitis viruses, HIV-1/2, cytomegalovirus, Epstein-Barr virus, rubella virus, parvovirus B19, and members of the Japanese encephalitis virus (JEV) serogroup), as well as specimens from influenza infected and HBV vaccinated individuals; (2) specimens contaminated with bacterial, yeast, or fungal pathogens (*Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Corynebacterium diphtheriae*, *Propionibacterium acnes*, *Candida albicans*, and *Pneumocystis carinii*) (healthy blood donations from the Community Blood Center of Greater Kansas City spiked to $10^6$ CFU/ml of each microorganism); (3) plasma samples from donors with autoimmune and other diseases (rheumatoid arthritis, rheumatoid factor, antinuclear antibody, lupus, multiple sclerosis, multiple myeloma, hypergammaglobulinemia [IgG and IgM], alcoholic cirrhosis, and elevated alanine aminotransferase levels); and (4) specimens containing potentially interfering substances (hemolyzed, icteric, and lipemic clinical specimens; and a custom analytical panel containing known high levels of bilirubin (2.5 mg/dl), lipids (3,000 mg/dl), hemoglobin (500 mg/dl), or serum albumin (6 g/dl)). The bacteria included in the interfering substance panel were chosen because they are all commonly found on skin, and therefore have an increased chance of being introduced during blood collection.

Analytical specimens were prepared by spiking either normal human serum or normal human plasma with the indicated substance or microorganism. JEV serogroup members were provided by Robert Lanciotti of the CDC (Division of Vector-Borne Infectious Disease, Arbovirus Diseases Branch, Fort Collins, CO). Clinical specimens were obtained from ProMedDx (Norton, MA), SeraCare Life Sciences (Milford, MA), BBI Diagnostics, and Teragenix (Ft. Lauderdale, FL). To assess the impact on assay sensitivity, aliquots of each specimen were spiked with tissue culture derived WNV (Chiron, a business unit of Novartis Vaccines & Diagnostics, Emeryville, CA) targeted at 150 c/ml, while the corresponding WNV negative specimen was used to test specificity. Approximately 10 individual specimens were tested for each potentially interfering substance described. Specimens were tested on both eSAS and the TIGRIS system, using three different reagent lots.

### Sensitivity and Specificity in Cadaveric Specimens

Cadaveric specimens were obtained from the American Red Cross (ARC) Tissue Services (Eagan, MN) and from SeraCare Life Sciences. All specimens were collected from donors between 1 and 22 hr post-mortem.

Specificity was assessed by testing 45 cadaveric specimens on eSAS and 51 specimens on the TIGRIS system. To test sensitivity, aliquots of the same specimens were spiked with tissue culture derived WNV (Chiron) targeted to 150 c/ml. Specimens from normal blood donors obtained from the Community Blood Center of Greater Kansas City were used as the control for both the sensitivity (WNV spiked) and specificity (unspiked) experiments. Specimens were divided among three reagent lots for each system.

### Sensitivity in the Presence of Anticoagulants and Following Freeze/Thaw Cycles

Blood specimens from normal donors were collected in serum collection tubes and each of the following plasma collection tubes: acid citrate dextrose (ACD), $K_2$EDTA and $K_3$EDTA, PPT™ ($K_2$EDTA plasma preparation tube; Becton-Dickinson), sodium citrate, citrate phosphate dextrose (CPD), and lithium heparin. Following collection, all specimens were spiked with tissue culture derived WNV (Chiron) targeted at 150 c/ml.

Fresh WNV spiked specimens were stored at 2–8°C and tested within 24 hr of collection (0 freeze/thaw cycles). Aliquots of each fresh specimen were then frozen for $\geq$4 hr at $\leq -65$°C and thawed for at least 2 hr at room

temperature either 1 or 4 times. Testing was performed on both eSAS and the TIGRIS system, using multiple reagent lots.

### Reproducibility of the WNV Assay on the TIGRIS System

Reproducibility studies were performed on eSAS as part of the clinical trial for the WNV assay and are not included in this report. Reproducibility of the WNV assay on the TIGRIS system was assessed by determining the intra- and inter-run, inter-instrument, and inter-reagent lot variability. The study involved testing assay calibrators, two WNV positive panel members and one WNV negative panel member. WNV panel members were prepared by spiking negative human serum with tissue culture derived WNV (Chiron) to a target concentration of 30 or 100 c/ml. Unspiked negative human serum was used as the negative panel member. Each assay run on the TIGRIS system consisted of two back-to-back worklists of 500 tests for a total of 1,000 tests. Each 500 test worklist consisted of 100 replicates of each WNV positive panel member along with 268 replicates of the negative panel member. Samples were evenly divided into five different brackets within the worklist; each bracket included three replicates of the WNV assay positive and negative calibrators. Eighteen total assay runs were performed by multiple operators using three different TIGRIS instruments with three reagent lots.

## RESULTS

### Sensitivity of the WNV Assay

The sensitivity of the WNV assay was evaluated for lineage 1 WNV using the Health Canada analytical sensitivity panel and the FDA WNV reference panel. The sensitivity of the assay for lineage 2 WNV was evaluated using the BBI qualification panel QWN701. The results for both eSAS and the TIGRIS system are shown in Tables I–III.

Probit analysis was performed for the lineage 1 WNV Health Canada panel (Table I). The 50% and 95% detection limits were similar for both systems: 3.4 and 4.0 c/ml for the 50% detection limit and 8.2 and 9.8 c/ml for the 95% detection limit for eSAS and the TIGRIS system, respectively. Results using the FDA WNV Reference Panel are shown in Table II, and again demonstrate 100% detection for all panel members with concentrations of 100 c/ml or greater, on both eSAS and the TIGRIS system. The detection of lineage 2 WNV is shown in Table III, and was demonstrated to be $\geq$97% at 30 c/ml for both systems.

### Specificity of the WNV Assay

The specificity of the assay was determined in normal, WNV negative blood donors for both fresh and frozen plasma specimens. The results are shown in Table IV. The specificity was $\geq$99.9% on both eSAS and the TIGRIS system, and was similar between fresh and frozen plasma specimens. No significant differences were observed between specimens collected in $K_3$EDTA tubes and specimens collected in PPT tubes (data not shown).

All initially reactive results were retested in duplicate to confirm the results and yielded valid and non-reactive results, indicating that the initial results were false positives. On eSAS there were five initially invalid reactions from the fresh samples, and seven initially invalid reactions from the frozen specimens, for overall initial invalid rates of 0.33% (5/1,525) and 0.44% (7/1,591), respectively. On the TIGRIS system, there were two initially invalid reactions from the fresh samples, and no invalid reactions from the frozen samples, for overall initial invalid rates of 0.13% (2/1,500) and 0% (0/1,500), respectively. In all cases, retesting of the initially invalid samples resulted in valid, non-reactive reactions, indicating that the specimens themselves were not inhibitory to the assay.

### Specimens Containing Potentially Interfering Substances

Possible interference of endogenous substances, microorganisms, and viruses other than WNV were

TABLE I. Detection of WNV Lineage 1 in the Health Canada Analytical Sensitivity Panel

| System | WNV (copies/ml) | # Reactive/# tested[a] | % Reactive | Average S/CO (%CV) | 50% detection limit (CI) | 95% detection limit (CI) |
|---|---|---|---|---|---|---|
| eSAS | 100 | 89/89 | 100 | 30.05 (9) | 3.4 copies/ml (1.8–7.2) | 8.2 copies/ml (5.5–21.5) |
| | 30 | 90/90 | 100 | 29.46 (10) | | |
| | 10 | 87/90 | 97 | 27.16 (25) | | |
| | 3 | 47/89 | 53 | 23.43 (35) | | |
| | 1 | 26/89 | 29 | 21.10 (49) | | |
| | 0 | 0/89 | 0 | 0.06 (120) | | |
| TIGRIS | 100 | 77/77 | 100 | 30.22 (14) | 4.0 copies/ml (1.7–8.8) | 9.8 copies/ml (6.5–27.3) |
| | 30 | 74/74 | 100 | 29.21 (18) | | |
| | 10 | 82/90 | 91 | 26.52 (27) | | |
| | 3 | 52/90 | 58 | 24.16 (36) | | |
| | 1 | 19/90 | 21 | 17.03 (61) | | |
| | 0 | 0/90 | 0 | 0.11 (101) | | |

S/CO, signal/cutoff ratio; %CV, percent coefficient of variation; CI, confidence interval.
[a]Only valid reactions were included.

TABLE II. Detection of WNV Lineage 1 in the FDA WNV Reference Panel

| | | eSAS | | TIGRIS | |
|---|---|---|---|---|---|
| WNV strain | Copies/ml | # Reactive/# tested[a] (%) | Average S/CO | # Reactive/# tested[a] (%) | Average S/CO |
| NY99 | 1,000 | 12/12 (100) | 32.5 | 10/10 (100) | 31.4 |
| NY99 | 500 | 12/12 (100) | 32.2 | 10/10 (100) | 30.7 |
| NY99 | 100 | 12/12 (100) | 31.5 | 10/10 (100) | 31.9 |
| NY99 | 50 | 12/12 (100) | 31.7 | 9/10 (90) | 31.1 |
| NY99 | 10 | 12/12 (100) | 29.2 | 8/10 (80) | 28.8 |
| NY99 | 5 | 11/12 (92) | 24.0 | 7/10 (70) | 19.1 |
| NY99 | 0 | 0/12 (0) | 0.15 | 0/9 (0) | 0.04 |
| Hu2002 | 1,000 | 12/12 (100) | 31.8 | 9/9 (100) | 29.7 |
| Hu2002 | 500 | 12/12 (100) | 32.2 | 10/10 (100) | 31.3 |
| Hu2002 | 100 | 12/12 (100) | 32.4 | 10/10 (100) | 30.6 |
| Hu2002 | 50 | 12/12 (100) | 31.6 | 9/10 (90) | 31.2 |
| Hu2002 | 10 | 12/12 (100) | 28.5 | 10/10 (100) | 29.4 |
| Hu2002 | 5 | 12/12 (100) | 25.1 | 10/10 (100) | 29.7 |
| Hu2002 | 0 | 0/12 (0) | 0.10 | 0/9 (0) | 0.03 |

S/CO, signal/cutoff ratio.
[a]Only valid reactions were included.

evaluated for their effect on the WNV assay. Specimens containing the potential interfering substances were evaluated on both eSAS and the TIGRIS system, comparing samples spiked with 150 c/ml WNV RNA to the unspiked control. The results are shown in Table V. No significant reduction in sensitivity or specificity was observed for any substance tested.

Notably, one of the JEV serogroup viruses tested was Kunjin virus, a strain of WNV found in Australia [Charrel et al., 2003]. Samples spiked with Kunjin virus were all reactive, indicating that the WNV assay also detects this strain of WNV. As Kunjin virus is a strain of WNV, the samples are excluded from the specificity results.

### Sensitivity and Specificity in Cadaveric Blood Specimens

The sensitivity of the assay in cadaveric blood specimens for both systems is shown in Table VIa. There were no non-reactive results, yielding a sensitivity rate of 100% for both eSAS and the TIGRIS system. One invalid result (1/51) was observed in the control (non-cadaveric, WNV spiked) sample set tested on the TIGRIS system.

The specificity of the assay in cadaveric blood specimens for both systems is shown in Table VIb. There were no reactive results, yielding a specificity of 100% for both eSAS and the TIGRIS system. One invalid result (1/45) was observed in the control sample set tested on eSAS.

The overall internal control failure rate across all specimens, reagent lots, and systems was 0% for the 192 cadaveric samples and 1.04% for the 192 control specimens, indicating that cadaveric samples do not lead to an increase in invalid results.

### Sensitivity in the Presence of Anticoagulants and Following Freeze/Thaw Cycles

The impact of the type of anticoagulant used and the effect of up to four freeze/thaw cycles on detection of WNV was evaluated with both eSAS and the TIGRIS system. Results are shown in Table VII.

The combined sensitivity for all anticoagulants tested in all three reagent lots was 100% for samples with 0 or

TABLE III. Detection of WNV Lineage 2 in the BBI Qualification Panel QWN701

| System | Copies/ml | # Reactive/# tested[a] | % Reactive | Average S/CO (%CV) |
|---|---|---|---|---|
| eSAS | 100 | 98/100 | 98 | 10.73 (18) |
| | 30 | 99/100 | 99 | 6.10 (45) |
| | 10 | 89/100 | 89 | 2.92 (69) |
| | 3 | 30/100 | 30 | 2.09 (54) |
| | 1 | 4/99 | 4 | 1.95 (39) |
| | 0 | 0/100 | 0 | 0.08 (82) |
| TIGRIS | 100 | 90/90 | 100 | 10.72 (19) |
| | 30 | 85/88 | 97 | 5.78 (45) |
| | 10 | 73/89 | 82 | 2.60 (66) |
| | 3 | 15/88 | 17 | 1.64 (45) |
| | 1 | 1/90 | 1 | 1.09 (n/a) |
| | 0 | 0/90 | 0 | 0.08 (113) |

S/CO, signal/cutoff ratio; %CV, percent coefficient of variation.
[a]Only valid reactions were included.

TABLE IV. Specificity in Normal Blood Donor Specimens

| System | Specimen type | # Tested[a] | # Non-reactive | # Reactive[b] | Specificity (%) |
|---|---|---|---|---|---|
| eSAS | Fresh plasma | 1,525 | 1,524 | 1 | 99.93 |
| | Frozen plasma | 1,591 | 1,590 | 1 | 99.94 |
| | Total | 3,116 | 3,114 | 2 | 99.94 |
| TIGRIS | Fresh plasma | 1,500 | 1,499 | 1 | 99.93 |
| | Frozen plasma | 1,500 | 1,500 | 0 | 100 |
| | Total | 3,000 | 2,999 | 1 | 99.97 |

[a]Number tested includes initially invalid results, all were valid when retested.
[b]Initial number reactive, does not include retesting results (all reactions non-reactive when retested).

1 freeze/thaws. After the fourth freeze/thaw cycle, there were two initially non-reactive samples on eSAS, one CPD tube (tested with reagent lot 2) and one $K_2$EDTA tube (tested with reagent lot 3). Both initially non-reactive samples were reactive in both replicates when retested in duplicate. The initial sensitivity on eSAS after four freeze/thaw cycles for all reagent lots combined was 99.2% (238/240).

Sensitivity was unaffected when specimens in different anticoagulants were tested after 0, 1, or 4 freeze/thaw cycles on the TIGRIS system, yielding sensitivity of 100% for all conditions tested.

### Reproducibility of the WNV Assay on the TIGRIS System

The in-house evaluation of the reproducibility of the WNV assay on the TIGRIS system was performed by testing 8,400 replicates of one negative and two positive WNV panel members. The standard deviation and percent coefficient of variation (%CV) for inter-instrument, inter-reagent lot, inter-worklist, inter-bracket (compares average signal for a set of panel members within a given worklist), and intra-worklist (compares sample to sample variation within a given panel) RLU values are shown in Table VIII. There were 63 invalid results (0.75%); all invalid reactions were excluded from the data analysis. Overall, the WNV assay on the TIGRIS system yielded 99.7% agreement (8,313 out of 8,337) with expected results for the three different panel members tested. The largest source of variability was seen with the intra-worklist data, where the %CV ranged from 5.24 to 15.53 for the different panels tested. This represents the variability between replicates, adjusting for instrument, reagent lot, worklist, and position variation.

## DISCUSSION

WNV is comprised of many individual strains that can be divided phylogenetically into two main lineages, lineage 1 and lineage 2. All oligonucleotides used in the WNV assay were developed in sequence regions that are conserved between the two lineages. In this report, standardized panels of both lineage 1 and lineage 2 strains were tested using the WNV assay on both eSAS and the TIGRIS system, showing sensitive detection of both lineages on either platform. For the Health Canada lineage 1 panel, the sensitivity of both platforms was 100% as low as 30 c/ml, with 95% detection limits predicted by probit analysis to be less than 10 c/ml. For the lineage 1 FDA WNV Reference

TABLE V. Detection of WNV in Samples Containing Potentially Interfering Endogenous Substances, Microorganisms and Viruses

| | # Reactive/# tested (% detected) | | | |
|---|---|---|---|---|
| | eSAS | | TIGRIS | |
| Condition | WNV negative samples | WNV spiked (150 c/ml) samples | WNV negative samples | WNV spiked (150 c/ml) samples |
| Endogenous substances[a] | 1/246 (0.41)[b] | 245/246 (99.6) | 0/247 (0) | 246/247 (99.6)[c] |
| Viruses other than WNV[d] | 0/411 (0) | 419/419 (100) | 0/270 (0) | 282/286 (98.6)[c] |
| Bacterial and fungal pathogens[e] | 0/210 (0) | 210/210 (100) | 0/211 (0) | 202/203 (99.5)[c] |
| Autoimmune and other disease states[f] | 0/258 (0) | 258/258 (100) | 0/202 (0) | 202/202 (100) |

[a]Analytical samples included normal serum spiked with: (a) hemoglobin, 500 mg/dl; (b) bilirubin, 20 mg/dl; (c) lipids, 3,000 mg/dl; (d) protein, 6 g/dl. Clinical specimens consisting of icteric, hemolyzed or lipemic plasma were also tested.
[b]Initial number reactive, does not include retesting results (reaction non-reactive when retested).
[c]Initial number reactive, does not include retesting results (all reactions reactive when retested).
[d]Viruses tested are detailed in Materials and Methods, and include human immunodeficiency virus types 1 and 2, hepatitis A, B, C, and G viruses, and multiple flaviviruses from the Japanese encephalitis virus serogroup. Samples spiked with Kunjin virus, a strain of WNV, were reactive and are excluded from the specificity results.
[e]Specimens were spiked to approximately $1 \times 10^6$ CFU/ml with one of the following microorganisms: *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Corynebacterium diphtheriae*, *Propionibacterium acnes*, *Candida albicans* or *Pneumocystis carinii*.
[f]Samples from patients with the following pathologies were evaluated: rheumatoid factor, rheumatoid arthritis, antinuclear antibody, lupus, multiple sclerosis, multiple myeloma, hyperglobulinemia, alcoholic cirrhosis, and elevated alanine aminotransferase levels.

TABLE VIa. Assay Sensitivity in WNV Spiked Cadaveric Blood Specimens

| | eSAS | | TIGRIS | |
|---|---|---|---|---|
| | Control[a] | Cadaveric | Control[a] | Cadaveric |
| Number tested | 45 | 45 | 50[b] | 51 |
| Mean analyte S/CO (%CV) | 33.16 (7) | 29.55 (7) | 23.81 (9) | 26.55 (7) |
| % Sensitivity | 100 | 100 | 100 | 100 |

%CV, percent coefficient of variation.
[a]Controls were normal blood donor specimens spiked with WNV (150 c/ml).
[b]Excludes one invalid result.

TABLE VIb. Assay Specificity in Unspiked Cadaveric Specimens

| | eSAS | | TIGRIS | |
|---|---|---|---|---|
| | Control[a] | Cadaveric | Control[a] | Cadaveric |
| Number tested | 44[b] | 45 | 51 | 51 |
| Mean analyte S/CO (%CV) | 0.12 (58) | 0.15 (70) | 0.20 (81) | 0.16 (69) |
| Mean IC S/CO (%CV) | 2.13 (6) | 2.07 (8) | 2.05 (9) | 2.16 (7) |
| % Specificity | 100 | 100 | 100 | 100 |

IC, internal control; S/CO, signal/cutoff ratio; %CV, percent coefficient of variation.
[a]Controls were normal blood donor specimens.
[b]Excludes one invalid result.

Panel, the sensitivity at 10 c/ml was 100% using the eSAS system, and 80–100% using the TIGRIS system. For the lineage 2 panel, the sensitivity was ≥97% at 30 c/ml using either system. The Kunjin strain of WNV was also tested as part of the viral interference studies on eSAS, and all reactions yielded reactive results. The effectiveness of the WNV assay for detection of strains found in diverse geographic areas suggests that the WNV assay is suitable for international use.

While it is of utmost importance in the blood bank setting to minimize false negative results, minimizing false positive results is important for a blood center's overall efficiency, avoiding retesting and the unnecessary deferral of blood donors. In this study, over 6,000 normal human blood donor specimens were tested to determine the specificity of the WNV assay. Specificity was found to be ≥99.9% using either eSAS or the TIGRIS system. Additional testing was performed to determine the sensitivity and specificity of the WNV assay under various possible donation conditions. The results demonstrate that the WNV assay is appropriate for screening under a wide range of commonly encountered conditions.

Screening donor blood for WNV is complicated by the need for the switch from mini-pool (MP) to individual donor (ID) screening in times of epidemic outbreak. Two guidelines for triggering the switch from MP to ID testing have recently been published [Custer et al., 2004; Stramer et al., 2005]. Although these guidelines differ somewhat, both are dependent upon the accuracy of the WNV assay; false negatives can delay the switch from MP to ID screening during the emergence of an

TABLE VII. Detection of WNV in Serum and Plasma Samples in Different Collection Tube Types After 0, 1, and 4 Freeze/Thaw Cycles

| | # Reactive/# tested | | | | | |
|---|---|---|---|---|---|---|
| | 0 Freeze/thaw cycle | | 1 Freeze/thaw cycle | | 4 Freeze/thaw cycles | |
| Tube | eSAS | TIGRIS | eSAS | TIGRIS | eSAS | TIGRIS |
| $K_3$EDTA plastic | 30/30 | 30/30 | 30/30 | 30/30 | 30/30 | 30/30 |
| PPT | 29/29 | 30/30 | 31/31 | 30/30 | 30/30 | 30/30 |
| Na citrate | 30/30 | 30/30 | 30/30 | 30/30 | 30/30 | 30/30 |
| ACD | 30/30 | 30/30 | 30/30 | 30/30 | 30/30 | 30/30 |
| Serum | 30/30 | 30/30 | 30/30 | 30/30 | 30/30 | 30/30 |
| $K_2$EDTA | 30/30 | 30/30 | 30/30 | 30/30 | 29/30 | 30/30 |
| CPD | 30/30 | 30/30 | 29/29 | 30/30 | 29/30[a] | 29/29 |
| Li heparin | 30/30 | 30/30 | 27/27 | 30/30 | 30/30 | 30/30 |
| Overall[b] | 239/239 | 240/240 | 236/236 | 240/240 | 238/240[a] | 239/239 |
| Initial % sensitivity | 100 | 100 | 100 | 100 | 99.2[a] | 100 |

[a]Initially non-reactive tests were retested in duplicate, all reactions were reactive when retested.
[b]Initially invalid reactions were excluded.

TABLE VIII. Reproducibility on the TIGRIS System

| WNV panel member | | | | SD (%CV) of RLU value | | | | |
|---|---|---|---|---|---|---|---|---|
| WNV (copies/ml) | # Replicates | Valid results[a] | % Agreement | Inter-instrument | Inter-reagent lot | Inter-worklist[b] | Inter-bracket | Intra-worklist |
| 0 | 4,787 | 4,749 | 100 | 0.02 (0.95) | 0.03 (1.43) | 0.00 (0.00) | 0.06 (2.86) | 0.11 (5.24) |
| 30 | 1,808 | 1,797 | 99.1 | 2.67 (8.84) | 0.72 (2.38) | 0.00 (0.00) | 1.36 (4.50) | 4.69 (15.53) |
| 100 | 1,805 | 1,791 | 99.6 | 1.87 (5.89) | 0.89 (2.80) | 0.00 (0.00) | 1.55 (4.88) | 3.27 (10.30) |
| Overall | 8,400 | 8,337 | 99.7 | | | | | |

%CV, percent coefficient of variation.
[a]Invalid results were excluded from the data analysis.
[b]Per CLSI/NCCLS guidelines (EP5-A, page 7) numbers <0 are recorded as 0.

outbreak, and false positives can delay switching back from ID to MP testing, increasing the burden on the testing laboratory. The sensitivity and specificity demonstrated here would allow the WNV assay to be used successfully in various testing algorithms in which individual donor testing is triggered based on regional incidence rates, as determined by pooled testing results.

The need for periodic ID-NAT screening for WNV places an extra demand on blood screening centers to maximize assay throughput. The automated TIGRIS system allows testing of up to 1,000 samples in 14 hr by a single operator, greatly increasing assay throughput over semi-automated systems. The sensitivity and specificity of the WNV assay using the TIGRIS system were determined under a variety of conditions, and are comparable to that determined for eSAS. Additionally, the reproducibility study on the TIGRIS system presented here yielded an overall percent agreement with expected results of $\geq$99.1% for all conditions tested. In combination, these results demonstrated the utility of the TIGRIS system for WNV blood screening.

As WNV is a relatively new health threat in the United States, the course that the virus will take over time is not predictable. Thus, the most effective screening strategies will have to be re-evaluated over time. The rapid introduction of the WNV assay in response to the 2002 epidemic is an example of the ability of regulatory agencies, blood testing organizations, and test manufacturers to respond quickly to unanticipated bloodborne pathogens [Epstein, 2005]. This successful response to WNV can be used as a model for responding to future threats to the safety of the blood supply.

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## REFERENCES

Busch MP, Caglioti S, Robertson EF, McAuley JD, Tobler LH, Kamel H, Linnen JM, Shyamala V, Tomasulo P, Kleinman SH. 2005. Screening the blood supply for West Nile virus RNA by nucleic acid amplification testing. N Engl J Med 353:460–467.

Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ. 2002. West Nile virus. Lancet Infect Dis 2:519–529.

Centers for Disease Control and Prevention (CDC). Division of Vector-Borne Infectious Diseases. 2006. 12 September 2006, posting date. West Nile virus; Statistics, Surveillance, and Control; Maps and Data. [Online] http://www.cdc.gov/ncidod/dvbid/westnile/surv & control.htm.

Charrel RN, Brault AC, Gallian P, Lemasson JJ, Murgue B, Murri S, Pastorino B, Zeller H, de Chesse R, de Micco P, de Lamballerie X. 2003. Evolutionary relationship between Old World West Nile virus

strains. Evidence for viral gene flow between Africa, the Middle East, and Europe. Virology 315:381–388.

Custer B, Tomasulo PA, Murphy EL, Caglioti S, Harpool D, McEvoy P, Busch MP. 2004. Triggers for switching from minipool testing by nucleic acid technology to individual-donation nucleic acid testing for West Nile virus: analysis of 2003 data to inform 2004 decision making. Transfusion 44:1547–1554.

Epstein JS. 2005. Insights on donor screening for West Nile virus. Transfusion 45:460–462.

Giachetti C, Linnen JM, Kolk DP, Dockter J, Gillotte-Taylor K, Park M, Ho-Sing-Loy M, McCormick MK, Mimms LT, McDonough SH. 2002. Highly sensitive multiplex assay for detection of human immunodeficiency virus type 1 and hepatitis C virus RNA. J Clin Microbiol 40:2408–2419.

Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL. 2005. Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis 11:1167–1173. [Online] http://www.cdc.gov/ncidod/EID/vol11no08/05-0289a.htm.

Iwamoto M, Jernigan DB, Guasch A, Trepka MJ, Blackmore CG, Hellinger WC, Pham SM, Zaki S, Lanciotti RS, Lance-Parker SE, DiazGranados CA, Winquist AG, Perlino CA, Wiersma S, Hillyer KL, Goodman JL, Marfin AA, Chamberland ME, Petersen LR. West Nile Virus in Transplant Recipients Investigation Team. 2003. Transmission of West Nile virus from an organ donor to four transplant recipients. N Engl J Med 348:2196–2203.

Orton SL, Stramer SL, Dodd RY. 2006. Self-reported symptoms associated with West Nile virus infection in RNA-positive blood donors. Transfusion 46:272–277.

Pealer LN, Marfin AA, Petersen LR, Lanciotti RS, Page PL, Stramer SL, Stobierski MG, Signs K, Newman B, Kapoor H, Goodman JL, Chamberland ME, West Nile Virus Transmission Investigation Team. 2003. Transmission of West Nile virus through blood transfusion in the United States in 2002. N Engl J Med 349: 1236–1245.

Saldanha J, Shead S, Heath A, Drebot M, West Nile Virus Collaborative Study Group. 2005. Collaborative study to evaluate a working reagent for West Nile virus RNA detection by nucleic acid testing. Transfusion 45:97–102.

Stramer SL, Fang CT, Foster GA, Wagner AG, Brodsky JP, Dodd RY. 2005. West Nile virus among blood donors in the United States, 2003 and 2004. N Engl J Med 353:451–459.