Overview of West Nile Virus Assays Under Commercial Development

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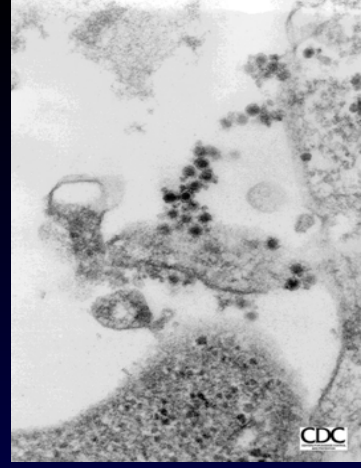
Division of Molecular Biology

Maryland DHMH Laboratories

Presentation Summary



- Brief overview of status of several commercial assays under development for the diagnosis of WNV infections (emphasize assays for testing human specimens)
- This presentation is not all inclusive
- Many of these assays are in the early stages of development and may or may not result marketable products

Impetus for Commercial WNV Test Development

- Unprecedented number of WNV infections in the US during the 2002 transmission season
  - Over 4,000 reported cases of human WN illness
  - Over 2,700 cases of WN meningoencephalitis
- Overburdened laboratories devoted significant resources to meet the demand for diagnostic human WNV testing
- A recognition that better testing tools in some areas were needed to met the increased diagnostic testing workloads
- In the late summer 2002 blood transfusion/organ donor transmission of WNV was first recognized

FDA Urges WNV Assay Development to Protect the Blood Supply

- September 20, 2002 **AABB meeting** Bethesda MD: FDA challenged manufacturers to develop WN testing platforms to protect the blood supply
- November 4-5, 2002 FDA **WNV Assay Development Workshop**: Several manufacturers presented preliminary assay development plans

West Nile Virus Antibody Detection Assays

- Role in blood donor screening?: Possible confirmation of NAAT(sero-conversion after detection of WNV RNA in donated unit), or for retrospective donor studies
- Need for more “user friendly” FDA licensed assay that can be widely applied for diagnostic testing
- Unique approaches (i.e., recombinant antigens, new detection technologies) may lead to improved performance in comparison to MAC ELISA
- Confirmatory/Supplemental assays or testing algorithms to replace reliance on PRNT??

Manufacturers with WNV Antibody Assays in development

- Focus Technologies
- Ortho Clinical Diagnostics
- Abbott Laboratories
- Chiron (recombinant antigens)

Focus Technologies' WNV IgG and IgM ELISA

Developed in our clinical reference laboratory and available soon as an in vitro diagnostic product

WNV IgM/IgG Assay Development

- The Focus clinical reference laboratory has tested over 40,000 specimens during the last 3 years for WNV antibodies (using two generations of in-house developed ELISA's)
- Licensed recombinant WNV technology from the CDC
- Initiated the development of a 3rd generation assay for a diagnostic product using recombinant proteins
- Plan to manufacture prototype kits in the spring of 2003
- Plan to conduct clinical trials during the 2003 WN transmission season
- Plan to submit clinical trial data to the FDA in fall of 2003

WNV ELISA Product Design

WNV IgM ELISA

IgM (mu) capture format

WNV recombinant protein (lyophilyzed)

Peroxidase-labeled monoclonal Ab

Cutoff, negative control, positive control

Specimen/Ag diluent, TMB, stop solution

WNV IgG ELISA

Indirect (Ag-coated plate) ELISA

Peroxidase-labeled anti-H IgG conjugate

Cutoff, negative control, positive control

Specimen diluent, TMB, stop solution

Assay Performance to Date

IgM ELISA

- 100% concordance for negative sera vs. ref lab assay
- 96% concordance for positive sera vs. ref lab assay
- 34 of 34 PHL screen/PRNT positives are positive by Dx product
- Reduced “false positives” due to high background (as determined by background subtraction) by 59%
- Final performance numbers pending final cutoff formulation, Index interpretation criteria, and expanded serum panel evaluations

IgG ELISA

- 95% of Southern California ARC donors are negative
- 95% concordance for negative samples vs. the lab assay
- 94% concordance for positive samples vs. the lab assay
- Final performance requires larger sample sizes. finalized cutoff formulation and Index interpretation criteria.

Ortho Clinical Diagnostics: WNV IgM Capture Assay

- **Assay developed with existing anti-HAV IgM capture microplate assay (96well) components**
- **< 2 hour assay (no pre-coating procedures)**
- **IgM capture: biotinylated mouse monoclonal(mm) anti-human IgM----(mm+IgM) immune-complex is captured on streptavidin coated microwells**
- **Antigen: recombinant WNV env antigen labeled with a HRP conjugated mouse anti-WNV (env) monoclonal bound to it (Ag binds to patient anti-WNV IgM captured on the microwell)**
- **Detection: Chemiluminescence (luminol derivative) 96 well plate reader**

Ortho Clinical Diagnostics: IgM Capture Assay Results

Sample ID	WN MAC IgM P/N	OCD rec Env ECi Format			
		#1		#2	
		Total signal	S/N	Total signal	S/N
MD-002	3.7	701	1.1	1307	0.9
MD-003	< 2.0	377	0.6	1423	0.9
MD-004	14.1	**7159**	**11.3**	**43374**	**28.3**
MD-010	15.3	**8417**	**13.3**	**16757**	**10.9**
MD-013	9.3	**6490**	**10.3**	**13215**	**8.6**
MD-015	7.6	**4396**	**6.9**	**9888**	**6.5**
MD-016	7.9	**16353**	**25.9**	**28096**	**18.3**
MD-024	neg	3227	5.1	5981	3.9
GC-1	nd	996	1.6	1759	1.2
GC-2	nd	767	1.21	2139	1.4
GC-3	nd	1562	2.5	1850	1.2
neg	nd	632	1	1534	1

West Nile Virus – Abbott Strategy

- Develop prototype IgM class antibody test for detection of antibodies to WNV and prototype NAT test to detect WNV RNA
- Studies will be performed to determine:
  - the overlap between RNA and WNV IgM detection
  - markers associated with acute, symptomatic infection
  - markers associated with recovery and donor reinstatement
- Several different proteins and assay formats will be evaluated over the next several weeks,EIA platform may be adapted to other platforms
- Need for characterized specimens are needed to optimize assay performance

CDC Proficiency Panel Test Results – Abbott Enzyme Immunoassay

Number	OD	S/N
1	1.074	39.8
2	0.035	1.3
3	2.000	74.1
4	0.170	6.3
5	2.000	74.1
6	2.000	74.1
7	0.043	1.6
8	2.000	74.1
9	0.036	1.3
10	1.040	38.5
11	0.037	1.4
12	1.935	71.7
13	0.033	1.2
14	2.000	74.1
15	0.329	12.2
16	2.000	74.1
17	0.076	2.8
18	2.000	74.1
19	0.031	1.1
20	0.039	1.4

The features of the WNV IgM EIA include low absorbance values (0.020 – 0.100) for most seronegative samples (data not shown) and high absorbance values (>1.00) for most positive samples

CHIRON | BLOOD TESTING
Trust Through Innovation

Fourth National Conference on West Nile Virus

New Orleans, Louisiana

February 8 – 12, 2003

"Production of Materials in Support of WNV Assay Development"

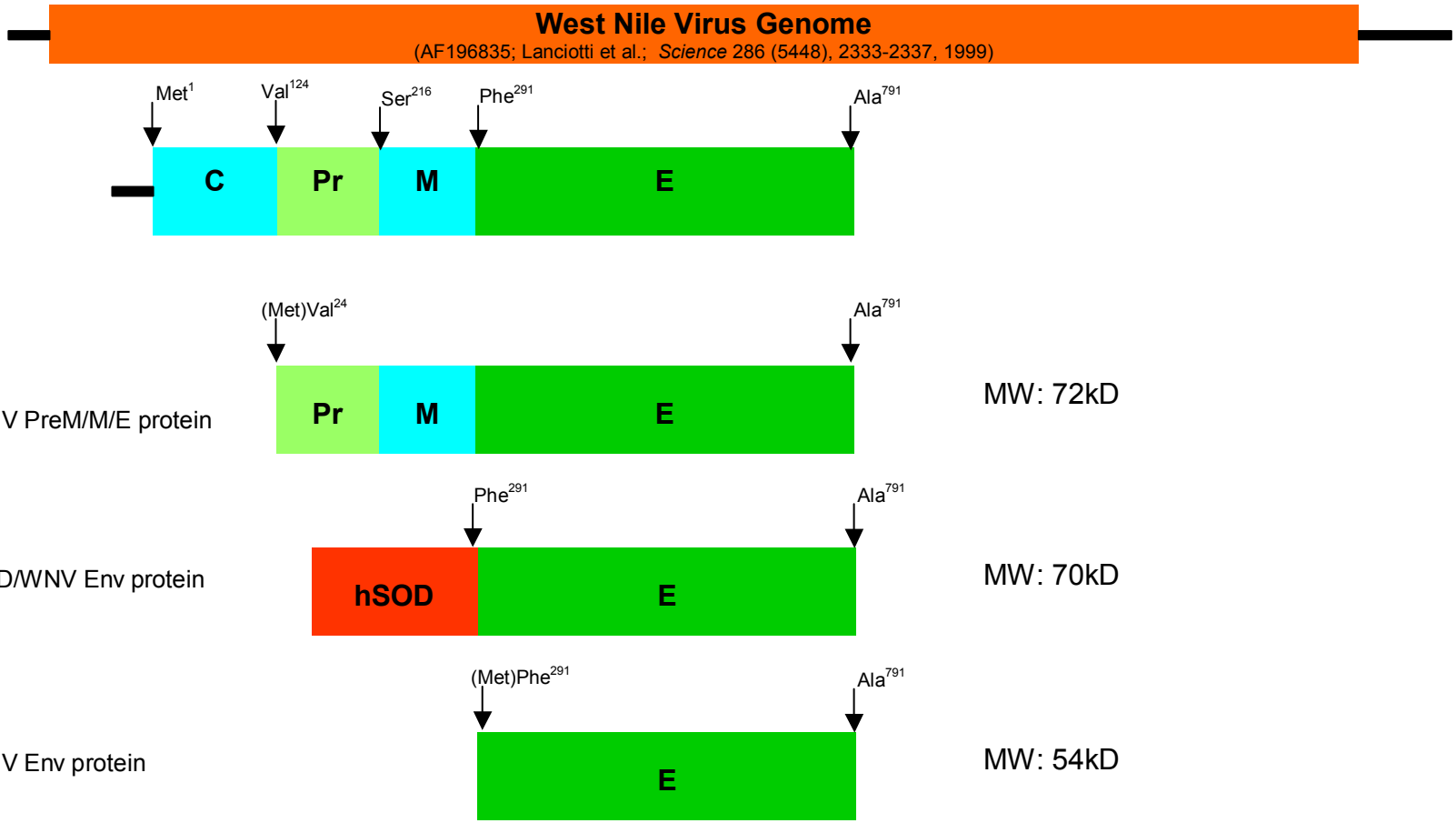
Blood Testing R&D

Chiron Corporation

Reagents for IgM/IgG Assay Development

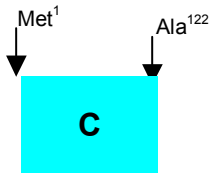
- **Cloning and yeast expression of West Nile virus recombinant proteins**
  - **A ~2 kb synthetic DNA fragment encoding for the WNV PreM/M/E region of WNV (New York strain) was cloned and expressed in *Saccharomyces cerevisiae AD3*.**
  - **A ~ 1.5 kb RT-PCR amplified fragment encoding for the WNV envelope protein was cloned into the yeast expression vector pBS24.1.**
  - **A ~370 bp synthetic DNA fragment encoding for the capsid protein of West Nile virus (New York strain) was cloned as SOD fusion for expression in *Saccharomyces cerevisiae*.**

Yeast Expression of West Nile Virus Recombinant Proteins



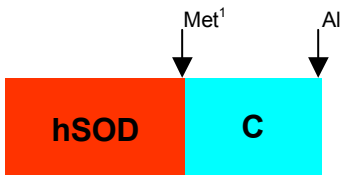
Yeast Expression of West Nile Virus Recombinant Proteins

WNV C protein



MW: 14kD

SOD/WNV C protein



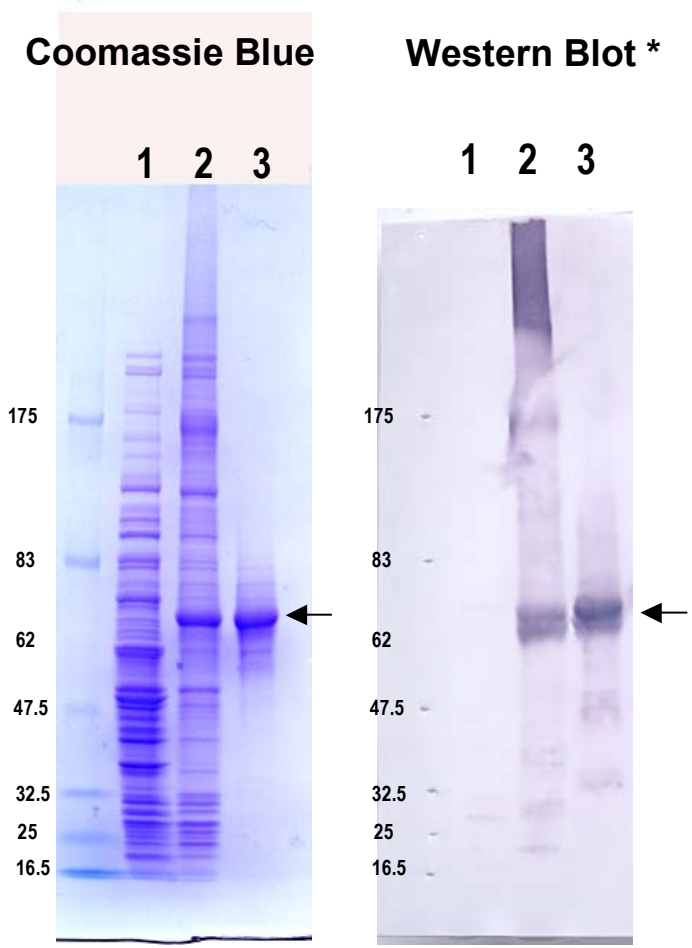
MW: 31kD

Reagents for IgM/IgG Assay Development

- **These recombinant West Nile virus proteins will facilitate development of diagnostic tests to detect IgM and/or IgG antibodies in the serum/blood of infected individuals.**

- **These recombinant proteins can also be used to generate Mo Abs for antigen test development.**

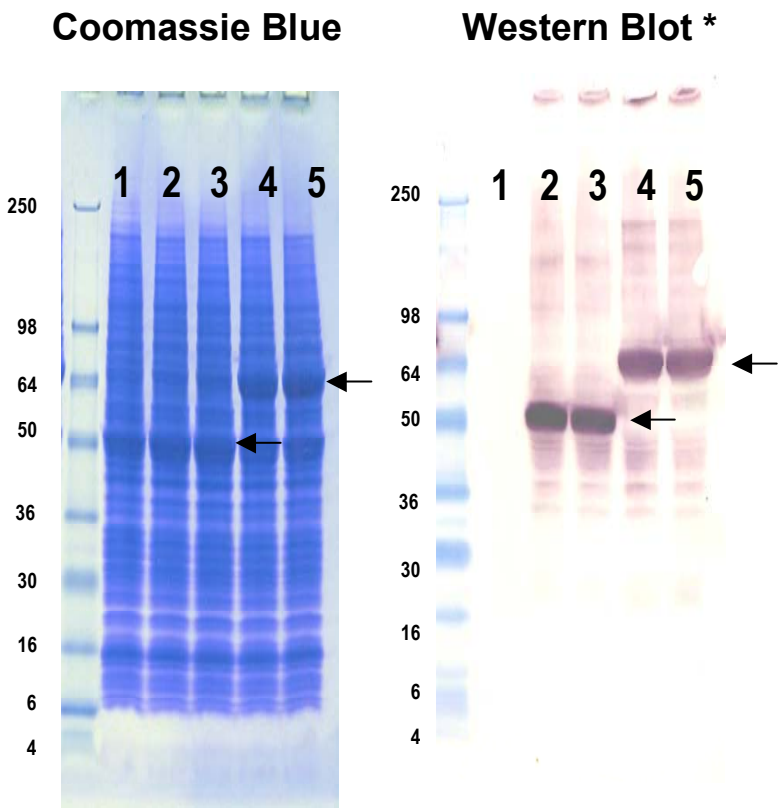
Expression of WNV PreM/M/E Recombinant Protein in *S. cerevisiae* AD3



1. Soluble Fraction
2. Insoluble Fraction
3. Purified Material

* MoAbs to Envelope protein of West Nile Virus/Kunjin Virus

Expression of WNV Env Recombinant Protein in *S. cerevisiae* AD3



1. AD3 Negative Control (total cell lysate)
2. WNV Env Protein
3. WNV Env Protein
4. SOD/WNV Env Protein
5. SOD/WNV Env Protein

* MoAbs to Envelope protein of West Nile Virus/Kunjin Virus

West Nile Virus Nucleic Acid Amplification Tests (NAAT)

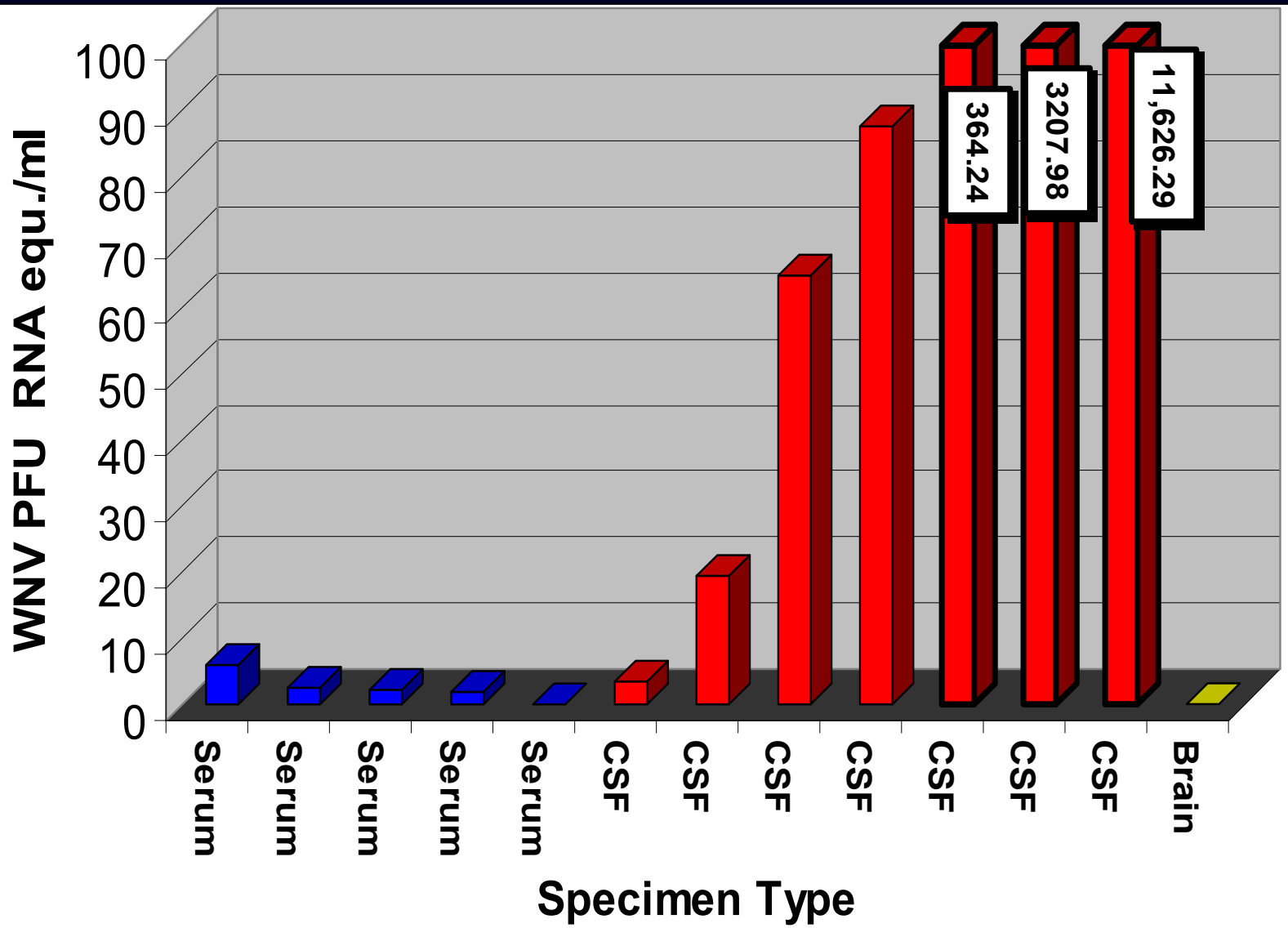
Diagnostic Testing

- Utility in diagnostic testing: Identifying viremic/ antibody negative "window phase" patients
- Low levels WNV RNA found in clinically ill, immuno-competent patients (i.e.,WN fever)
- Higher persistent levels of WNV RNA found in immuno-compromised patients
- Improved sensitivity with better quality of diagnostic samples or virus concentration methods?
- WNV RNA(-) results are meaningless

Maryland DHMH Laboratory Human WNV PCR Testing : *2002 Summary*

- Testing of clinically ill individuals:
  (encephalitis, meningitis, WN fever)
- **CSF: 412 specimens tested**: **7 WNV(+)** [from 4 individuals]
- All acute (<8 days post on-set of illness) serum/plasma specimens were tested
- **Serum/Plasma: 276 samples tested**: **5 WNV(+)** [from 5 individuals]
- The initial acute sample from 4 of 6 individuals were WNV RNA(+)/antibody:IgM (-) ("window phase")

Relative Levels of WNV RNA In Human Samples



West Nile Virus Nucleic Acid Amplification Tests (NAAT)

Blood /Organ Donor Screening

- Variable WNV RNA levels in sub-clinical WNV infections (low levels WNV RNA have been found in blood products associated with transfusion transmission cases)
- Practical issues of incorporating into existing NAAT platforms that are now used to screen blood donors for HIV/Hep. C
  - Utility of testing pooled samples?

Manufacturers with WNV NAT Assays in development

- Roche Molecular Systems
- Gen-Probe
- Abbott Laboratories
- Boston Biomedica Inc. (BBI) (bird/mosquito testing only?)

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Diagnostics

West Nile Virus Testing Development - Roche Update

James L. Gallarda, Ph.D.

Roche Molecular Diagnostics

West Nile Virus Testing - Logistic Issues

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Diagnostics

- Target start date for Canada and the U.S. - July 1, 2003
- Timeframe and the associated, necessary resources are unprecedented
- Roche seeking to identify synergies with global automation efforts
- System comprises
  - PCR reagents and controls
  - System hardware
  - System software

TaqMan 5' Nuclease Kinetic Assay
Fluorescence Energy Transfer Probe

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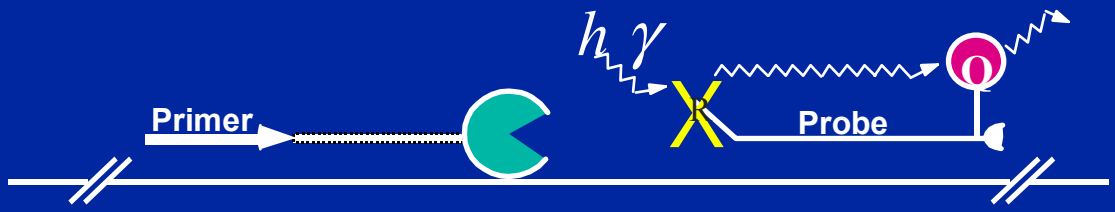
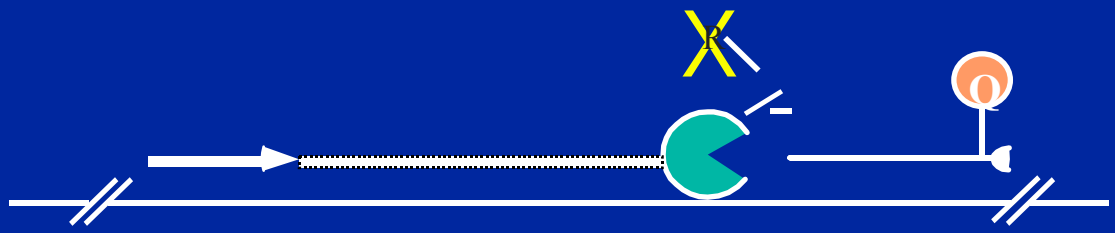
Diagnostics



R Fluorescence Emission
Quenched



R = Reporter
Q = Quencher

R Fluorescence Emission
Detected

Current Sensitivity Performance (< 30 C/mL)

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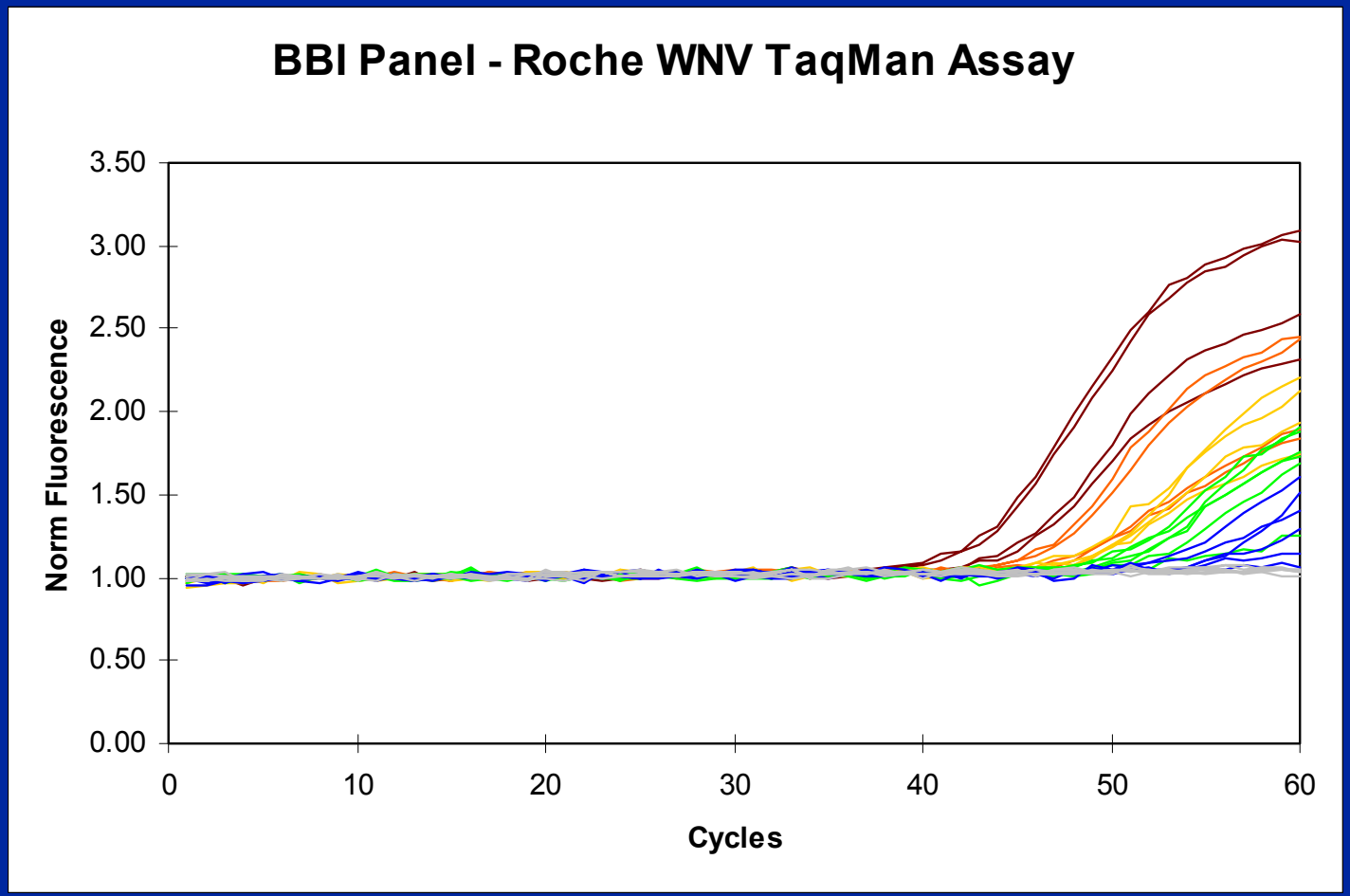
Diagnostics



Preferred System - Fully Automated

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Diagnostics

Hamilton Pipettor

COBAS TaqMan (96/48)

COBAS AmpliPrep

Studies in Progress

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Diagnostics

- Specificity Studies
  - Analytical Specificity
  - Population from low endemic area
  - Population from high endemic area
- Limit of Detection (LOD) Studies
- Whole System Failure, Precision, Reproducibility
- Interfering Substances

Procleix® WNV Assay: Transcription-mediated Amplification Assay for the detection of West Nile virus RNA

Jeff Linnen and Cristina Giachetti
Gen-Probe Incorporated
San Diego, CA

Objectives of the West Nile Virus Assay Development Program

- To develop and manufacture a TMA-based assay for detection of West Nile virus in blood, plasma and organ/tissue donor specimens
  - This program will support linked donor-recipient WNV epidemiological studies
  - Blood screening under an IND beginning in the US in summer of 2003.

WNV Assay Development Goals

- Analytical sensitivity: at least 95% detection at 50 copies/mL
- Detection of genetic variants of WNV (e.g Lineage 1, including Kunjin virus, and Lineage 2 strains) with similar sensitivity
- Analytical specificity: > 99.5 %
- Internal Control for validating each reaction
- Completely compatible with Procleix (semi-automated) and fully automated TIGRIS™ instrument platforms

Procleix Semi-Automated System: Assay Protocol

Pipetting	Sample Processing	Amplification (TMA)	Detection (HPA)	Results
Calibrators, Specimens, and TCR	Viral Lysis, RNA Capture, and Washes	Amp Rgt., Oil, and Enzyme	Probe Hybridization Selection	Automatic RLU Reading
TECAN	TCS	Water baths	Water bath	Luminometer

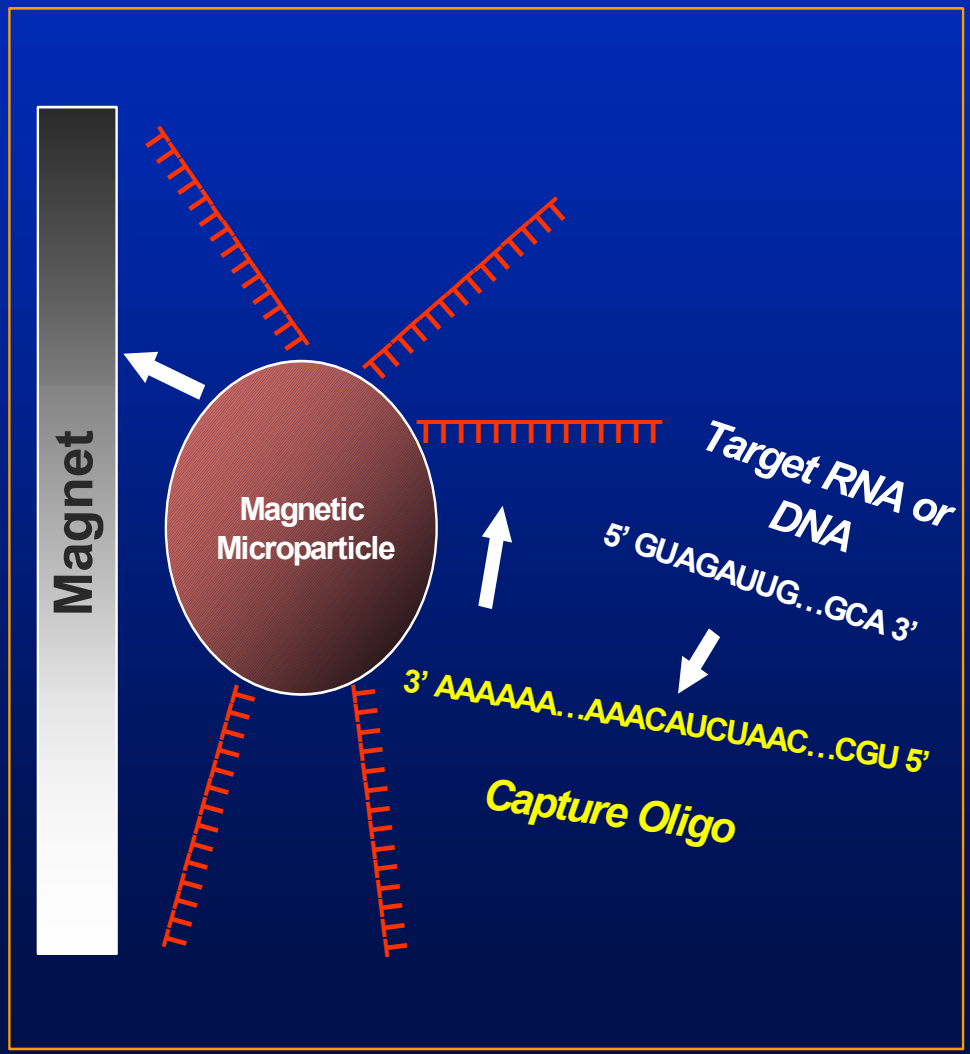
TECAN

Target Capture System (TCS)

Luminometer

Specimen Processing

Target Capture/Magnetic Microparticle Separation



Viral Lysis

- Treat specimens with heat and detergent
- Release nucleic acid

Nucleic Acid Capture

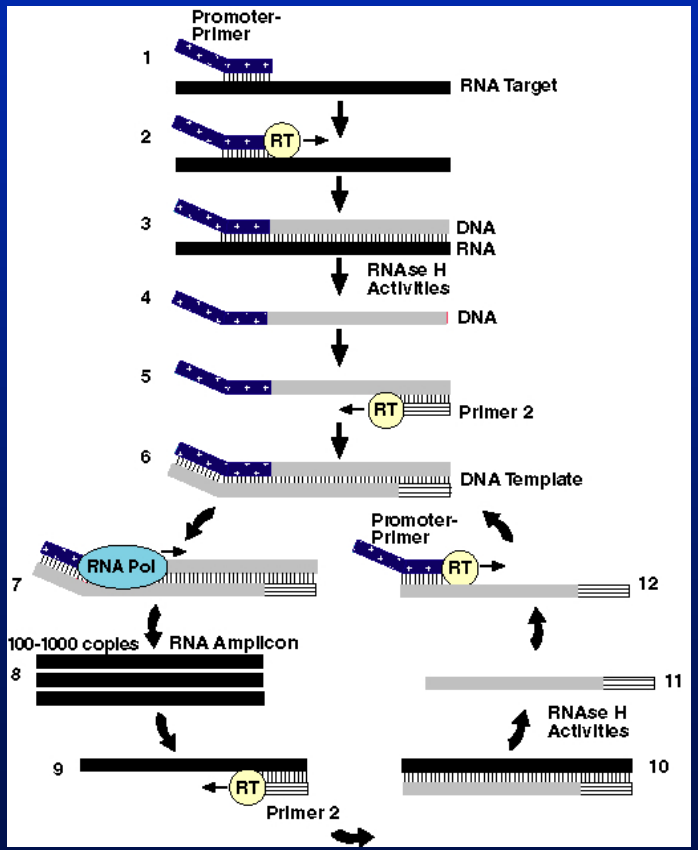
- Hybridize target sequence to capture probes
- Hybridize capture probe to oligomer sequence bind to magnetic particle

Removal of unwanted specimen

- Apply magnetic field to separate target from residual sample
- Remove residual specimen by washing

Amplification

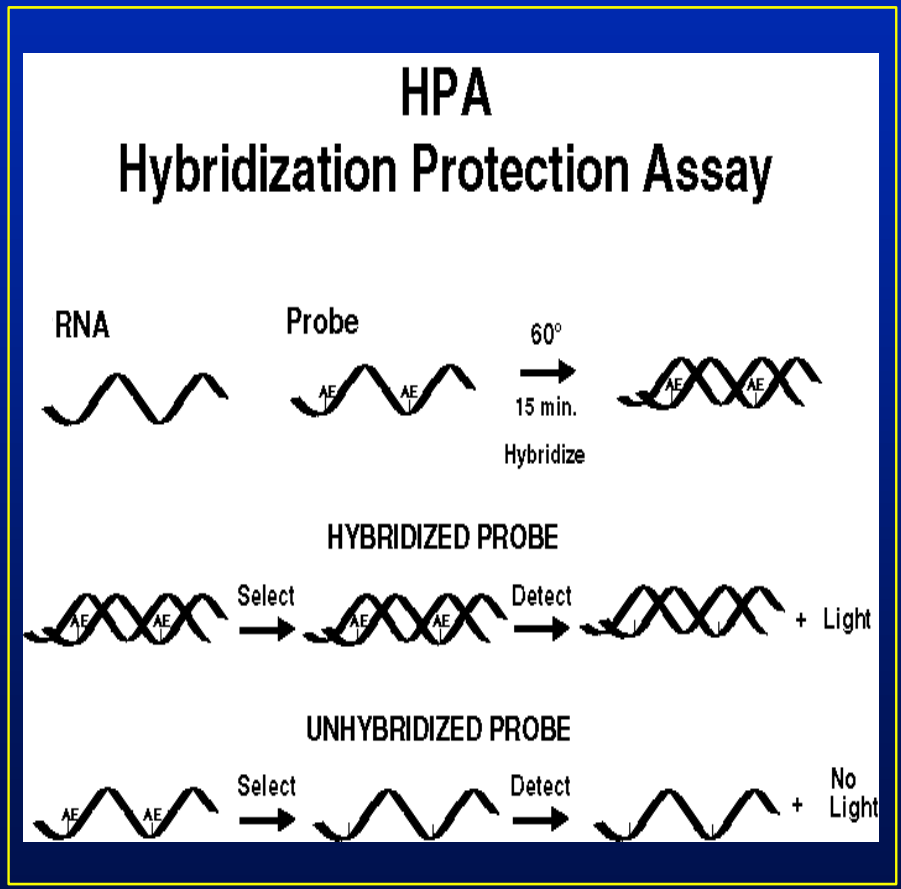
Transcription-Mediated Amplification (TMA)



- Utilizes two enzymes:
  - Reverse Transcriptase
  - T7 RNA Polymerase
- Amplifies RNA or DNA
- Produces RNA amplicon
- Exponential amplification (> billion fold amplification in less than one hour)
- Isothermal, simplifies automation

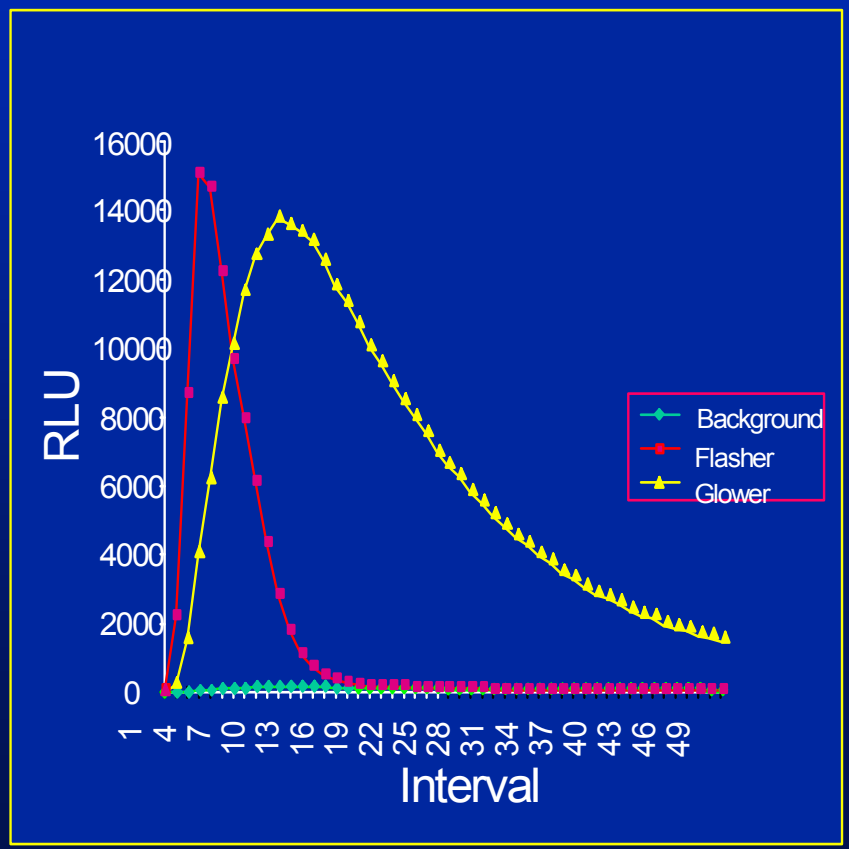
Detection

Hybridization Protection Assay (HPA)



- Utilizes Acridinium Ester (AE) labeled probes
- Reaction Steps:
  1. **Hybridization**
     AE-labeled probe hybridizes to target RNA in solution
  2. **Selection**
     Label on unhybridized probe is hydrolyzed, label on hybridized probe is protected
  3. **Detection**
     Label on protected hybridized probe is detected by chemiluminescence

Detection (cont.)
Dual Kinetics Analysis (DKA)



- Used to differentiate Internal Control (IC) signal from target signal
- Utilizes Acridinium Ester (AE) labeled probes with differential kinetics of light-off
- Ortho Fluoro Acridinium Ester labeled probe = flasher probe, hybridizes to IC
- 2'Methyl Acridinium Ester labeled probes = Glower probes, hybridize to West Nile virus amplicon
- ETF Algorithm deconvolutes light-off and calculates each signal

Detection of WNV using Synthetic RNA Transcript

WNV RNA (copies/mL)	N	% detection	95% detection [95% CI]*	50% detection [95% CI]*
600	20	100	7.6 copies/mL [6.1 to 10.6]	3.5 copies/mL [2.5 to 4.5]
200	30	100		
60	30	100		
20	29	100		
6	26	79.3		
2	20	43.8		
0.3	20	0		
0	30	0		

* Probit analysis using SAS

Detection of WNV using CDC Viral Lysate Standard (Lineage 1)*

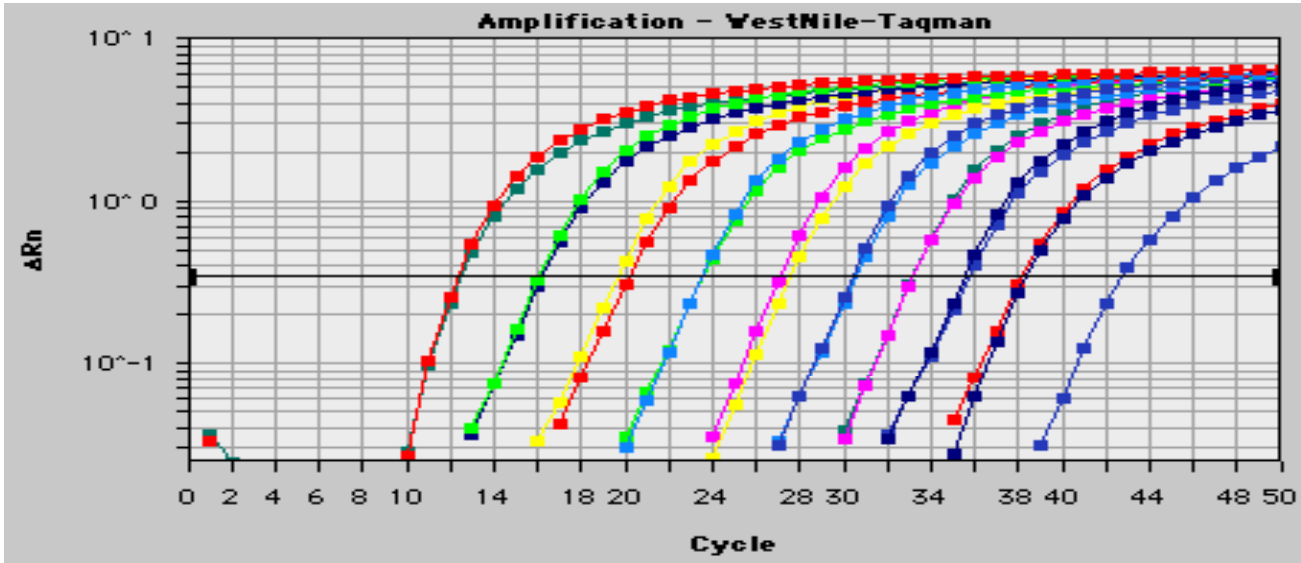
RNA Dilution	% Detection (N =50)	95% Detection (dilution level)	50% Detection (dilution level)
1×10^{-4}	100	2.7×10^{-6} dilution	4.5×10^{-7} dilution
1×10^{-5}	100		
3.2×10^{-6}	96		
1×10^{-6}	76		
3.2×10^{-7}	38		
Negative Control	0		

*Provided by Dr. R. Lanciotti

Conclusions

- **Analytical sensitivity for detection of WNV to date:**
  - 95% detection of CDC standard at **2.7×10^{-6} dilution**
  - 50% detection of CDC standard at **4.5×10^{-7} dilution**
  - 95% detection of WNV transcript at **7.6 copies/mL**
  - 50% detection of WNV transcript at **3.5 copies/mL**
- **Demonstrated sensitive detection (down to 10 c/mL) of West Nile virus Lineage 2 isolate (genetic variant of the US strain)**
- **Analytical sensitivity suggests feasibility of pooled testing (pool size of 16).**

Boston Biomedica, Inc.
West Nile Virus RNA Detection



- Closed-tube, real-time fluorescent TaqMan assay
- Broad linear dynamic range, 10^1 – 10^{10} copies/ml
- Simultaneous amplification and detection of internal control and specimen (2 – color system)
- Excellent inter- and intra-assay reproducibility
- Low background and high sensitivity

Bird Brain and Mosquito Pool Testing RNA (PCT + TaqMan) vs. Virus Culture

	Crow Brain				Mosquito Pool	
ID	PCT: RNA Titer	Cell Culture		ID	PCT: RNA Titer	Cell Culture
249	0	-		1735	0	-
251	0	-		1742	0	-
255	0	-		1782	0	-
257	0	-		4183	0	-
265	0	-		7432	870	+
754	2,100,000	+		7465	8,800	+
755	450,000	+		7438	7,000	+
756	130,000	+		10395	0	-
760	140,000	+		1042	0	-
761	82,000	+		10466	4,300	+

Development of Standardized WNV Reference Reagents

- Standardized Quantified WNV Stocks: (virus particle count, genomic equivalents/ml, recombinant molecules, non-infectious clones) : Required to uniformly compare analytical sensitivities of NAT assays
- Sero-conversion panels (longitudinally collected samples from plasmaphoresis donors): Needed to access the biological sensitivity of serological and NAAT assays
- WNV Ab(+) sera/plasma of sufficient volumes for assay development and proficiency testing

BBI WNV Control and Panels

- **Manufactured from**
  - **Cultured West Nile Virus (Lineage I or II)**
- **Heat-treated:**
  - **No detectable infectivity in Vero cells**
- **Diluted in defibrinated plasma:**
  - **To mimic virus in human plasma**

BBI WNV Qualification Panels

- **15 Member Panel:**
  - 30 – 10,000 copies/ml; 3 negative
  - Randomly assorted, semi-blinded
- **Two Qualification Panels:**
  - WNV Lineage I - NY99 [available soon]
  - WNV Lineage II -Uganda 1937 [now available]
- **Intended Usage:**
  - Validate the sensitivity, specificity and reproducibility of blood screening Assays
  - Proficiency assessment
  - Train laboratory personnel and improve overall test performance
  - Confirm detection of both major lineages of WNV

BBI

Chiron Corporation:

Reagents for Nucleic Acid Testing

- **A number of West Nile virus RT-PCR amplified fragments are being cloned in pGEM-4z for *in vitro* synthesis of RNA transcripts.**

- **Three RNA transcripts of different genomic regions have been prepared to support the TMA assay being developed in Gen-Probe.**
  - **5'NC/C/Pre M/M (~900 nt)**
  - **E/NS1/NS2a (~1500 nt)**
  - **NS5/3'NC (~1000 nt)**

Chiron Corporation:

Reagents for Nucleic Acid Testing

- **A genetically engineered non-infectious full-length West Nile Virus genome is being cloned in vectors suitable for RNA production.**

- **Once fully characterized, this full length West Nile virus transcript could be used as a standard for nucleic acid test evaluation.**

Concluding Remark

- Hopefully cooperation and synergy between industry, regulatory agencies, public and private laboratories will result in improved testing technologies for the diagnosis of WNV infections

Acknowledgements: Industry

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