Current Laboratory Protocols for West Nile Virus

Robert S. Lanciotti
Arbovirus Diseases Branch
Diagnostic & Reference Laboratory
Fort Collins, Colorado

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West Nile Virus Diagnostic Assays

- **Serological Assays for WN Virus**
  - **Acute & convalescent serum, csf.**
    - IgM ELISA (CDC, FOCUS, PanBio, Abbott)
    - IgG ELISA (CDC, FOCUS)
    - Blocking ELISA (avian & mammals)
    - Plaque Reduction Neutralization (PRNT)
    - IFA
    - IgA ELISA
    - Microsphere Immunoassay (CDC & NYSDH)

- **Virus Detection Assays**
  - **Acute csf, tissues, donated blood, environmental surveillance.**
    - Real Time Fluorescent RT-PCR (CDC, Roche, & Reference Labs)
    - TMA (GenProbe)
    - NASBA (BioMerieux)
    - Virus Isolation
    - Antigen Detection (ELISA & Dipstick)

Testing for West Nile Virus

	Bird Surveillance	Mosquito Surveillance	Veterinary Diagnostic	Human Diagnostic
Test Target	Virus	Virus	Antibody	Antibody
Sample Type	Tissues, oral swabs	Mosquito pools	serum	Serum, plasma, csf tissues
Available Tests	TaqMan RT-PCR NASBA RT-PCR Isolation in Vero VecTest	TaqMan RT-PCR NASBA RT-PCR Isolation in Vero VecTest	IgM ELISA Plaque Reduction Neutralization	IgM ELISA IgG ELISA Plaque Reduction Neutralization IgA ELISA IFA
Comments	Birds have high viremia; 10^6 - 10^9	Mosquito pool titers vary; VecTest will detect approx. 65%	Tissues from fatal equine cases tested by RT-PCR	Tissues from fatal human cases tested by RT-PCR. Plasma/serum/csf can be tested by NAT.

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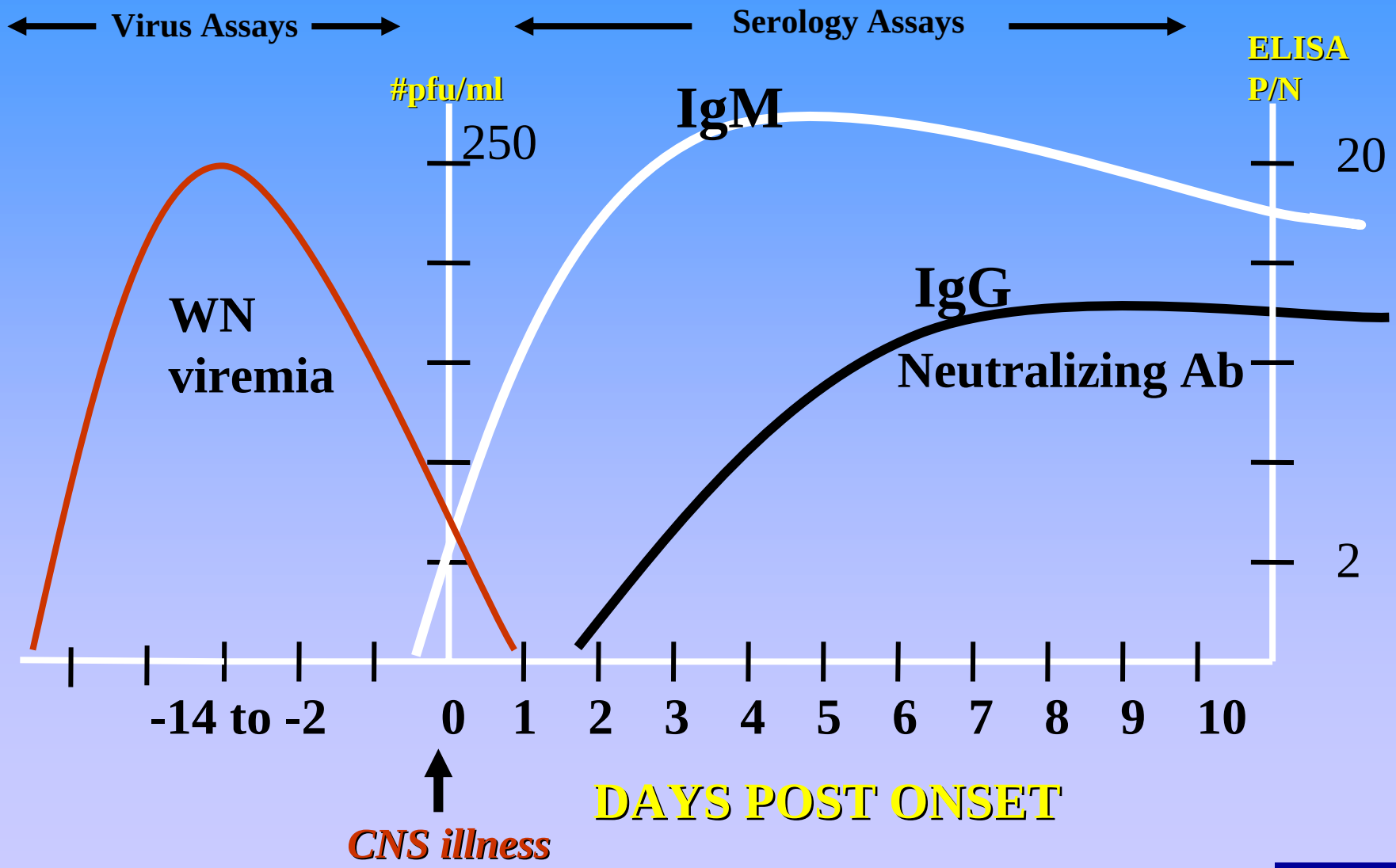
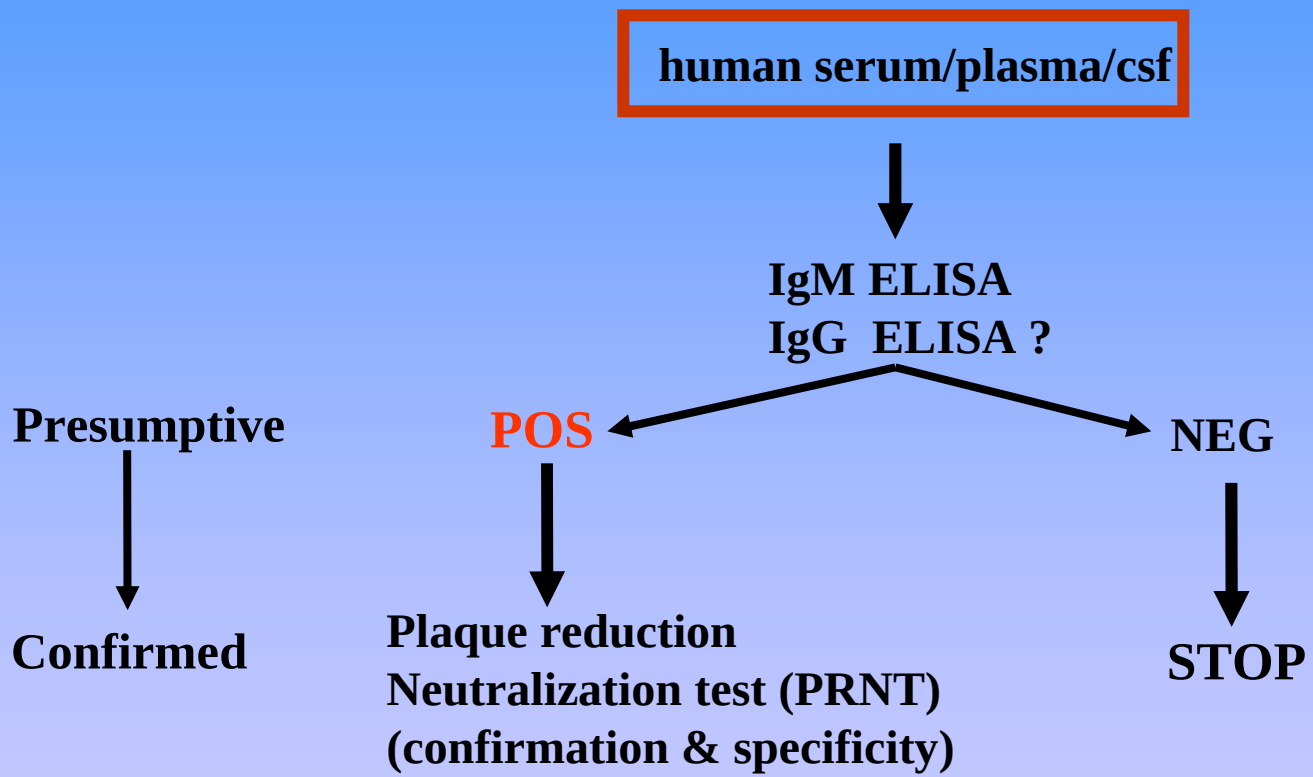
Theoretical Depiction of WNV Human Viremia & Immune Response



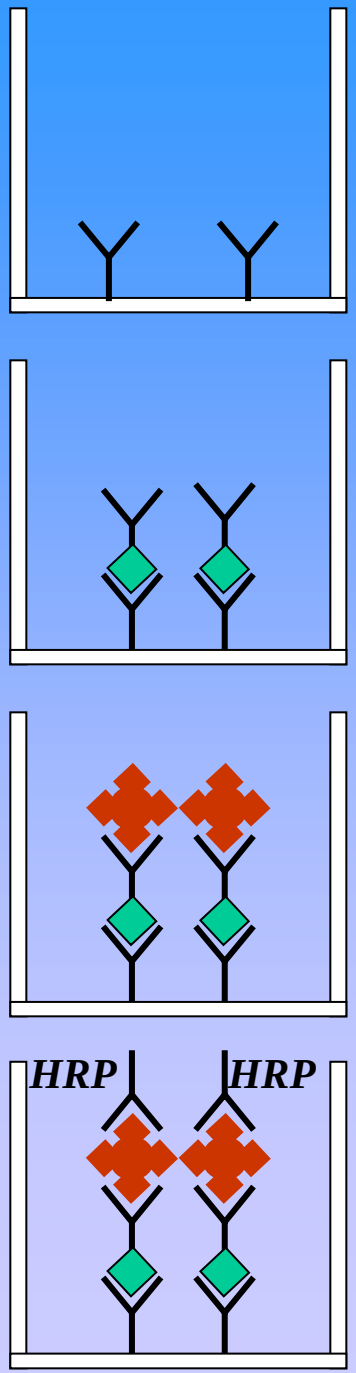
Recommended Serological Testing Algorithm for Arboviruses



Why Run the IgG ELISA?

- Secondary flavivirus infections
- Old versus recent infections
  - IgG POS & IgM NEG indicates a previous flavivirus infection
- Additional Confirmation of IgM assay

IgM Capture ELISA



1. Coat With Goat anti-Human IgM
   - 4° Overnight
2. Add Patient Serum @ 1:400
   - 37° 1 Hour
3. Add West Nile Recombinant Antigen
   - 4° Overnight
4. Add HRP anti-Flavivirus McAb
   - 37° 1 Hour

Patient OD/Negative OD >3 = POS

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Flavivirus Cross-reactivities of IgM from WN Patient Serum*

Serum	SLE	JE	WN	DEN2	YF	POW
1	4.96	7.75	16.74	2.45	1.82	1.56
2	4.8	13.77	16.68	4.13	2.14	1.75
3	5.45	9.67	16.08	4.09	1.61	1.44
4	4.76	10.07	17.19	3.32	1.62	1.3
Positive Control	6.5	8.2	6.34	7.45	3.96	4.5

* 1:400 screening dilution ; POS = > 3

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Complete Serological Analysis

Patient	Days P.I.	IgM (WN)		Plaque Reduction Titer WN	SLE	DEN2	JE
CSF	8	26.91		nd	nd	nd	nd
S1	9	9.1		160	20	<10	10
S2	34	6.7		1280	20	<10	20
Positive Control	n.a.	9		>5120	2560	2560	320

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WN Serological Data

Typical Human WN Case

	Days	IgM P/N		IgG P/N		PRNT	
Sample	**post-onset**	**WN**	**SLE**	**WN**	**SLE**	**WN**	**SLE**
Typical WN Case							
acute serum	8	12.75	4.00	1.37	2.04	1:80	1:20
conv. serum	31	11.35	4.21	6.38	5.76	1:1280	1:80

In primary flavivirus infections ;

➢*Martin et al 2002*: **IgM P/N to WN is 2-5X greater than SLE.**

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Analysis of 1,336 IgM Positive Serum Specimens for WN to SLE Ratio

WN/SLE ratio	% WN Cases	% SLE Cases	% Unresolved	Total # specimens
< 1.00 SLE>WN	32%	68%	0%	34
1.00-1.99	85.8%	6.7%	7.5%	120
2.00-2.99	93.5%	3.6%	2.9%	139
3.00-3.99	93.1%	1.9%	5%	159
4.00-4.99	97.1%	0.7%	2.2%	139
>5.00	98.8%	0%	1.2%	745

Longevity of Human WN Virus-Reactive IgM in Serum

Days P.I.	N	Positive MAC-ELISA		Total (%)	Ave. P/N (Range)
		Positive (%)	Equivocal		
200	22	13 (60)	4	17 (77)	6.0 (3.0-10.8)
300-400	21	9 (43)	2	11 (52)	4.0 (31.-6.5)
500	12	5 (42)	2	6 (60)	5.0 (3.1-6.9)

WN Serological Data

Secondary Flavivirus Infection

	WNV IgM	SLE IgM	WNV PRNT	SLE PRNT	JE PRNT	YF PRNT
CASE 1	7.1	5.8	1:2560	1:2560	1:5120	1:640
	WNV IgM	**DEN IgM**	**WNV PRNT**	**SLE PRNT**	**DEN PRNT**	**YF PRNT**
CASE 2	33.2	2.4	1:2560	1:1280	1:640	1:640

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WN Serological Data

2002 WN Case Tested in 2003

	WNV IgM	**SLE IgM**	**WNV IgG**	**SLE IgG**	**WNV PRNT**	**SLE PRNT**	**WNV IgA**
DAY 7	5.2	NEG	12.0	3.4	1:160	1:10	NEG
DAY 25	5.0	NEG	11.2	3.2	1:160	1:10	NEG

West Nile Virus IgA Assay

- 95% WN IgM positive serum samples are IgA positive days 11 – 40
- No IgA positives after day 51

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WN Human Serological Data

Lessons Learned 1999-2003

- IgM detectable in serum & csf by CNS illness onset (99%); not WN fever; IgG Positive by day 7 Post-Onset
- In primary WN cases: ELISA reactivity is 2-5X higher to WN than to SLE
  - PRNT may not be necessary to confirm all WN IgM positives
- IgM Persistence > 1 Year in 50% cases in 1999 study
  - WNV IgM positives detected in endemic areas could be previous years cases; additional laboratory resting is necessary
  - IgA can be an additional marker for recent infection along with IgM
- Secondary flavivirus infections are problematic
  - High PRNT to several flaviviruses; no clear “winner.”

WN EIA Serological Reagents

- IgM & IgG EIA Kits from FOCUS & PanBio (FDA approved)
- WN antigen from
  - FOCUS for Public Health Labs; 2004; not likely for 2005 & beyond
  - Hennessey Research Associates
- SLE antigen from CDC
  - Hoping for commercial partners
- HRP conjugate & IgG coating antibody from CDC
  - Commercial sources possible

http://www2a.cdc.gov/ncidod/dvbid/misc/index.asp

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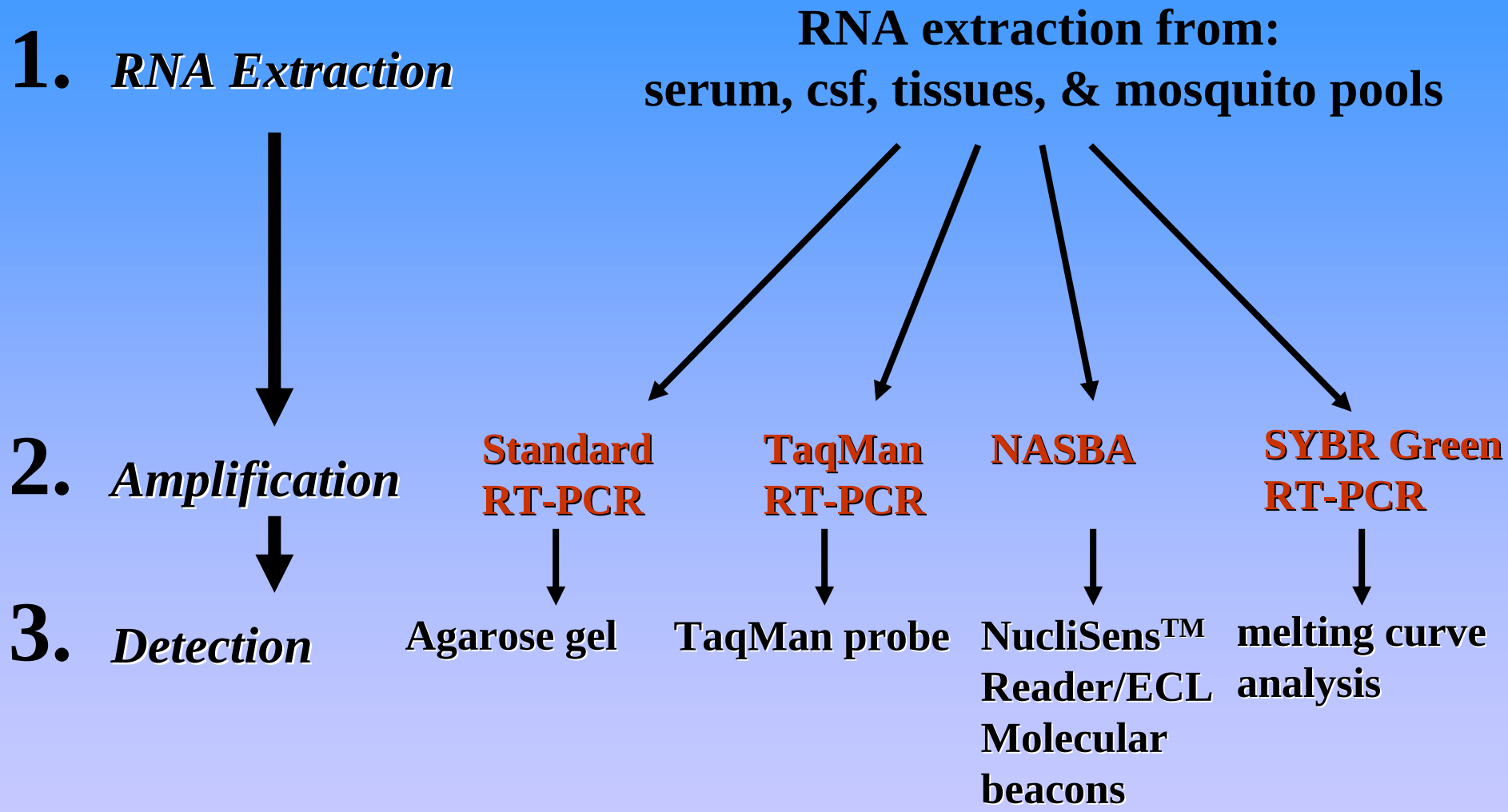
CDC Molecular Amplification Assays



Laboratory Safety Issues

CDC Implementation of *Biosafety in Microbiological & Biomedical Laboratories;* 4th Ed.

- West Nile is a BSL3 virus
  - **ELISA:** Biosafety Cabinet (BSC) until serum is washed, then BSL2
  - **PRNT:** BSL3
    - YF/WN chimera virus attenuated available from CDC
  - **Virus Isolation:** BSL3
  - **PCR:** BSC until viral lysis buffer is added, then BSL2
  - **Antigen (Dipstick) Assays:** BSC until detergent lysis buffer is added, then BSL2
  - **Animal Necropsy:** BSL3

Sample Preparation for Molecular Amplification Assays

Mosquito pool (n=50)
Tissues

Disruption In QIAGEN Mixer Mill

Homogenized Lysate

Serum/plasma
CSF/breast milk

RNA extraction & purification in QIAGEN BioRobot or kits

RNA in H_2O

RNA in H_2O

Molecular Amplification Test

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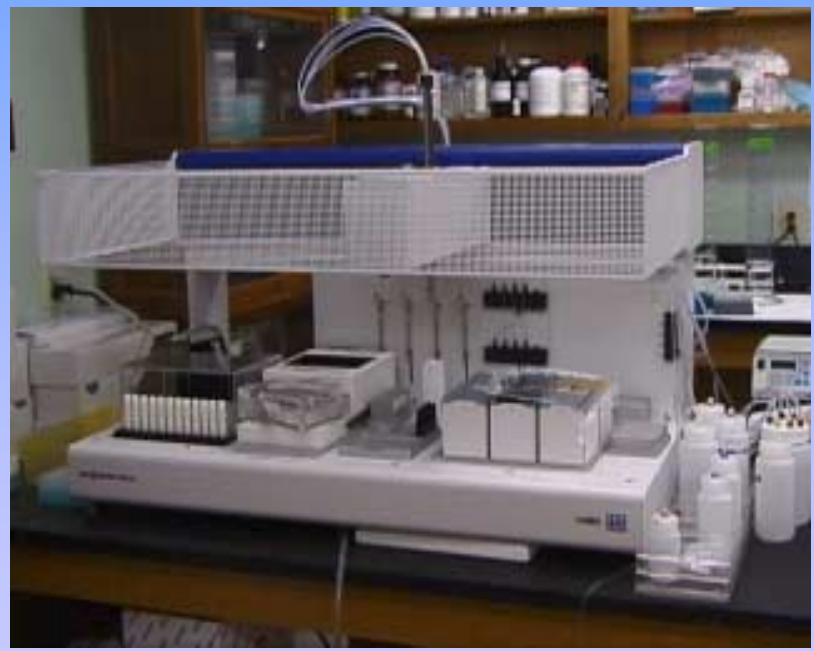
RNA Extraction & Purification

- **Chemical/Phase Separation**
  - guanidine isothiocyanate, phenol/chloroform, ethanol precipitation. (Home-made;TRIzol)
  - 40 samples per day
- **Silica-gel Kits**
  - column (QIAGEN)
  - beads (Nuclisens, Bio-101)
  - 80 samples per day
- **Robotics QIAGEN 9604**
  - 300 samples per day



CDC TaqMan Testing Algorithm

- ✓ Extract RNA (100 ul to 500 ul)
- ✓ TaqMan with ENV primer set + internal control
- ✓ Ct < 37 positive; Ct 37 – 45 equivocal
- ✓ All positives & equivocal are repeated with a second primer set; using newly extracted RNA

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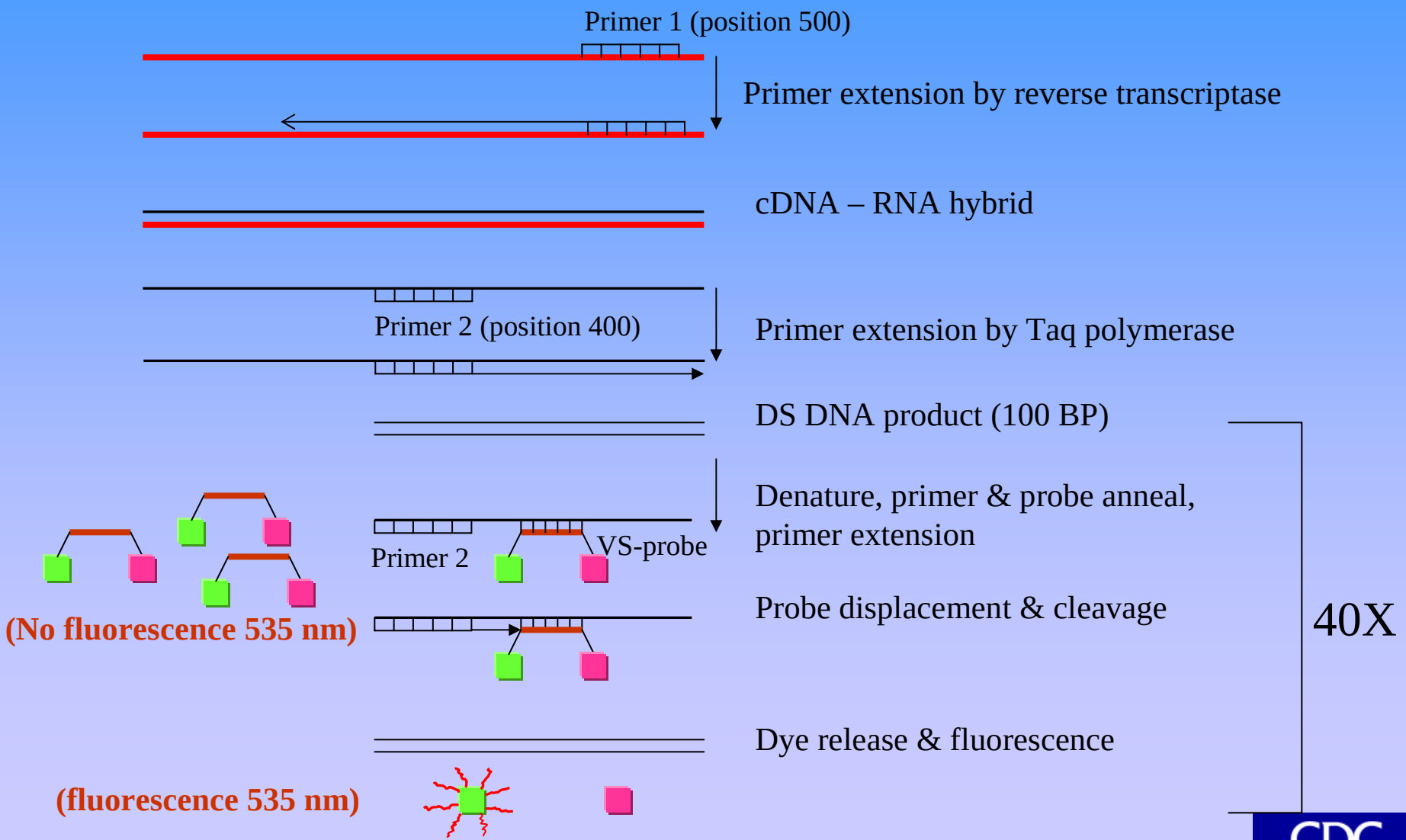
TaqMan RT-PCR



WNV TaqMan Detection Limit

Plaque forming units (pfu)

ENV set

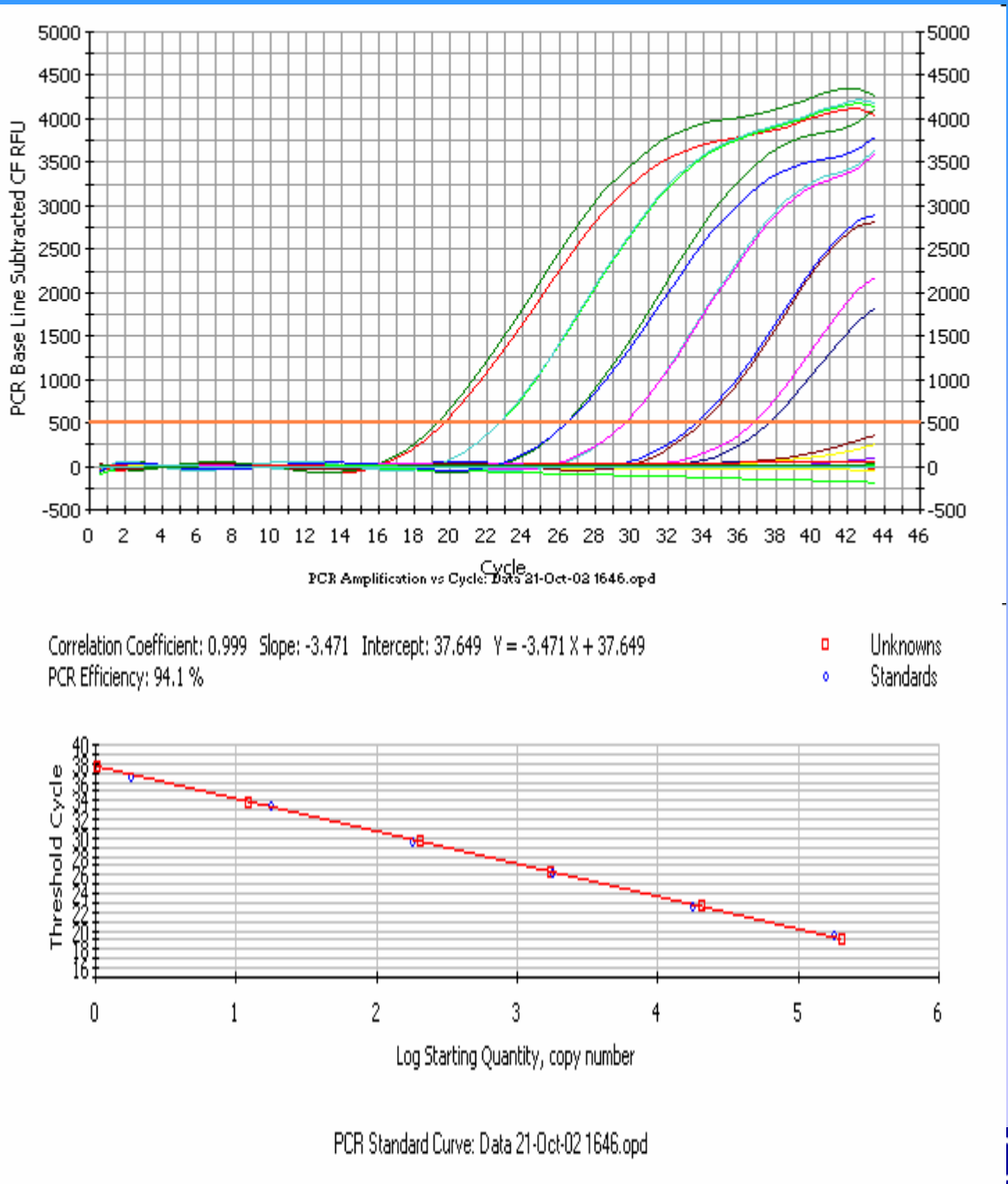
0.10 pfu/ml or
40 copies/ml

3'NC set

0.4 pfu/ml or
160 copies/ml

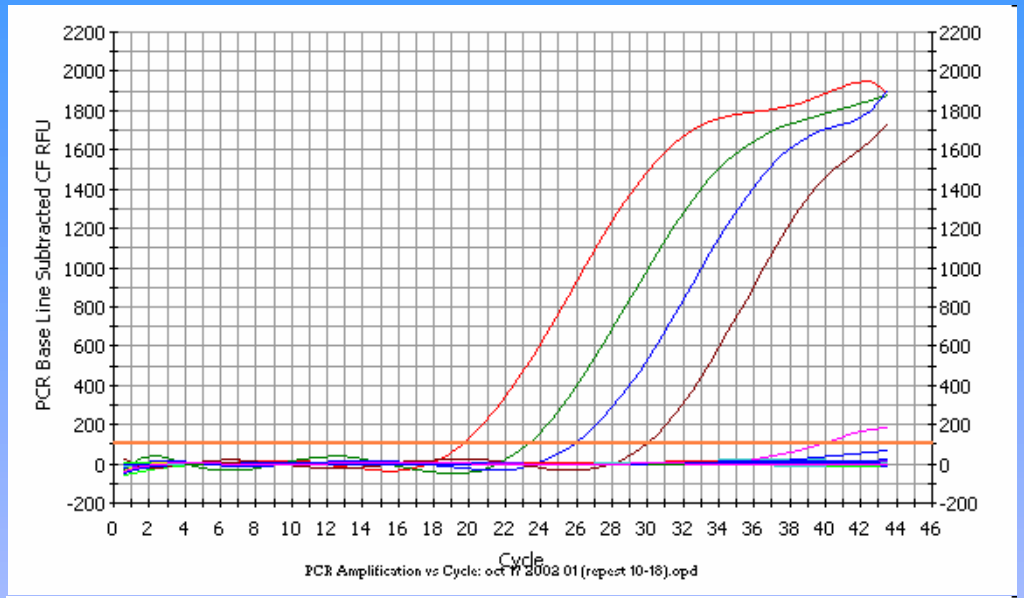
NS5 set (Lipken)

0.2 pfu/ml or
80 copies/ml

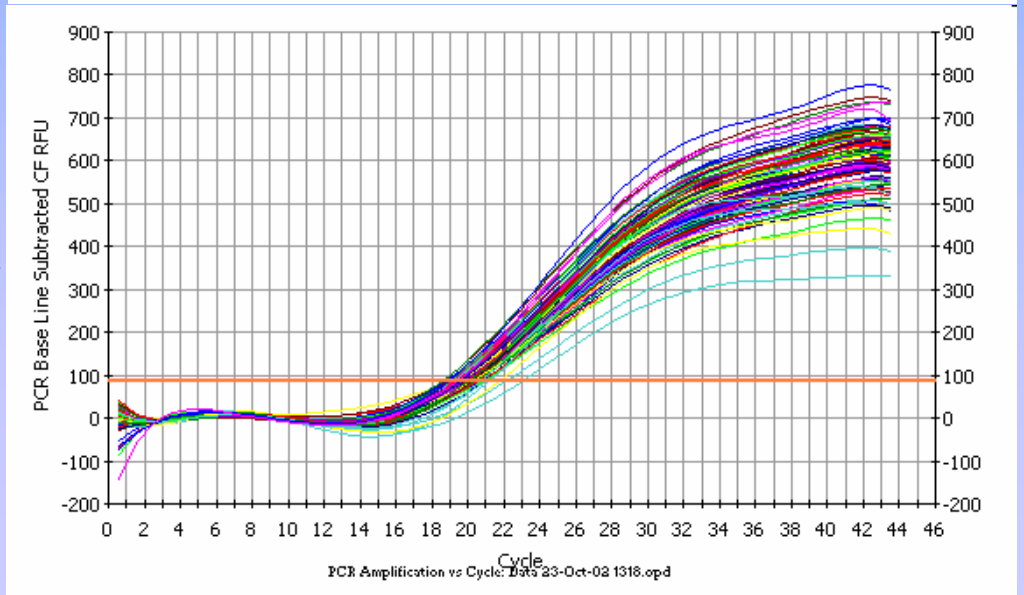


WN Virus TaqMan Assay With HEX-Labeled Internal Positive Control

WN virus primer/probe set



HEX internal control primer/probe set



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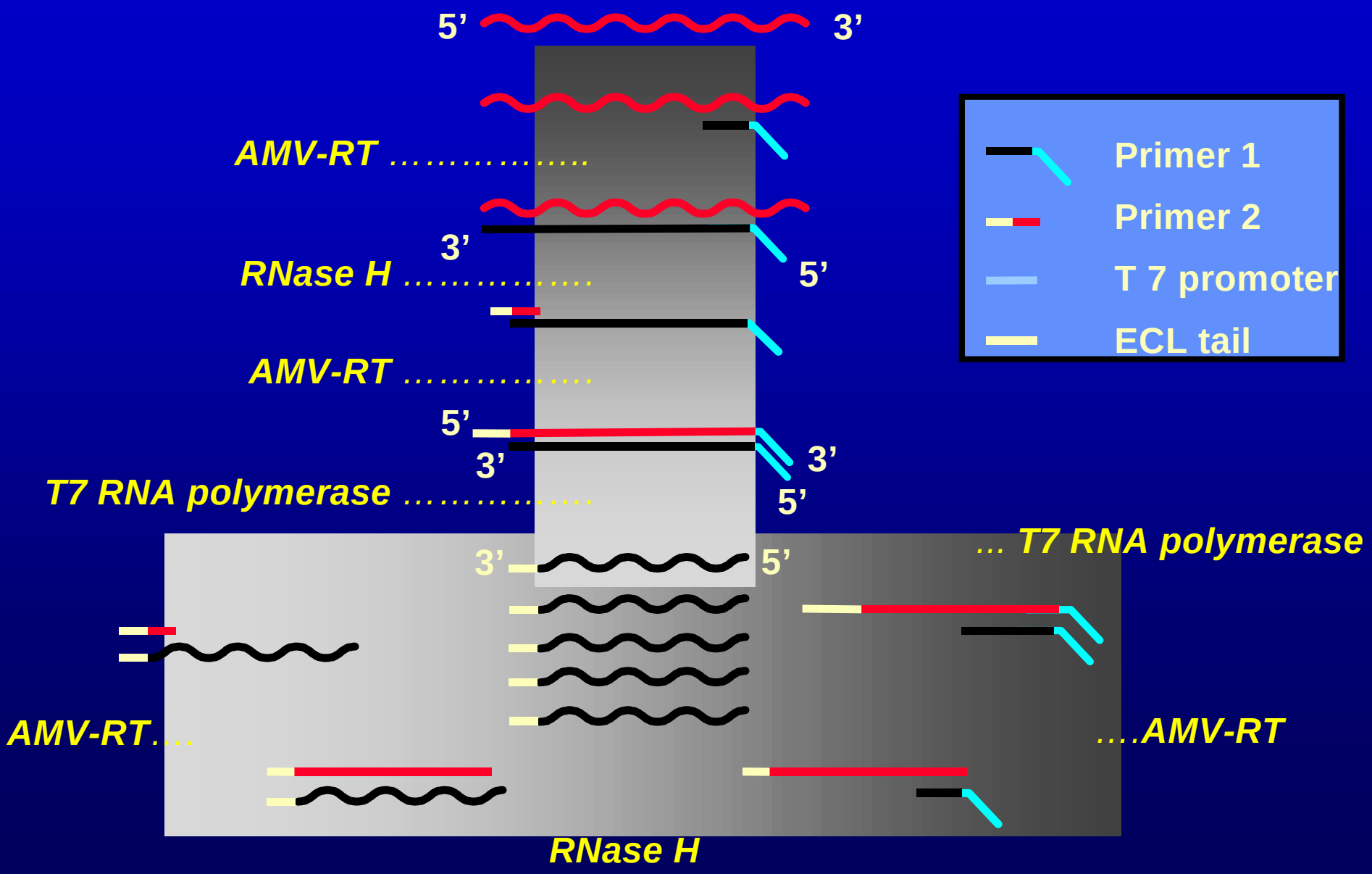
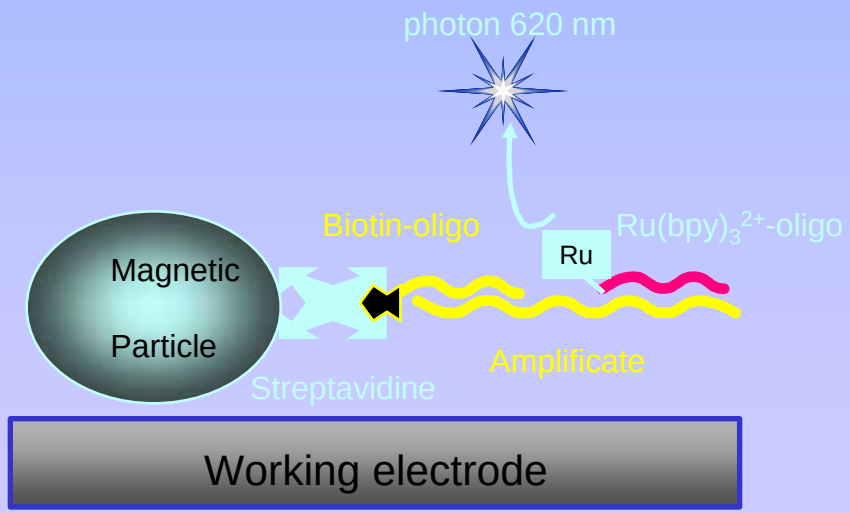
NASBA – Nucleic Acid Sequence Based Amplification



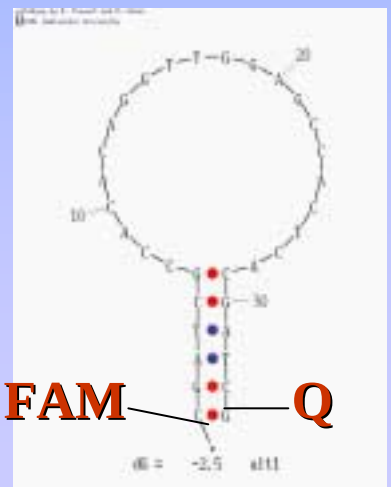
NASBA Detection Formats

SS RNA

NASBA ECL Reader



NASBA Molecular Beacon



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Sensitivity of WN Virus NASBA & TaqMan Assays

	TaqMan		NASBA		NASBA	
#pfu/ml	Ct	Interp.	ECL	Interp.	MB	Interp
1,000,000	16.21	pos	1653417	pos	9.44	pos
100,000	19.72	pos	1187613	pos	12.01	pos
10,000	23.42	pos	1810790	pos	12.27	pos
1,000	26.53	pos	1666084	pos	14.81	pos
100	30.01	pos	1211426	pos	19.21	pos
10	33.62	pos	1209491	pos	21.42	pos
1	35.28	pos	326954	pos	45	neg
0.1	37.12	pos	5782	pos	45	neg
0.01	45	neg	110	neg	45	neg

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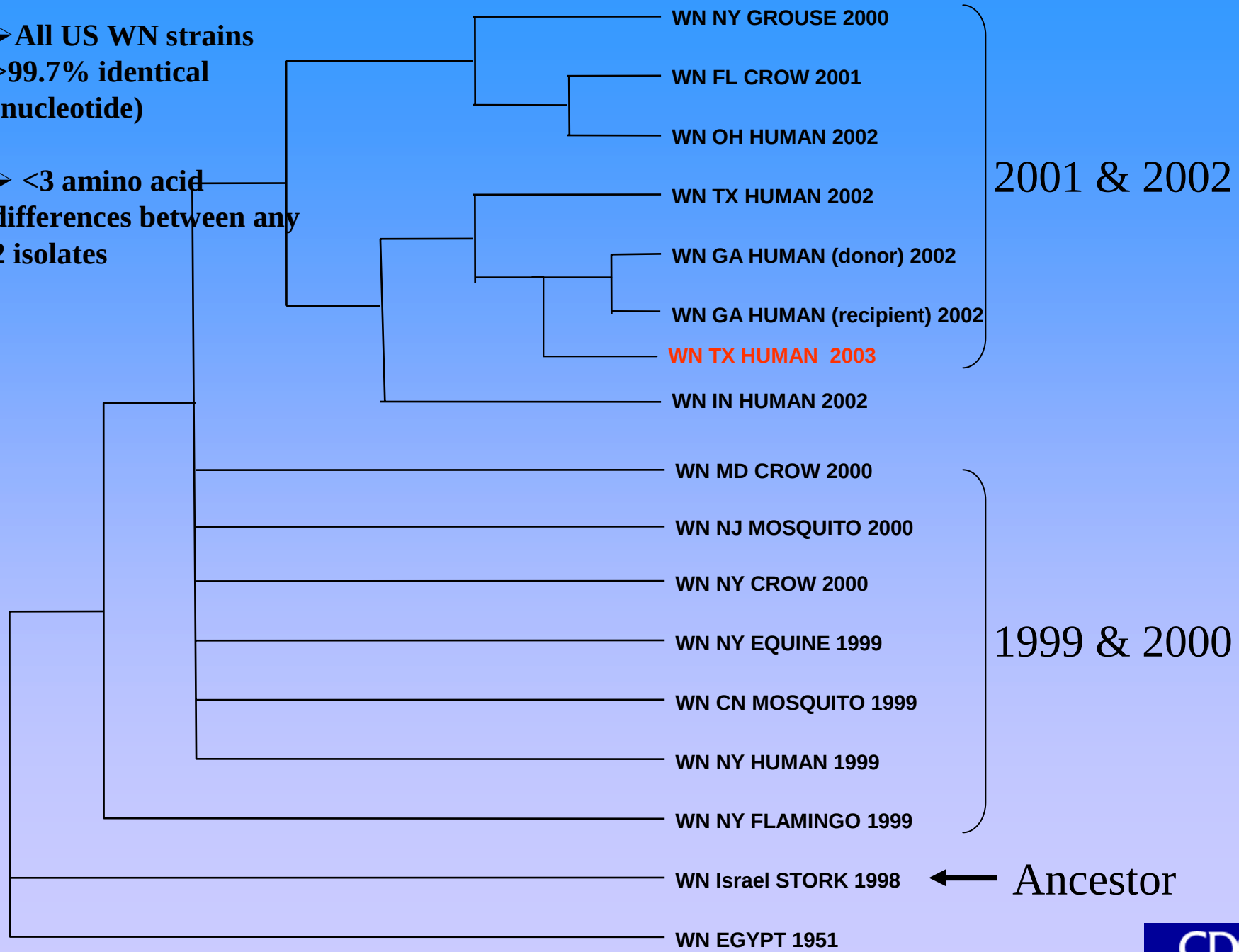
- **All US WN strains >99.7% identical (nucleotide)**
- **<3 amino acid differences between any 2 isolates**



West Nile Virus NAT Testing Summary

- Most sensitive NAT tests are TaqMan & NASBA
- Use of internal, negative, & copy number controls is critical to validating the assay
  - Copy number WN virus controls in human plasma available from Boston Biomedica.
- WN virus strains in the U.S. are highly conserved; 99.7% identical.
  - Only 1 mutation in 9 primers commonly used

WNV Isolates From Humans: 1999 - 2002

- 1999: No WNV isolated
- 2000: No WNV isolated
- 2001: 1 virus isolated csf (NY State Lab)
- 2002: 16 WNV isolated CDC + 1 from MD Dept. Health
  - 5 serum/plasma
  - 3 csf
  - 4 brain tissue
  - 1 liver
- 2003: Numerous isolates from donated blood

WN Human Viremia

Data Summary

- **Human viremia is low:**
  - Transfusion studies: <1-200 pfu/ml (100 – 80,000 copies/ml
  - Average 40 pfu/ml (16,000 copies/ml)
  - Virus isolation is rare (approx. 1 in 5)

- **Human viremia is short-lived**
  - Not detectable in most cases by day 1 of illness onset
  - IgM and virus rarely detected together

Special Thanks to the CDC Arbovirus Diagnostic Lab Staff

Denise Martin
Kathy Wolff
Trudy Chambers
Jason Velez
Amy Lambert
Jane Johnson
Olga Kosoy
Brandy Russell
Amanda Noga
Barbara Johnson
Janeen Laven
Roseyln Hochbein