Serological and Molecular Amplification Assays for West Nile & Other Arboviruses

Arbovirus Diseases Branch
Diagnostic & Reference Laboratory
Fort Collins, Colorado

Medically Important Arboviruses in the United States

Family/Genus	Pathogens
Togaviridae/Alphavirus	Eastern equine encephalitis
ss + RNA +; 70 nm particle	Western equine encephalitis
	Venezuelan equine encephalitis
Flaviviridae/Flavivirus	St. Louis encephalitis
ss + RNA; 40-60 nm particle	Powassan
	West Nile
	Dengue
Bunyaviridae/Bunyavirus	
California serogroup	California encephalitis
ss -RNA; 3 segment genome	La Crosse encephalitis
	Jamestown Canyon
	Snowshoe hare
	Cache Valley (bunyamwera)
Reoviridae/Coltivirus	Colorado tick fever
ds RNA	

Eastern Equine Encephalitis Human cases: 1964-2002

- 191 cases
- 5 cases/year
- no epidemic years
- 40% FL & GA

Western Equine Encephalitis Human cases: 1964-2002

- 640 cases
- some epidemic years
- 65% cases 1964-66 & 1975
- 8 cases/year non epidemic
- 4 cases since 1990

La Crosse Virus Encephalitis
Human cases: 1964-2002

- **2910 cases**
- **76 cases/year**
- **children < 16**
- **other CAL viruses**

St. Louis Encephalitis Human cases: 1964-2002

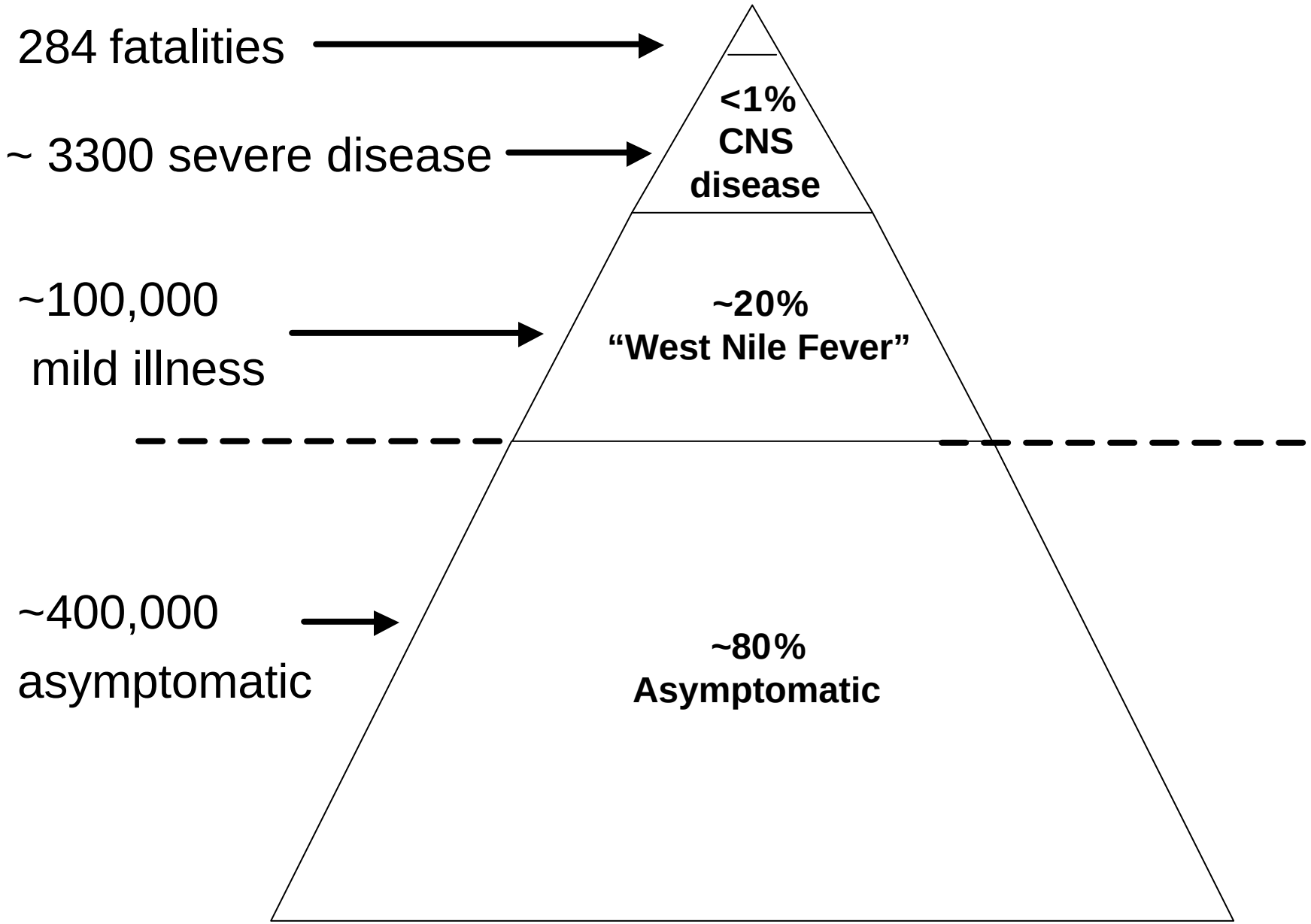
- 4561 cases
- Epidemic cycles
- 50% 1975 & 1976
- 70% TX, IL, OH, IN, FL, MS

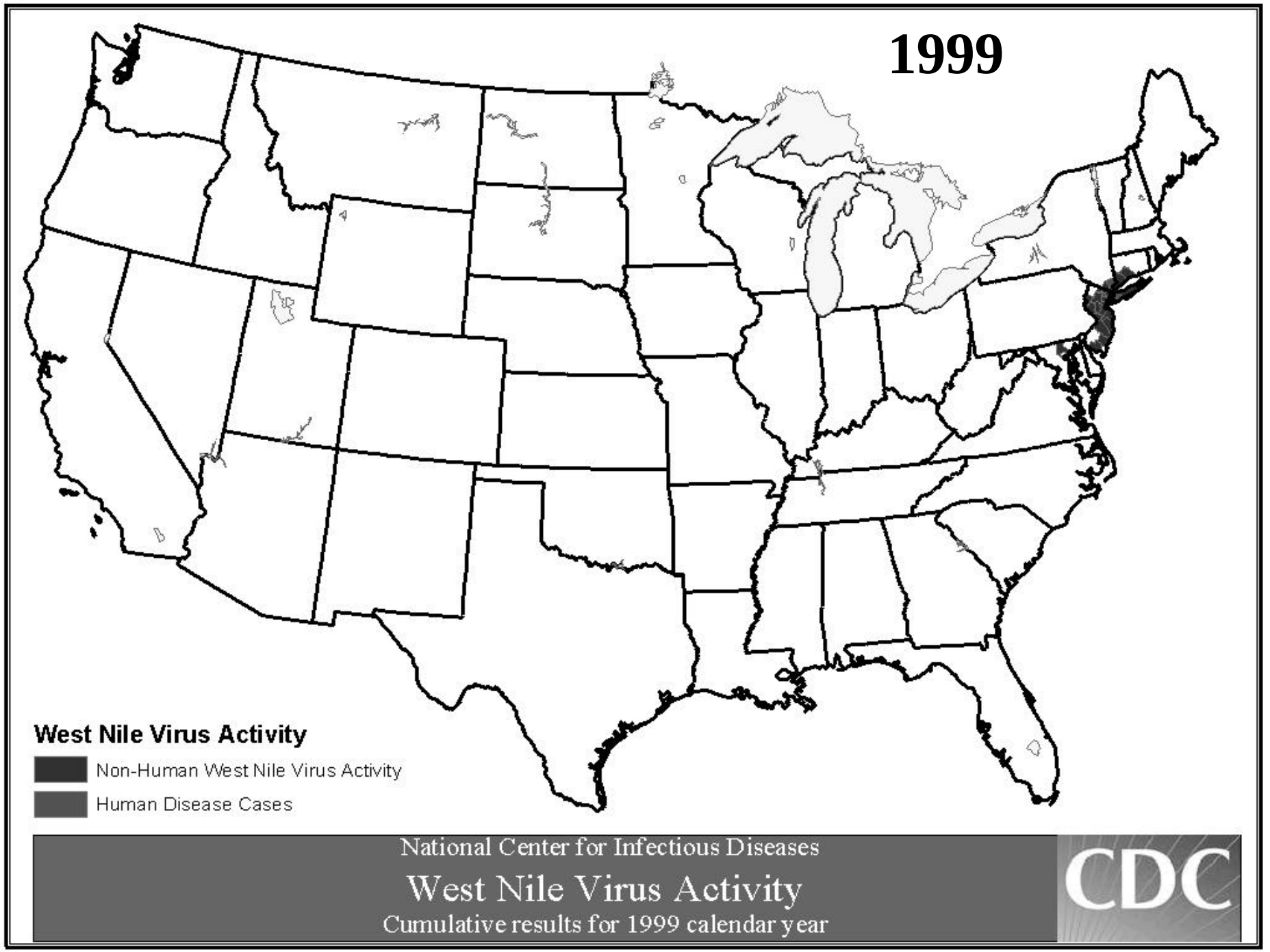
Reported WNV Disease Cases in Humans, United States, 1999-2003

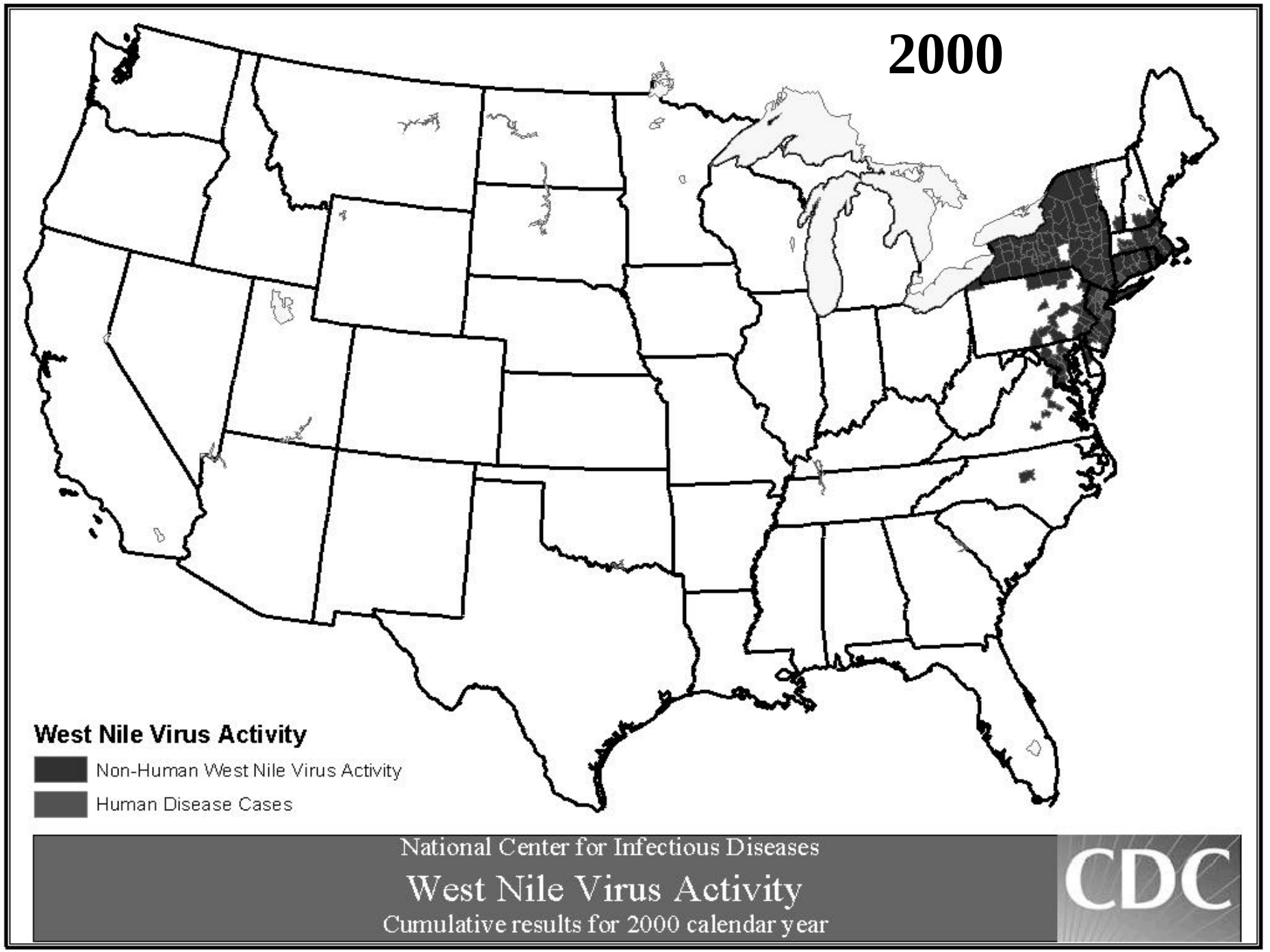
Year	# Cases	# States	# Counties	Onset Date Range
1999	62	1	6	2 AUG – 24 SEP
2000	21	3	10	20 JUL – 27 SEP
2001	66	10	39	13 JUL – 7 DEC
2002	4,156	39*	740	19 MAY – 19 DEC
2003	8,977	46	1048	28 MAR – 30 NOV

* Plus D.C.

WNV Human Infection “Iceberg” in 2002




1999
West Nile Virus Activity
Non-Human West Nile Virus Activity
Human Disease Cases
National Center for Infectious Diseases
West Nile Virus Activity
Cumulative results for 1999 calendar year
CDC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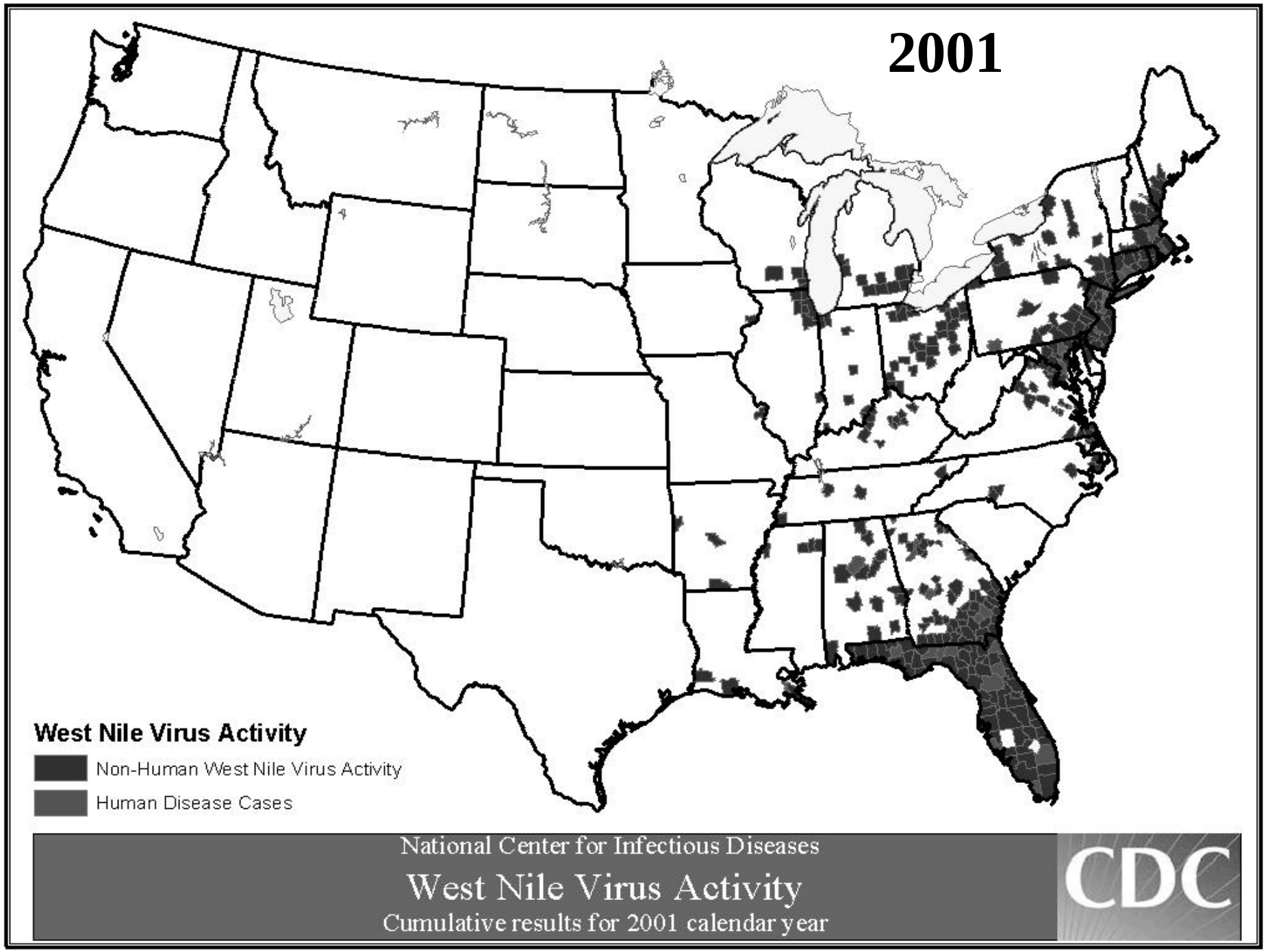
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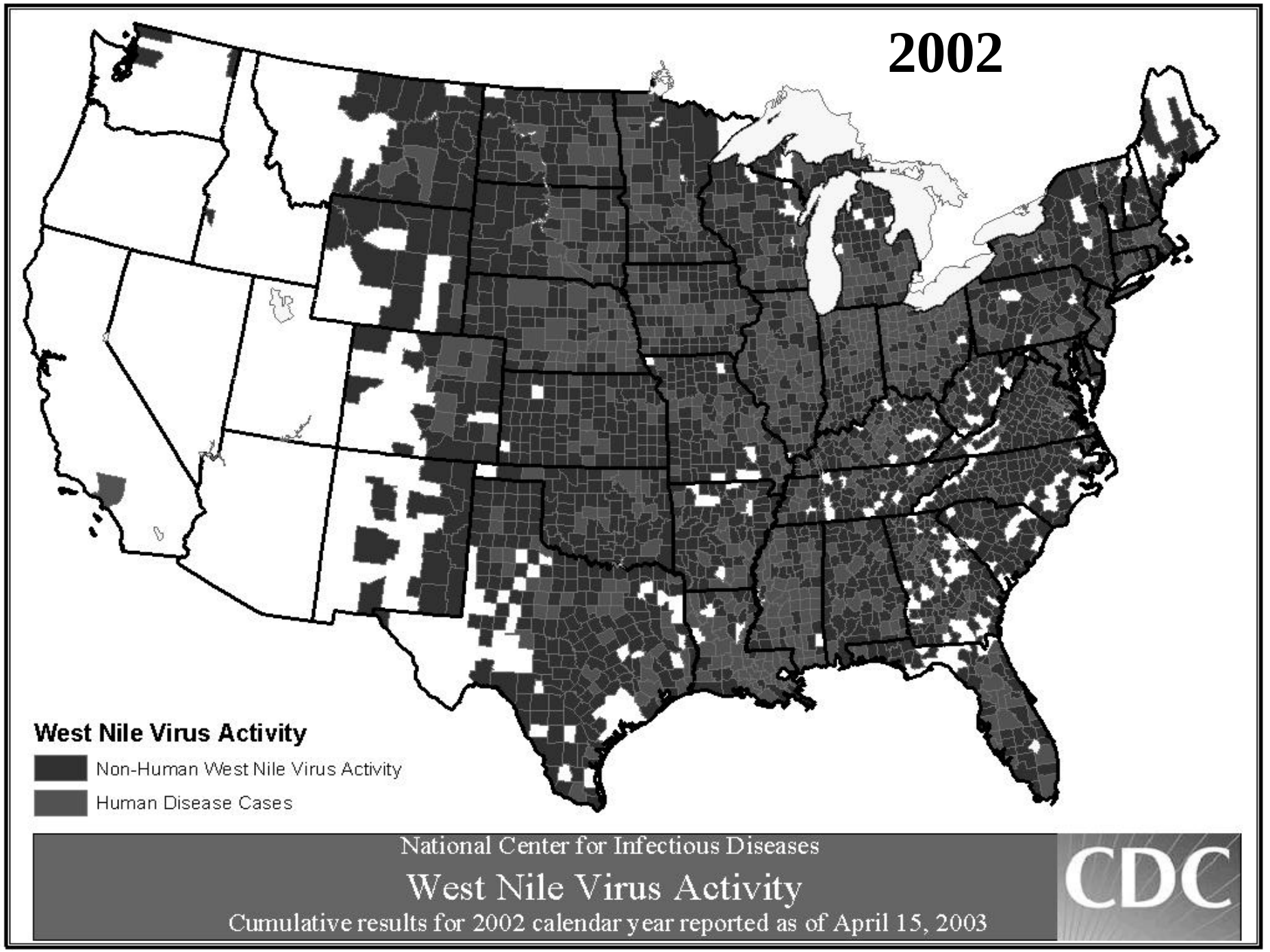
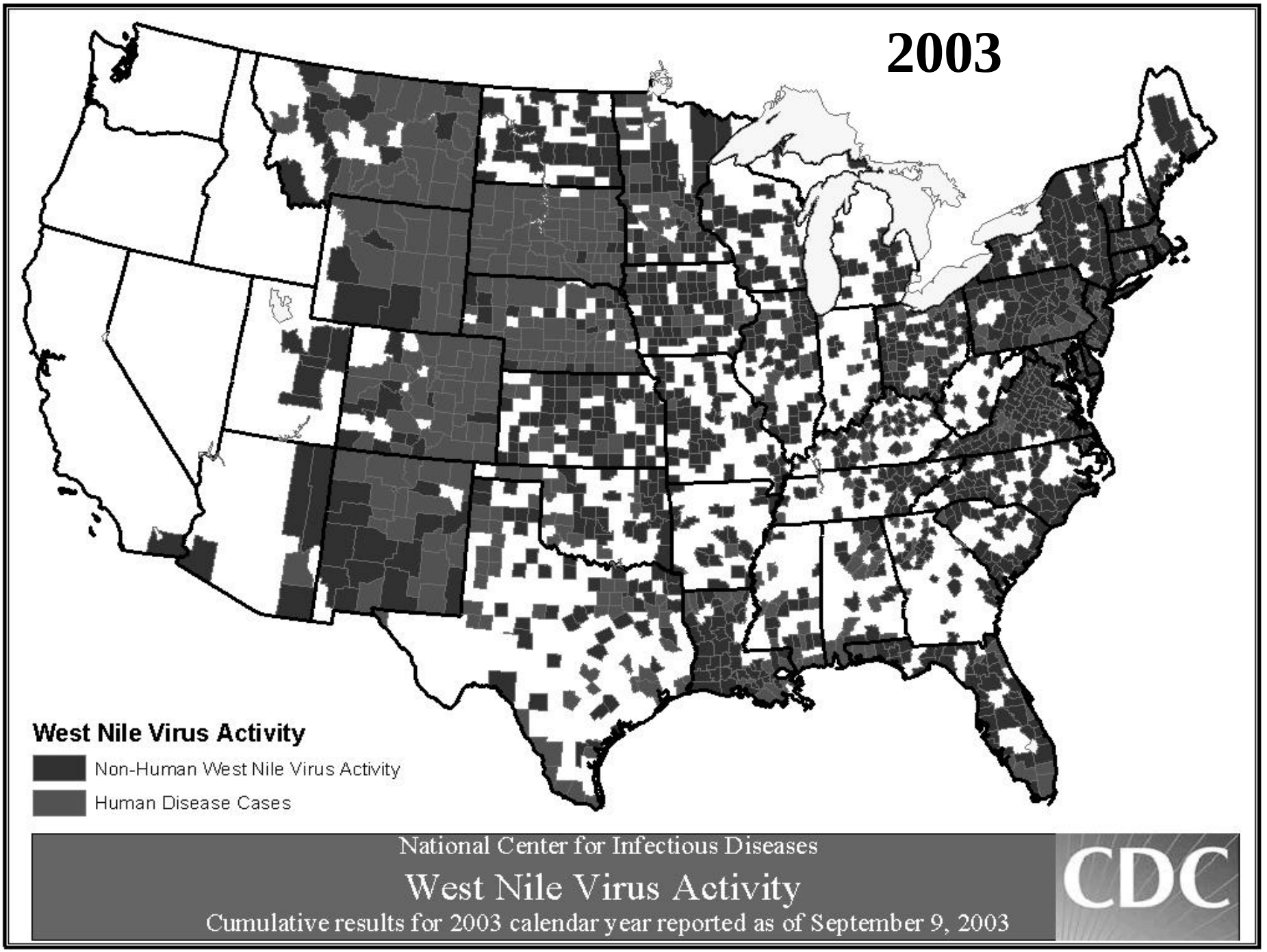
Non-Human West Nile Virus Activity
Human Disease Cases
National Center for Infectious Diseases
West Nile Virus Activity
Cumulative results for 2001 calendar year
CDC

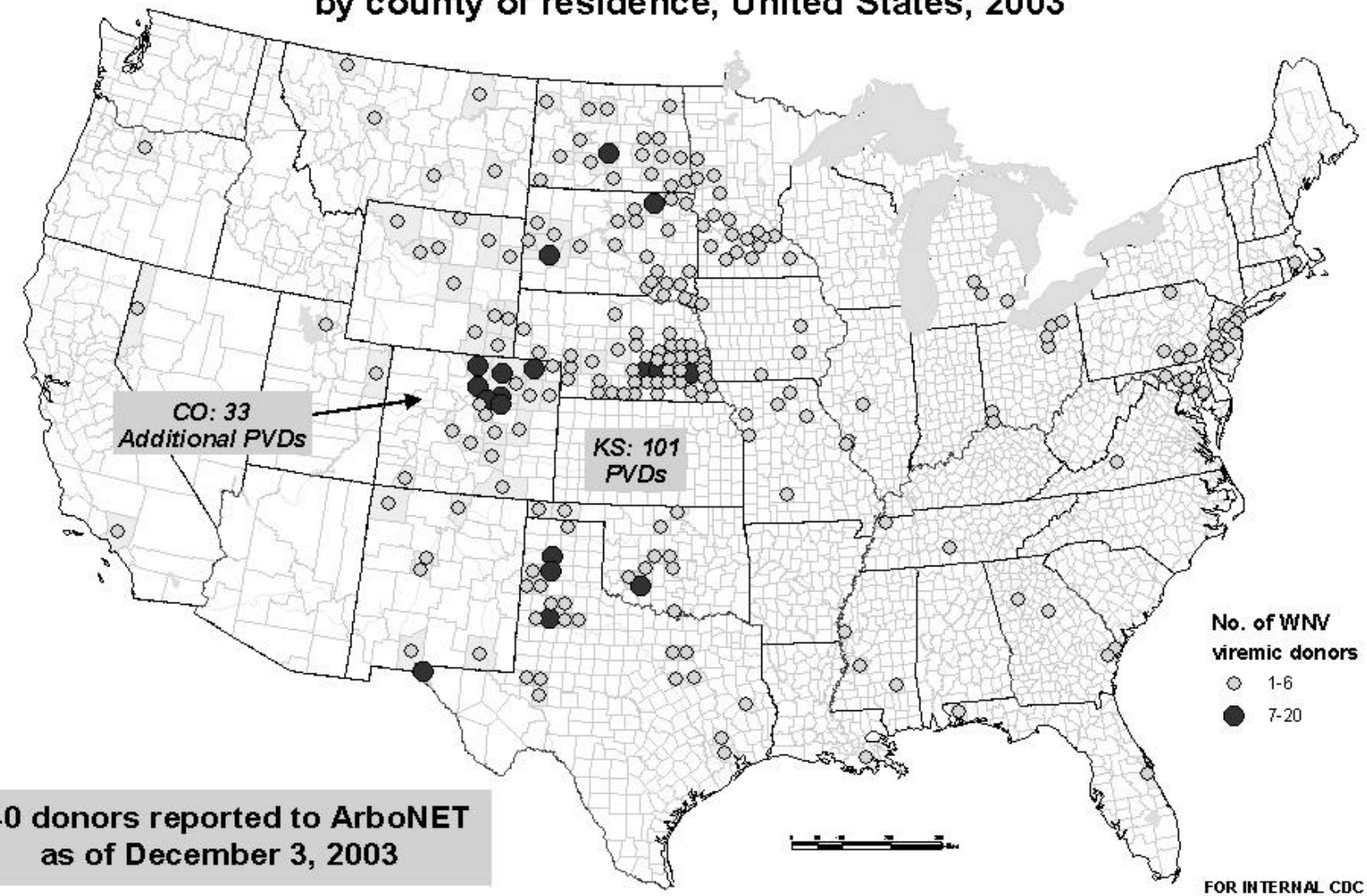

2002
West Nile Virus Activity
Non-Human West Nile Virus Activity
Human Disease Cases
National Center for Infectious Diseases
West Nile Virus Activity
Cumulative results for 2002 calendar year reported as of April 15, 2003
CDC


2003
West Nile Virus Activity
Non-Human West Nile Virus Activity
Human Disease Cases
National Center for Infectious Diseases
West Nile Virus Activity
Cumulative results for 2003 calendar year reported as of September 9, 2003
CDC

Novel Modes of West Nile Virus Transmission, 2002

- Transplanted organs
  - One donor to four recipients
- Transfused blood
  - 23 confirmed cases in 2002, many more likely
  - WNV NAT screening in Blood Banks began in July; >700 positive
- Breast milk
  - One case, infant asymptomatic
- Transplacental transmission
  - One case, severe outcome to infant
- Occupational exposure

West Nile viremic blood donors,*
by county of residence, United States, 2003



740 donors reported to ArboNET as of December 3, 2003

FOR INTERNAL CDC USE ONLY

** Reported to CDC/ArboNET by state and metropolitan health departments. Reports are presumptive and confirmatory investigations may be ongoing. Some viremic donors known to the blood banks and health departments may not be reported here. For more summary information on reported viremic blood donors, go to the MMWR online at http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5232a3.htm.*

ArboNET

CDC

West Nile Virus Diagnostic Assays

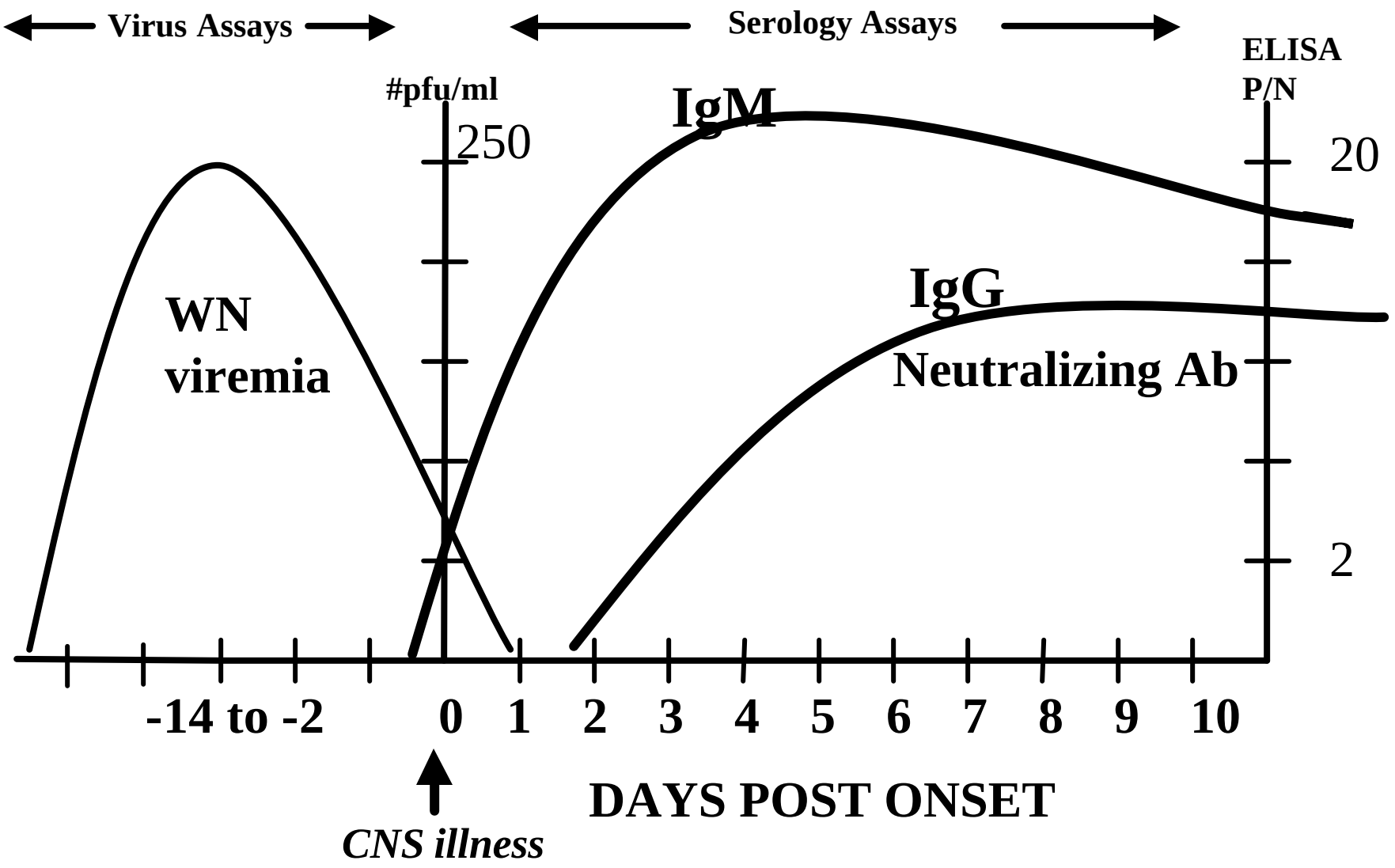
- **Serological Assays for WN Virus**
  - **Acute & convalescent serum, csf.**
    - IgM ELISA (CDC, FOCUS, PanBio, Abbott)
    - IgG ELISA (CDC, FOCUS)
    - Blocking ELISA (avian & mammals)
    - Plaque Reduction Neutralization (PRNT)
    - IFA
    - IgA ELISA
    - Microsphere Immunoassay (CDC & NYSDH)

- **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

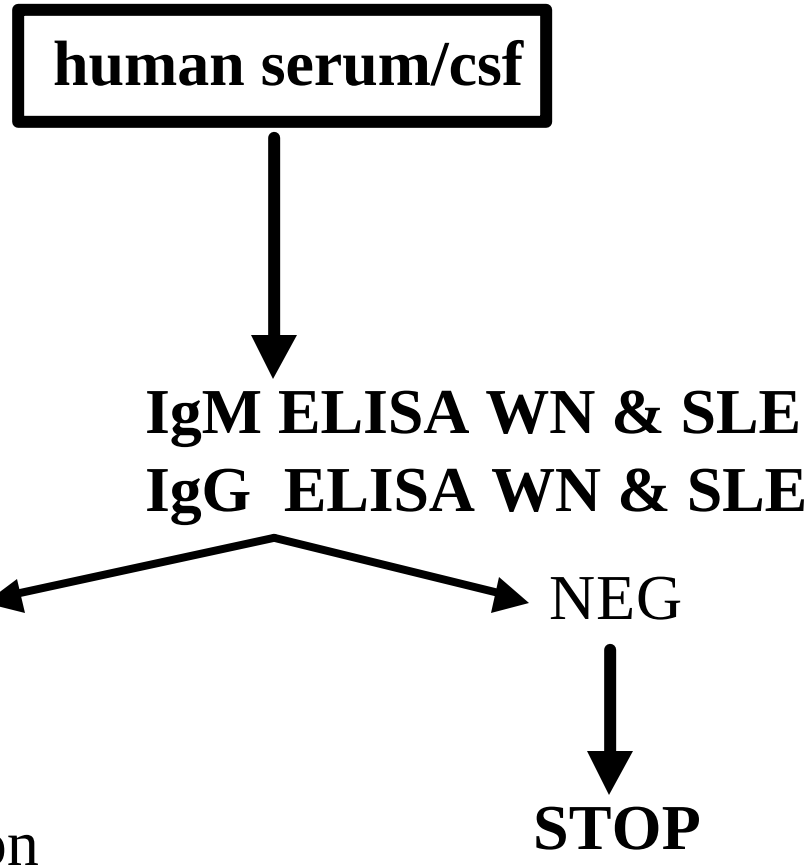
	Bird Surveillance	Mosquito Surveillance	Veterinary Diagnostic	Human Diagnostic
Test Target	Virus	Virus	Antibody	Antibody
Sample Type	Tissues, oral swabs	Mosquito pools	serum	Serum, plasma, csf tissues
Available Tests	TaqMan RT-PCR NASBA RT-PCR Isolation in Vero VecTest	TaqMan RT-PCR NASBA RT-PCR Isolation 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.

Theoretical Depiction of WNV Human Viremia & Immune Response



Serological Testing Algorithm for West Nile Virus

National Case Definition
Confirmed:
IgM pos csf
IgM pos serum + PRNT
>4-fold increase PRNT titer



Plaque reduction
Neutralization test (PRNT) with:
SLE, WN, (other flaviviruses)

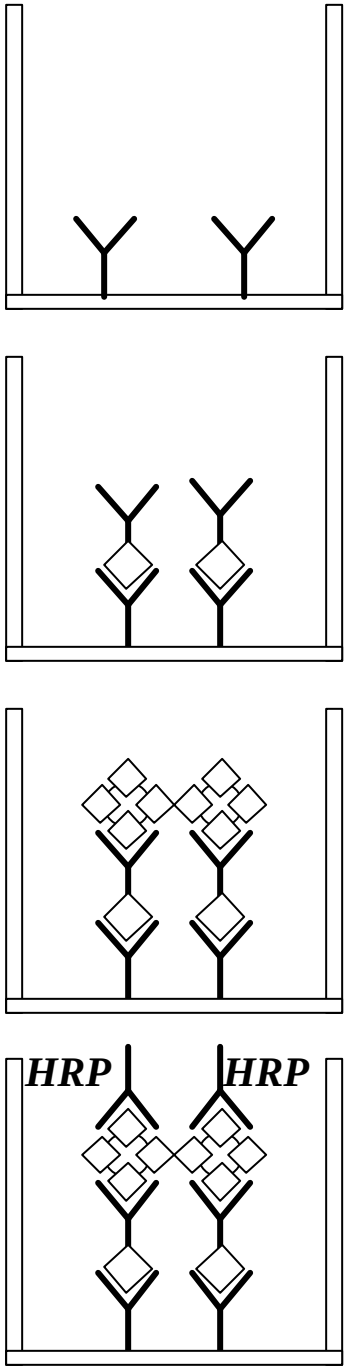
Why Run the IgG ELISA?

- Secondary flavivirus infections

- Old versus recent infections
  - IgG POS & IgM NEG indicates a previous flavivirus infection

- Additional Confirmation of IgM assay
  - Seroconversion in paired specimens

- **IgG for early season testing and/or special cases; not during a confirmed WN epidemic**



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

Interpretation of Results

- P/N: O.D. patient serum/O.D. negative control serum.
- P/N > 3 = positive
- P/N < 2 = negative
- P/N 2-3 = equivocal

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

CDC
Centers for Disease Control and Prevention

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.**

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)

CDC

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

<u>West Nile Virus IgA Assay</u>

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

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

WN Human Serological Data

Lessons Learned 1999-2003

- IgM remains the front-line screening assay
  - 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 testing 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

IgM & IgG ELISA Technology Transfer

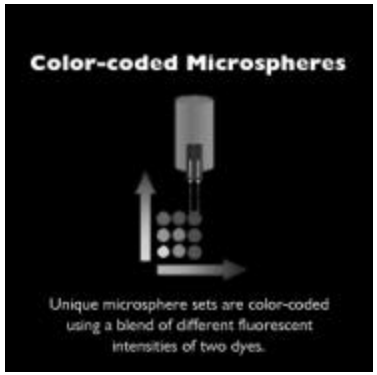
- CDC Training Course
  - Trained > 60 Public Health Laboratories
- Proficiency Panel
  - 100% agreement IgM ELISA
  - 92% agreement IgG ELISA (false neg's)

WN Serological Assays

Future Directions

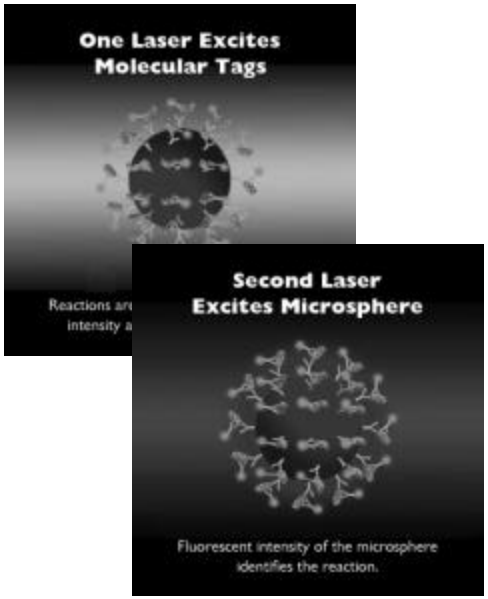
- Automation of IgM & IgG ELISA
- Reagent Stability
- Incubation Times
- Luminex Assay
- Commercial Assays

Microsphere-based assay to detect IgM to WN and SLE viruses in human serum



Beadsets are coupled to 6B6C-1 → One beadset is reacted with WNV antigen and the other with SLEV antigen → Add reacted beadsets to IgG-depleted serum and anti-IgM R-PE.

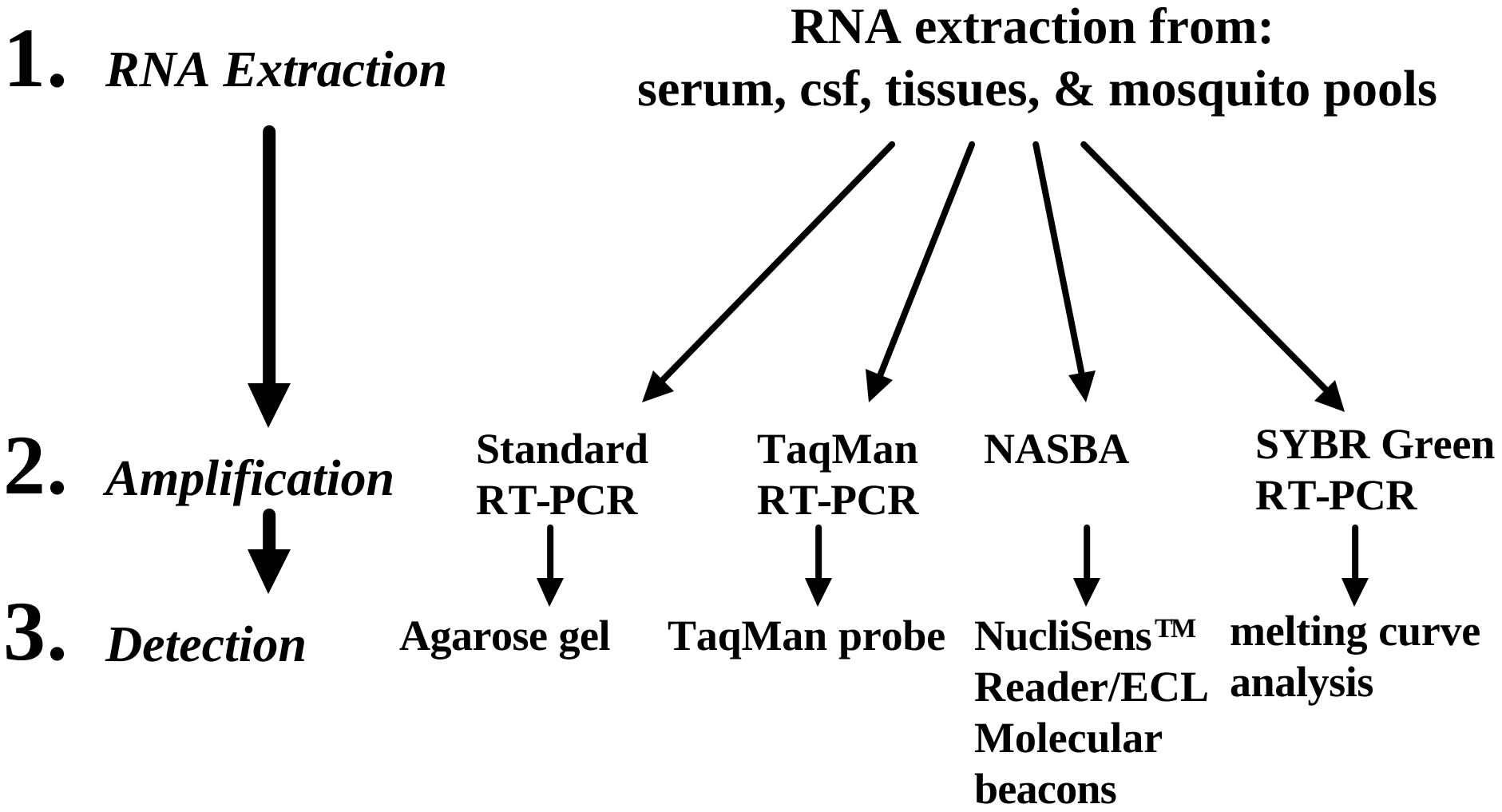
- The assay gives concurrent WN and SLE virus IgM values
- All samples reacted on viral and control antigens
- Time of reaction 1.5 hours
- Cut-off determination and validation in progress



Detection of WNV and SLEV IgM in microsphere-based duplexed immunoassay

Serum Sample	MFI (32) WN	MFI (57) SLE	MAC-ELISA P/N WN	MAC-ELISA P/N SLE
1 (WN)	7464	408	37.3	4.3
2 (WN)	430	38	5.8	0.9
3 (SLE)	46	2296	2.9	7.5
4 (SLE)	152	836	3.3	6.6
5 (NEG)	17	41	0.8	0.9
6 (NEG)	36	56	1.2	0.8

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

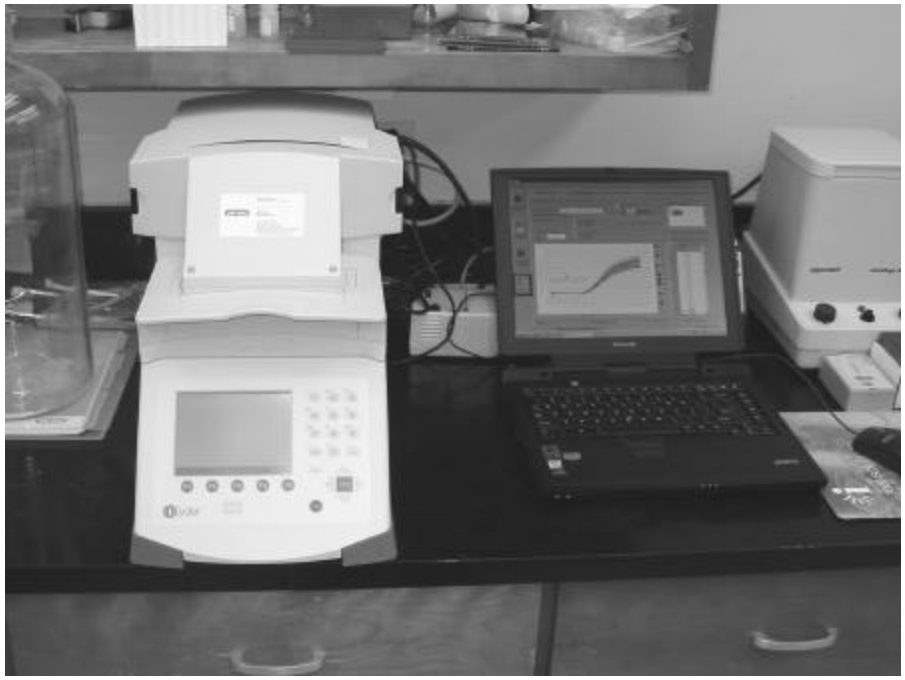
RNA in H_2O

RNA in H_2O

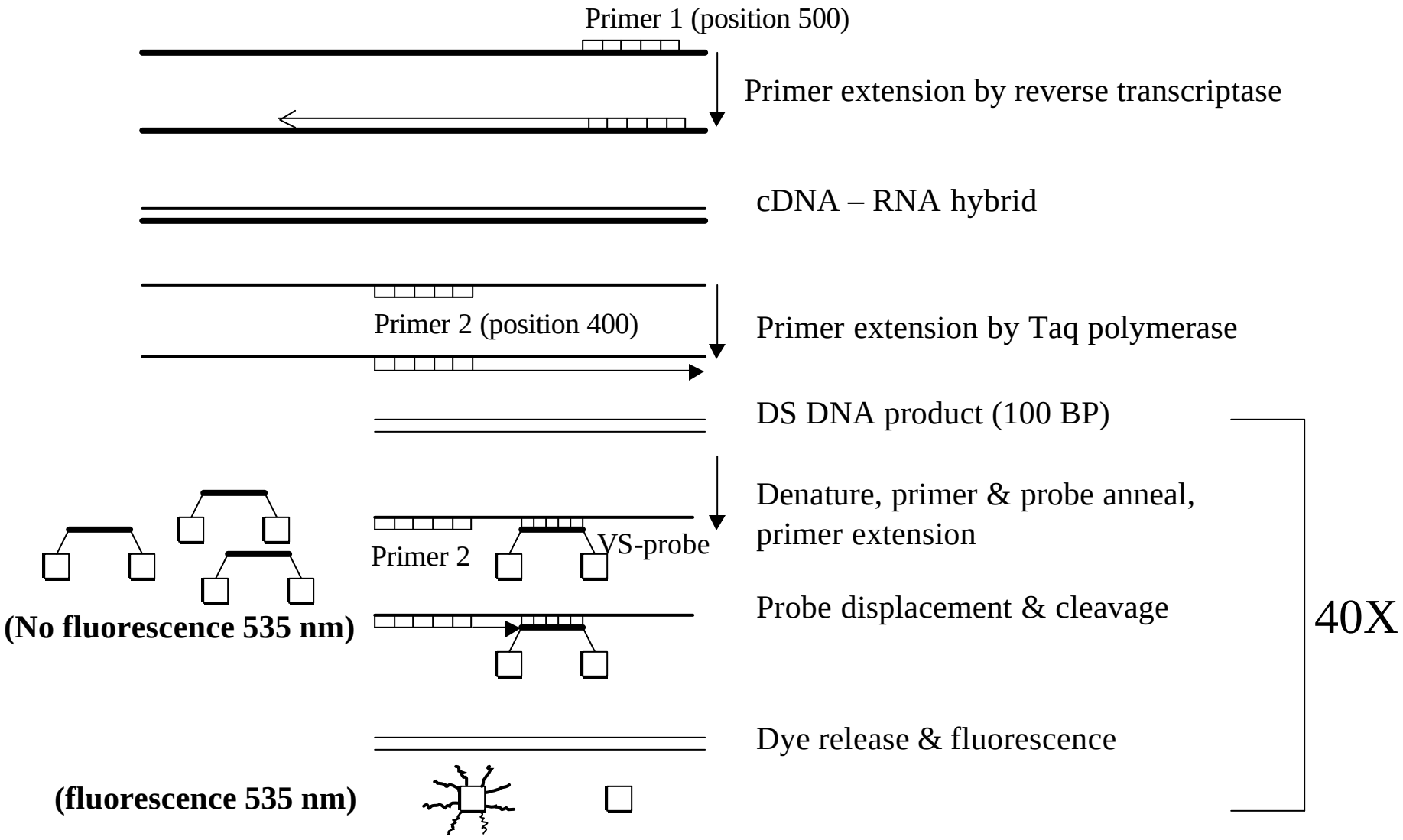
Molecular Amplification Test

CDC TaqMan Testing Algorithm

- ✓ Extract RNA (100 ul to 1 ml or >)
- ✓ TaqMan with ENV primer set + internal control
- ✓ Ct < 38 positive; Ct 38 – 45 equivocal
- ✓ All positives & equivocal are repeated with a second primer set; using newly extracted RNA



TaqMan RT-PCR



WNV TaqMan Detection Limit

Plaque forming units (pfu)

ENV set

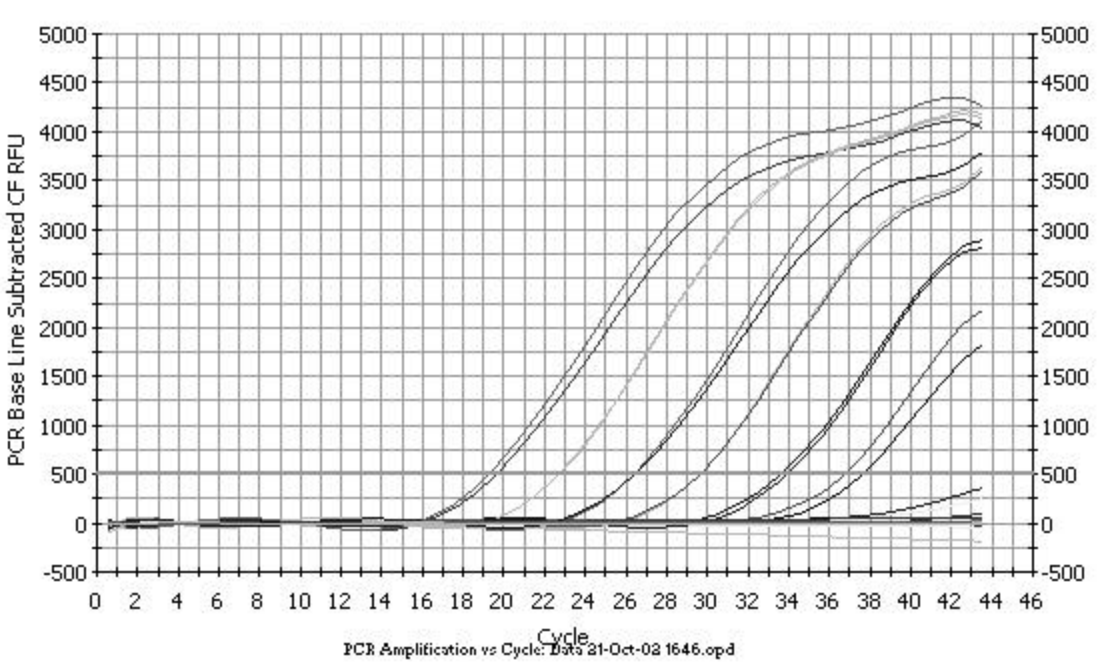
0.10 pfu/ml or
40 copies/ml

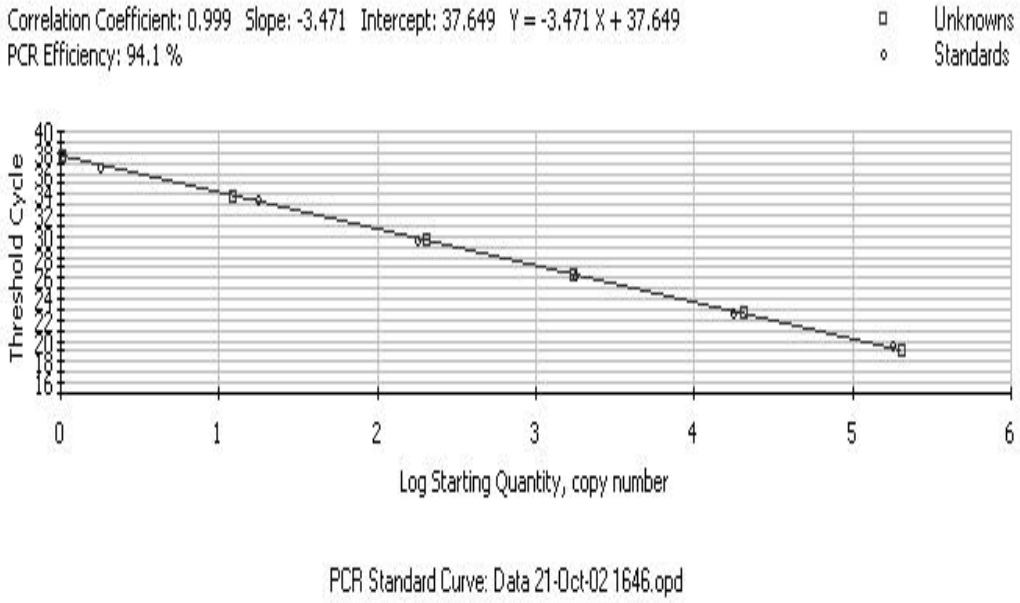
3'NC set

0.4 pfu/ml or
160 copies/ml

NS5 set (Lipken)

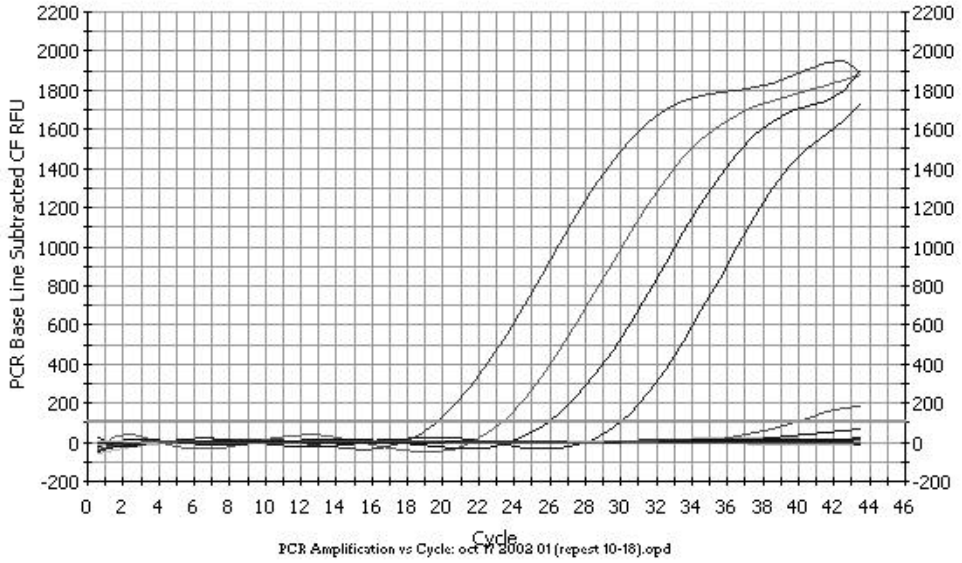
0.2 pfu/ml or
80 copies/ml



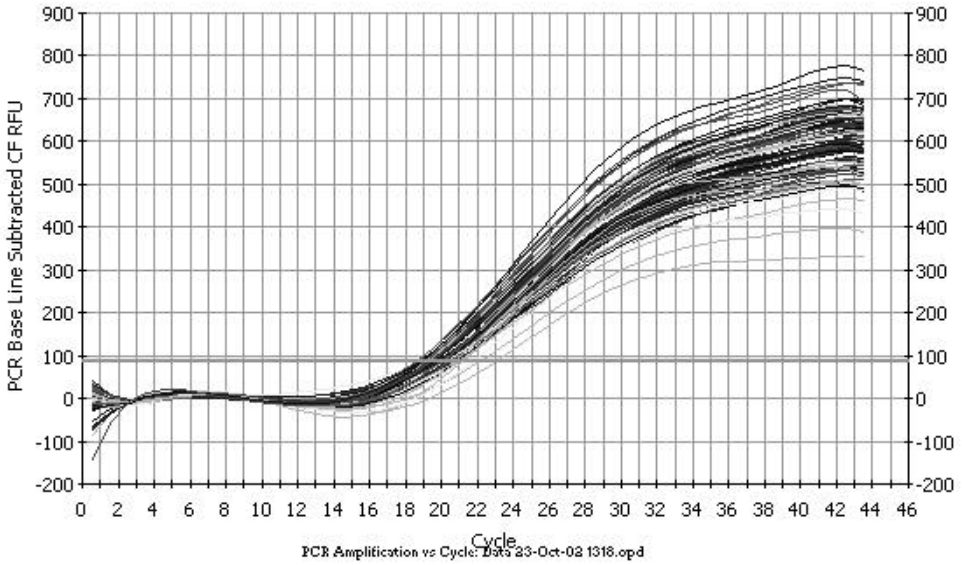


WN Virus TaqMan Assay With HEX-Labeled Internal Positive Control

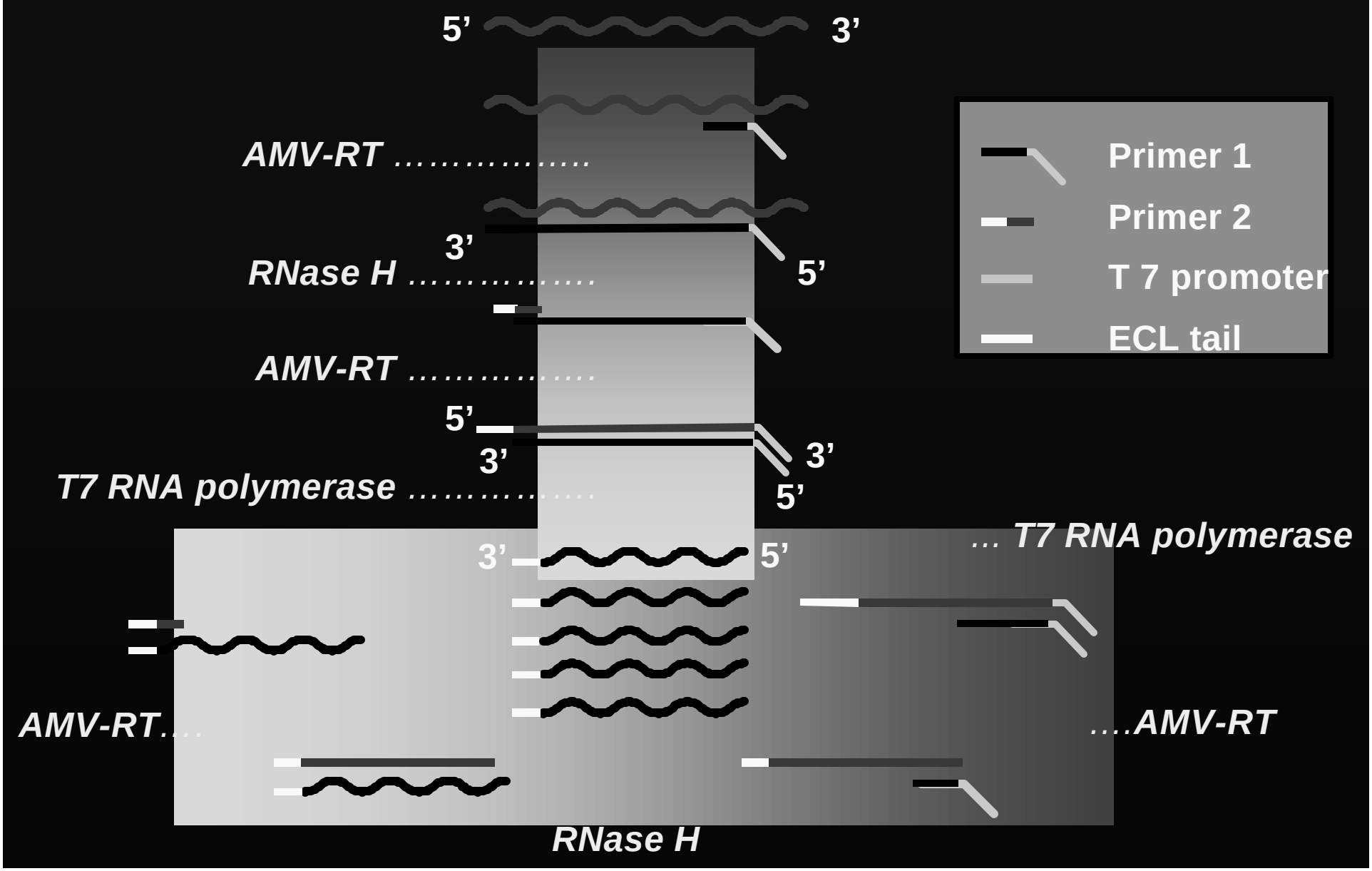
WN virus primer/probe set



HEX internal control primer/probe set



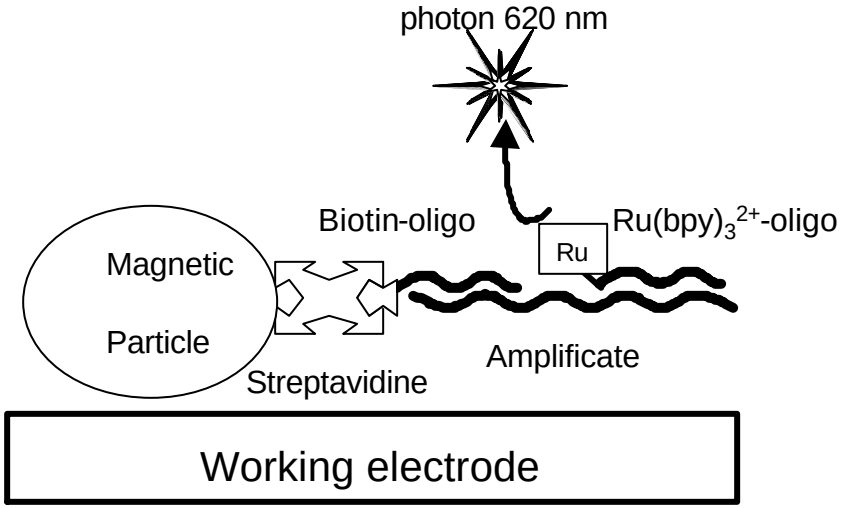
Basic Kit Amplification Principle



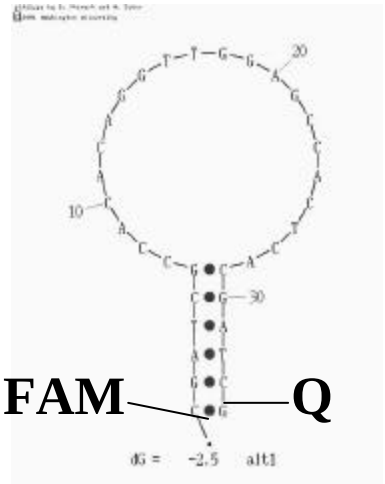
NASBA Detection Formats

SS RNA

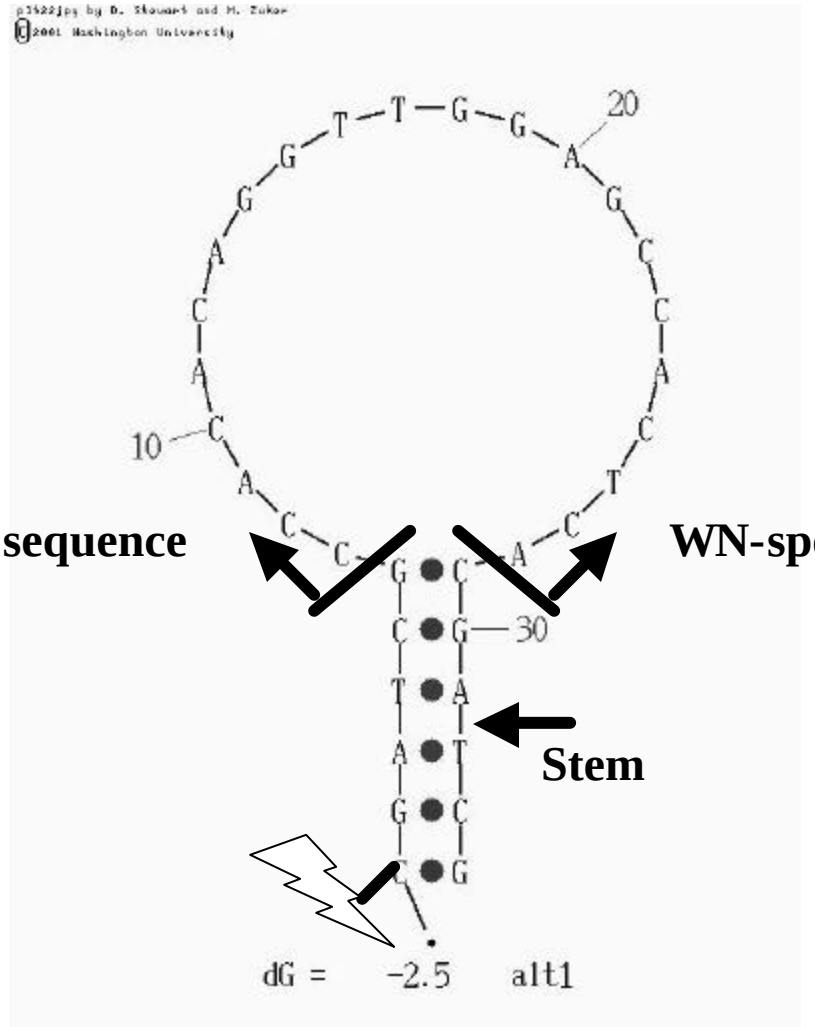
NASBA ECL Reader



NASBA Molecular Beacon



Molecular Beacon Probe for WN Virus NASBA Assay



WN-specific sequence

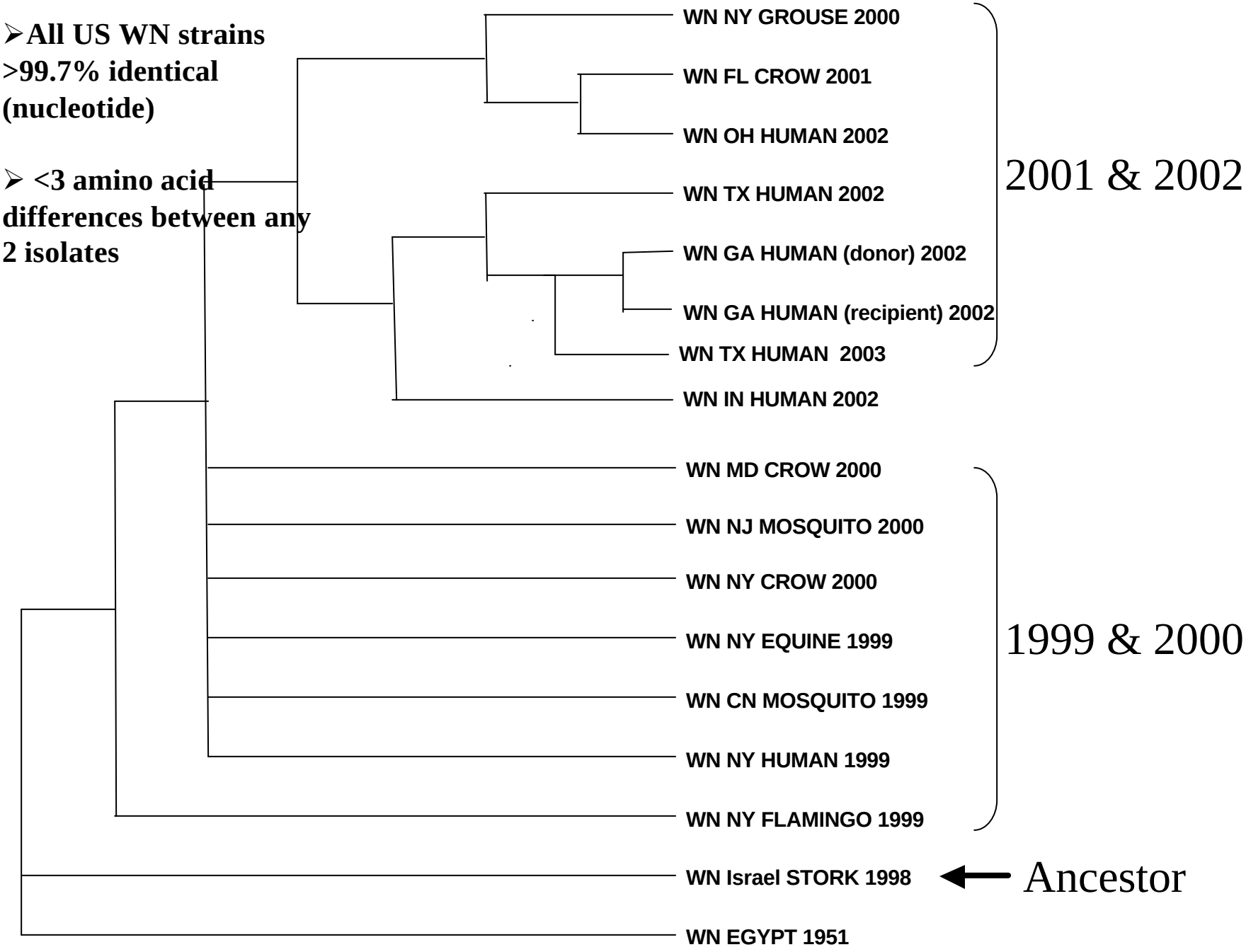
WN-specific sequence

Stem

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

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



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**

West Nile Virus Testing Summary

- Most sensitive virus detection tests are TaqMan (quantitative) & NASBA (TMA)
- 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
- Virus isolation

WN Human Viremia

Data Summary

- **Human viremia is low:**
  - Transfusion studies: 1-130 pfu/ml
  - Average 24 pfu/ml
  - Virus isolation is rare

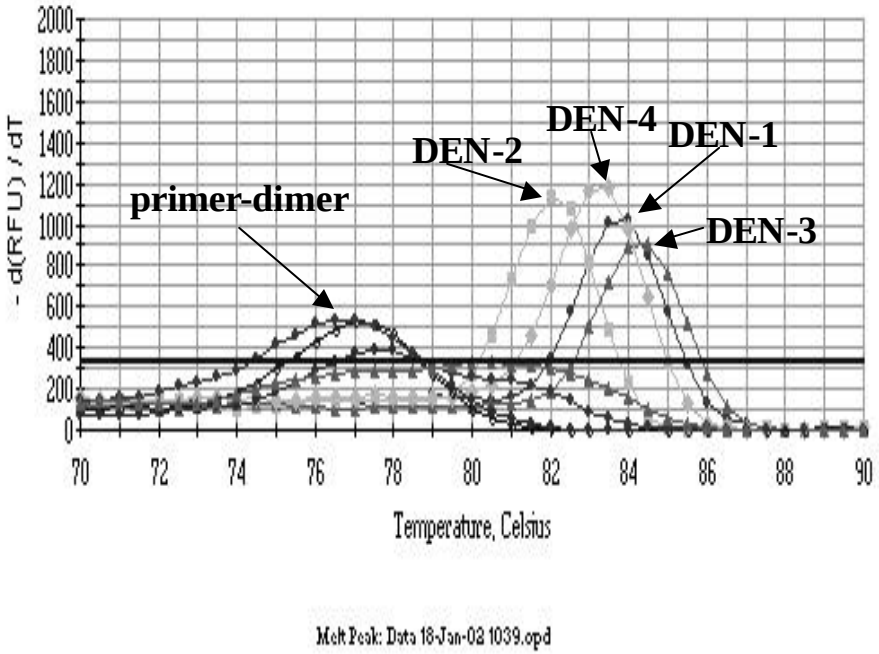
- **Human viremia is short-lived**
  - Not detectable by Day 1 of onset
  - 2 TaqMan Positives/ 100 Acute IgM positives

- **Viremia is absent when IgM is detectable**
  - 2 IgM & TaqMan positives in transfusion studies
  - Israel study
  - 2002 LA Fever Study

Diagnostic & Reference Section

SYBR Green Consensus Assays

- Flavivirus primers (*Chang & Kuno)*
- California & Bunyamwera serogroup (*Kuno)*
- Dengue
- Alphavirus



Special Thanks to the CDC Arbovirus Diagnostic Lab Staff

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Kathy Wolff	**Jane Johnson**	**Barbara Johnson**
Trudy Chambers	**Olga Kosoy**	**Janeen Laven**
Jason Velez	**Brandy Russell**	**Roseyln Hochbein**