

# 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

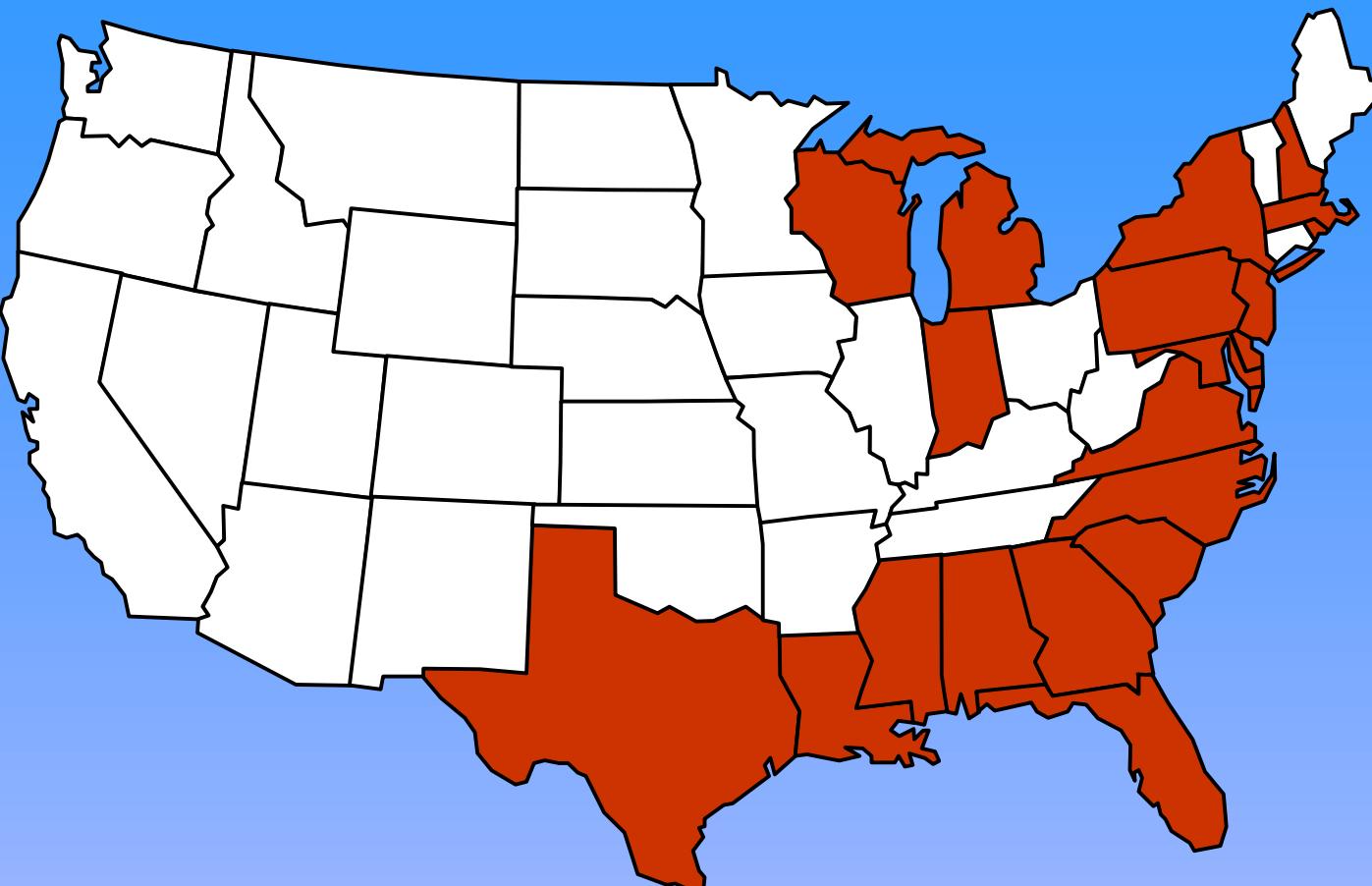
Family/Genus	Pathogens
Togaviridae/ <b>Alphavirus</b>	<b>Eastern equine encephalitis</b>
ss + RNA +; 70 nm particle	<b>Western equine encephalitis</b>
	<b>Venezuelan equine encephalitis</b>
Flaviviridae/ <b>Flavivirus</b>	<b>St. Louis encephalitis</b>
ss + RNA; 40-60 nm particle	<b>Powassan</b>
	<b>West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

# Medically Important Arboviruses in the United States

Pathogen	Vector	Host	Cases (1964-2002)	Human Disease
<b>EEE</b>	Mosquito	Avian	191	Encephalitis; CF-50% (children)
<b>WEE</b>	Mosquito	Avian	640	Encephalitis; CF-< 5% (children)
<b>SLE</b>	Mosquito	Avian	4561	Encephalitis; CF-10% (elderly)
<b>WN</b>	Mosquito	Avian	2870 (>4000)	Encephalitis; CF-10% (elderly)
<b>LAC</b>	Mosquito	Small mammals	2910	Encephalitis; CF < 1% (children)

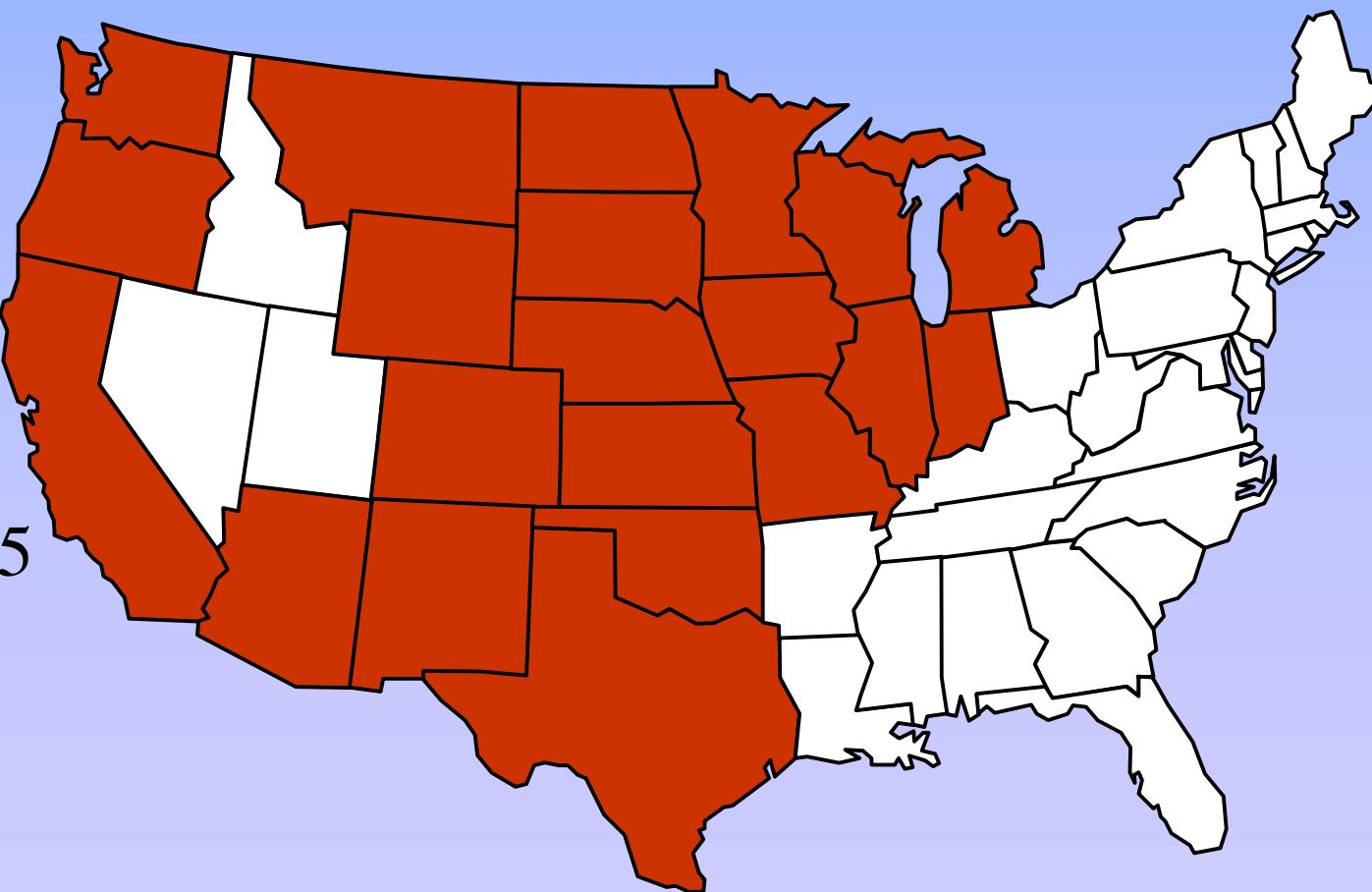
## **Eastern Equine Encephalitis Human cases: 1964-2002**

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



## **Western Equine Encephalitis Human cases: 1964-2002**

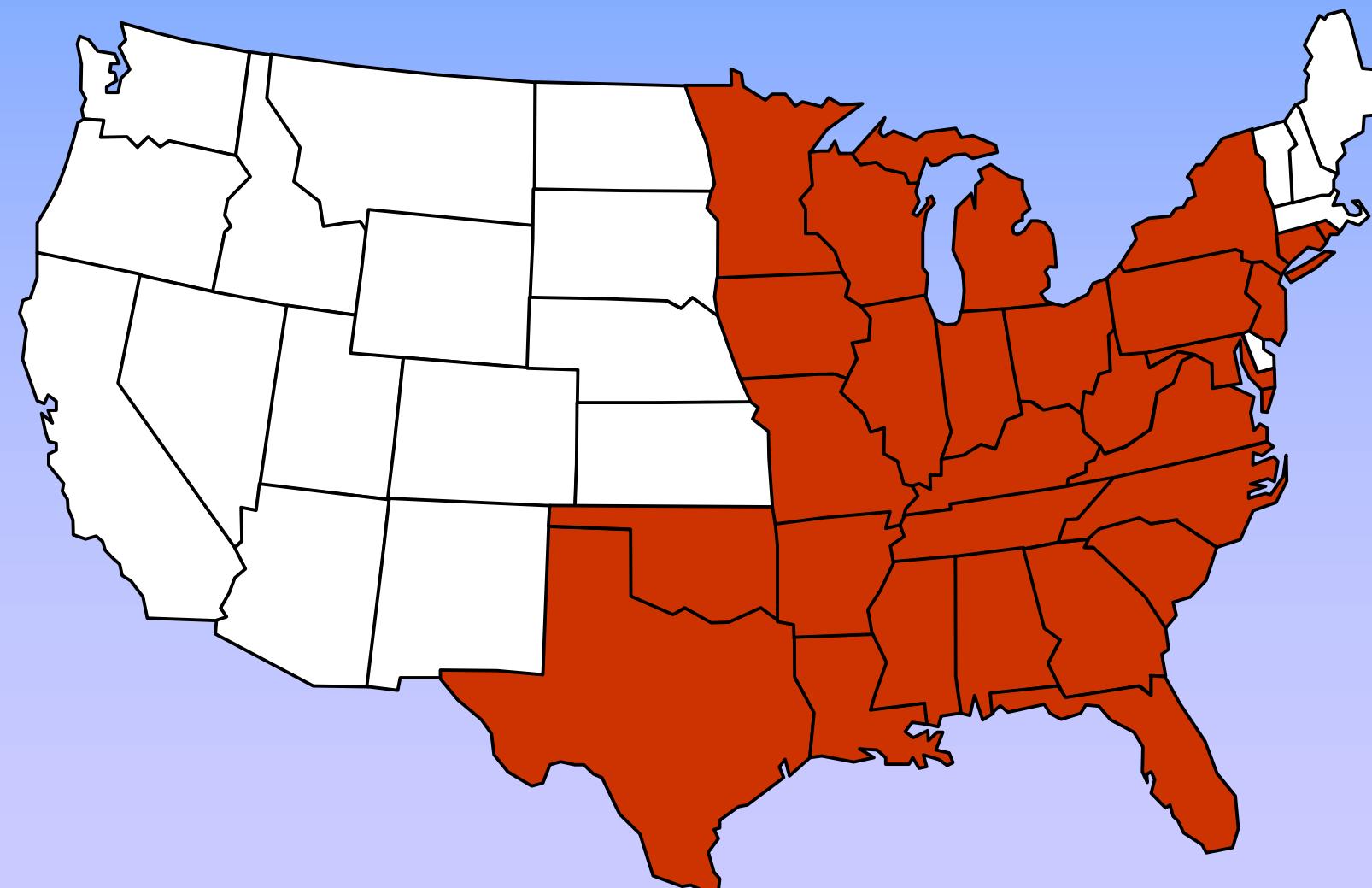
- 640 cases
- some epidemic years
- 65% cases 1964-66 & 1975
- 8 cases/year non epidemic
- 4 cases since 1990



# **La Crosse Virus Encephalitis**

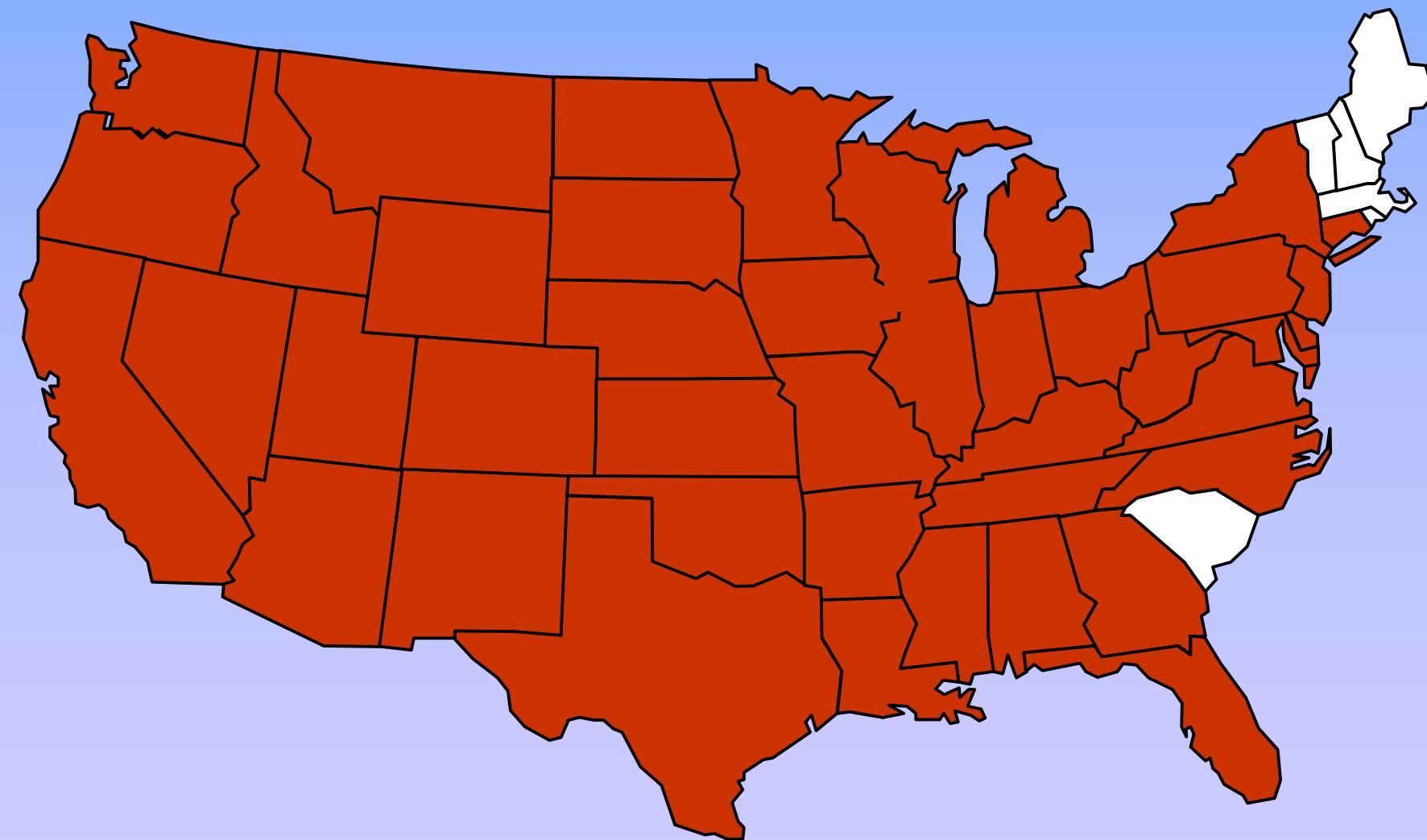
## **Human cases: 1964-2002**

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

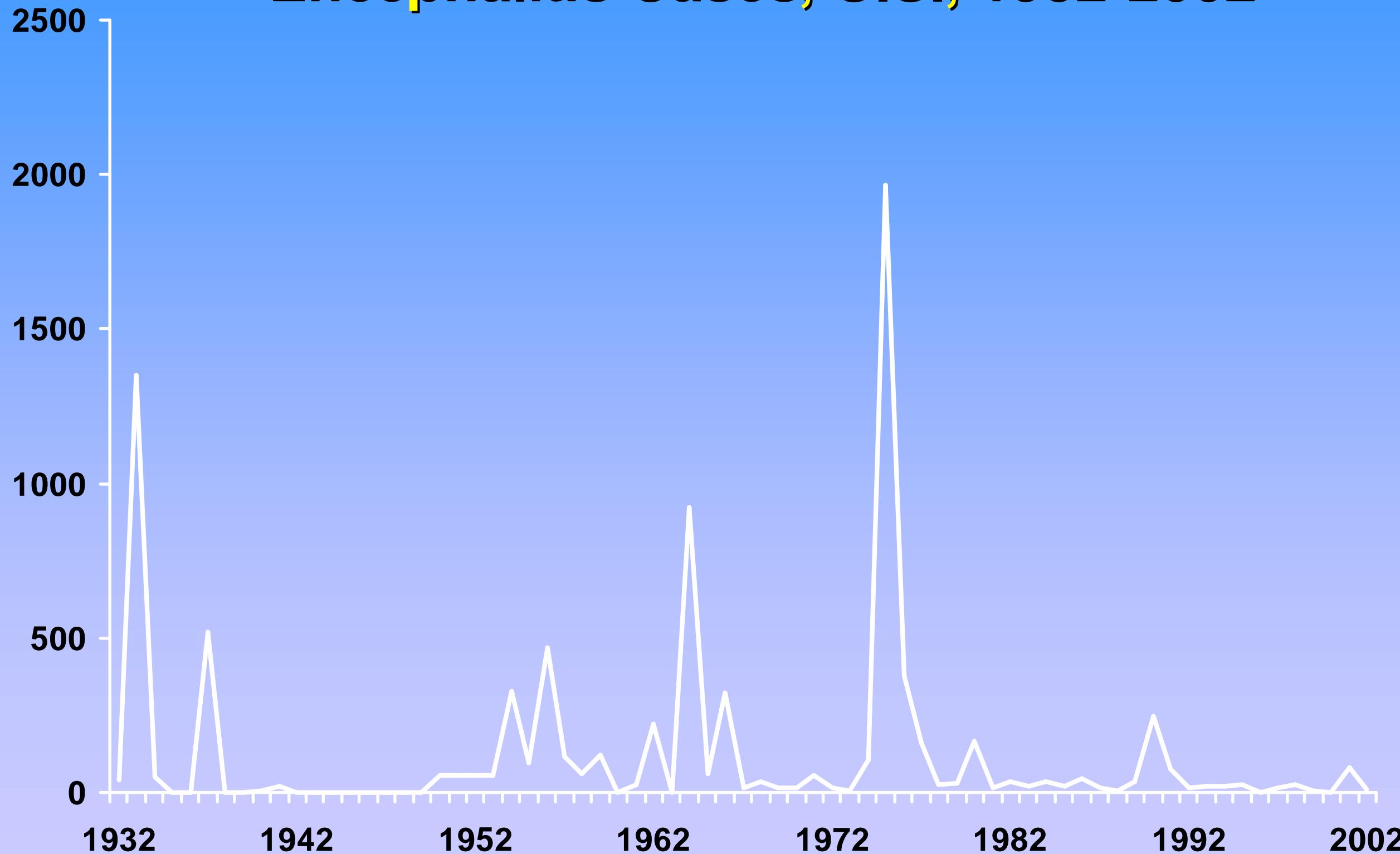


# St. Louis Encephalitis Human cases: 1964-2002

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

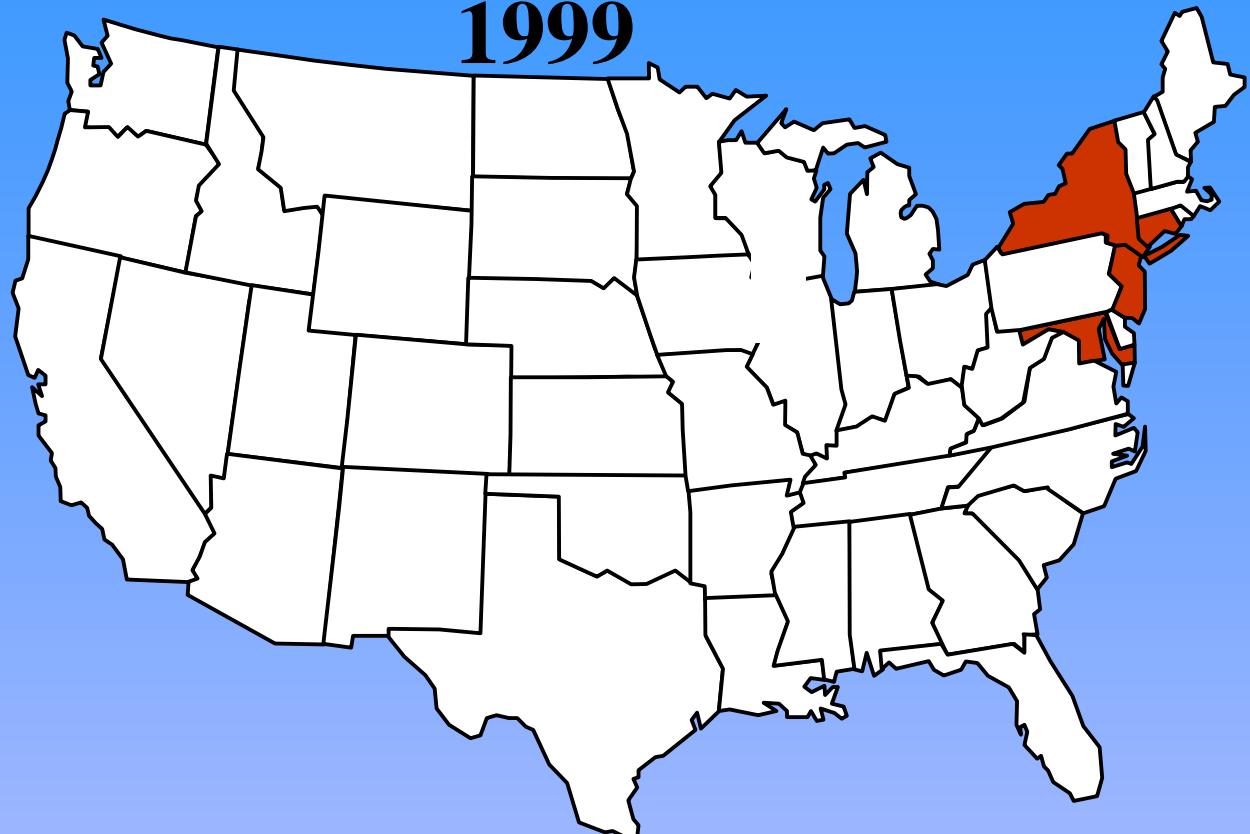


# **Reported / Estimated Number of St. Louis Encephalitis Cases, U.S., 1932-2002**

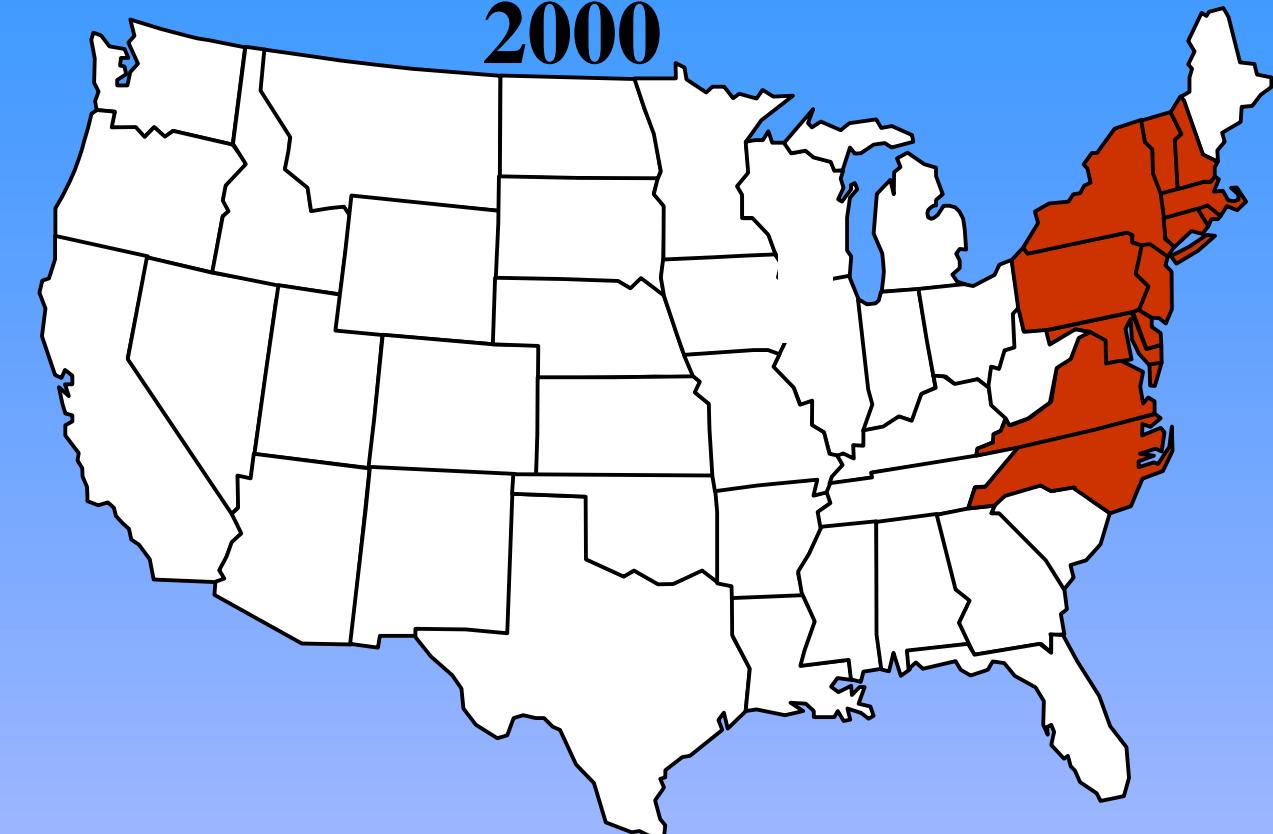


# Spread of WN Virus in the US

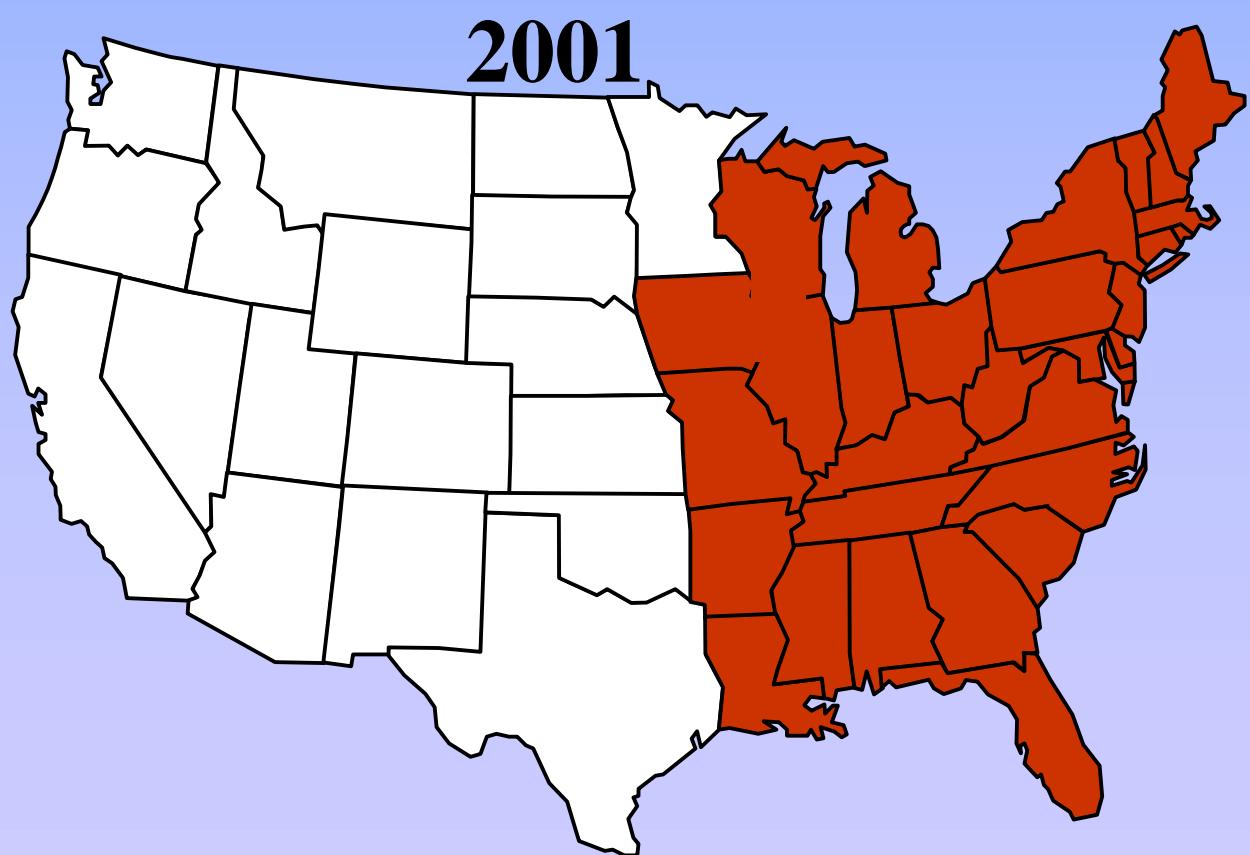
1999



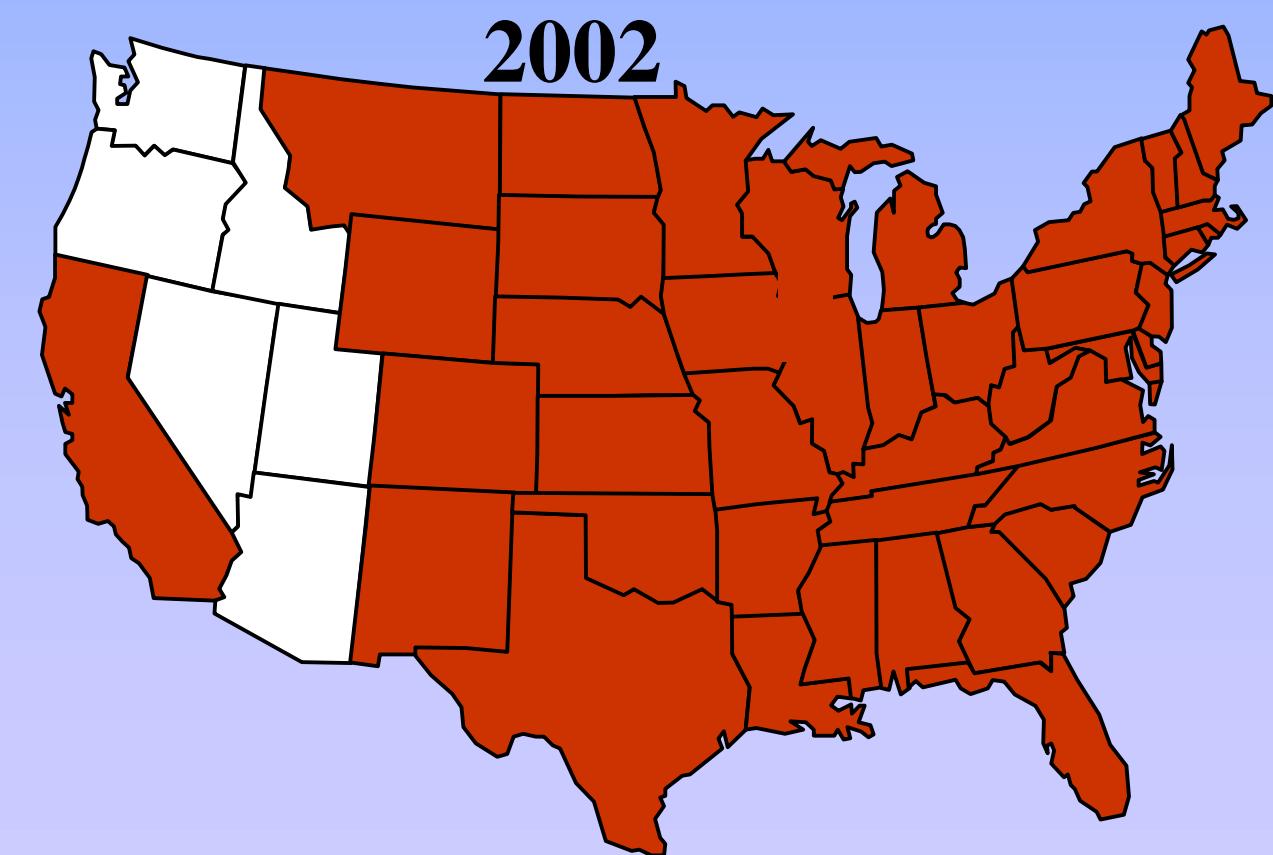
2000



2001



2002



# CDC Tests for WN Virus

Specimen	1 <sup>st</sup> Choice	Other	Comments
<b>Human serum/CSF</b>	IgM ELISA Plaque Reduction Neutralization	TaqMan/NASBA Virus Isolation	TaqMan (57%) for acute CSF; <10% serum
<b>Human tissue</b>	TaqMan/NASBA	Virus Isolation	Fatal WN cases: TaqMan/NASBA positive ~ 100%
<b>Donated blood products</b>	TaqMan/NASBA		
<b>Non-Human</b>			
<b>Avian tissue</b>	TaqMan/NASBA Virus isolation	VecTest/ Ag. Cap. ELISA/RT-PCR	Ag.-based tests require 1000 pfu
<b>Mosquito pool</b>	TaqMan/NASBA Virus isolation	VecTest/Ag. Cap. ELISA/RT-PCR	

# Serological Testing Algorithm for West Nile Virus

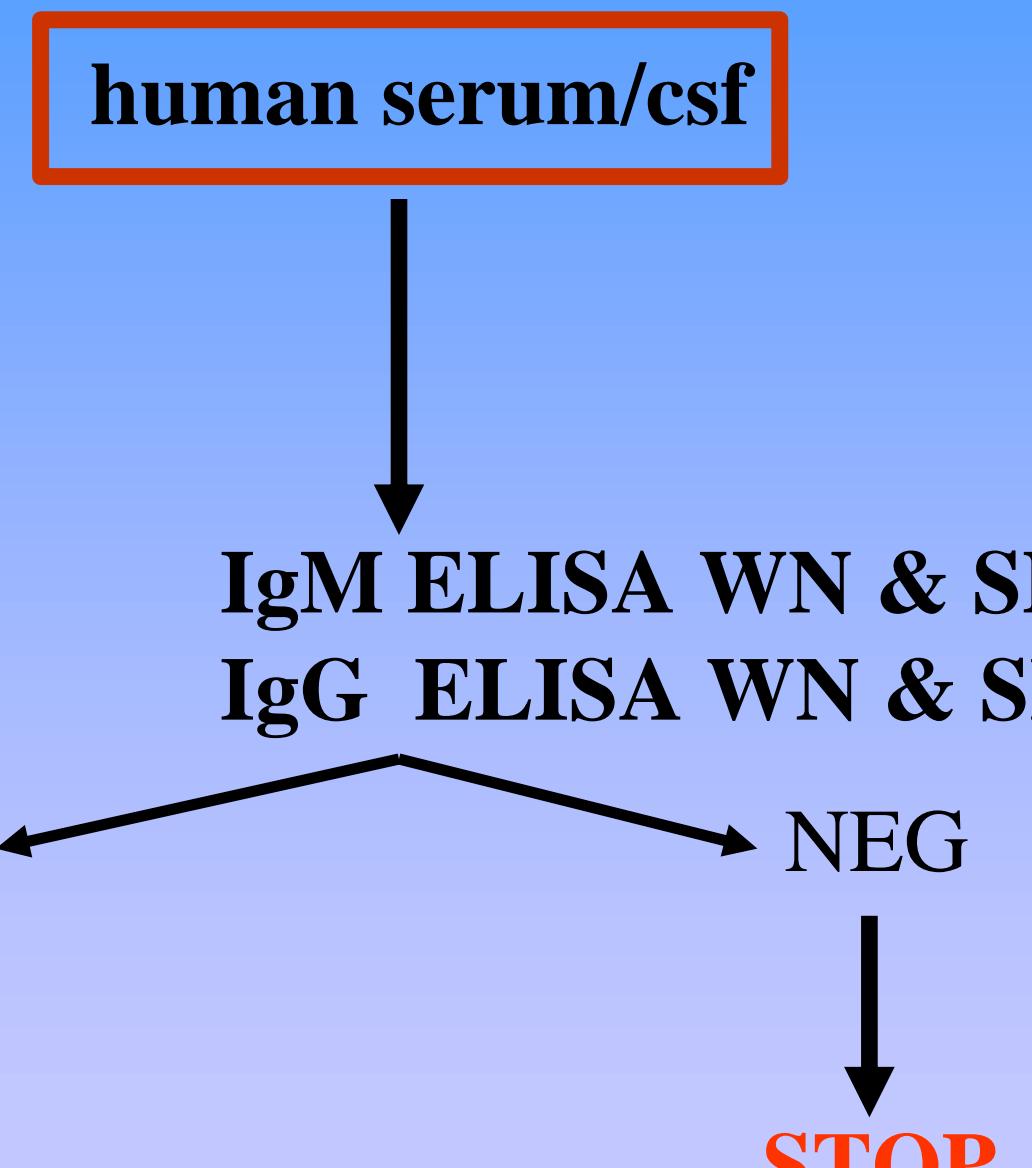
## National Case Definition

### Confirmed:

*IgM pos csf*

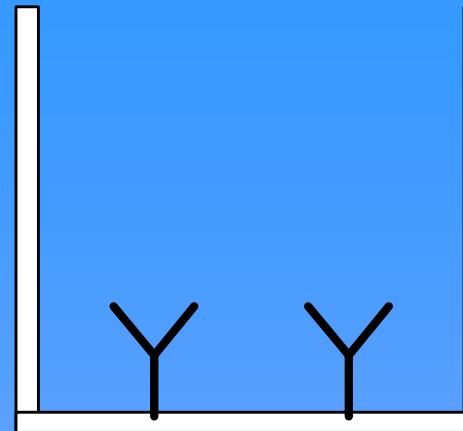
*IgM pos serum + PRNT*

*>4-fold increase PRNT titer*

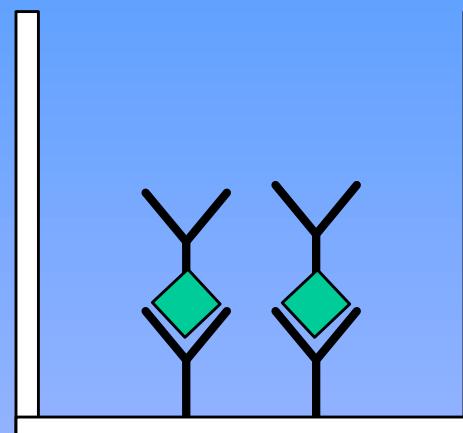


Plaque reduction  
Neutralization test (PRNT) with:  
SLE, WN, (other flaviviruses)

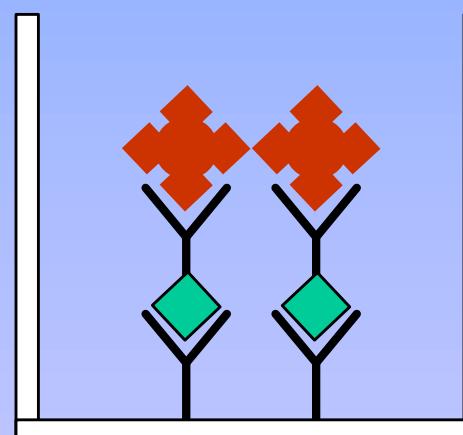
# IgM Capture ELISA



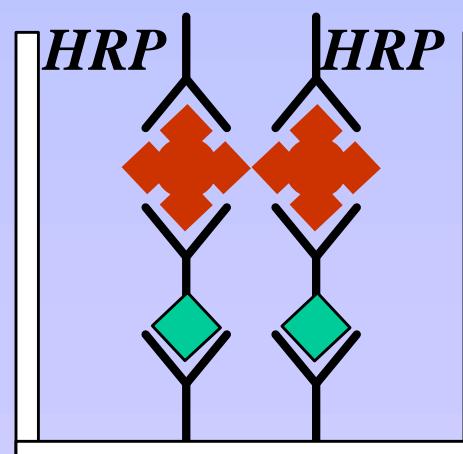
1. Coat With Goat anti-Human IgM
  - 4° Overnight



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

	IgM	IgG	PRNT
EEE	0.92	1.88	NEG
SLE	4.42	2.86	1:20
LAC	1.26	1.12	NEG
WN	9.97	4.62	1:160

# WN Serological Data

## *Typical Human WN Case*

Sample	Days post-onset	IgM P/N		IgG P/N		PRNT	
		WN	SLE	WN	SLE	WN	SLE
<u>Typical WN Case</u>							
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 3-5X greater than SLE.

➤ 2002 data: Use 2X criteria WN to SLE ratio: only 1 exception in 417 WN confirmed cases.

# WN Serological Data

Sample	Days post-onset	IgM P/N		IgG P/N		PRNT	
		WN	SLE	WN	SLE	WN	SLE
<b><u>Typical WN Case</u></b>							
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
<b><u>Secondary flavivirus infection</u></b>							
acute serum	0	1.40	1.42	4.27	2.62	1:40	1:40
conv. serum	10	6.10	2.20	22.90	ND	1:5120	1:2560

# 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-	21	9 (43)	2	11 (52)	4.0
400					(31.-6.5)
500	12	5 (42)	2	6 (60)	5.0 (3.1-6.9)



# WN Human Serological Data

## *Lessons Learned 1999-2002*

- IgM Detectable in serum & csf by onset (99%)
  - 6 exceptions serum of 800 – 1999 - 2002 cases
  - 10 exceptions csf of 800 - 1999 - 2002 cases
- IgG Positive by day 7 Post-Onset
- P/N 3-5X Higher to WN than SLE
- IgM Persistence > 1 Year
- Secondary Flavivirus Infections are Problematic
  - Low IgM; high PRNT to several flaviviruses

# **CDC IgM ELISA Assay**

## **Good Points**

- Sensitive
- Specific (WN & SLE P/N ratio)
- IgM Positive in csf & serum at onset

## **Bad Points**

- Cross-reactivity among flaviviruses
- Limited utility in secondary infections
- Two day test
- IgM persistence

# 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



# Molecular Amplification Assays

## 1. *RNA Extraction*

RNA extraction from:  
serum, csf, tissue, & mosquito pools

## 2. *Amplification*

**Standard  
RT-PCR**

Agarose gel

**TaqMan  
RT-PCR**

TaqMan probe

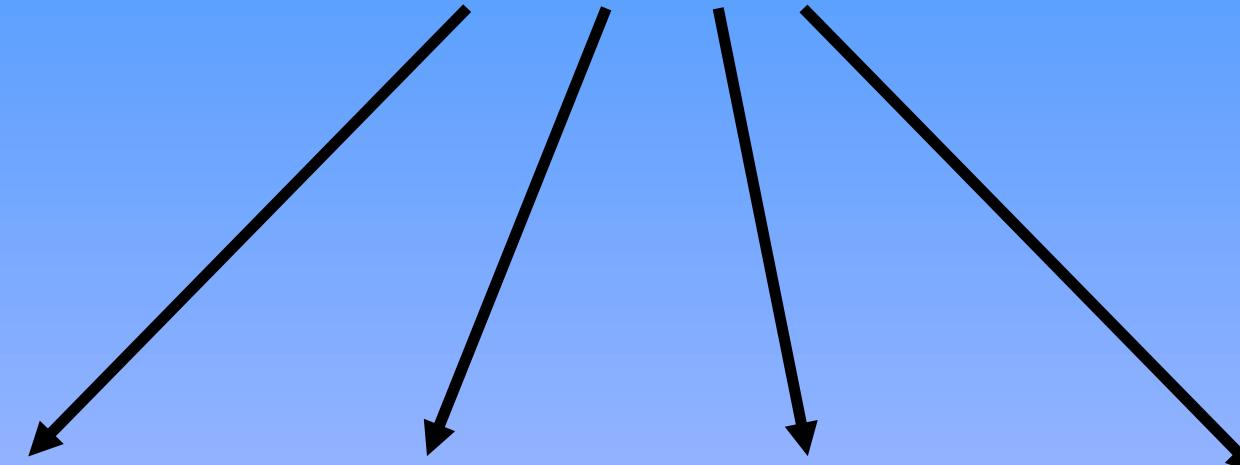
**SYBR Green  
RT-PCR**

melting curve  
analysis

**NASBA**

NucliSens™  
Reader/ECL  
Molecular  
beacons

## 3. *Detection*



# 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



# RNA Extraction & Purification

- **Chemical/Phase Separation**

- guanidine isothiocyanate, phenol/chloroform, ethanol precipitation. (Home-made; TRIzol)
- 40 samples per day

- **Silica-gel Kits**

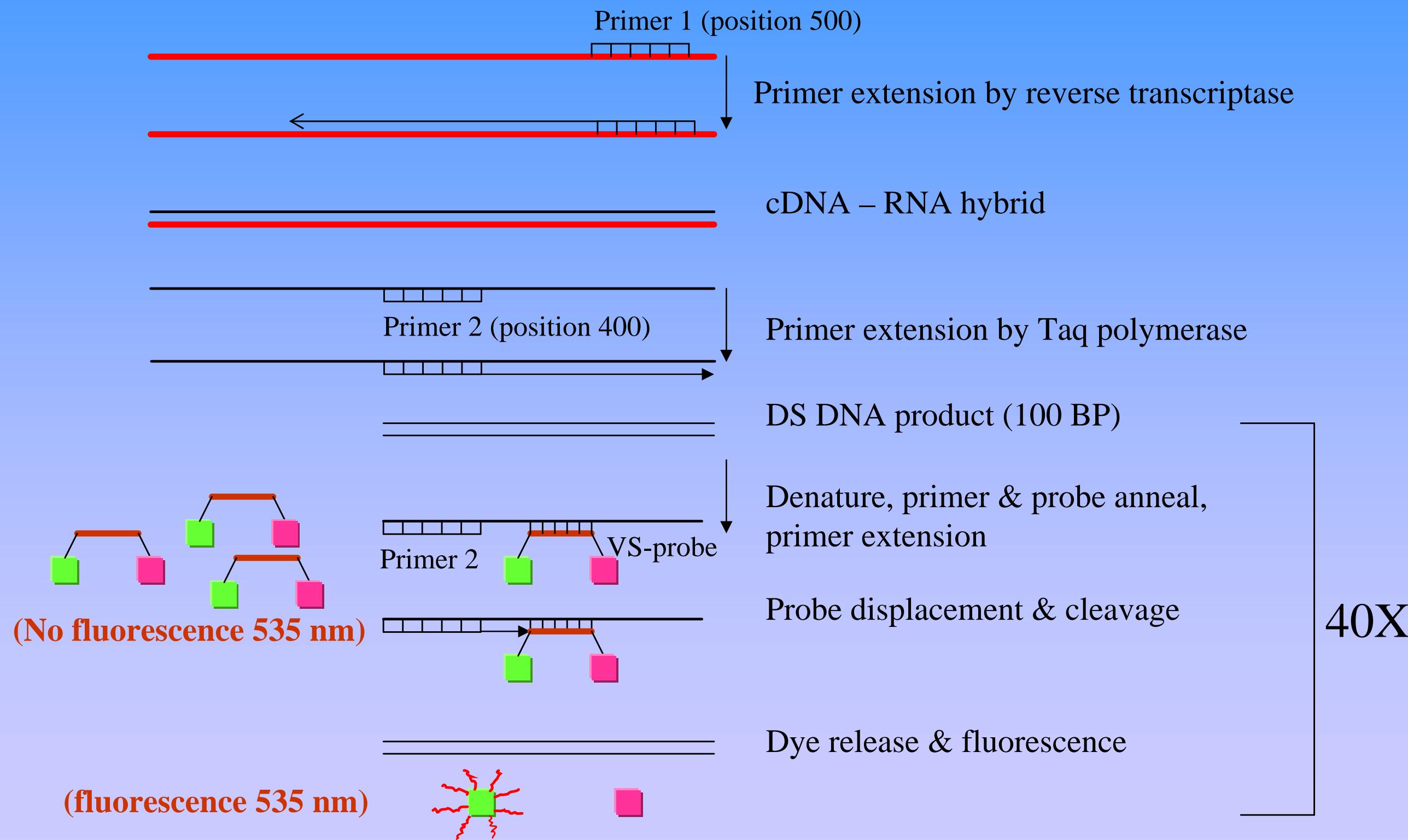
- column (QIAGEN)
- beads (Nuclisens, Bio-101)
- 80 samples per day

- **Robotics QIAGEN 9604**

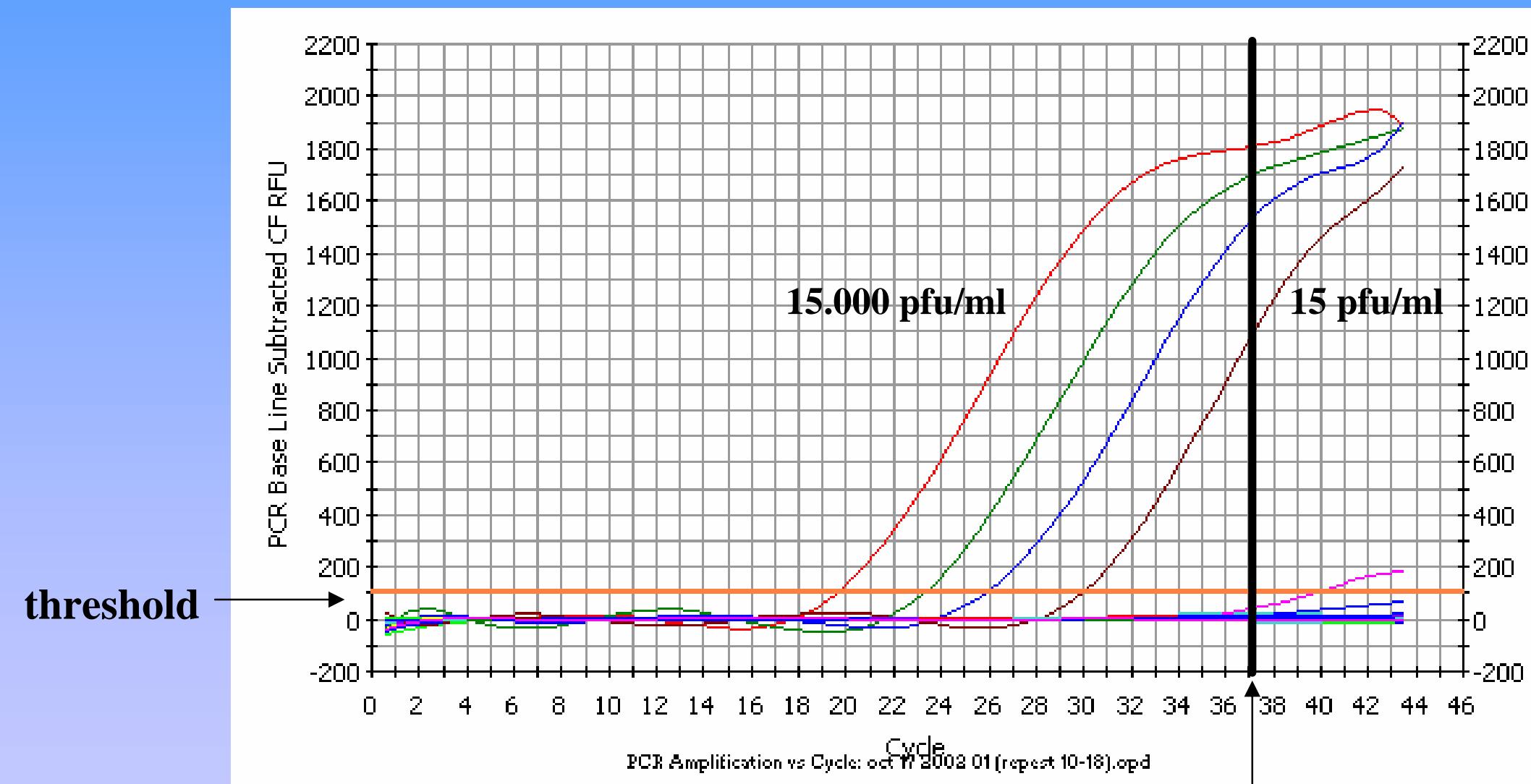
- 300 samples per day



# TaqMan RT-PCR



# TaqMan RT-PCR of West Nile Virus Dilutions



Ct = 37

# Detection Limit

Plaque forming units (pfu)

C<sub>t</sub> = 37

ENV set

**0.80 pfu/ml (100 ul)**

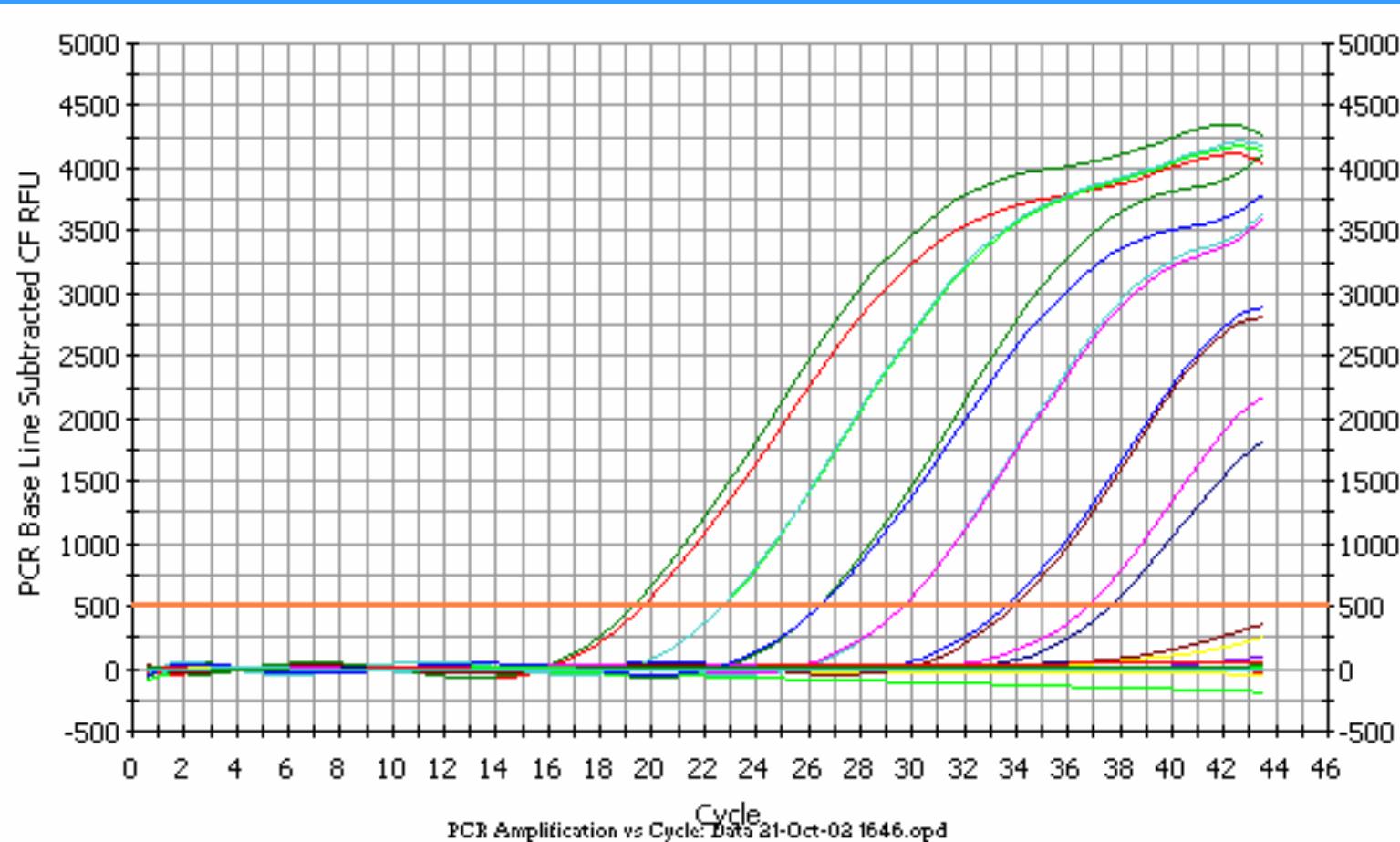
**0.10 pfu/ml (500 ul)**

3'NC set

**3.2 pfu/ml**

NS5 set (Lipken)

**1.2 pfu/ml**

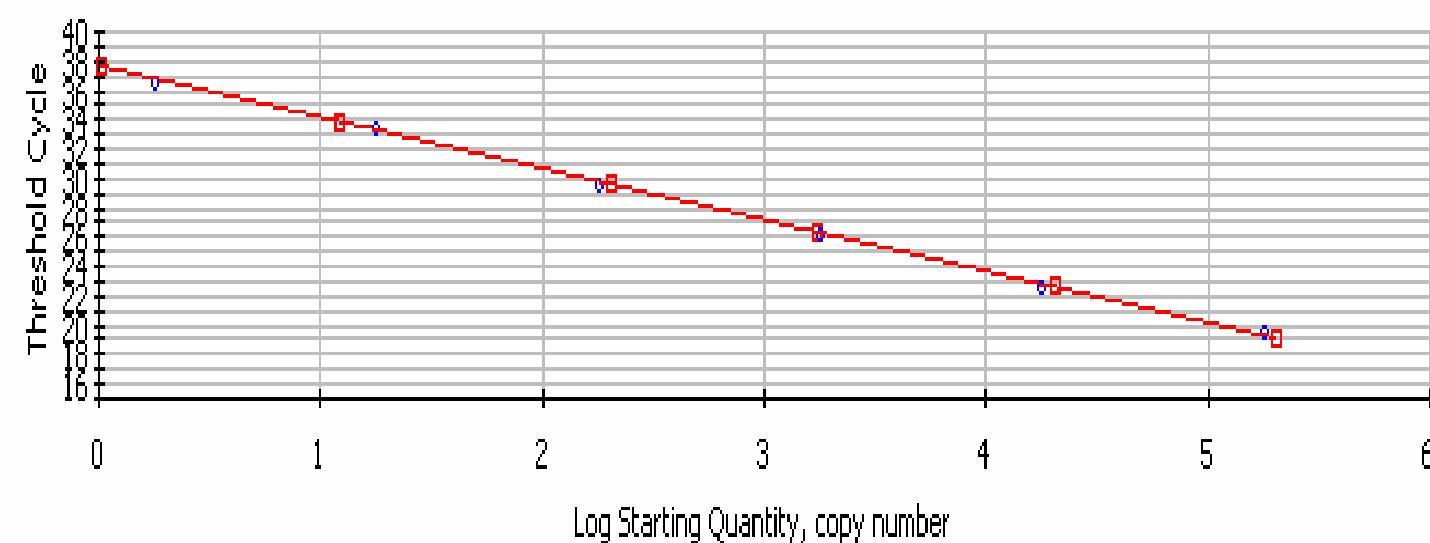


Correlation Coefficient: 0.999 Slope: -3.471 Intercept: 37.649 Y = -3.471 X + 37.649

PCR Efficiency: 94.1 %

□ Unknowns

○ Standards



# WN Virus TaqMan Assay Detection Limit

## Plasmid

1,000 copies/ml

**5 copies**

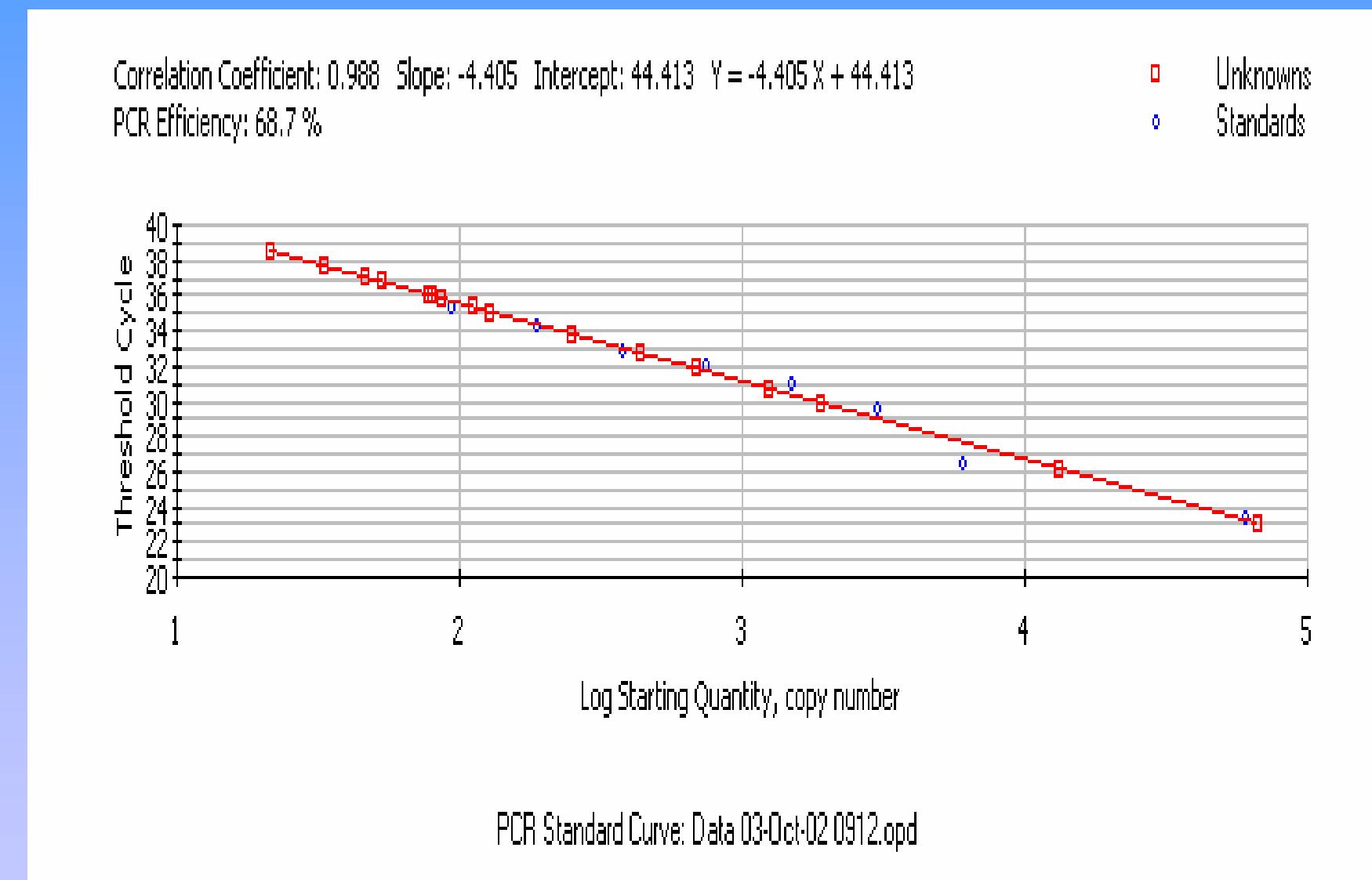
## DS DNA

2,400 copies/ml

**12 copies**

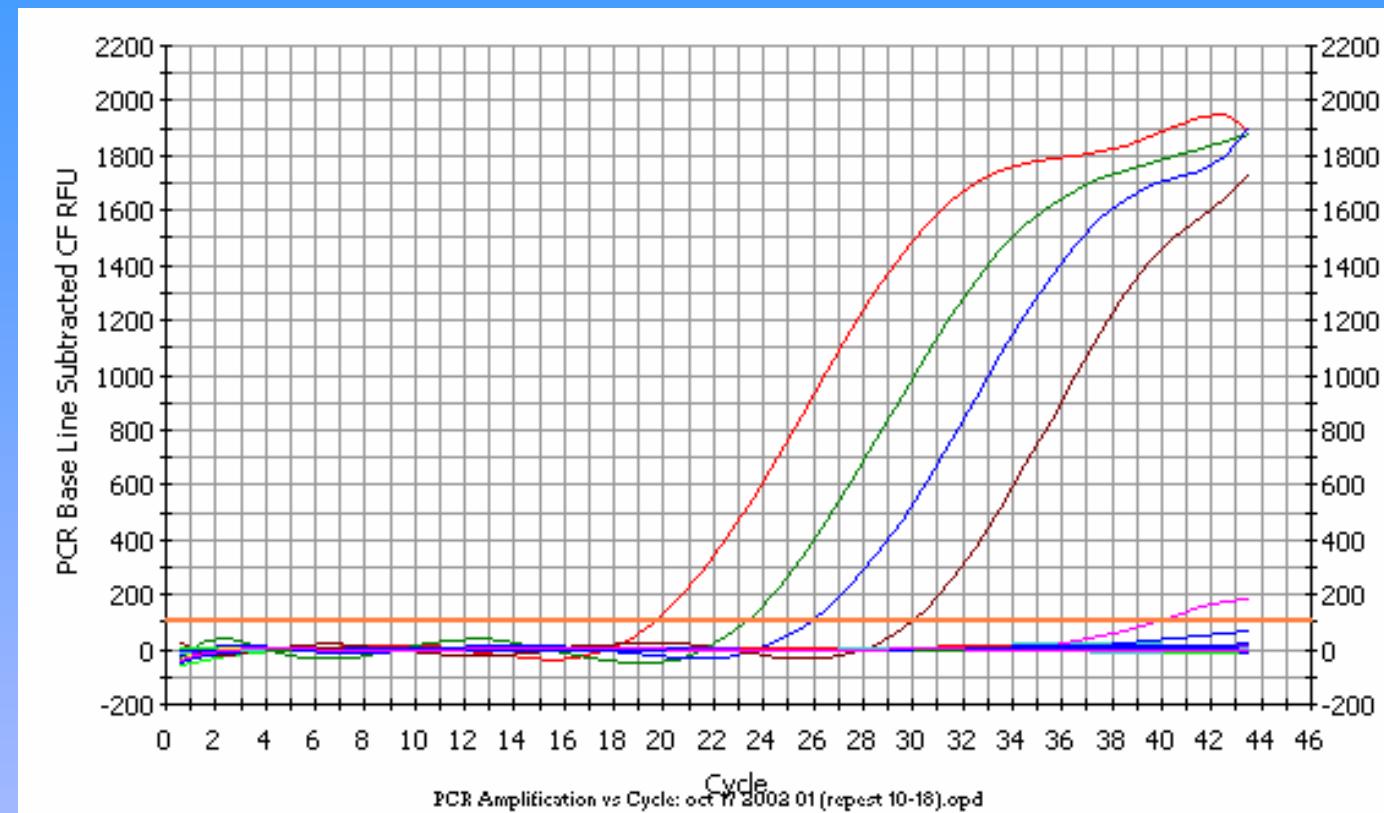
## RNA (Kramer)

**37 copies**

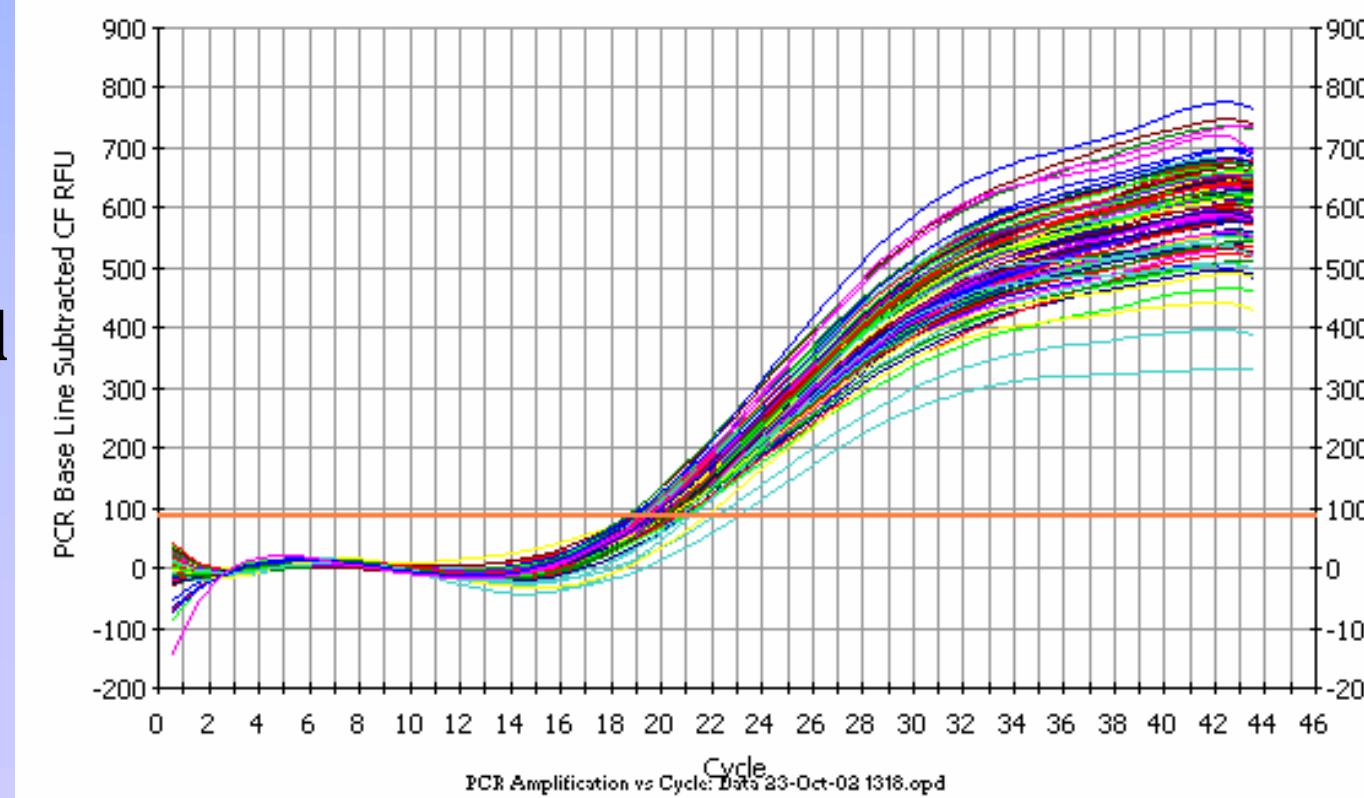


# WN Virus TaqMan Assay With JOE-Labeled Internal Positive Control

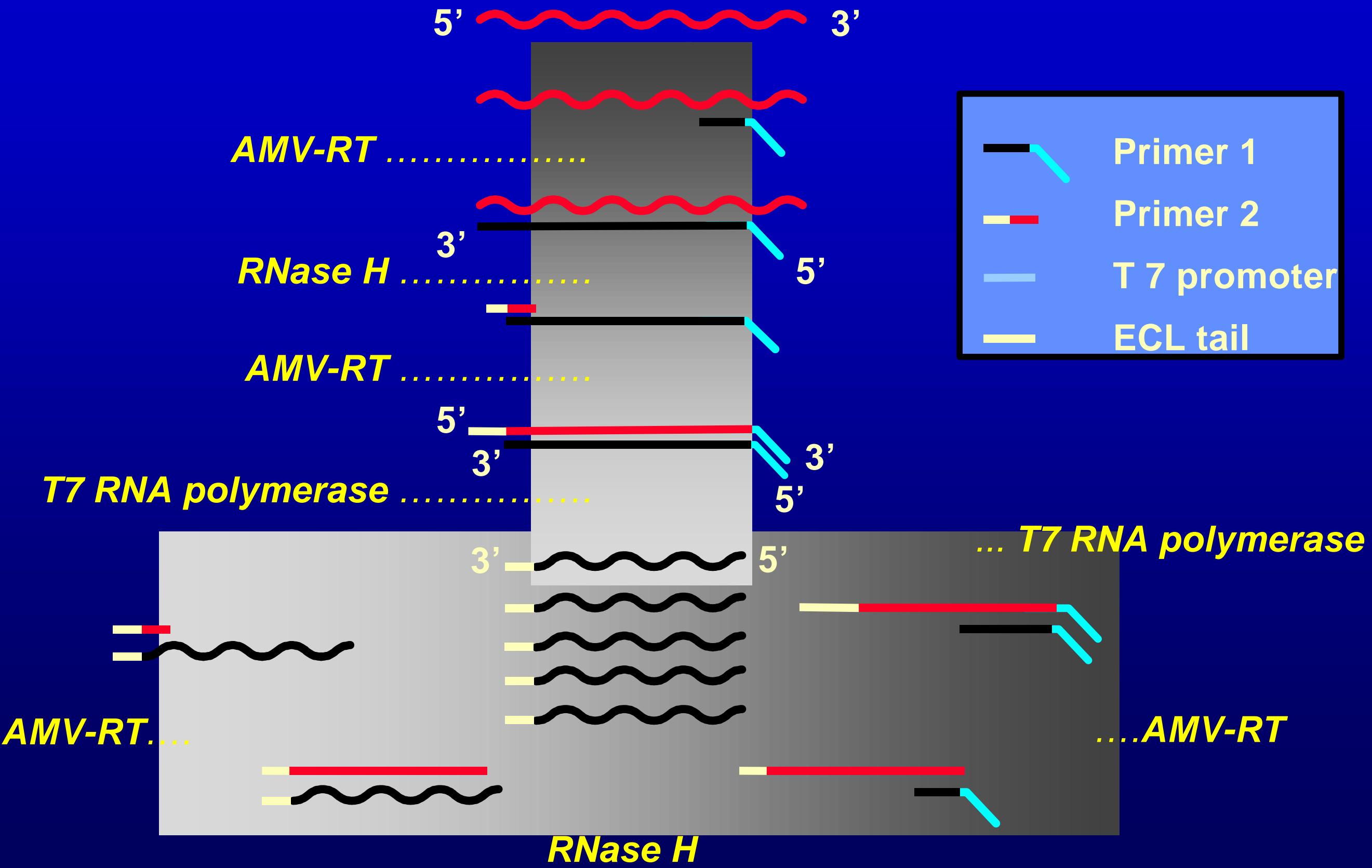
WN virus  
primer/probe set



HEX internal control  
primer/probe set

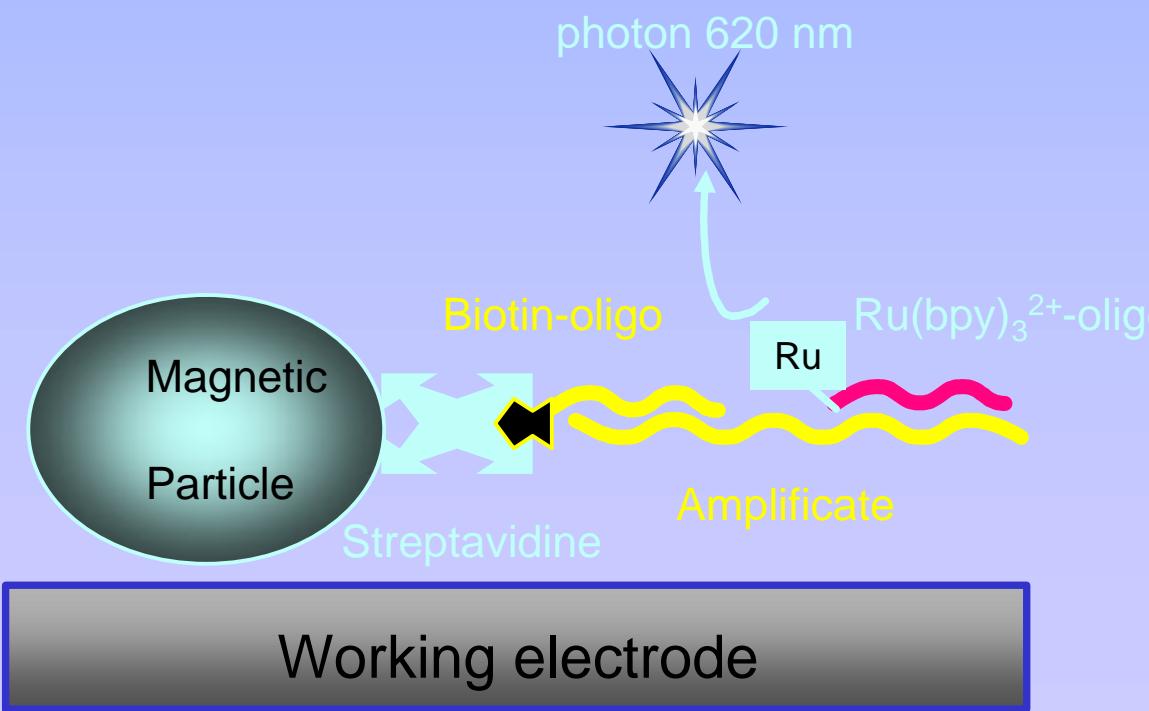


# Basic Kit Amplification Principle

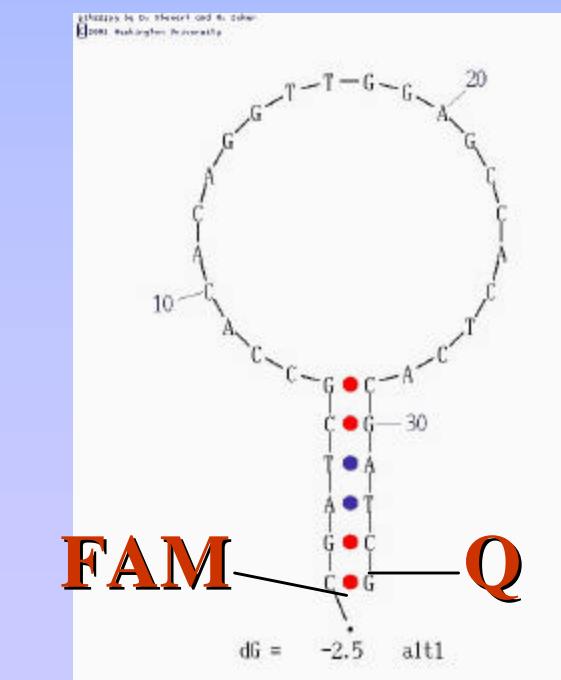


# NASBA Detection Formats

NASBA ECL Reader

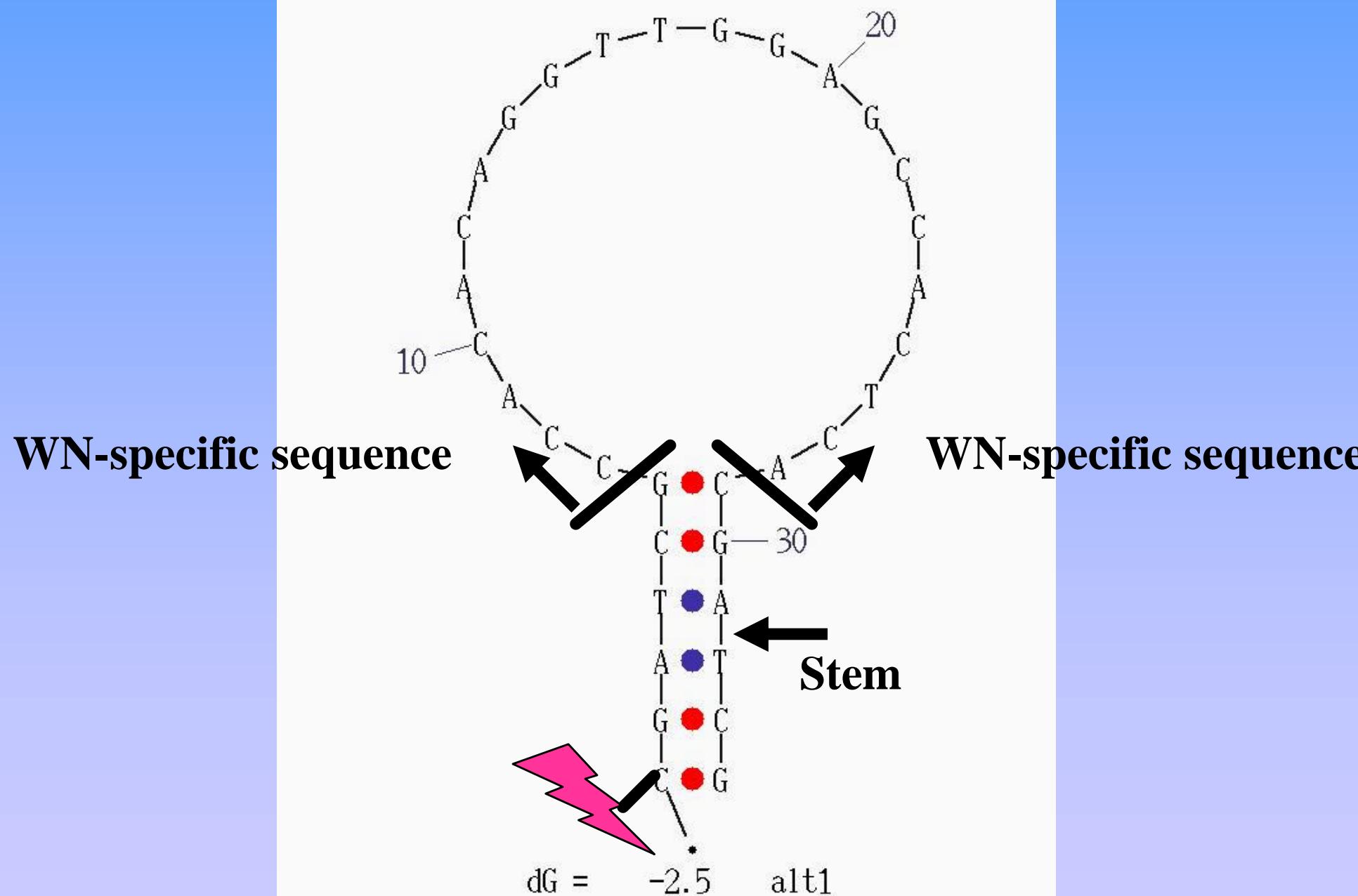


NASBA Molecular Beacon



# Molecular Beacon Probe for WN Virus NASBA Assay

plt22.jpg by D. Stewart and M. Zuker  
©2001 Washington University

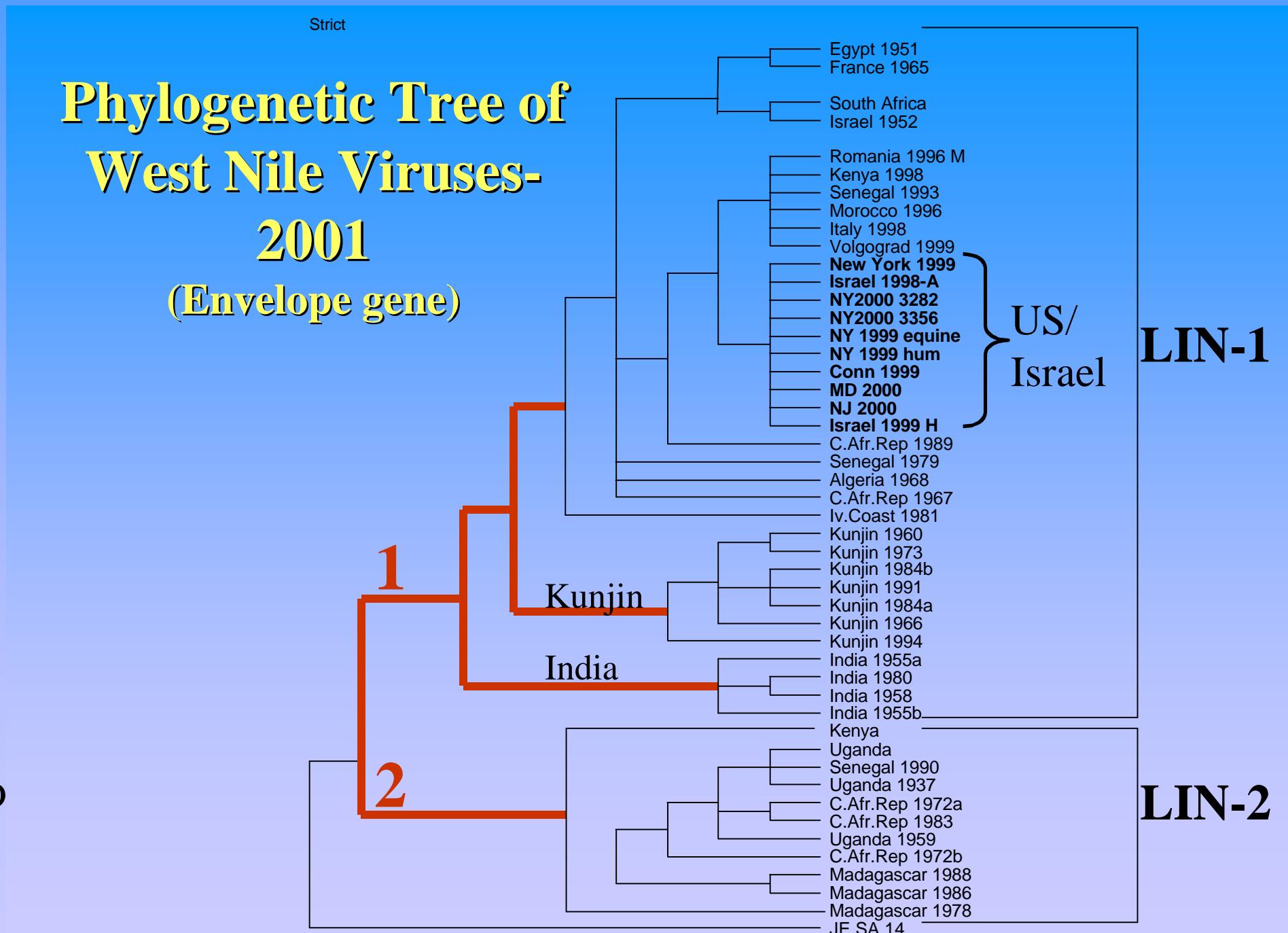


# Sensitivity of WN Virus NASBA & TaqMan Assays

#pfu/ml	TaqMan		NASBA		NASBA	
	Ct	Interp.	ECL	Interp.	MB	Interp
1,000,000	17.88	pos	1653417	pos	9.44	pos
100,000	20.9	pos	1187613	pos	12.01	pos
10,000	24.17	pos	1810790	pos	12.27	pos
1,000	27.75	pos	1666084	pos	14.81	pos
100	31.21	pos	1211426	pos	19.21	pos
10	34.07	pos	1209491	pos	21.42	pos
1	36.32	pos	326954	pos	45	neg
0.1	45	neg	5782	pos	45	neg
0.01	45	neg	110	neg	45	neg

# Molecular Evolution of WN Virus Strains in the U.S.

- All US WN strains >99.8% identical (nucleotide)
- <3 amino acid differences between any 2 isolates
- WNV NY1999 & WNV FLA 2002: 25 nucleotide differences & 1 amino acid substitution



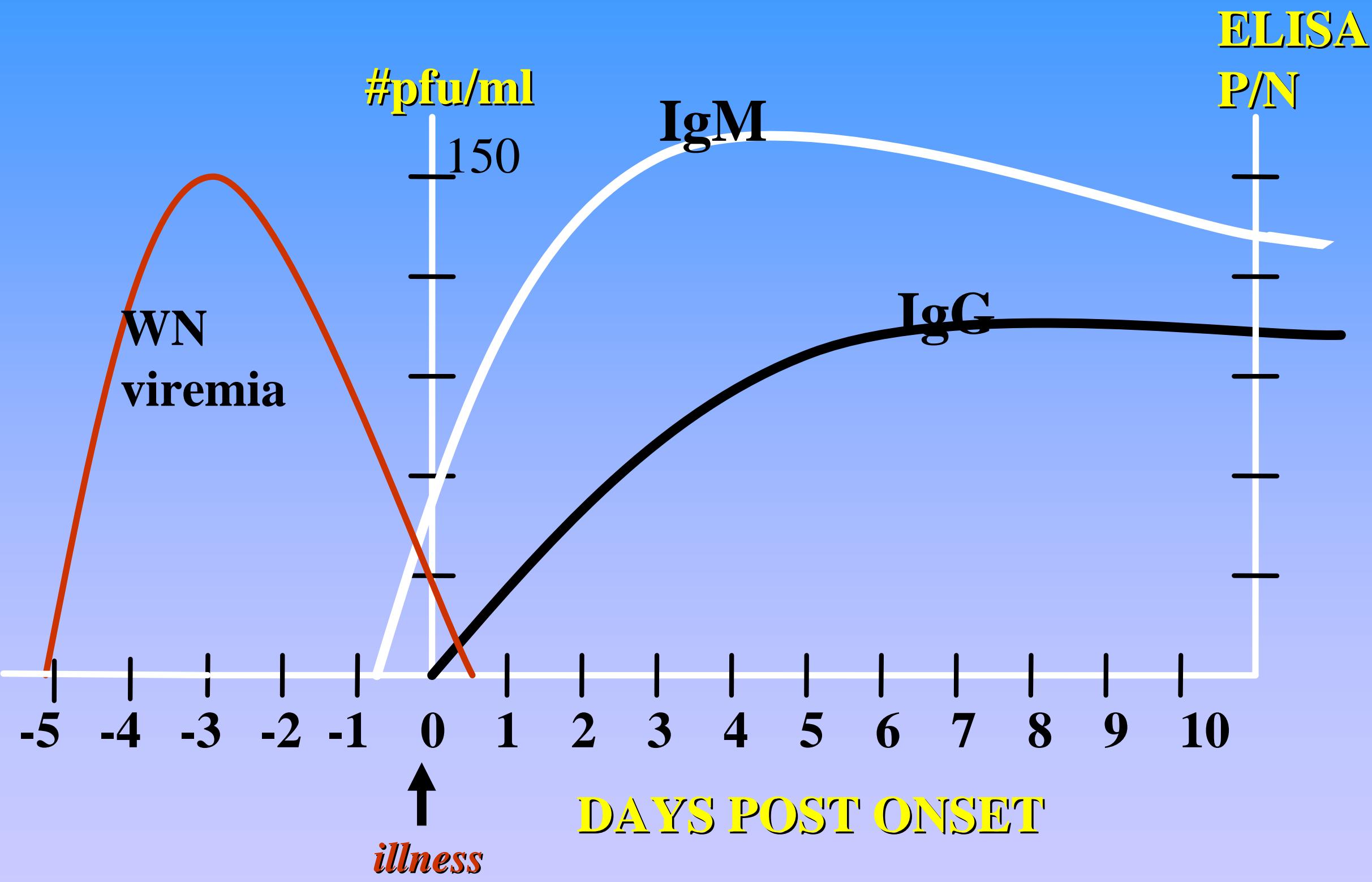
# **WNV Isolates From Humans: 1999 - 2002**

- 1999: No WNV isolated
- 2000: No WNV isolated
- 2001: 1 virus isolated csf (NY State Lab)
- 2002: 13 WNV isolated CDC + 1 from MD Dept. Health
  - 5 serum/plasma
  - 3 csf
  - 4 brain tissue
  - 1 liver

# 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



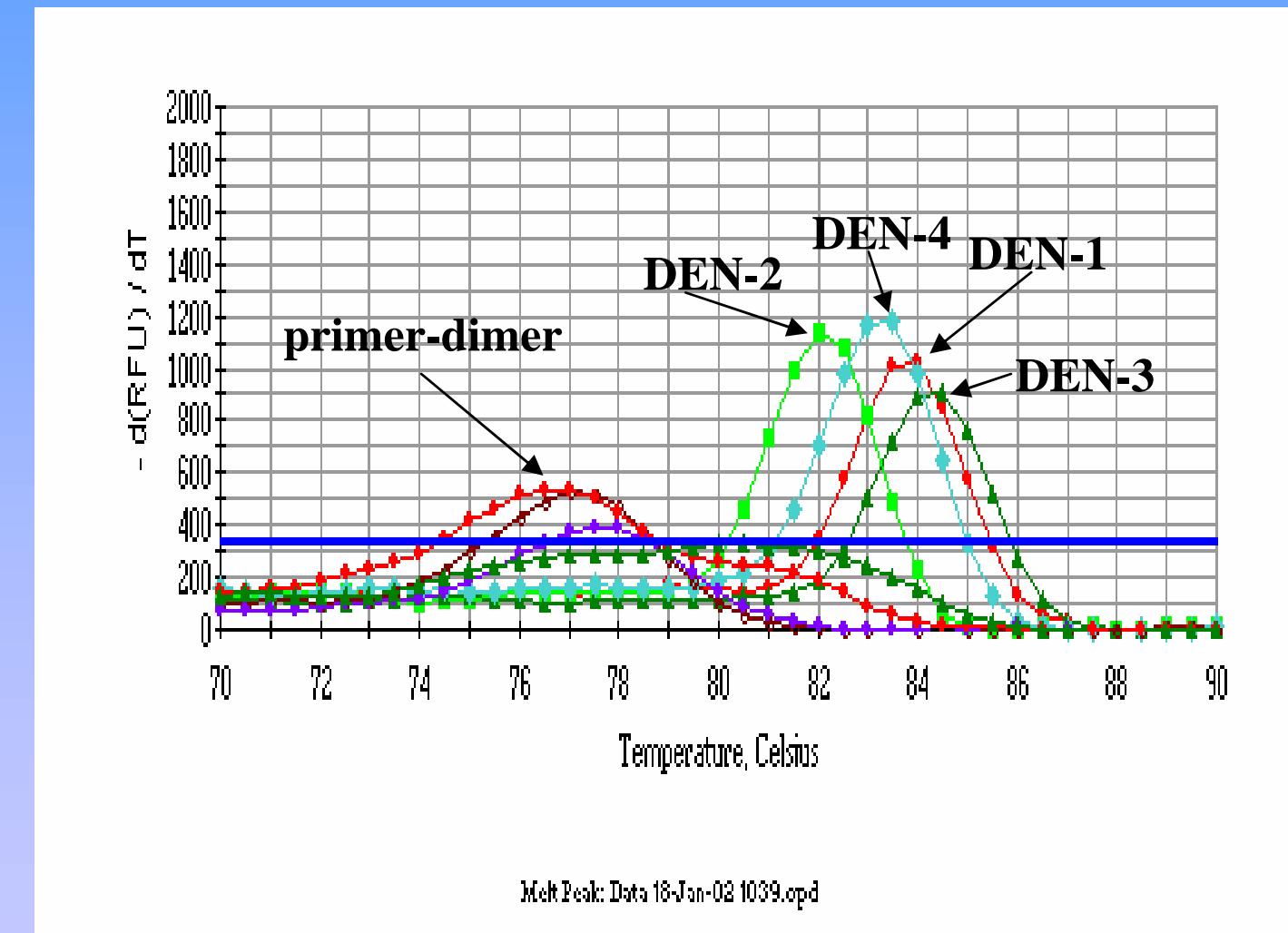
# TaqMan Technology Transfer

- CDC Training Course
  - Trained > 60 Public Health Laboratories
- Proficiency Panel
  - 76% Complete Agreement
  - False positives
  - Failure to detect the lowest positive

# Diagnostic & Reference Section

## SYBR Green Consensus Assays

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



# Diagnostic & Reference Section

## *TaqMan & NASBA Assays*

Viral Target	Sensitivity	Specificity/Comments
WN	< 0.1 pfu	Lineage 1 WN
SLE	< 0.15 pfu	All NA & SA SLE
EEE	< 0.10 pfu	NA EEE only
WEE	< 0.35 pfu	All NA & SA WEE; TaqMan > sensitivity
LAC	< 1 pfu	15 LAC strains; no other CAL serogroup
In Progress		
DEN	< 0.1 pfu	Multiplex with serotype probes
SYBR Green	< 1 pfu	Consensus assays for DEN, alphavirus, flavivirus, CAL serogroup bunyavirus.
VEE		