

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

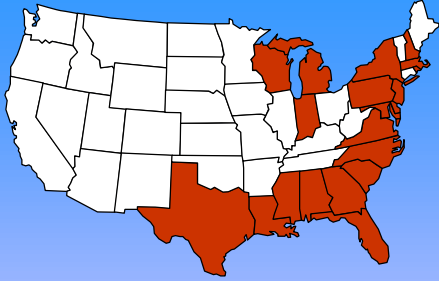
Family/Genus	Pathogens
Togaviridae/Alphavirus	Eastern equine encephalitis
ss + RNA +; 70 nm particle	Western equine encephalitis
	Venezuelan equine encephalitis
Flaviviridae/Flavivirus	St. Louis encephalitis
ss + RNA; 40-60 nm particle	Powassan
	West Nile
	Dengue
Bunyaviridae/Bunyavirus	
California serogroup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

Medically Important Arboviruses in the United States

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

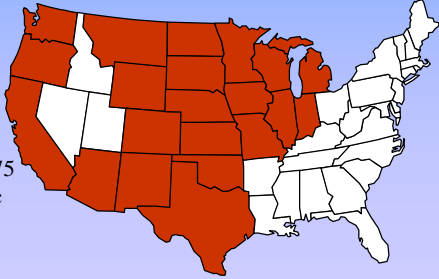
Eastern Equine Encephalitis Human cases: 1964-2002

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



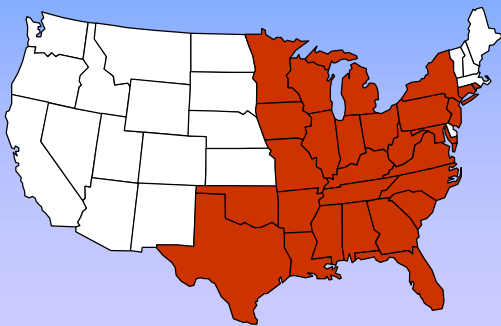
Western Equine Encephalitis Human cases: 1964-2002

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



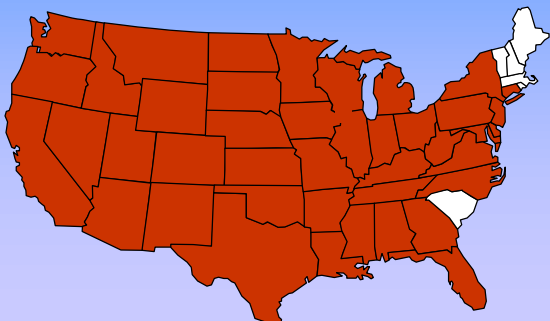
La Crosse Virus Encephalitis Human cases: 1964-2002

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

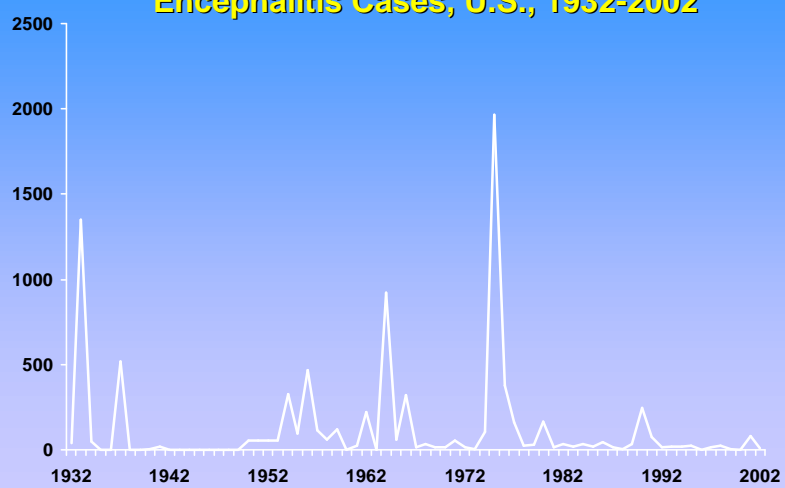


St. Louis Encephalitis Human cases: 1964-2002

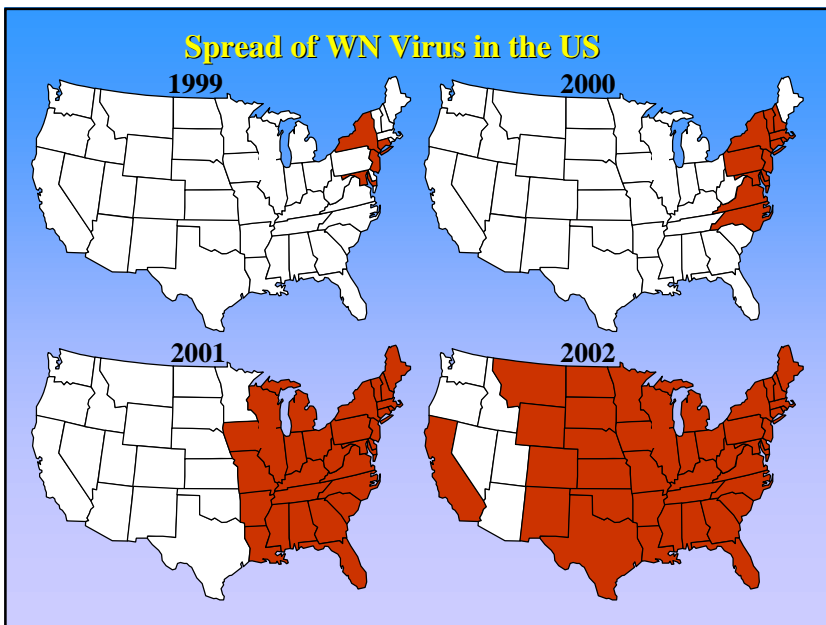
- 4561 cases
- Epidemic cycles
- 50% 1975 & 1976
- 70% TX, IL, OH, IN, FL, MS



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



Spread of WN Virus in the US



CDC Tests for WN Virus

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

human serum/csf

IgM ELISA WN & SLE
IgG ELISA WN & SLE

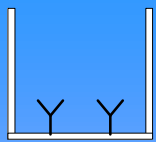
POS

NEG

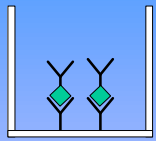
Plaque reduction
Neutralization test (PRNT) with:
SLE, WN, (other flaviviruses)

STOP

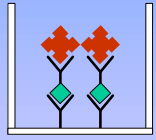
IgM Capture ELISA



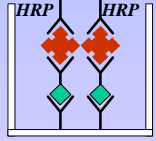
1. Coat With Goat anti-Human IgM
➤ 4° Overnight



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

	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
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 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
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
Secondary flavivirus infection							
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)
300-400	21	9 (43)	2	11 (52)	4.0 (3.1-6.5)
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

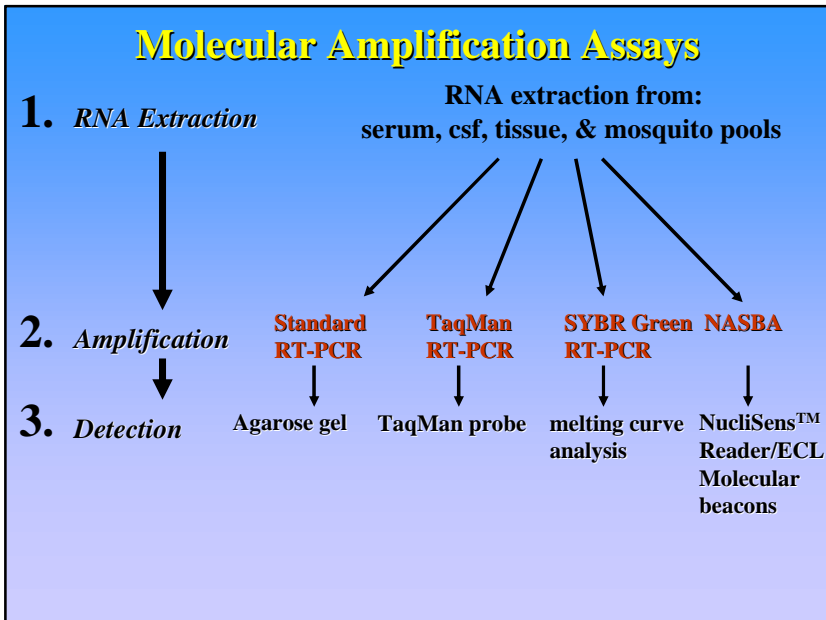
- CDC Training Course
 - Trained > 60 Public Health Laboratories
- Proficiency Panel
 - 100% agreement IgM ELISA
 - 92% agreement IgG ELISA (false neg's)

WN Serological Assays

Future Directions


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






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



WN Virus TaqMan Assay Detection Limit

Plasmid

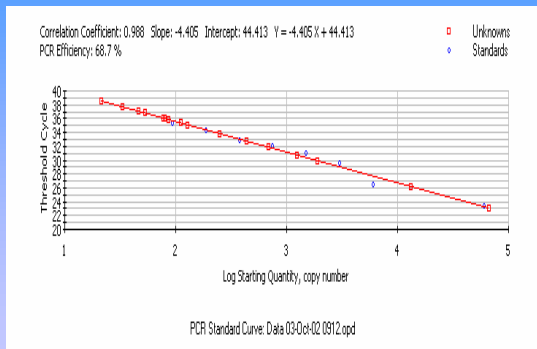
1,000 copies/ml
5 copies

DS DNA

2,400 copies/ml
12 copies

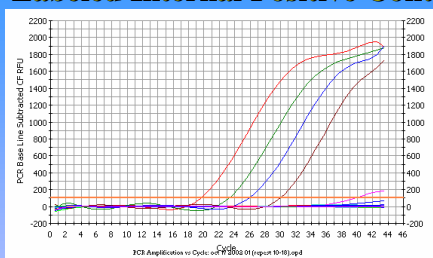
RNA (Kramer)

37 copies

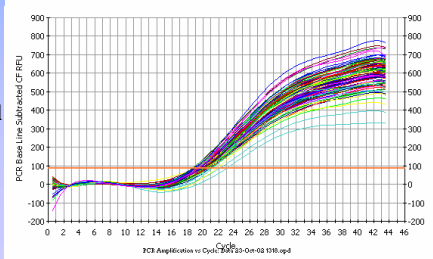


WN Virus TaqMan Assay With JOE-Labeled Internal Positive Control

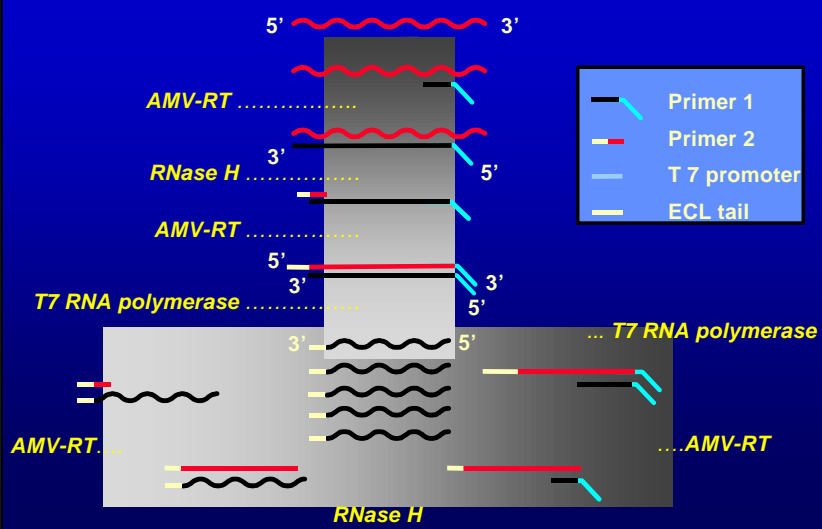
WN virus
primer/probe set



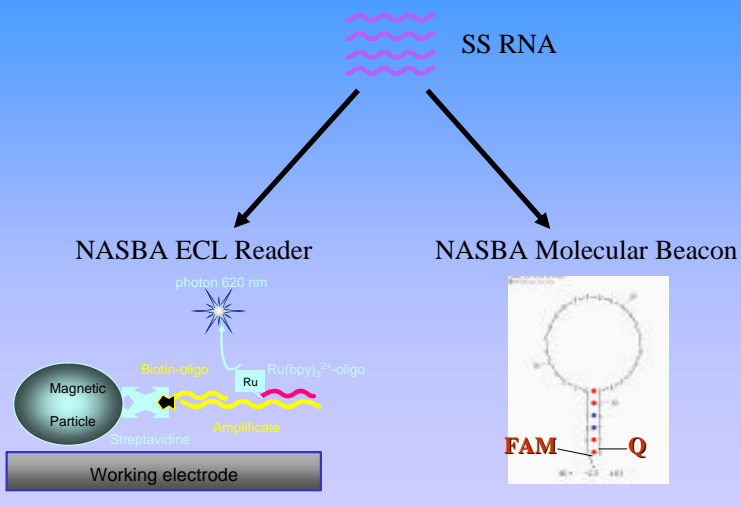
HEX internal control
primer/probe set



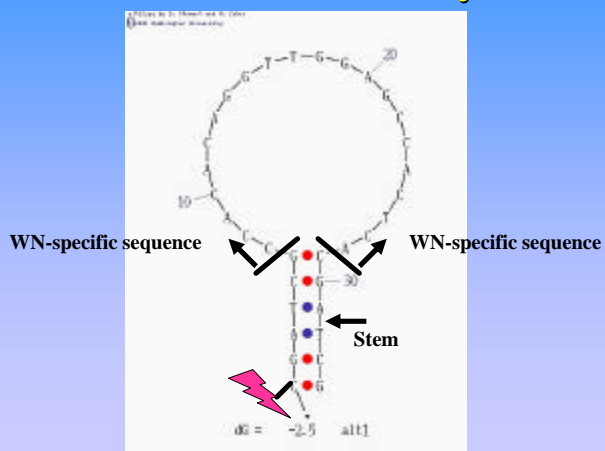
Basic Kit Amplification Principle



NASBA Detection Formats



Molecular Beacon Probe for WN Virus NASBA Assay

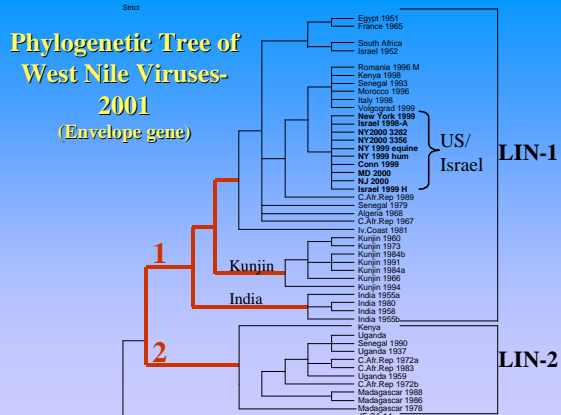


Sensitivity of WN Virus NASBA & TaqMan Assays

#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



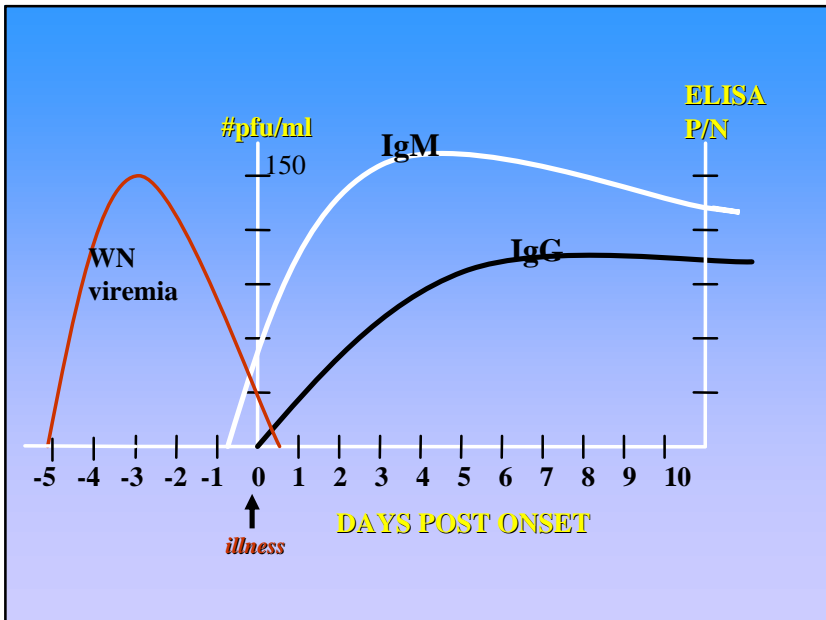
WNV Isolates From Humans: 1999 - 2002

- **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

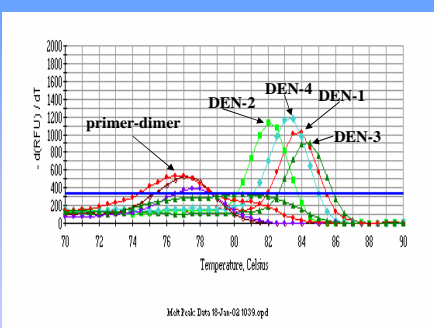


TaqMan Technology Transfer

- 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		
