Serological and Molecular Amplification Assays for West Nile Virus

Arbovirus Diseases Branch

Diagnostic & Reference Laboratory

Fort Collins, Colorado

CDC

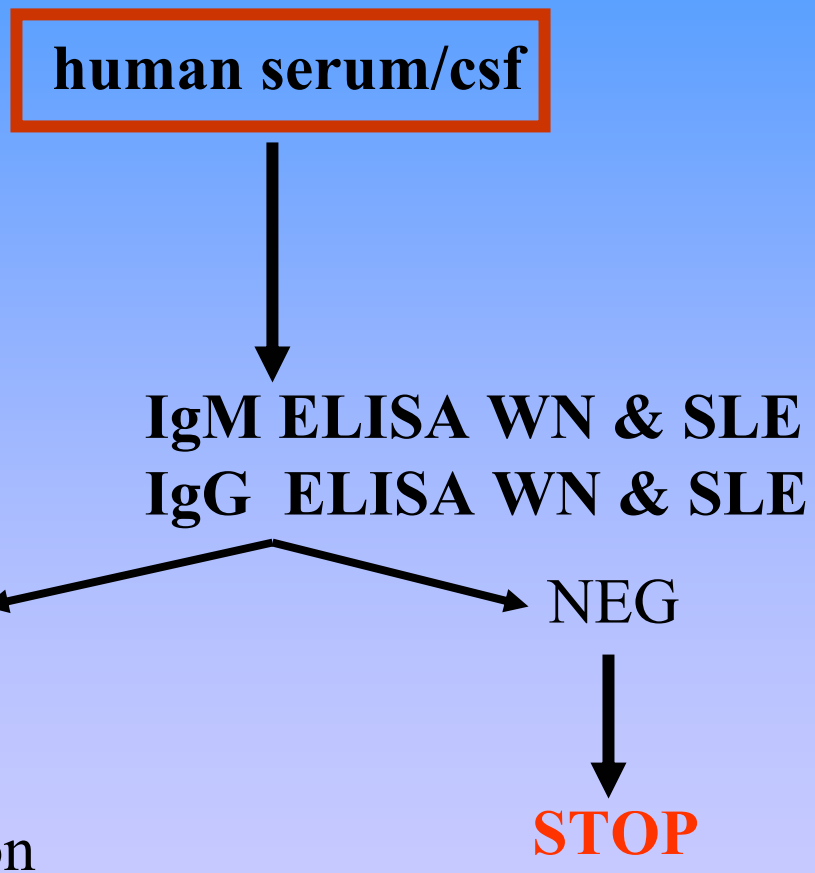
Centers for Disease Control and Prevention

CDC Tests for WN Virus

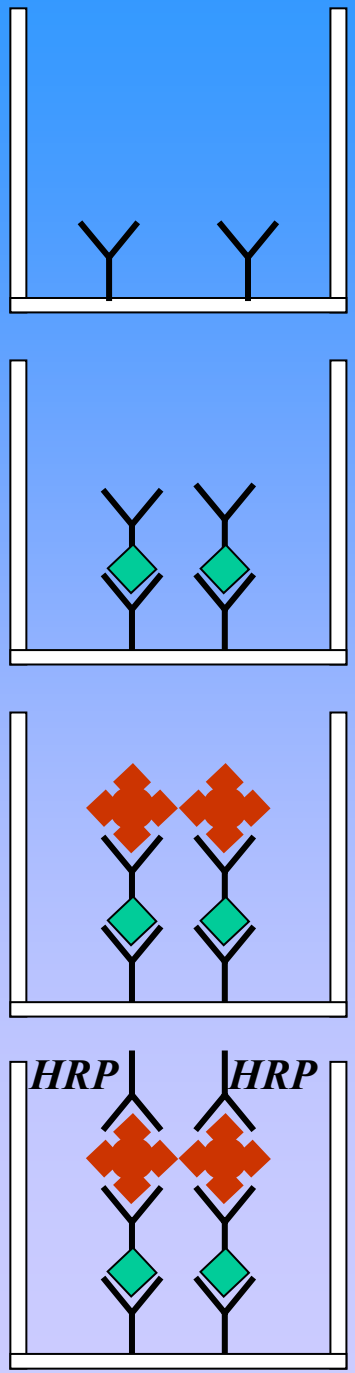
Specimen	1st Choice	Other	Comments
Human serum/CSF	ELISA Plaque Reduction Neutralization	TaqMan/NASBA Virus Isolation	TaqMan (57%) for acute CSF; <10% serum
Human tissue	TaqMan/NASBA	Virus Isolation IHC	Fatal WN cases: TaqMan/NASBA positive ~ 100%
Non-Human	**1st Choice**	**2nd Choice**	
Avian tissue	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



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

ELISA Assay must be standardized in each lab

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

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Complete Serological Analysis

Patient	Days P.I.	IgM (WN)	IgG (SLE)	WN	SLE	DEN2	JE
CSF	8	26.91	1.78	nd	nd	nd	nd
S1	9	9.1	4.16	160	20	<10	10
S2	34	6.7	4.62	1280	20	<10	20
Positive Control	n.a.	9	6.5	>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 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

	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
Secondary flavivirus infection?							
acute serum	4	1.59	1.42	3.12	2.62	<1:10	<1:10
conv. serum	15	9.01	3.96	10.00	9.90	1:640	1:320

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)

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WN Human Serological Testing Algorithm

- Early Season: Before WN Cases in a Geographic Region.
  - IgM & IgG ELISA with WN, SLE, other arboviruses
  - PRNT with WN, SLE, & others?

- After WN Confirmed Cases in a Geographic Region:
  - IgM ELISA with WN & SLE (***probable***)
  - PRNT, IgG ?
    - SLE ?
    - Travel?
    - P/N WN < 2X SLE

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

CDC IgM ELISA Assay

Good Points

- Sensitive
- Relatively Specific (WN & SLE P/N ratio)
- Technology Transferable

Bad Points

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

IgM & IgG ELISA Technology Transfer

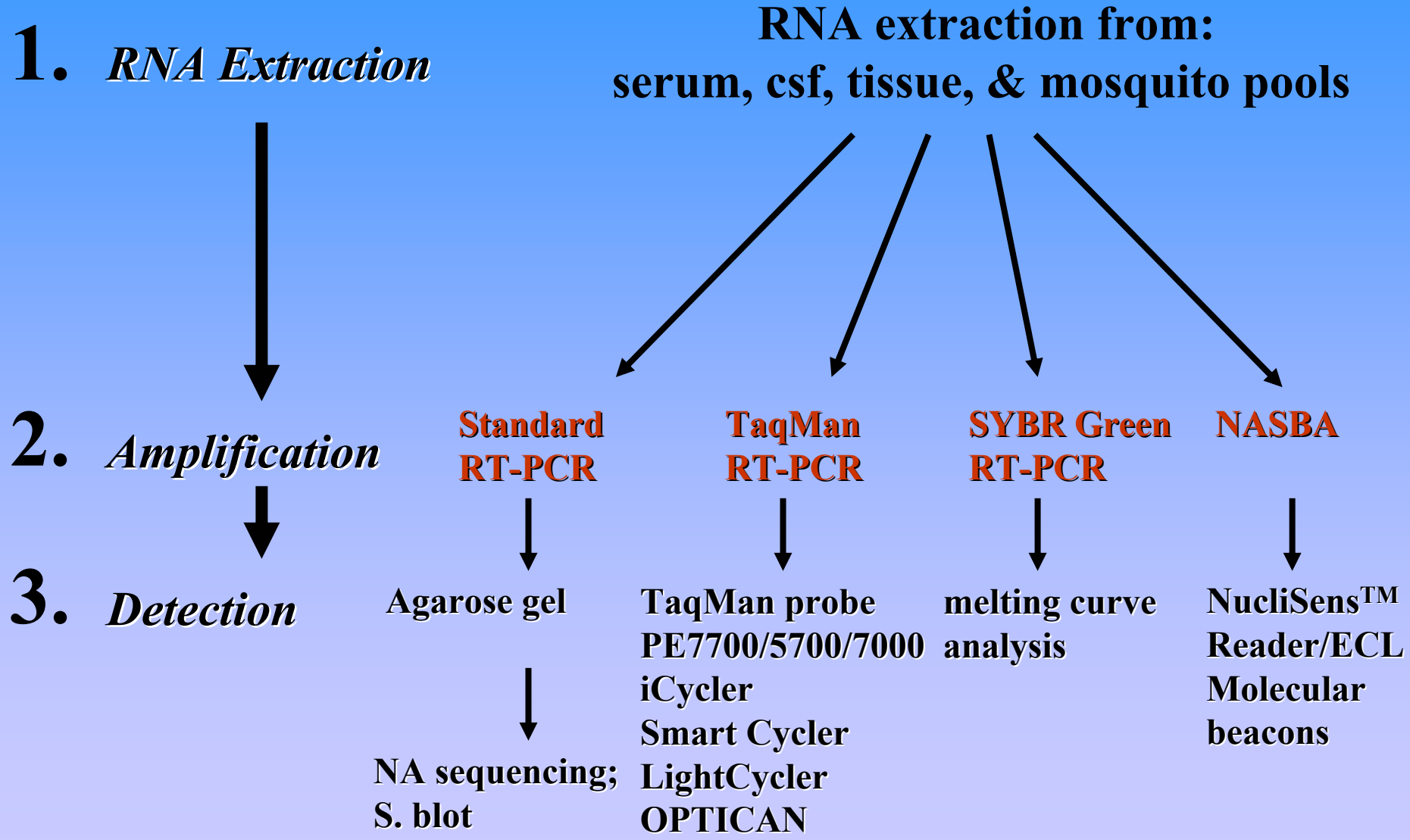
- CDC Training Course
  - Trained > 60 Public Health Laboratories
- Proficiency Panel
  - 100% agreement IgM ELISA
  - 92% agreement IgG ELISA (false neg's)

WN Serological Assays

Future Directions

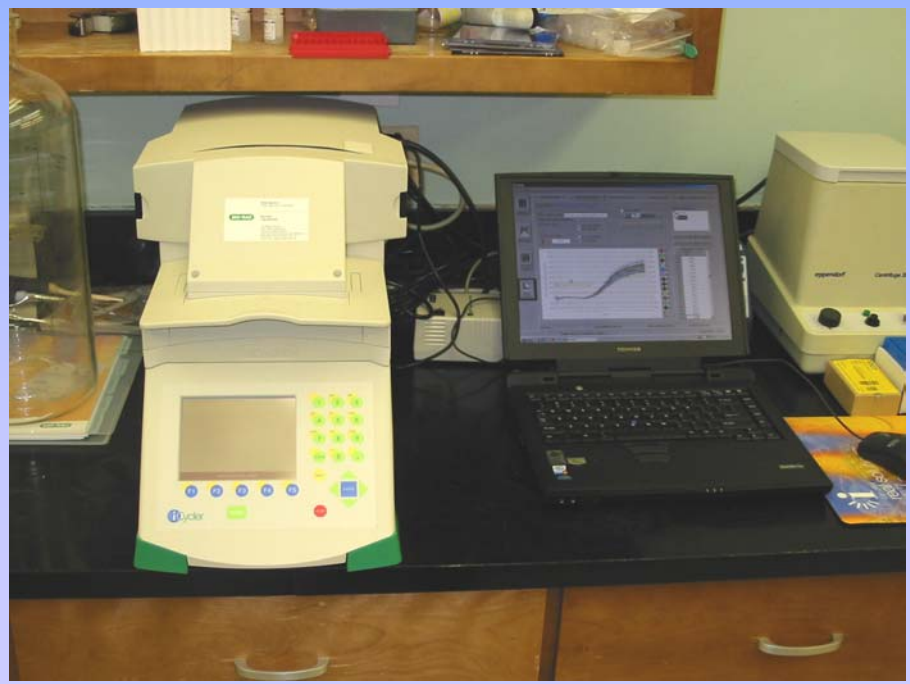
- Automation of IgM & IgG ELISA
- Reagent Stability
- Incubation Times
- Luminex Assay
- Commercial Assays (flavivirus)

Molecular Amplification Assays



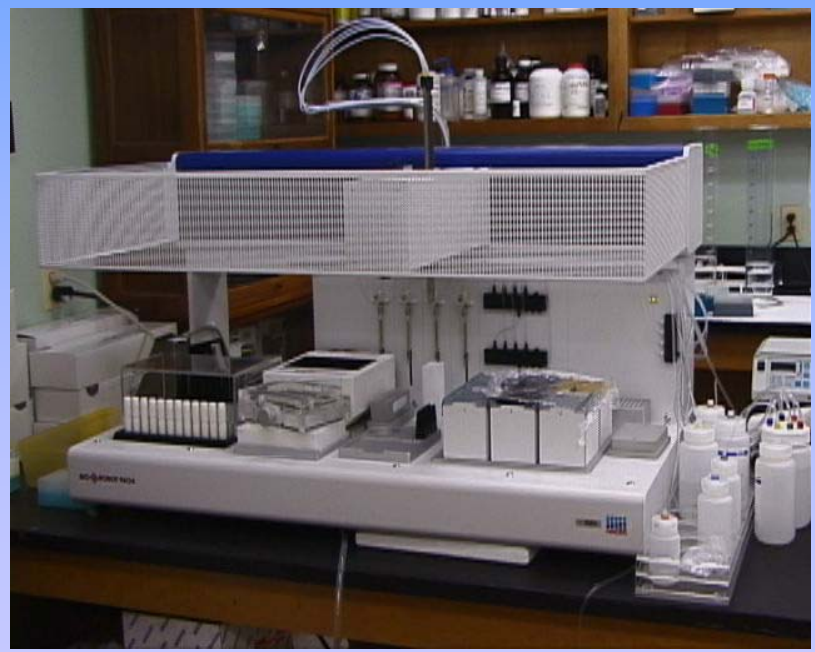
CDC TaqMan Testing Algorithm

- ✓ 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

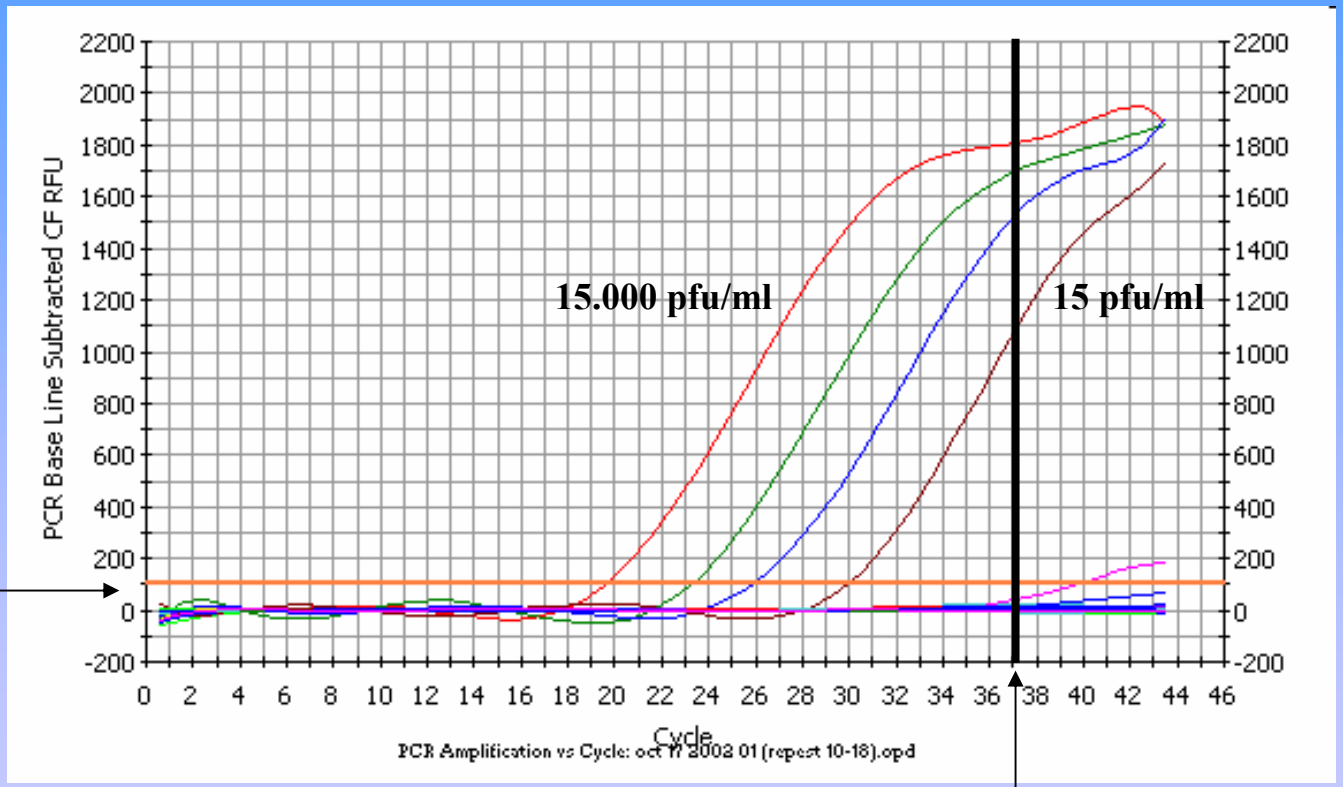


RNA Extraction & Purification

- **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



TaqMan RT-PCR of West Nile Virus Dilutions



threshold

Ct = 37

Detection Limit

Plaque forming units (pfu)

Ct = 37

ENV set

0.80 pfu/ml (100 ul)

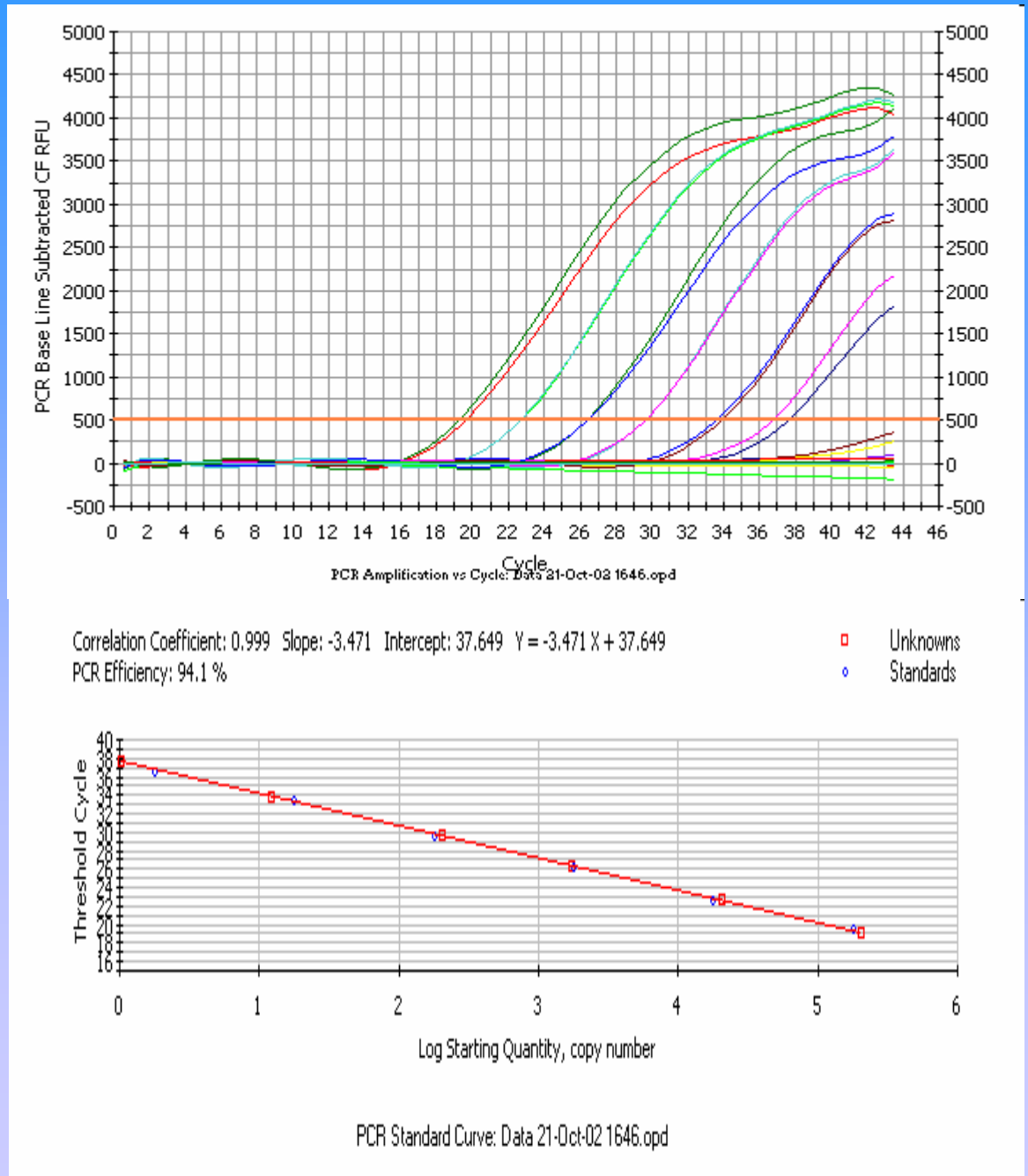
0.10 pfu/ml (500 ul)

3'NC set

3.2 pfu/ml

NS5 set (Lipken)

1.2 pfu/ml

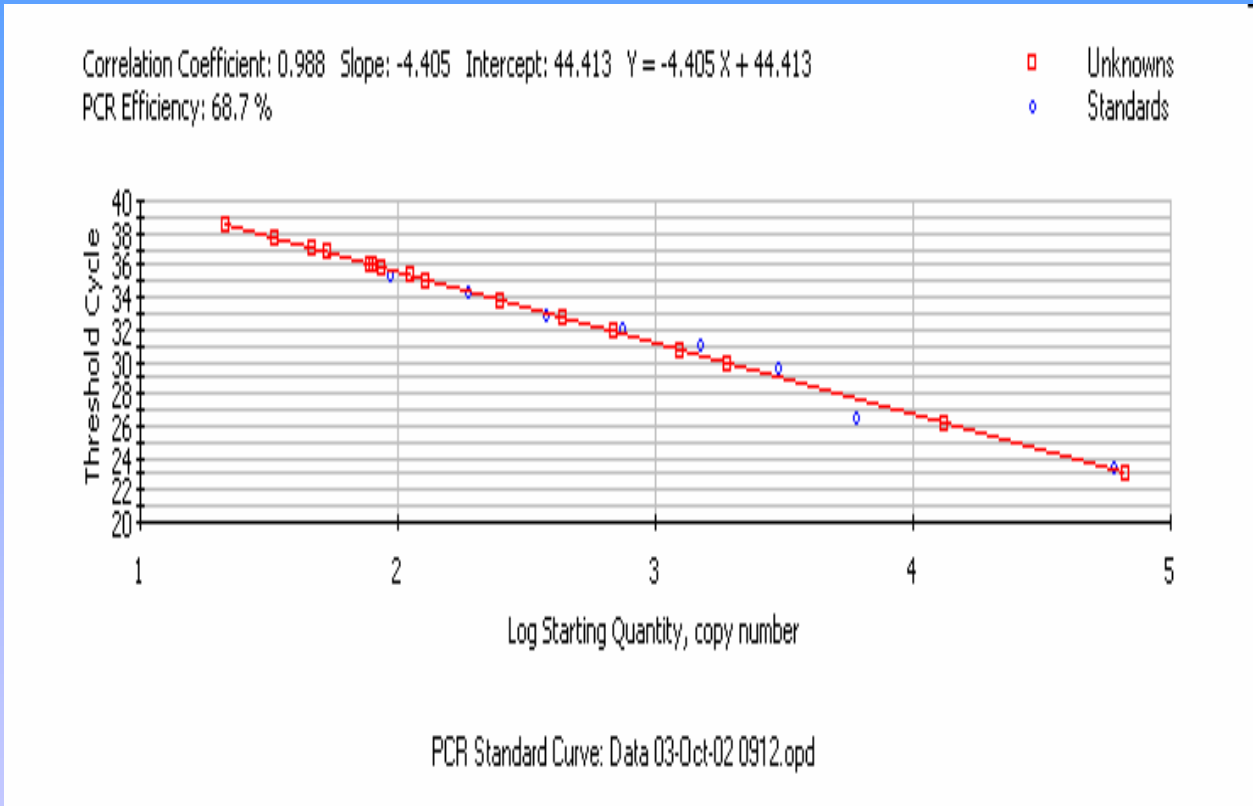


WN Virus TaqMan Assay Detection Limit

Plasmid
5 copies

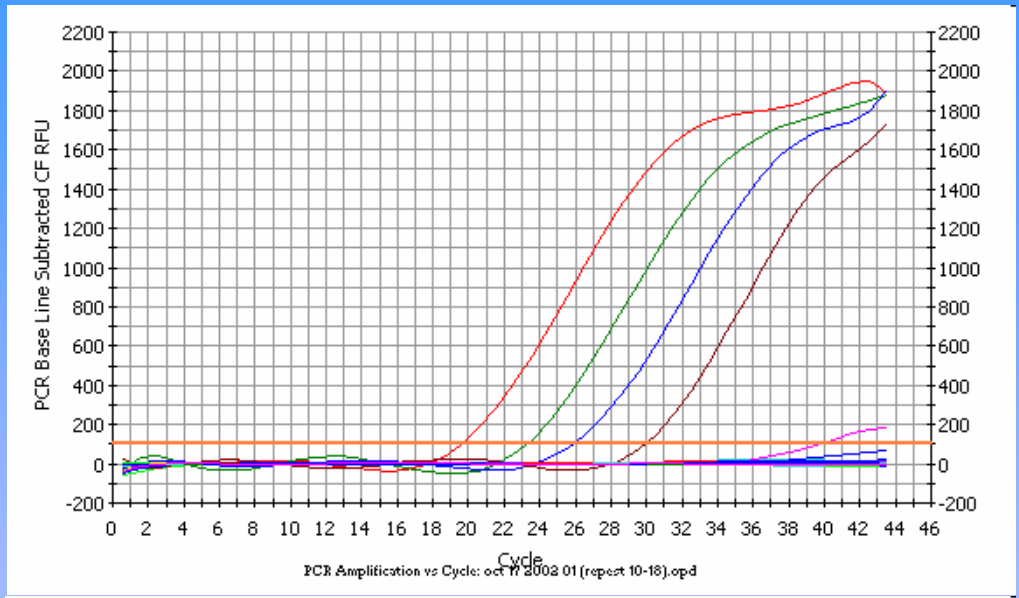
DS DNA
12 copies

RNA (Kramer)
37 copies

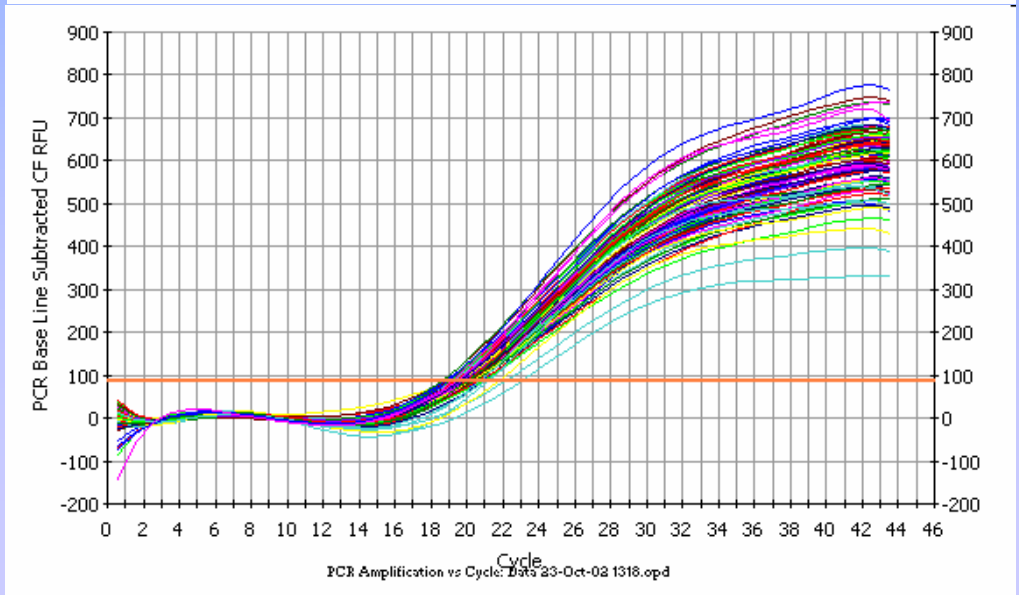


WN Virus TaqMan Assay With JOE-Labeled Internal Positive Control

WN virus primer/probe set



HEX internal control primer/probe set



Sensitivity of WN Virus NASBA & TaqMan Assays

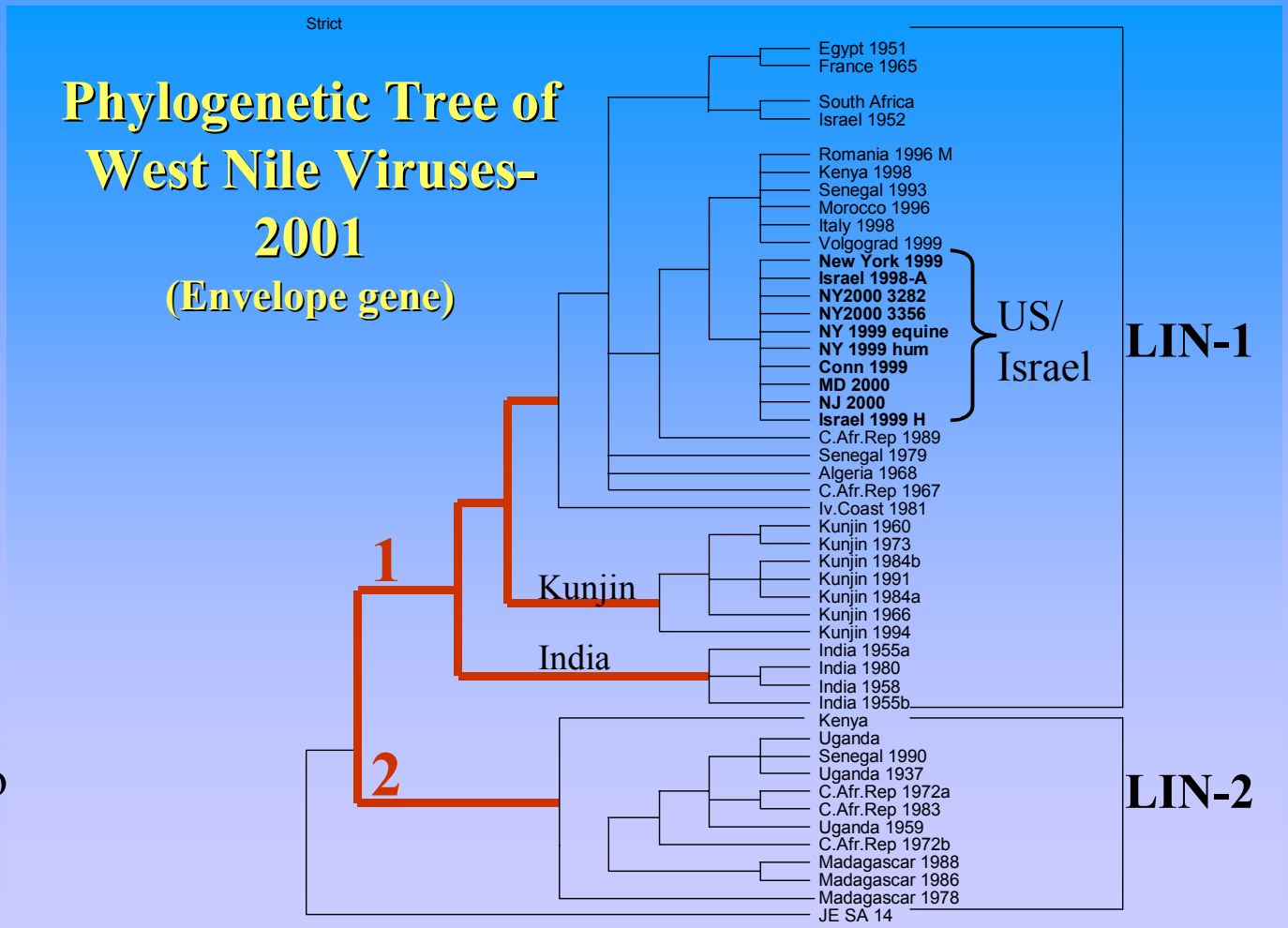
	TaqMan		NASBA		NASBA	
#pfu/ml	Ct	Interp.	ECL	Interp.	MB	Interp
1,000,000	17.88	pos	1653417	pos	9.44	pos
100,000	20.9	pos	1187613	pos	12.01	pos
10,000	24.17	pos	1810790	pos	12.27	pos
1,000	27.75	pos	1666084	pos	14.81	pos
100	31.21	pos	1211426	pos	19.21	pos
10	34.07	pos	1209491	pos	21.42	pos
1	36.32	pos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

Specificity of WNV NASBA & TaqMan Assays

			TaqMan				NASBA
	10,692 probe (3'NC)			1186 Probe (ENV)			
WN Virus strains	**Ct**	**Rn**	**Interp.**	**Ct**	**Rn**	**Interp.**	**ECL**
WNV-Romania-1996H	24.63	1.66	**pos**	45	0.24	neg	**469251**
WNV-Romania-1996M	29.02	1.25	**pos**	26.04	0.98	**pos**	**313605**
WNV-Egypt-1951	25.54	1.63	**pos**	45	0.14	neg	**437541**
WNV-Italy 1998	23.82	1.52	**pos**	23.97	0.89	**pos**	**237753**
WNV-Kenya 1998	21.38	1.75	**pos**	21.68	0.88	**pos**	**226175**
Kunjin	20.58	1.49	**pos**	45	0.23	neg	109
Other Viruses							
dengue-2	45	0.39	neg	45	0.29	neg	27
yellow fever	45	0.47	neg	45	0.19	neg	8
St. Louis enceph.	45	0.43	neg	45	0.17	neg	1
Japanese enceph.	45	0.42	neg	45	0.29	neg	7
Murrey Valley enceph.	45	0.35	neg	45	0.18	neg	2
eastern equine enceph.	45	0.42	neg	45	0.28	neg	1
western equine enceph.	45	0.46	neg	45	0.23	neg	12
Powassan	45	0.43	neg	45	0.19	neg	29
Lacrosse	45	0.42	neg	45	0.2	neg	1

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

Virus Isolates are Important!

- **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**

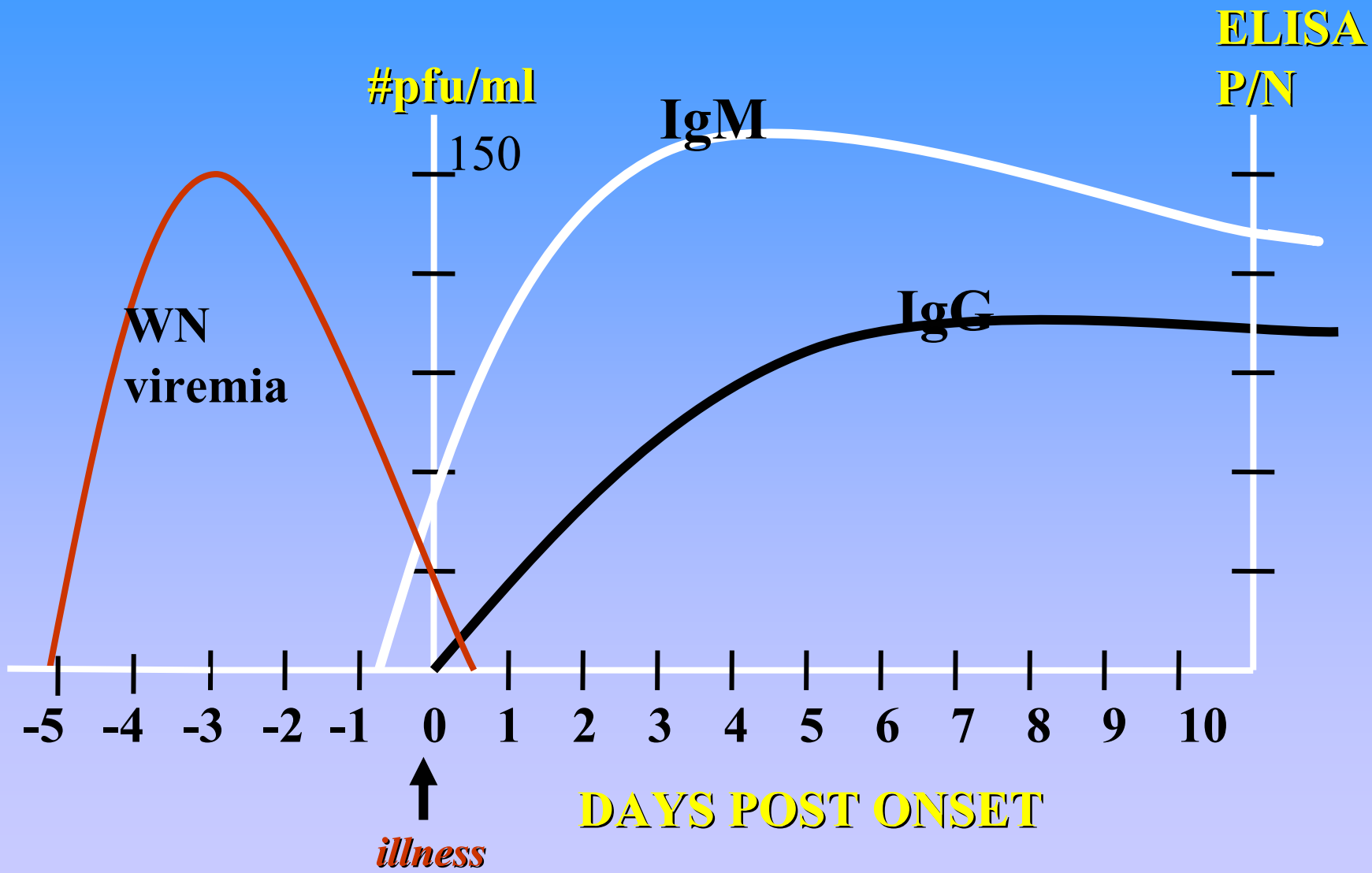
WN Human Viremia

Data Summary

- **Human viremia is low:**
  - Transfusion studies: 1-130 pfu/ml
  - Average 24 pfu/ml
  - Virus isolation is rare (asymptomatic IgM neg donors)

- **Human viremia is short-lived**
  - Rarely 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


ELISA
P/N
#pfu/ml
150
IgM
IgG
WN
viremia
-5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
DAYS POST ONSET
illness

TaqMan Technology Transfer

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

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
  - **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

Reagent Production & Shipping

- **CDC Reagent Production**
  - 1995 - 1999: 100 – 150 Reagent Requests/year
  - 2002 - 560 reagent requests
  - No change in personnel or policy
- **Commercial Partners** – patent license agreements for WN antigen production.
  - Abbott Laboratories; Focus Technologies; GenBio; Hennessey Research Associates; Immucor; InBIOS; RMZ; Biotech Corporation; Rapid Medical Diagnostic Corp.
- **USDA Permit** for WN RNA lysate
- **Select Agent Issues**
- **Vaccine Strains** (Acambis) for WN & SLE PRNT

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