AFRIMS DENGUE DIAGNOSITICS

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JITMM

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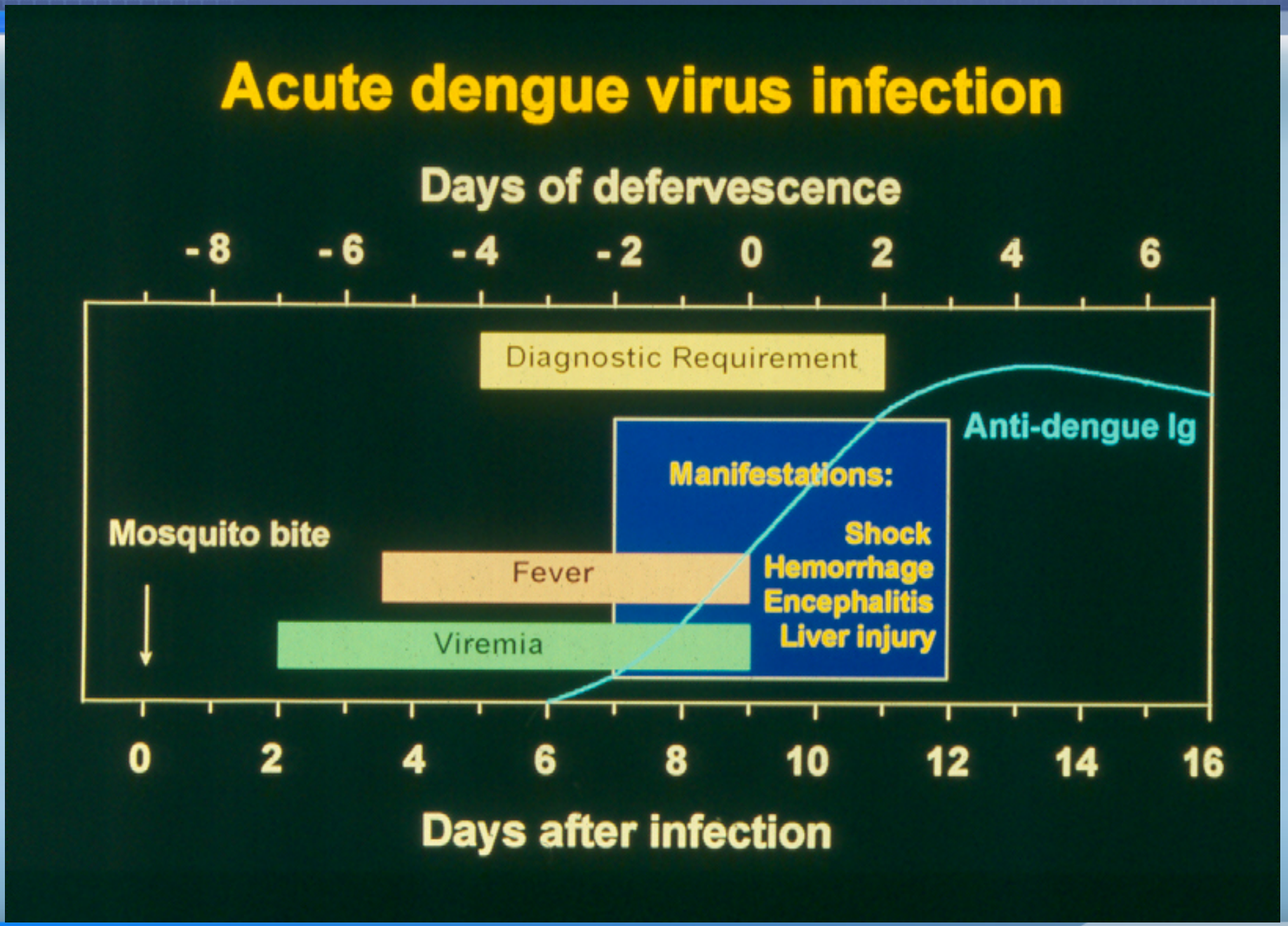
Diagnostic Goals

- Detection of infecting virus or its components

OR

- Measurement of antiviral antibody


Acute dengue virus infection
Days of defervescence
-8
-6
-4
-2
0
2
4
6
Diagnostic Requirement
Anti-dengue Ig
Manifestations:
Shock
Hemorrhage
Encephalitis
Liver injury
Mosquito bite
Fever
Viremia
0
2
4
6
8
10
12
14
16
Days after infection

Dengue Diagnosis

Virus isolation

Mosquito inoculation (intrathoracic)

Toxorhynchites splendens

Mosquito cell culture

C6/36 (*Aedes albopictus*)

Molecular techniques

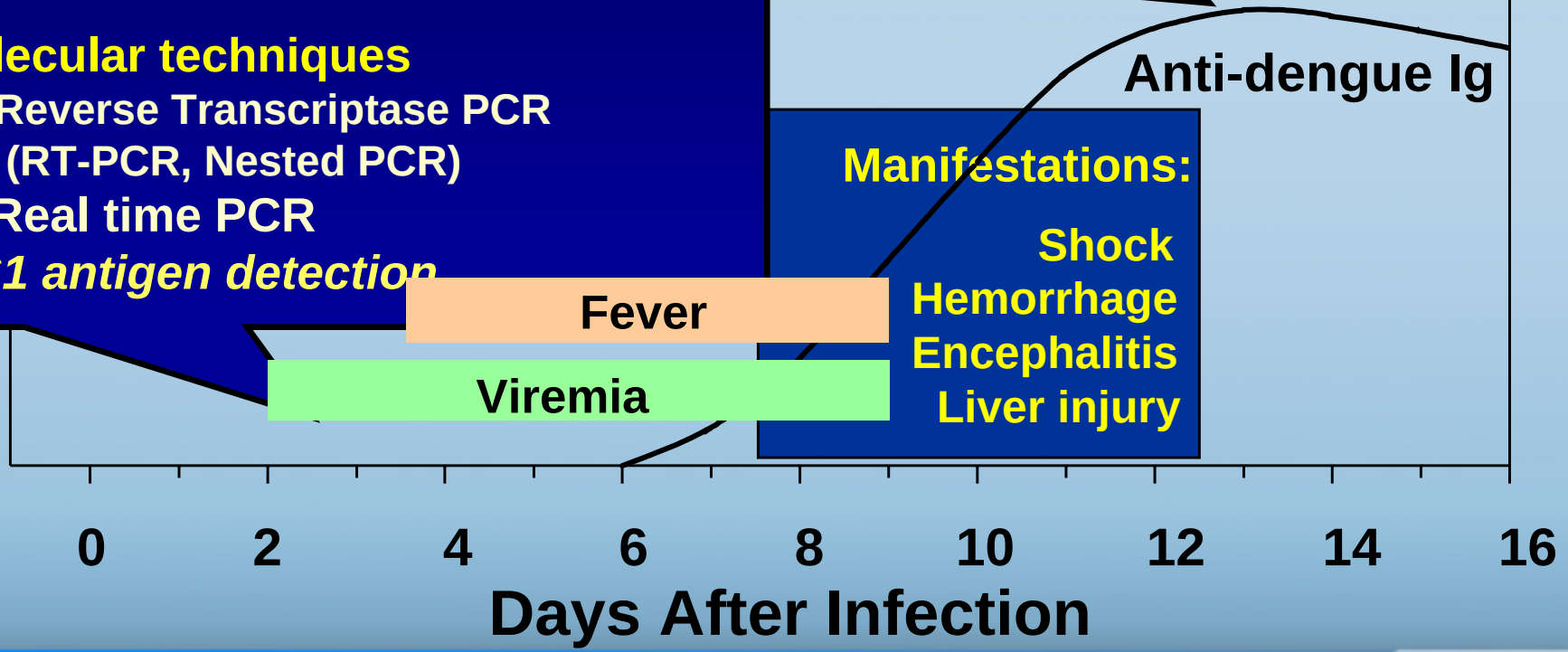
Reverse Transcriptase PCR (RT-PCR, Nested PCR)

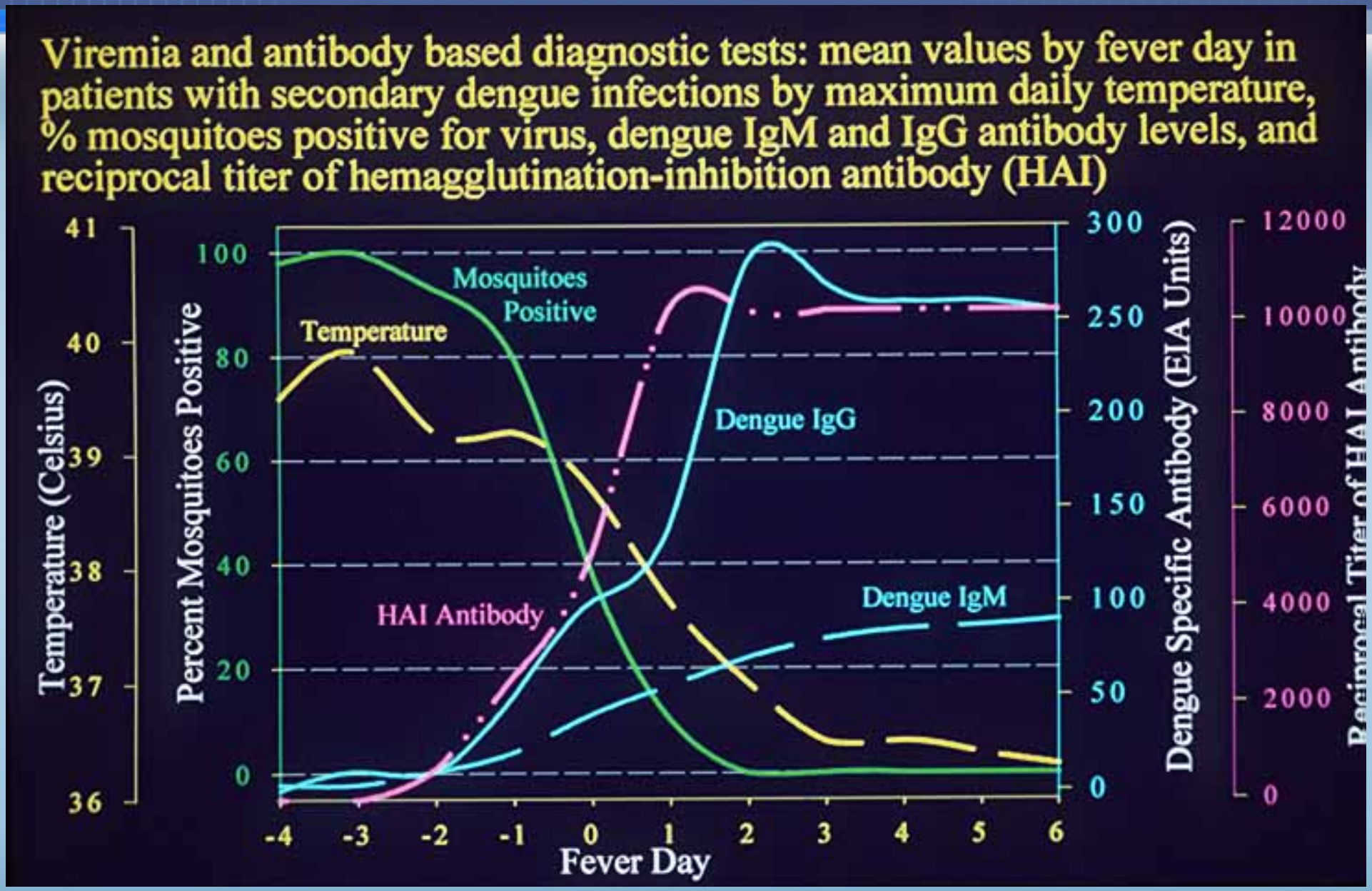
Real time PCR

NS1 antigen detection

Hemagglutination Inhibition (HI)

IgM and IgG ELISA





Vaughn DW et al. J. Infect Disease 1997 (2) : 322-30

Diagnostics at AFRIMS

- Serology
  - IgM/IgG ELISA
  - HAI
  - Plaque Reduction Neutralization
- Antigen
  - NS-1
- NA
  - Nested PCR
  - Real-time PCR (Quantitative)
  - Sequencing
- Viral Isolation
  - Direct C6/36 cells
  - Mosquito Inoculation

What to do?

1) Laboratory diagnosis of clinical cases
   - IgM/IgG -acute/convalescent (Serological diagnosis)
   - Nested PCR (serotype)

   <u>Discordant results</u>

   Repeat assay

   Viral isolation

   HAI

What to do?

2) Surveillance (Cohort-Study)

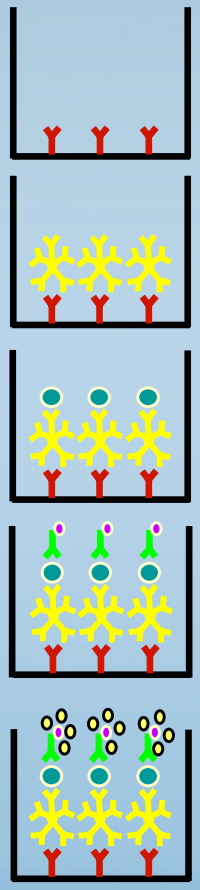
- Off-Season
  - HAI (asymptomatic infections)
  - PRNT (baseline)
- Acute infection
  - EIA (Serological Diagnosis)
  - Nested PCR
  - Real-time PCR (Viral Load Study) (selected)
  - PRNT of symptomatic and asymptomatic infections (selected)
  - Viral Isolation (Selected)
  - Sequencing (Selected)

What to do?

3. Outbreaks
   - EIA (Often on single specimens)
   - Nested PCR (used for serotyping and to aid in serological diagnosis)
   - Sequencing

Assays- EIA

- Used for all dengue studies



1. **Coat plate with goat anti-human IgM OR IgG**
2. **Add test specimens (serum or CSF), Negative and Positive controls**
3. **Add dengue antigens (dengue 1- 4)**
4. **Add human anti-flavivirus IgG- HRP**
5. **Add substrate, incubate, stop reaction, OD at 492**

Specimen type	Results	interpretation
Paired	IgM:IgG >1.8 and IgM ≥ 40	Acute Primary Infection
Paired	IgM:IgG <1.8 and IgM ≥ 40	Acute Secondary Infection
Paired	IgM:IgG <1.8 and IgM≥ 40 ***IF*** IgM<40 IgG rise between S1 and S2 to >100 ***IF*** IgG <100 confirm with HI	Acute Secondary Infection

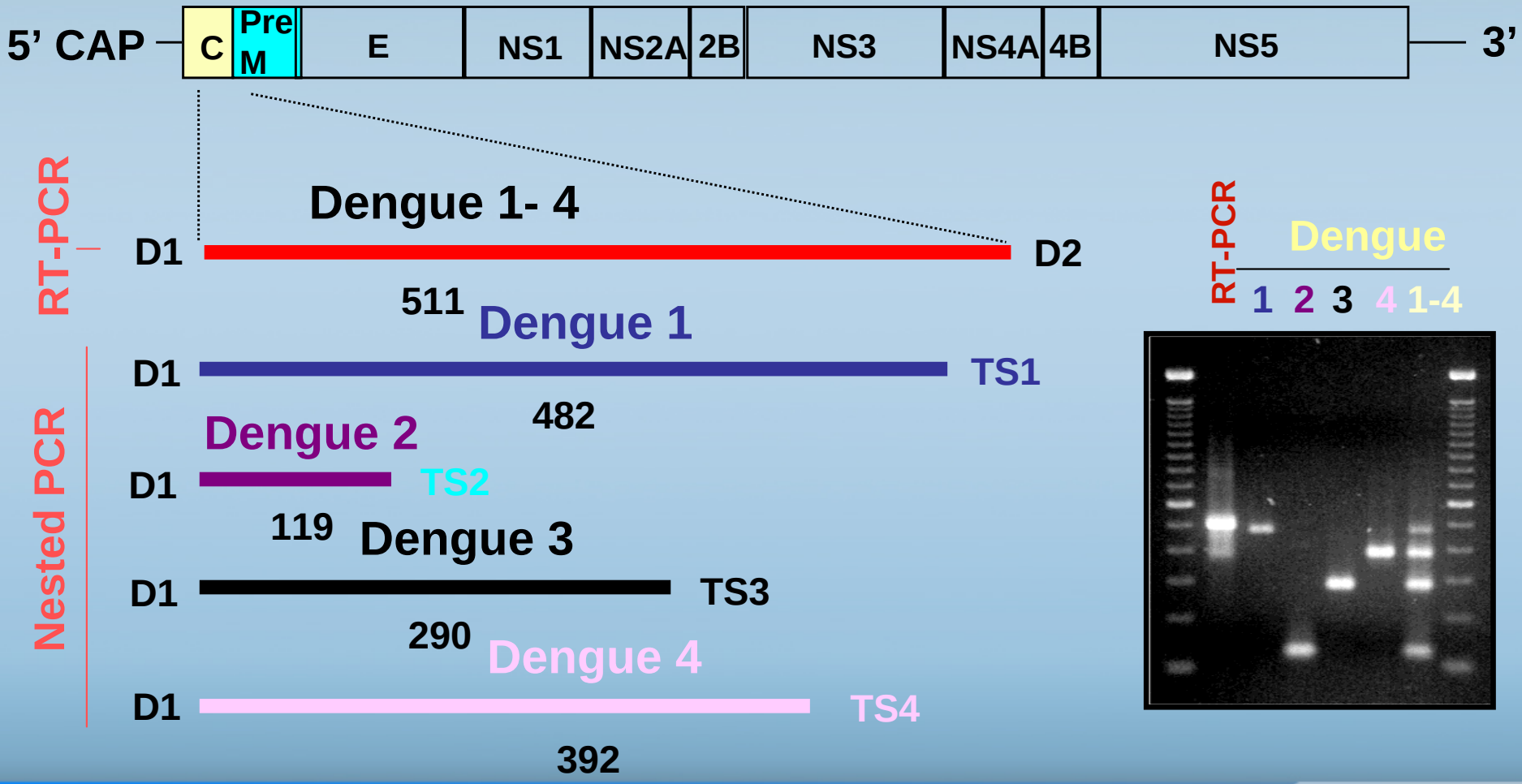
Specimen type	Results	Interpretation
Paired	IgM <40 AND IgG >100 ***IF*** S1 <40 and IgG >100 and S2 IgG drop to less than 100	Recent Secondary Infection
Paired	IgM<40 and IgG >100	Suggestive Secondary Infection
Single	IgM>40	Acute Dengue Infection
Single	IgM <40 And IgM:IgG <1.8 and PCR Positive	Secondary Infection

- Acute and convalescent sera interval should be ≥ 7 days to make serologic diagnosis as “No Evidence of Recent Flavivirus Infection”

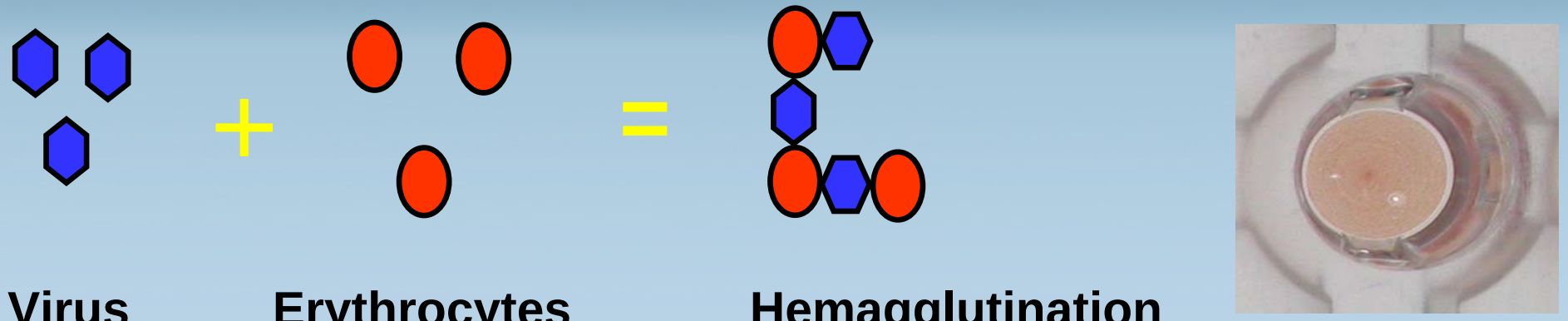
Dengue Virus PCR

Lanciotti et al., (1992) J. Clin. Microbiol. 30: 545

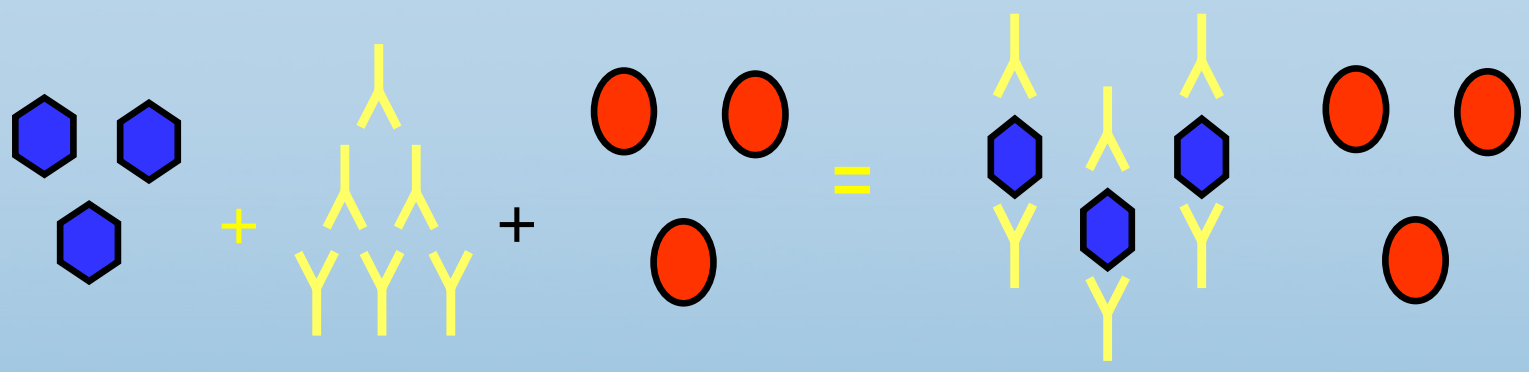
RT-PCR ---> Nested PCR with type specific primers



Hemagglutination Inhibition Test



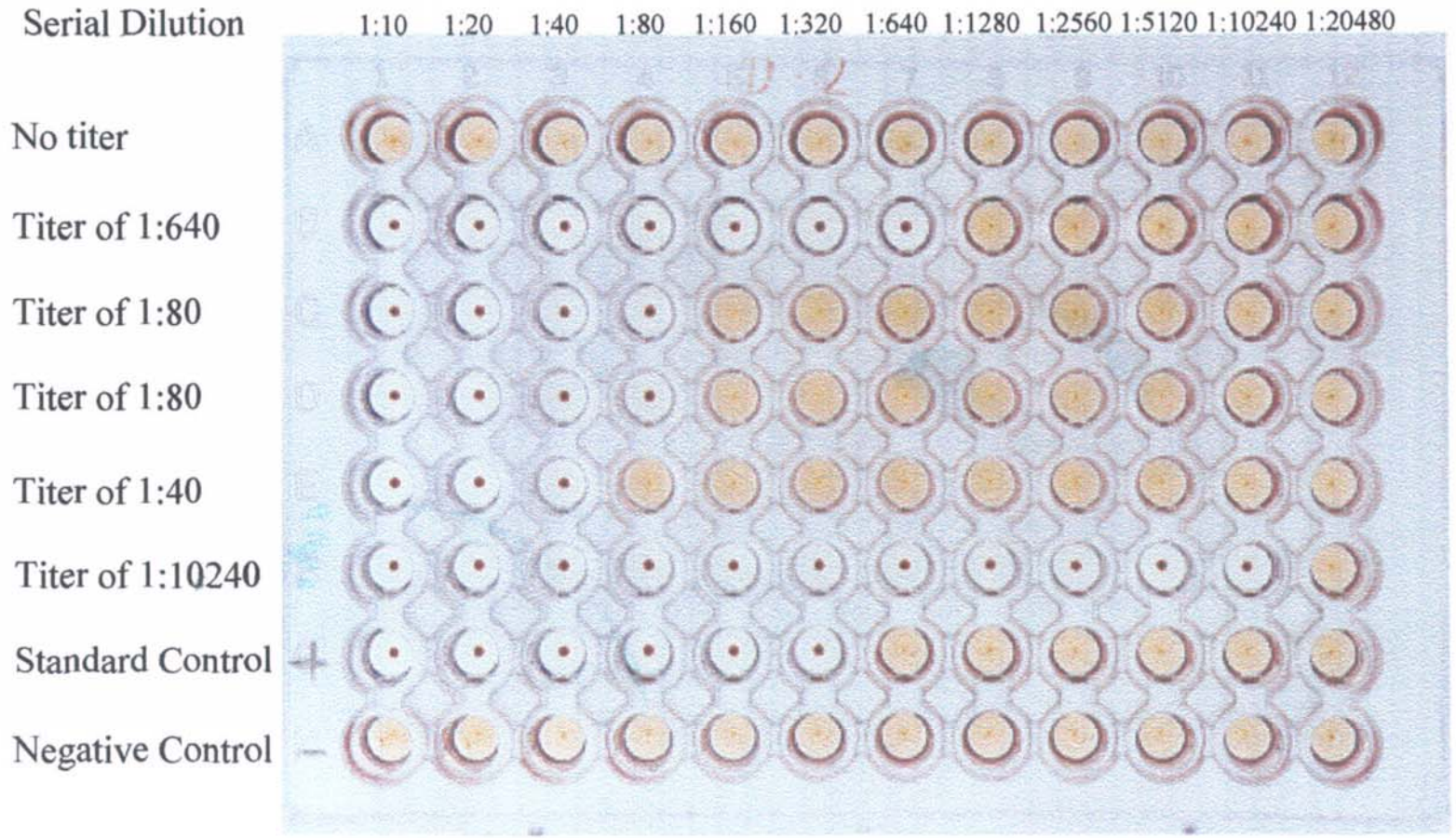
Virus Erythrocytes Hemagglutination



Antibody

No Hemagglutination
(Hemagglutination Inhibition)

Figure Four: Example of Six Patients with Hemagglutination Inhibition Titers to den-2.



Interpretation of the dengue hemagglutination-inhibition test

Antibody response	Specimen Interval	Conversion titer; any dengue Ag	Interpretation
≥ 4x rise	≥ 7 days	≤1:1280	Acute flavivirus infection, primary
		>1:1280	secondary
No change	Any	>1:1280 specimen	Recent flavivirus infection, secondary

Isolation and Identification of Dengue Viruses

Specimens
- **Serum**
- **CSF**
- **Tissue**

Toxorhynchites splendens
(intrathoracic inoculation)

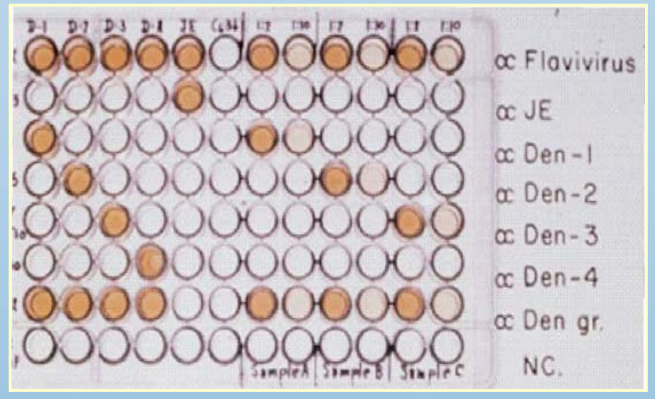
10-14 days

Head squash

Indirect Immunofluorescence Assay

Negative

Positive



Grind mosquito body

C6/36 cells

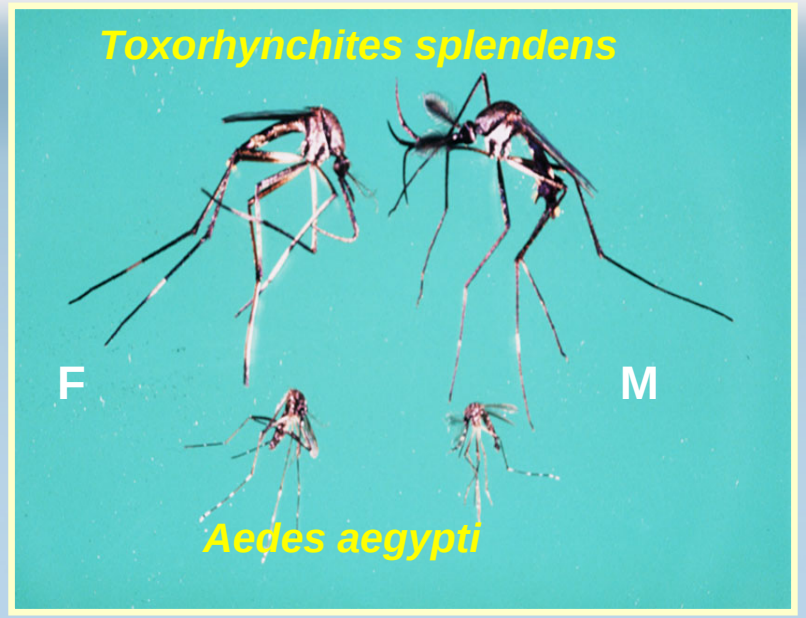
7 days

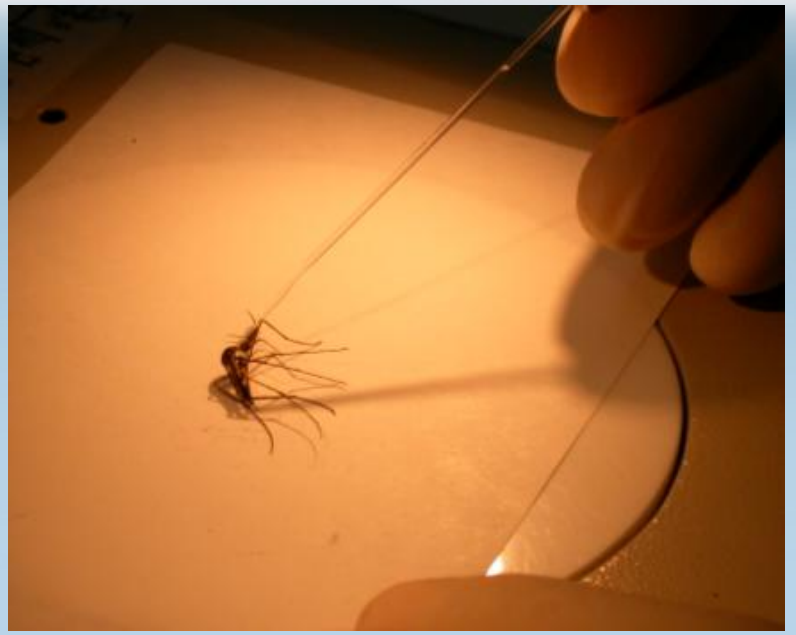
Cell culture fluid

Storage at -70° C

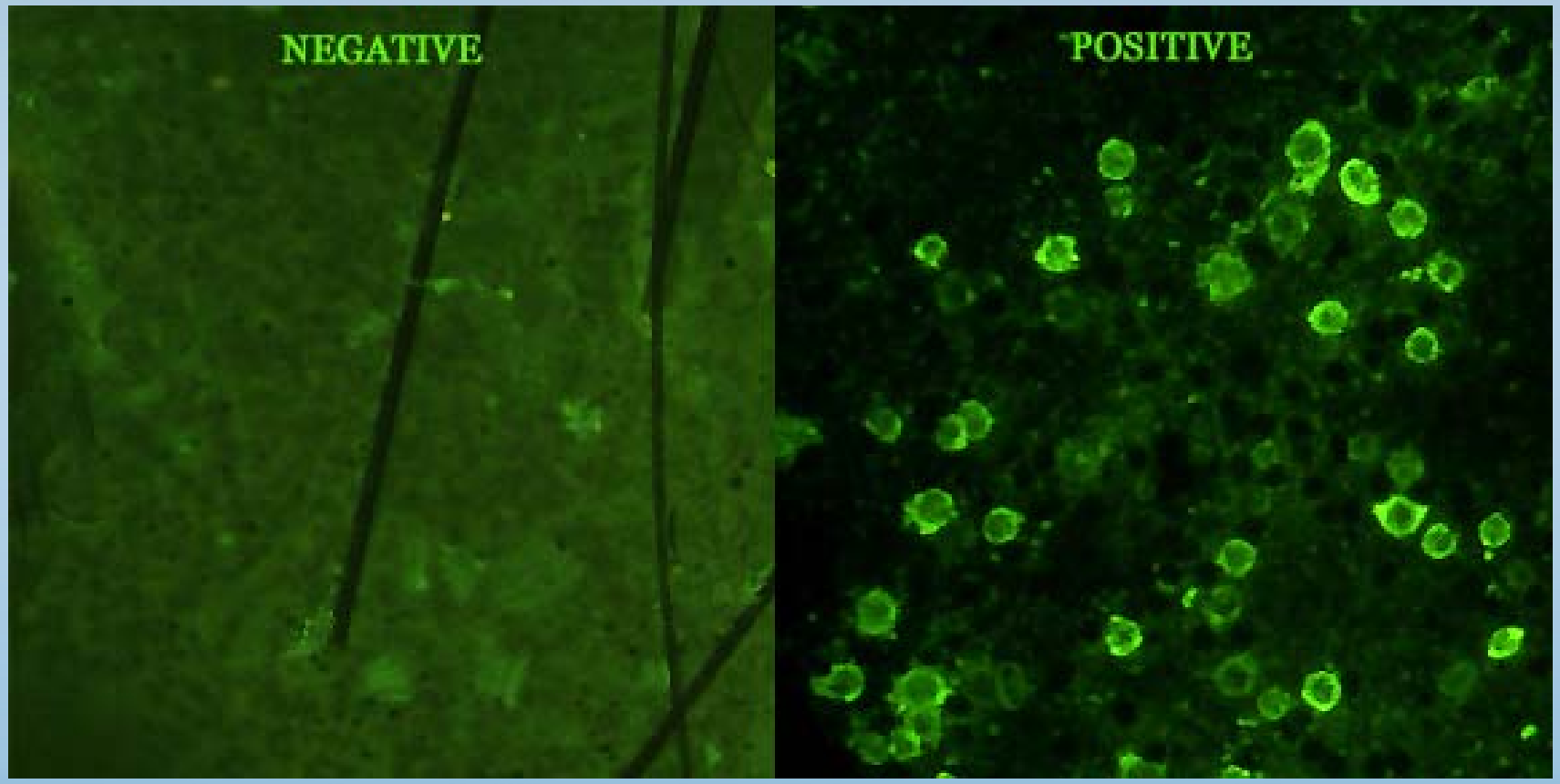
Typing by ELISA using mouse monoclonal Ab

Toxorhynchites splendens (intrathoracic inoculation)





Indirect Immunofluorescence Assay



Dengue Virus Isolation in C6/36 Cells

Diluted Serum/Plasma → C6/36 monolayers

5-7 days (at 28°C-35°C)

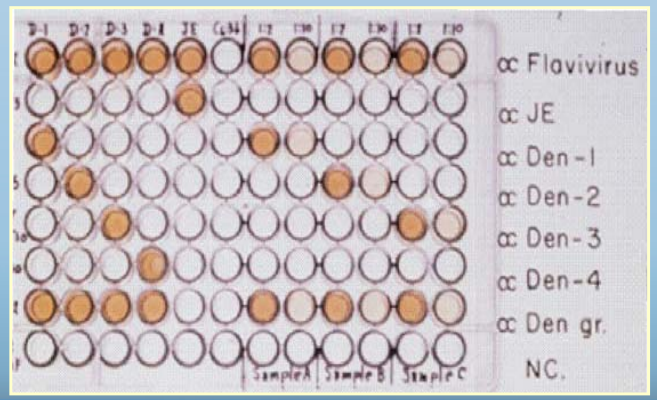
1st passage C6/36 cell fluid → C6/36 monolayers

5-7 days (at 28°C-35°C)

2nd passage C6/36 cell fluid → C6/36 monolayers

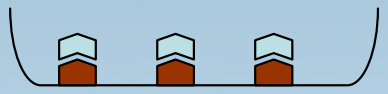
5-7 days (at 28°C-35°C)

3rd passage C6/36 cell fluid → Identification by Typing ELISA

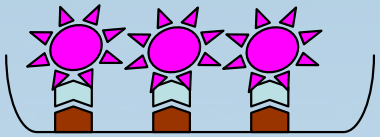


Dengue Virus Identification by Typing ELISA with Dengue Specific Monoclonal Antibodies

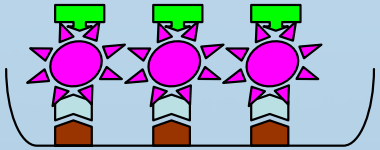
Coat plate with goat anti-mouse IgG



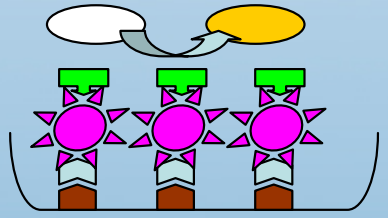
Add specific mouse monoclonal antibodies



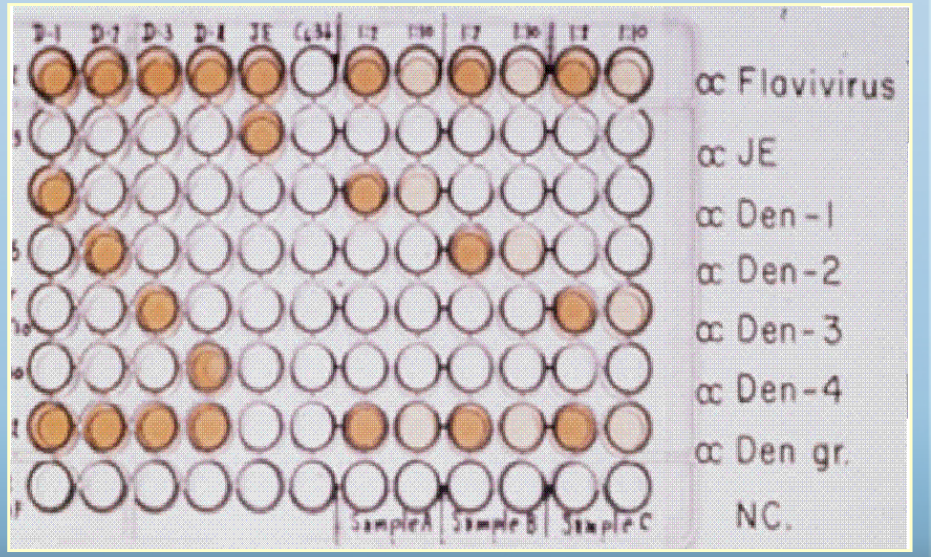
Add test sample, Dengue1-4 antigen, and normal cell fluid (negative control)



Add human anti-flavivirus HRP



Add substrate, stop reaction, and read O.D. at 492 nm.



Plaque Reduction Neutralization test

- Mixture of serum and reference virus or clinical isolate to determine if sample has antibodies that can stop virus from infecting cultured cells
  - Used to assess presence of neutralization antibodies
  - Can be used to determine seroconversion
  - Single specimen: provides prior dengue virus serotype exposure (limited by cross-reactivity between dengue serotypes and between flaviviruses soon after infection)
  - Paired specimens: 4-fold rise for any given serotype suggests an intervening dengue virus infection

Plaque Reduction Neutralization Test (PRNT)

3-5 4 fold dilutions of heat inactivated test sera +

Reference viruses:50 PFU

- Dengue 1(16007), Thailand
- Dengue 2(16681), Thailand
- Dengue 3(16562), Philippines
- Dengue 4(1036), Indonesia
- JE(SA-14-14-2), China

↓

LLC-MK2 or Vero cells

↓ 4-7 days

Stain with neutral red, plaque count

↓

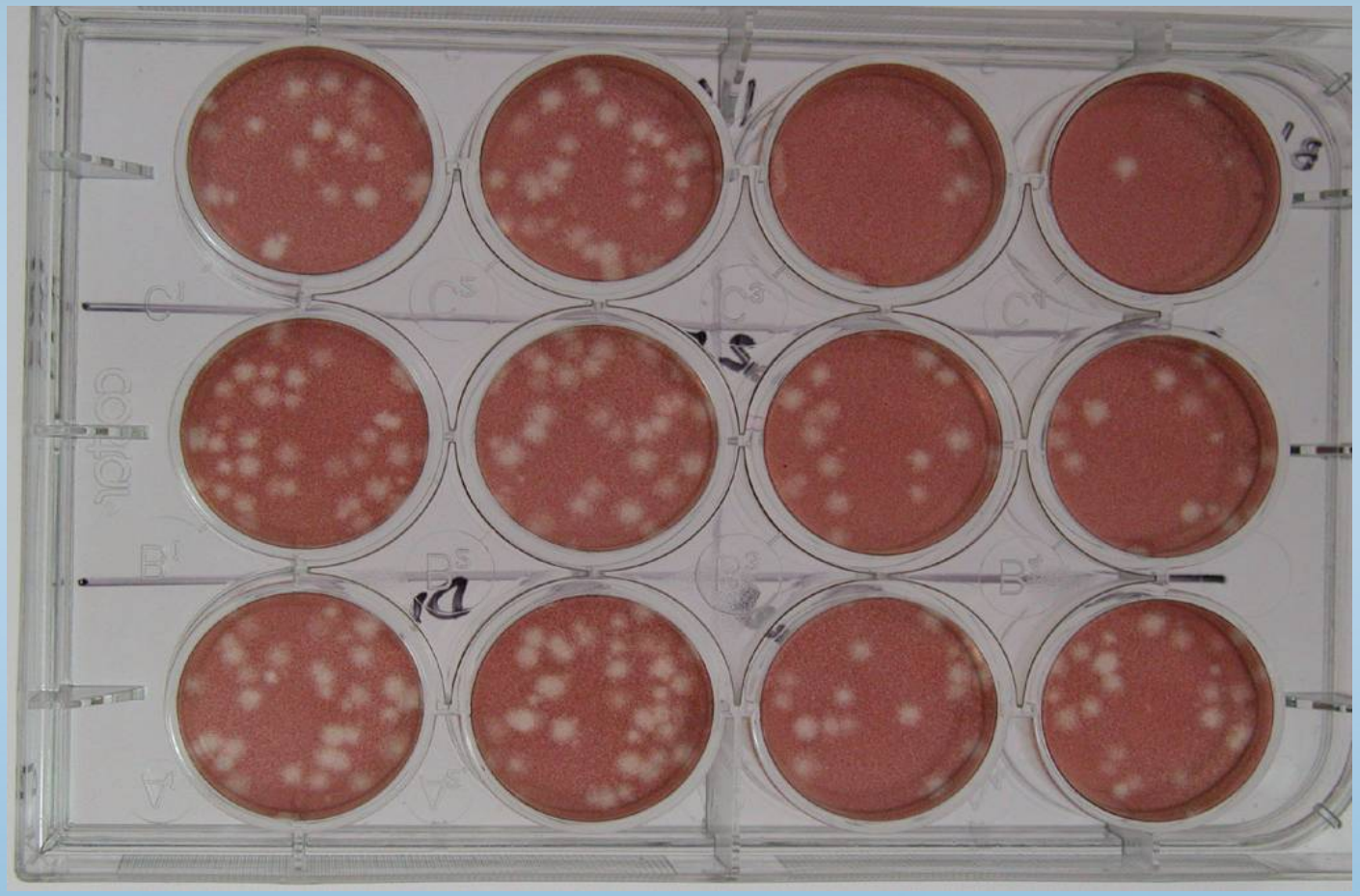
50% plaque reduction by Probit analysis

DEN-1 viral plaques per well and serial dilution of test sera

1:640

1:2,560

Virus control



1:10

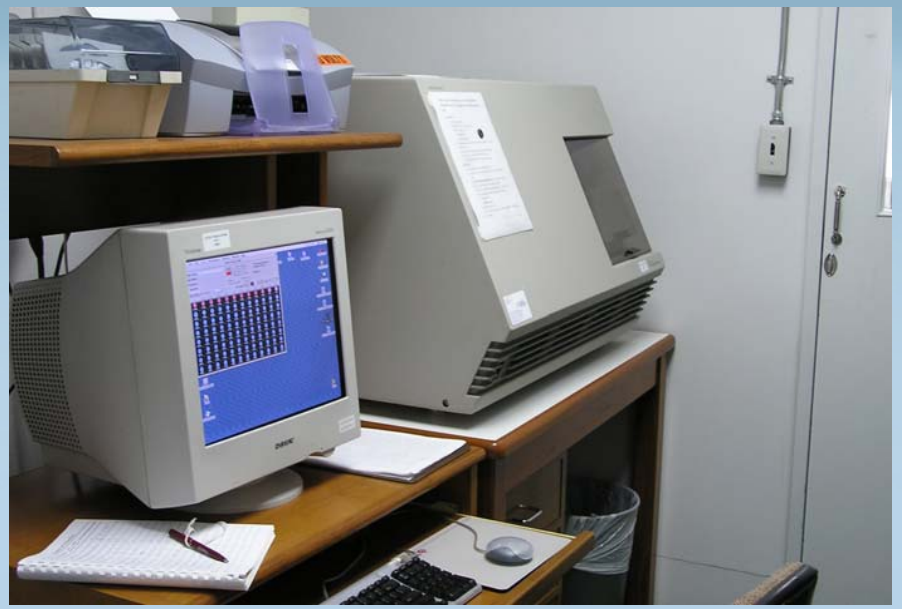
1:40

1:160

Variation of PRNT

- Single Dilution Neutralization
  - 1:30 dilution
  - Used for screening
  - Used as dengue serotype sero-survey
- Microneutraliztion
  - Can be used as PRNT
  - Can be automated
  - Readout by spectrophotometer
  - High Throughput

Real time PCR



Low Risk of Contamination

Data Acquisition

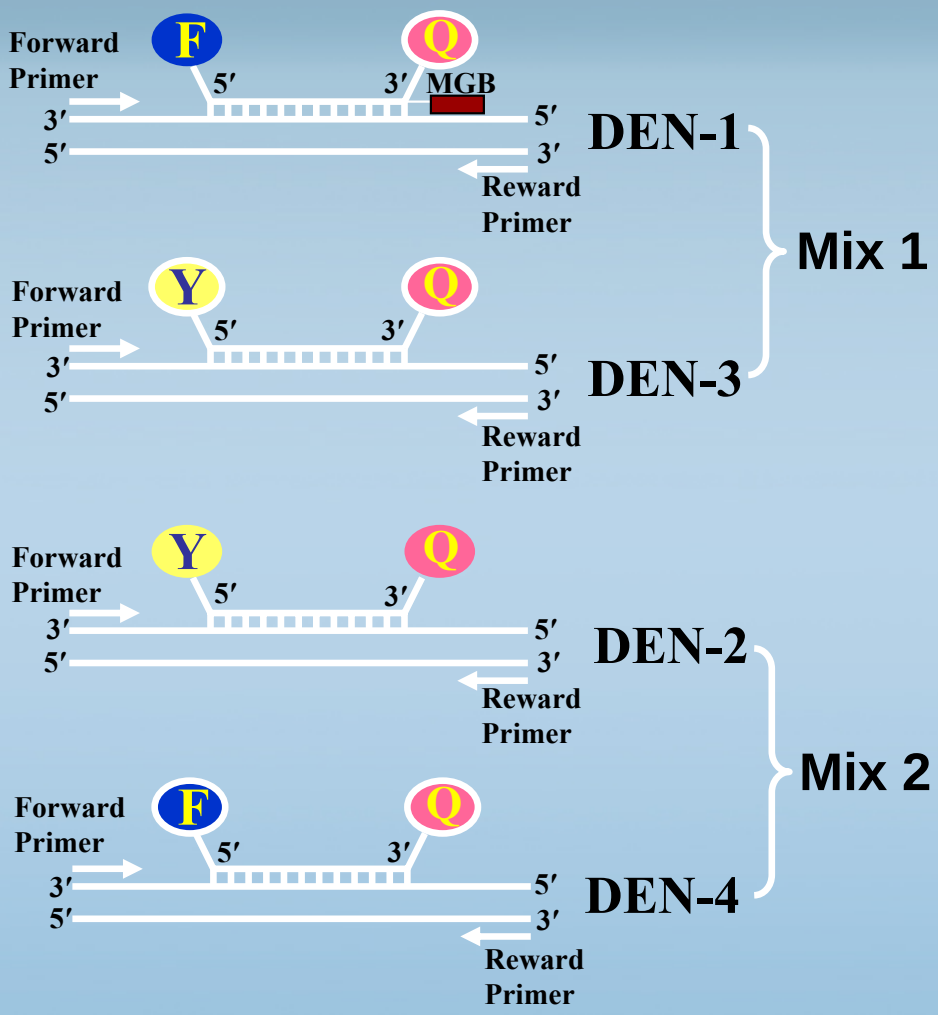
Amenable to Validation

Cost

More sophisticated equipment

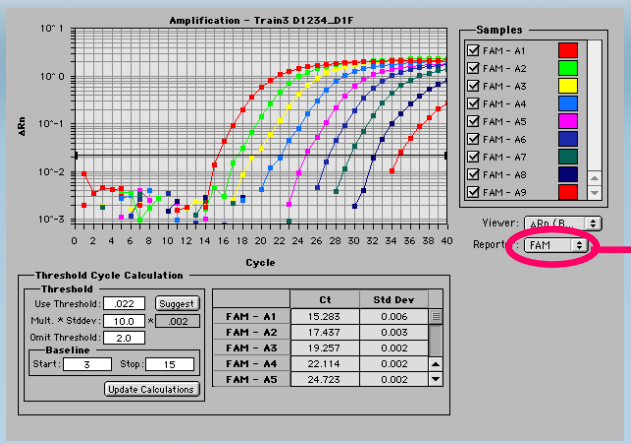
Dengue Quantitative Real-Time PCR

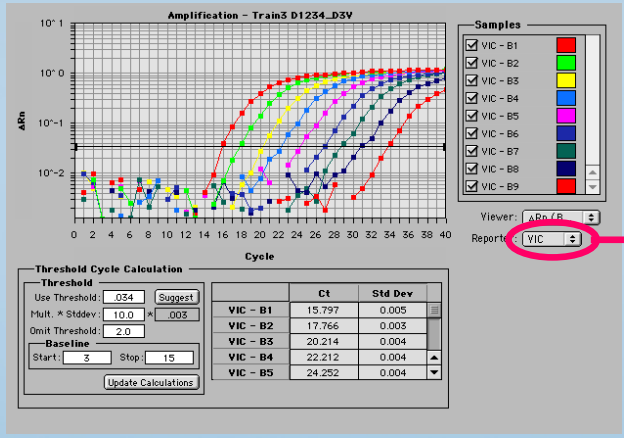
DEN 5′-noncoding region

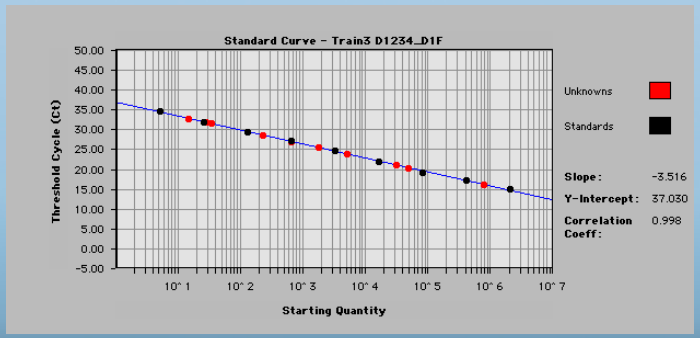


F=FAM Y=YAKIMA YELLOW

Q=Dark Quencher





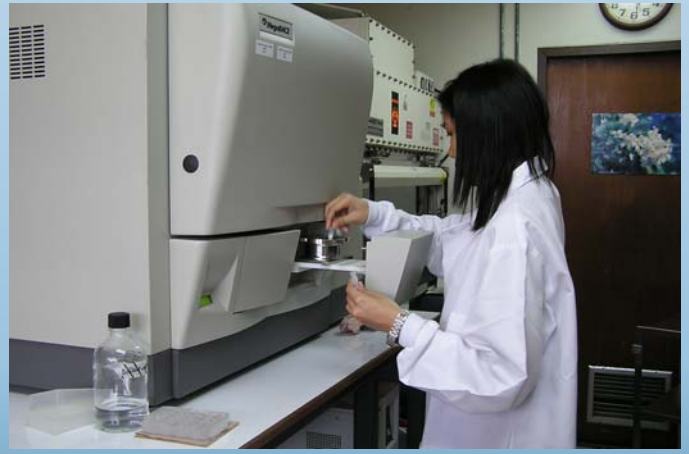


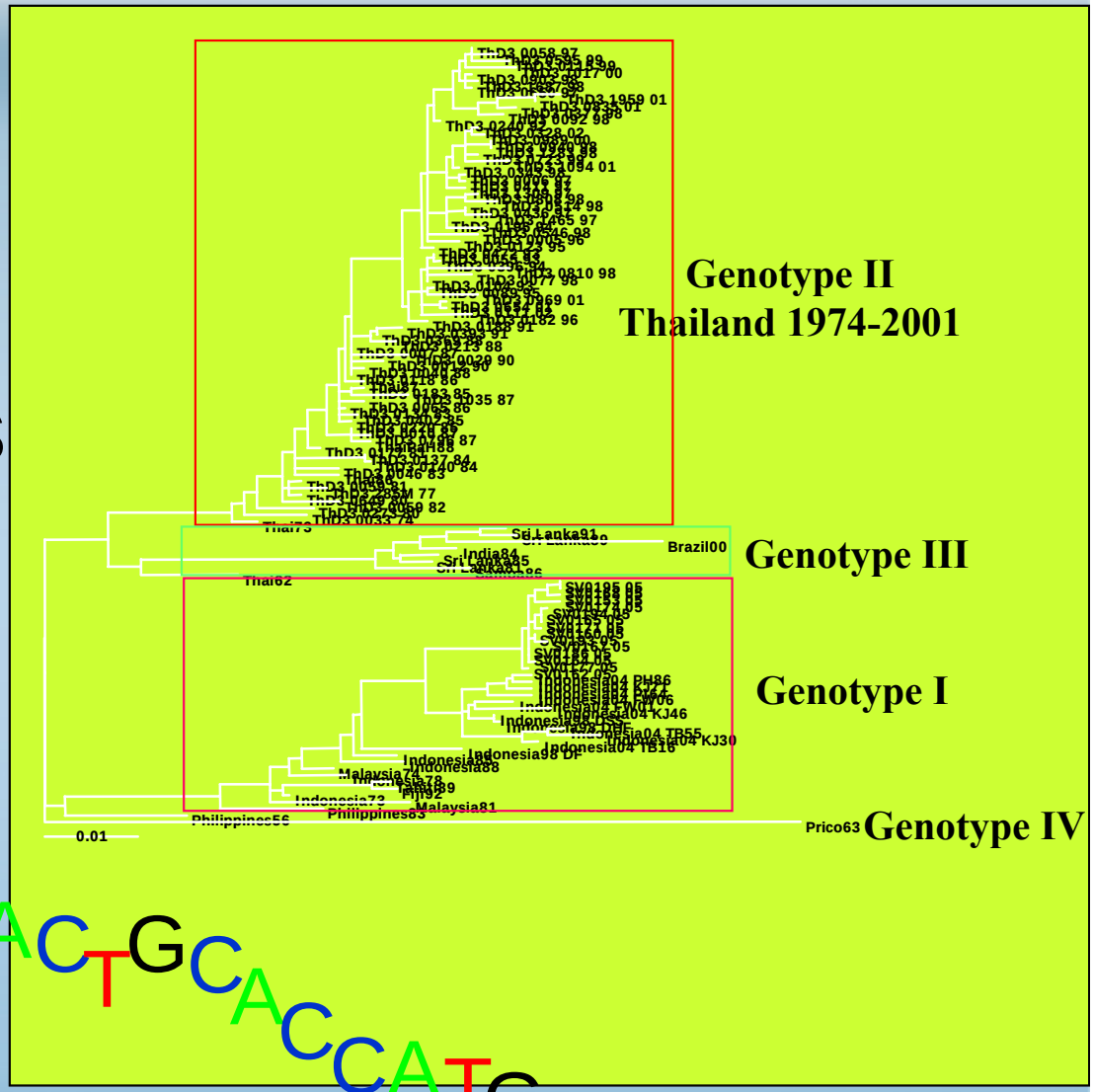
Detection of Dengue NS1 antigen

- Commercial NS1 Ag Detection Kits used at AFRIMS
- Commercial EIA and Rapid antigen are available but are not used in AFRIMS sponsored s

Genetic Sequencing

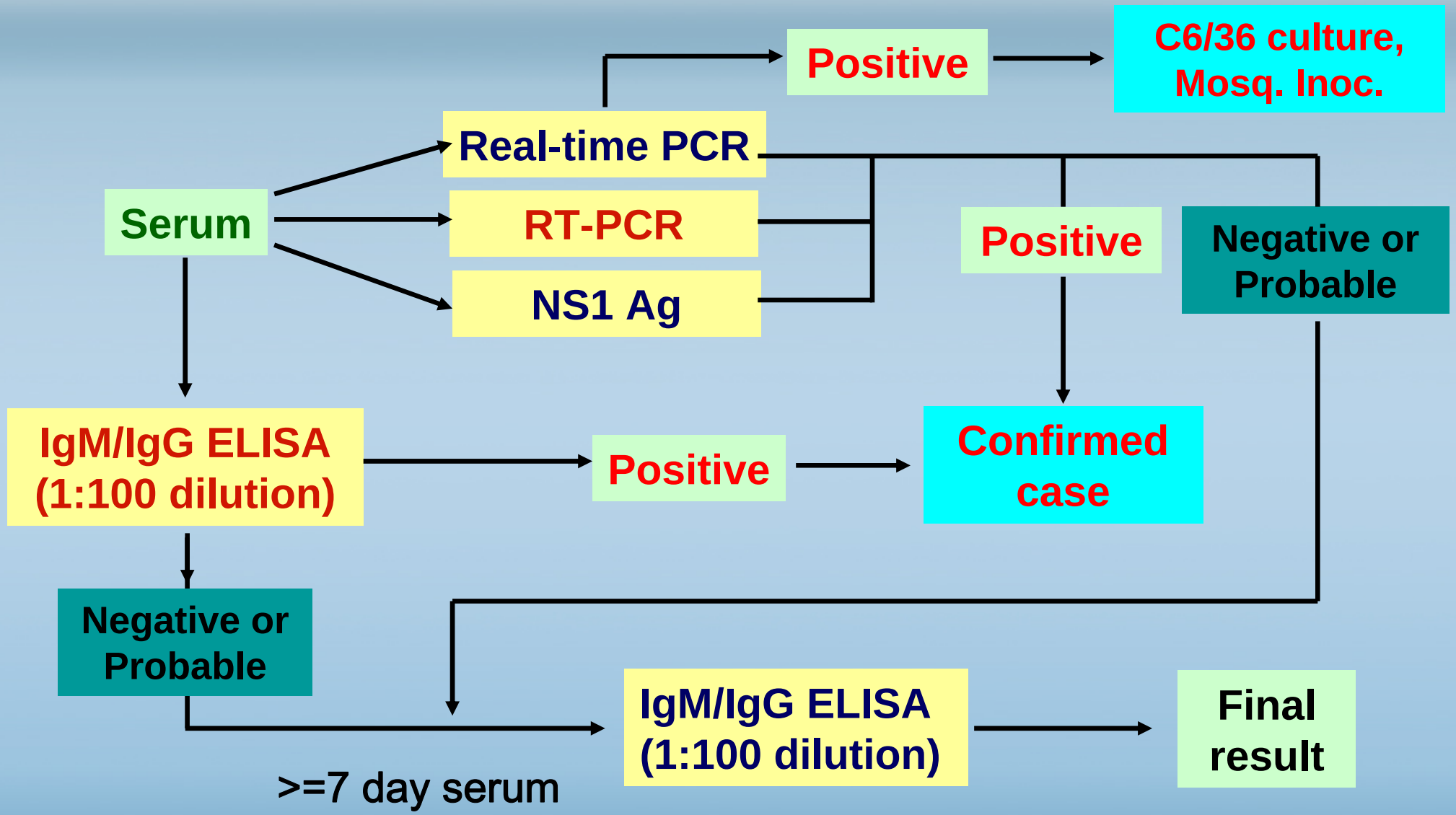
- Evolution Studies
- Genotyping
- Genetic Pathogenesis





AGTTACTGCACCATG

Flow Chart of Laboratory Diagnosis of Dengue Virus Infection



Questions