

BIOANALYTICAL CHARACTERIZATION AND SEPARATION OF DENGUE VIRUS

Biologics & Vaccine Analytics, MMD

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28Sep2020



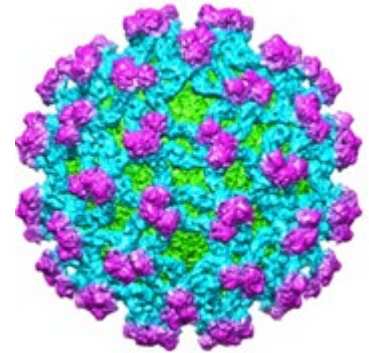
Outline

1. Introduction to Dengue virus
2. Introduction to CE
 - a) CZE-UV separations of Dengue Virus
 - b) CZE-LIF separations of antibody – dengue conjugation reactions
3. Cryo EM images of Dengue Virus
4. SDS-PAGE analysis of Dengue proteins
5. Conclusions & future directions

Dengue Virus – A Flavivirus

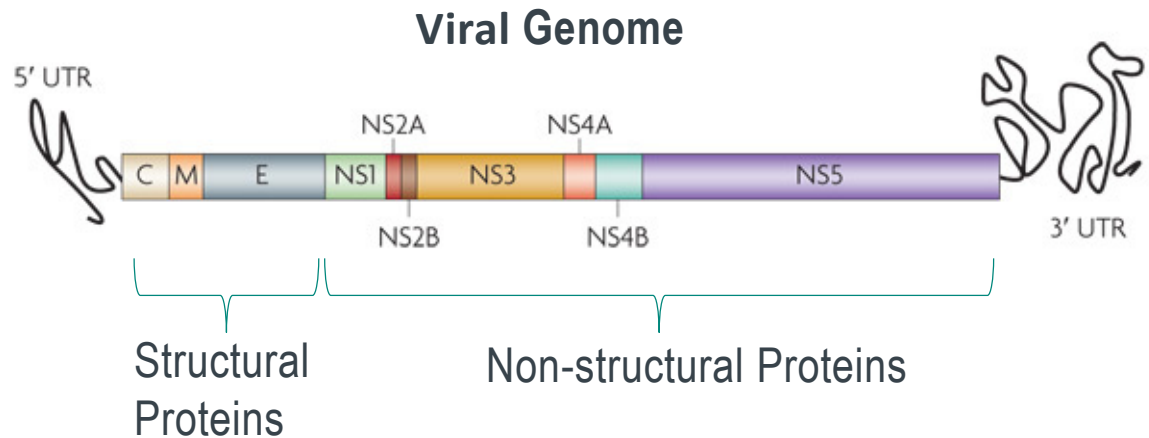
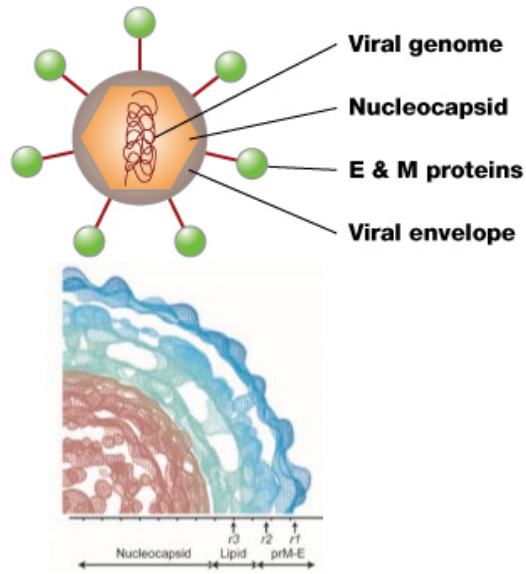
Dengue infection is a global health threat with a potential to affect at least 3.6B people

- **Family:** Flaviviridae
- **Genome:** Positive sense, single stranded RNA, 11Kb in size
- **Transmission:** Female *Aedes* mosquito
- **Incubation period:** Three to 14 days
- **Disease:** Dengue fever, dengue hemorrhagic fever and dengue shock syndrome
- **Epidemiology:** Globally an estimated 390 million infections occur annually
 - Out of 390M, 96M are symptomatic and reminder 294M are asymptomatic
- Each serotype provides specific long-term immunity and short term cross – immunity.



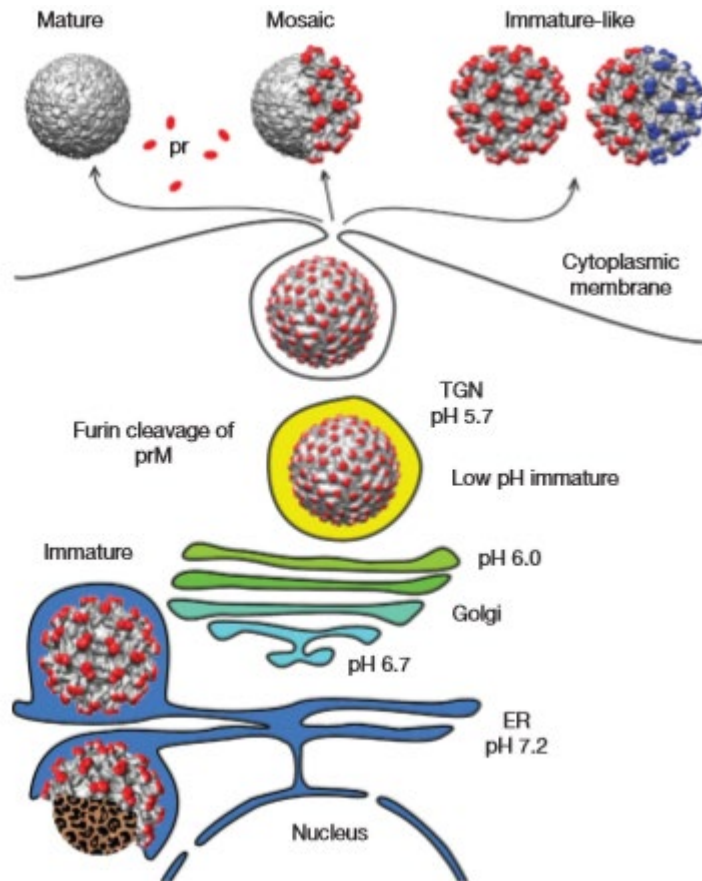
- Bäck, Anne Tuiskunen, and Ake Lundkvist.. *Infection ecology & epidemiology* vol. 3 (2013); 1-22.
- Gubler, Duane J. *The American journal of tropical medicine and hygiene* vol. 86,5 (2012): 743-744
- Mathieu, trauchessec et al. *Vaccine* vol 37 (2019), 3580-3587

Structure of Dengue Virus:



- Dengue virus is roughly spherical in shape
- Inside the virus, nucleocapsid is made of viral genome and C proteins
- Nucleocapsid is surrounded by viral envelope, a lipid bilayer
 - Embedded in viral envelope are E & M proteins than span through lipid bilayer
 - E & M form a protective outer layer that controls entry of virus into human cells

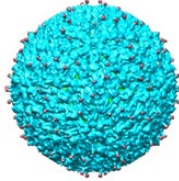
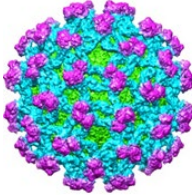
Schematic of Flavivirus Maturation Pathway



- Replication of RNA in cytoplasm
- Immature particles are formed by non-covalent association of prM-E heterodimers
- Virus export to low pH trans golgi network
 - Rearrangement of heterodimers into smooth surface
 - Cleavage of PrM by enzyme *Furin*
 - Forms mature particles

Dengue Virus Particle Characteristics

Table 1: Dengue viral particle characteristics

Viral Particle	Mature	Immature	Partially Immature
Surface morphology	Smooth	Spiky	Mix of smooth and spiky
Diameter	~50 nm	~60 nm	~50-60 nm
Infectivity	Infectious	Non-infectious	Can be infectious
Presence of Pr part of PrM protein	Absent	Present	Can be present
			

- Jiraphan Junjhon et al.; *Journal of Virology*, 2010, 84(16): 8353-8358.
- Guzman, M. G., Halstead, S. B., Artsob, H., Buchy, P., et al. *Nature reviews. Microbiology* 2012, 8(12): 7- 16

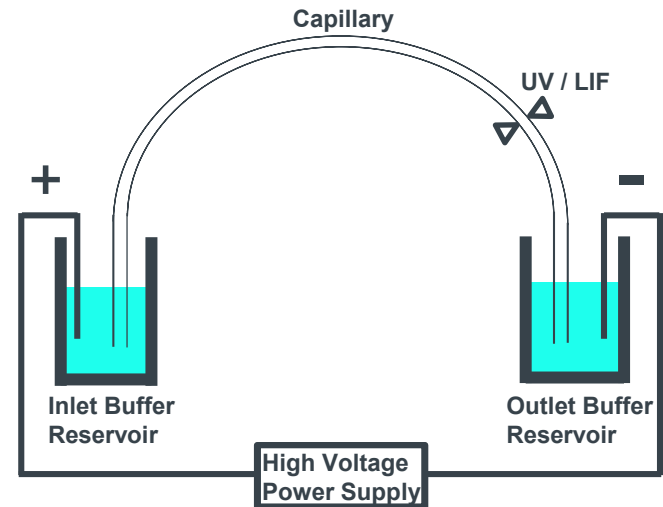
Goal:

“To develop a method that can quantify mature to immature particle ratio that can be used for process development and characterization to demonstrate process consistency”

Capillary Electrophoresis

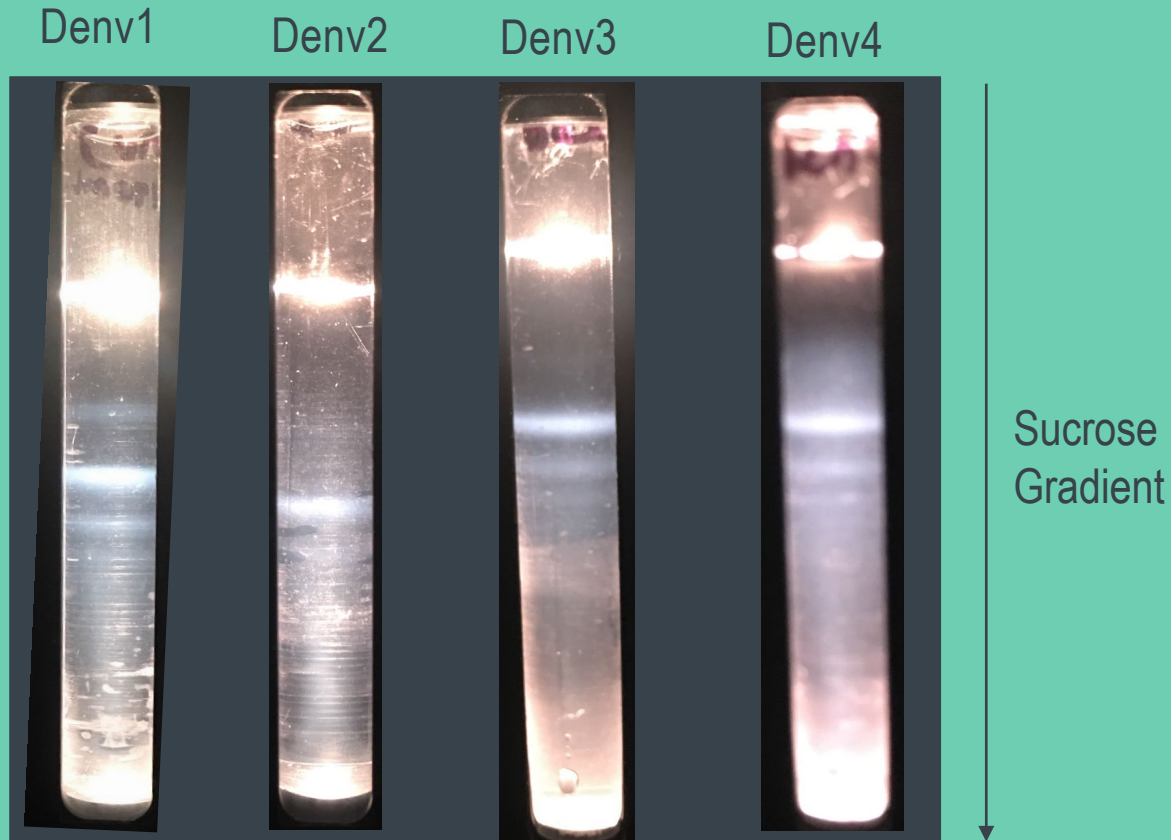
Advantages of Capillary Electrophoresis

- Couples to multiple detection systems
 - Mass Spectrometry
 - Fluorescence
 - UV-VIS
 - Electrochemical
- Small volume analysis
- Fast separation
- Automated (programmable)
- High throughput analysis

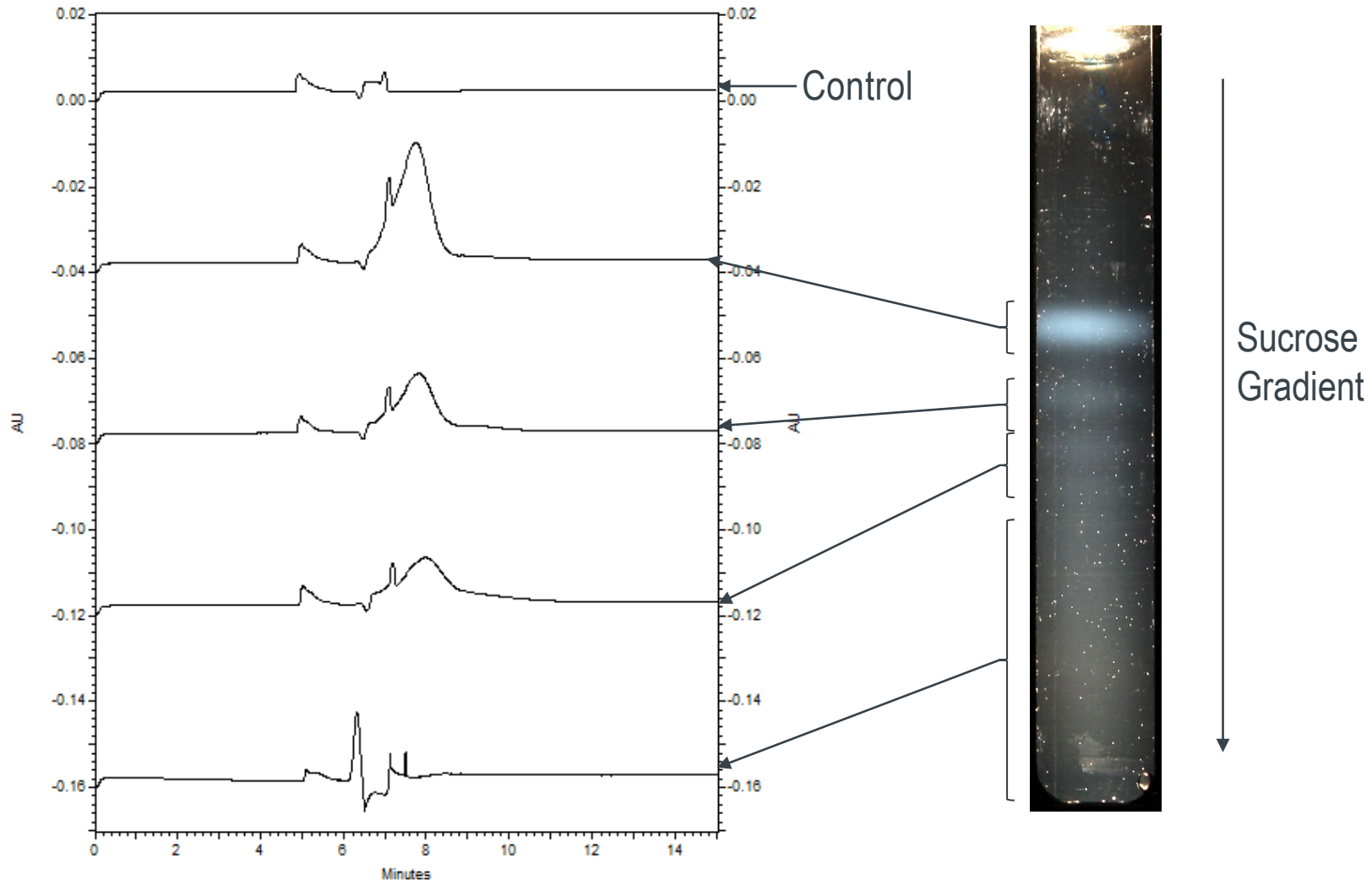


PA 800 Plus

Dengue Virus Purification by Sucrose Density Gradient Centrifugation



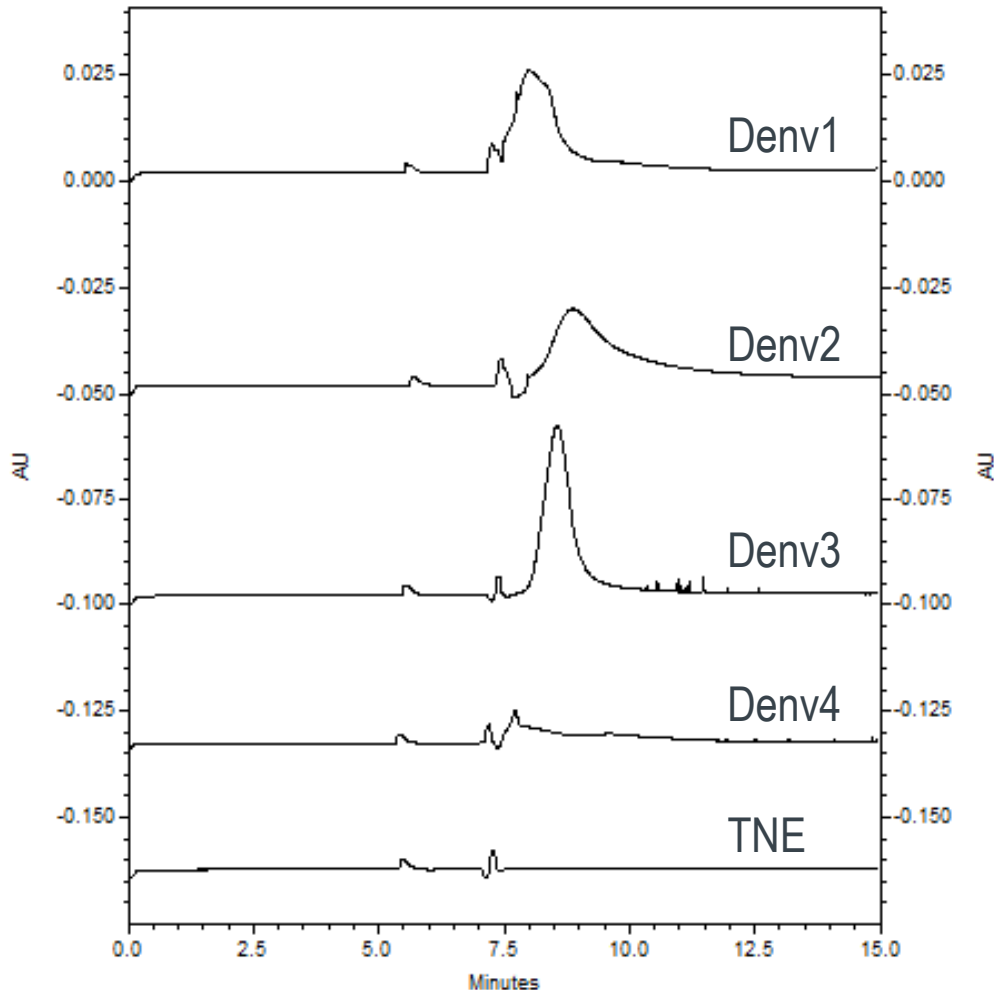
Separation of DENV3 Virus by Capillary Zone Electrophoresis



Hypothesis 1: The mature particles are in very low abundance to be seen

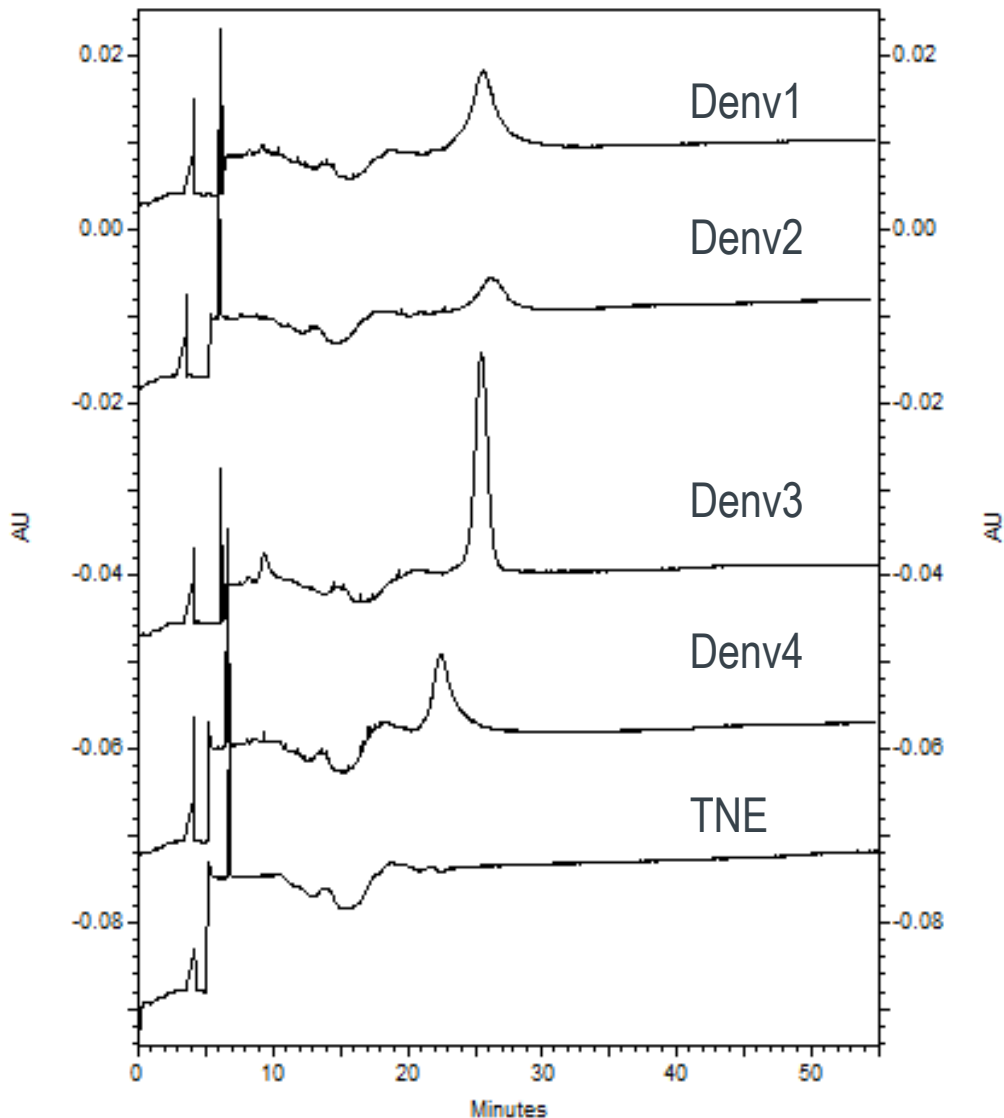
Hypothesis 2: The mature virus particles are not resolved from the immature virus particles

Separation of DENV1,2,3,4 Serotypes after Sucrose Gradient Centrifugation by CZE



- Denv1 – Asymmetric peak profile
- Denv3 – Symmetric peak profile
- Denv2,4 – Peak tailing
- Ionic interactions
- Hydrophobic interactions
- TNE buffer injected into BGE serves as a control
- Triplicate runs has an RSD in area of <4% and time <1%

Separation of Dengue Virus with Neutral Capillary

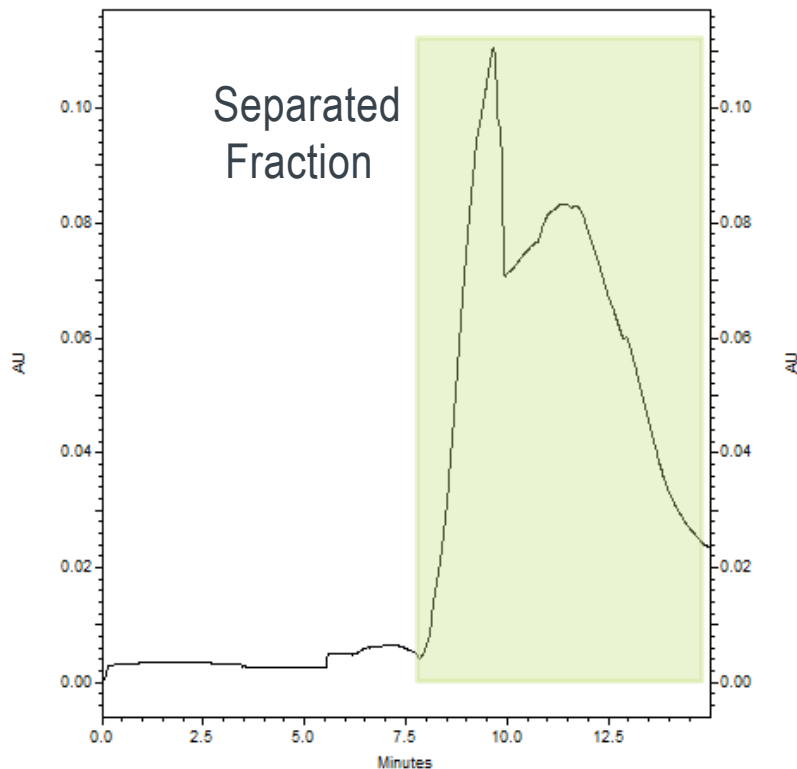


- Separation based on charge only
- No electro-osmotic flow
- Gaussian peak profile and peak tailing was removed using a neutral capillary

PCR Analysis Confirms Separated Peak by CZE is Dengue Virus

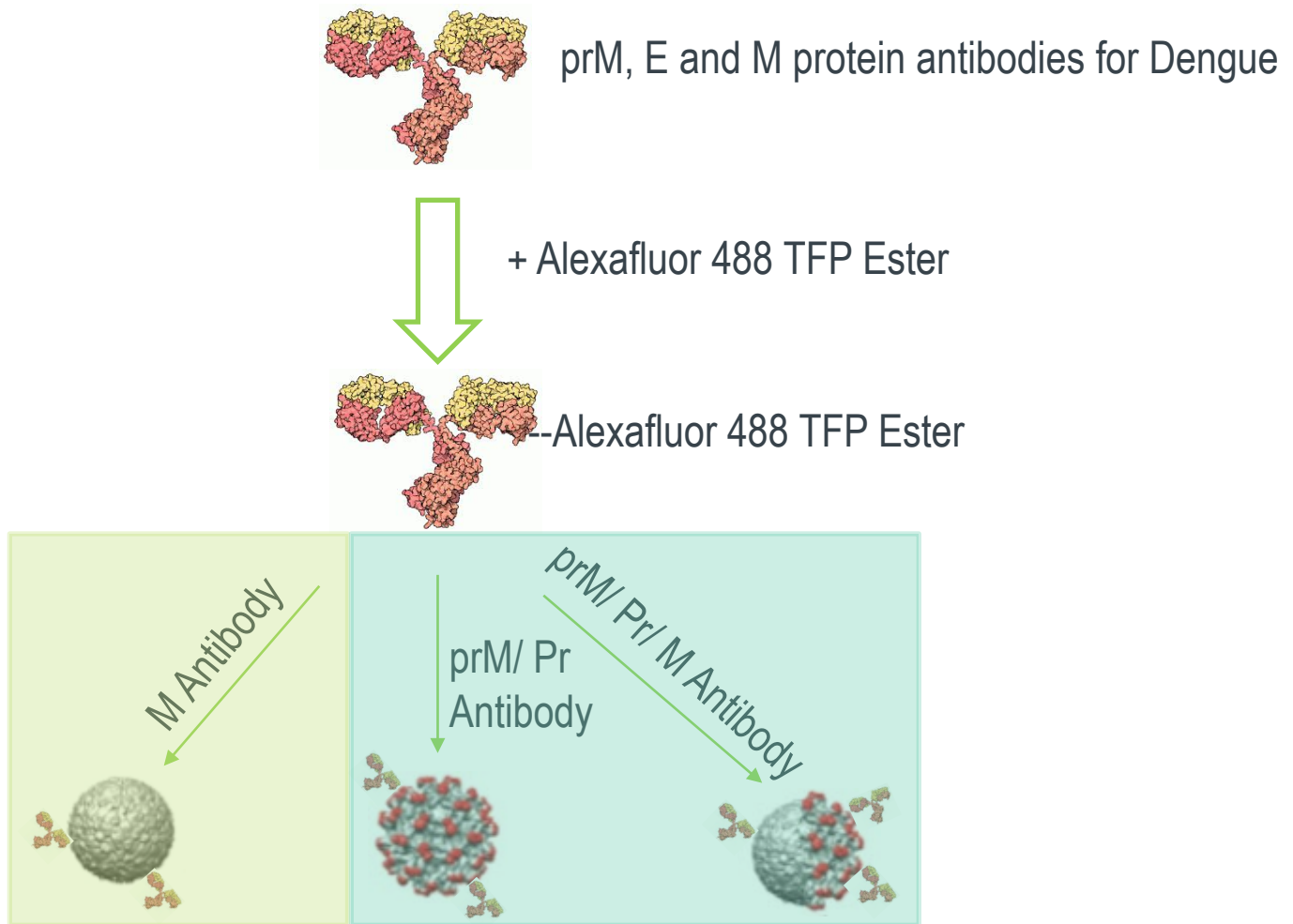
Sample	Avg Ct
Denv3 Positive Control (n=10)	11.5
Denv3 Separated Fraction (n=6)	19.6

$$\text{Cycle Turnover} \propto \frac{1}{\text{Amount of nucleic acid}}$$

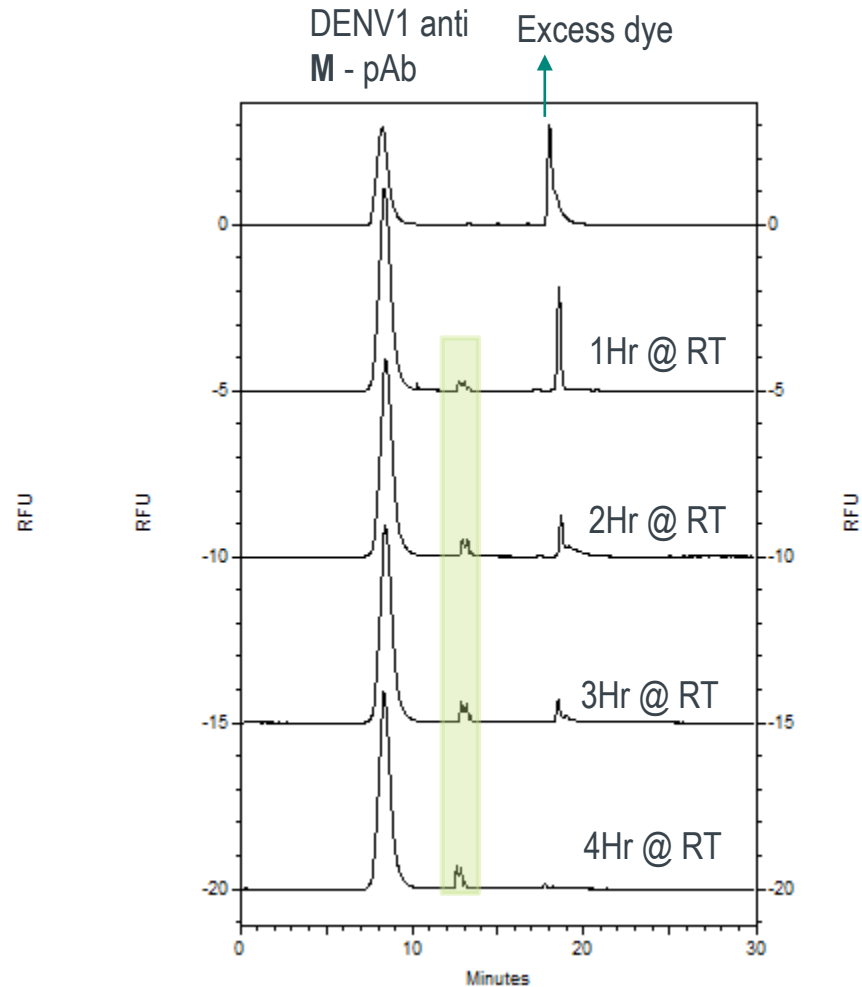
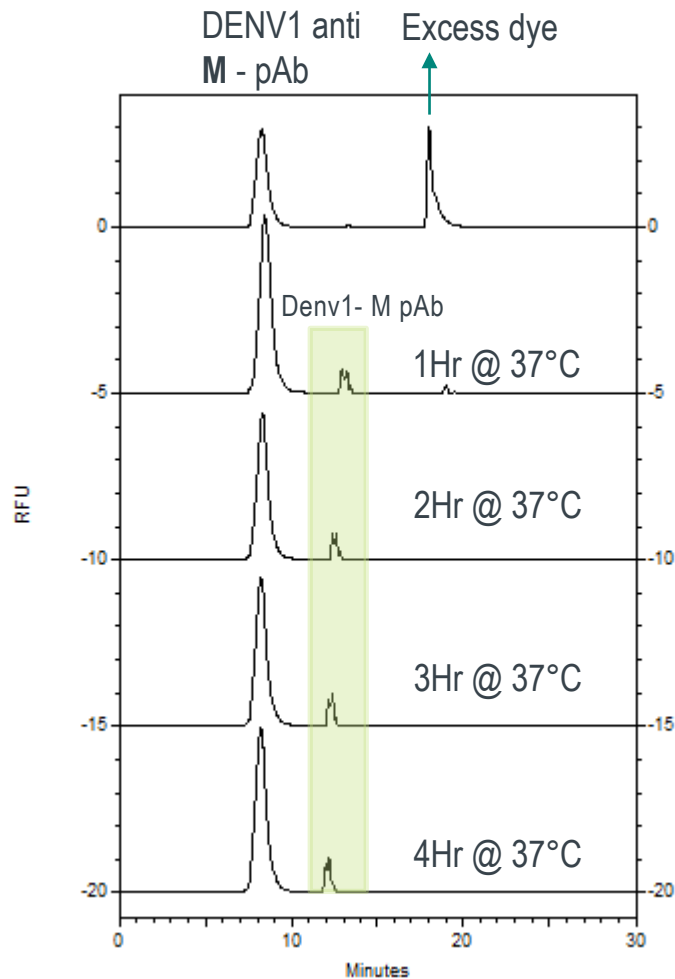


- Fraction collected from multiple runs
- Increased the sample injection

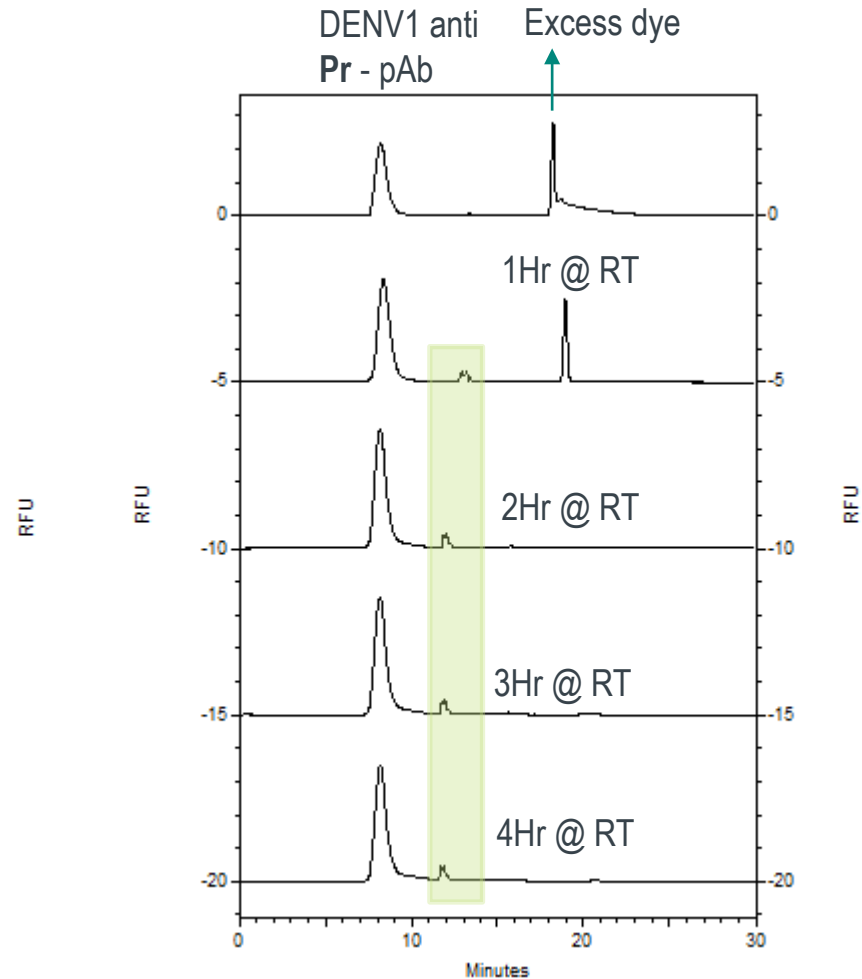
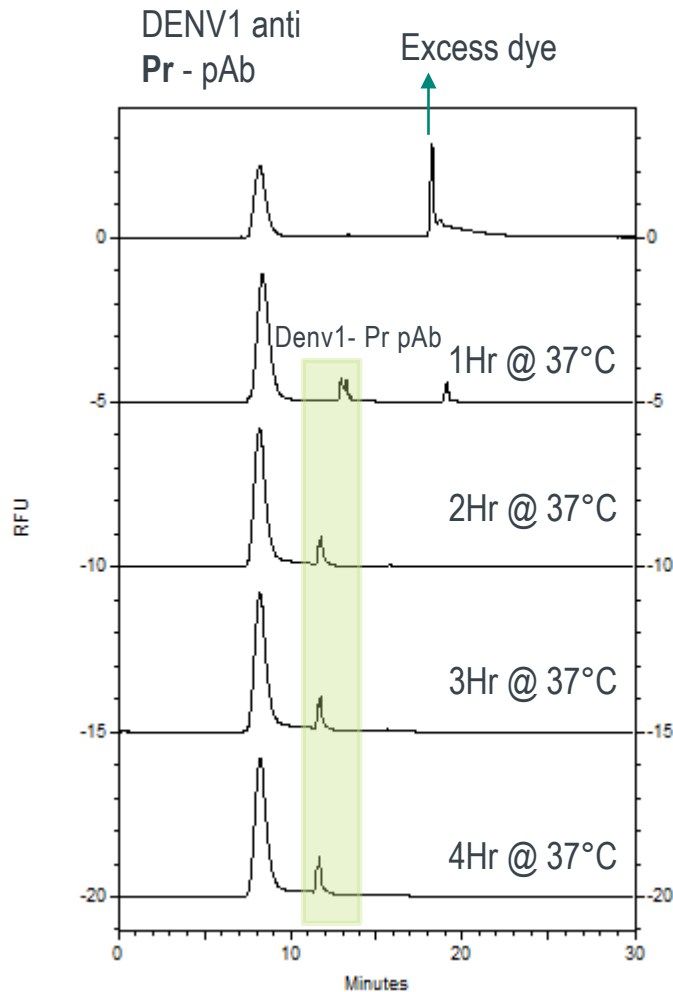
Antibody Dengue Virus Conjugation Reactions



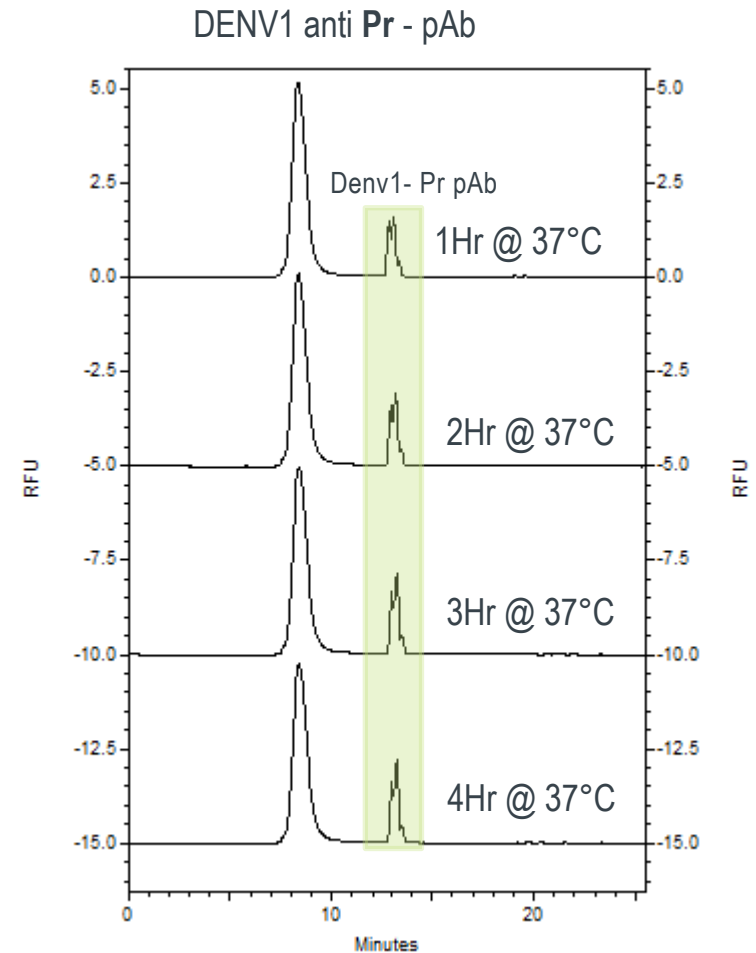
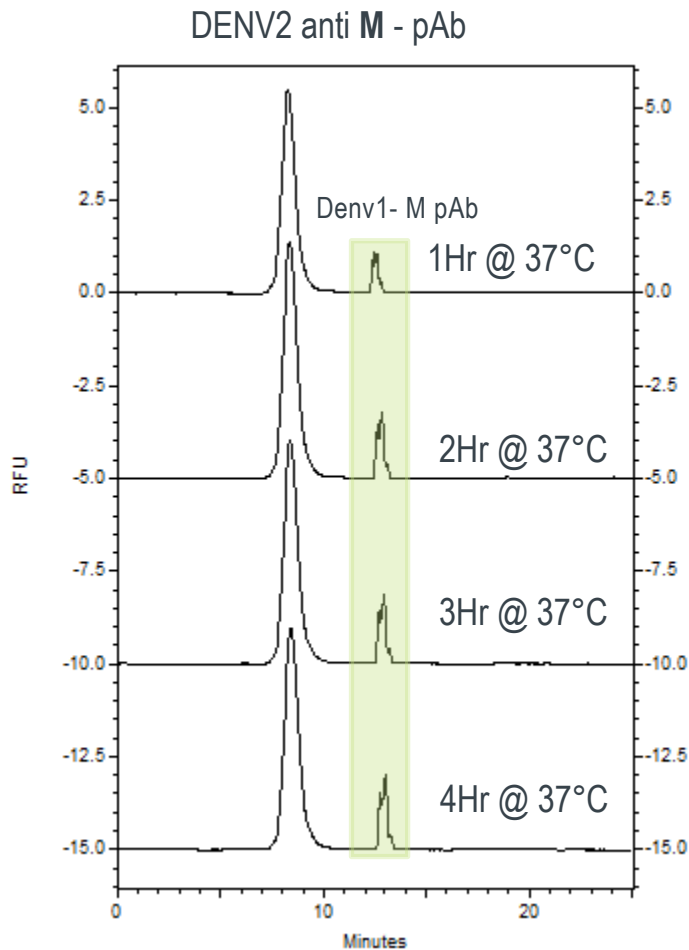
The Antibody Conjugation to Denv1 Increased with Increase in Time at 37°C and RT for M pAb



The Antibody Conjugation to Denv1 Increased with Increase in time at 37°C and RT for Pr pAb



The Antibody Conjugation to Denv1 Significantly Increased with Increase in Time at 37°C for M and Pr pAb After a 2-day 4°C Incubation



Key Takeaways

- Separation of Dengue virus is achieved by CZE technique
- Sucrose gradient centrifugation resulted in pure form of virus
- Antibody of Pr and M cannot be compared as the true binding co-efficient is not known
- Virus conjugation to antibody occurs best at 37°C with incubation time ≥ 240 min and additional 4°C incubation increases the overall conjugation yield.
- mAb are required to differentiate mature and immature particles

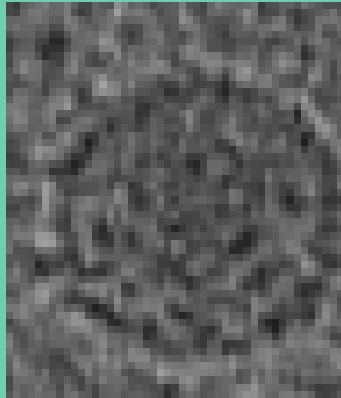
Cryo EM Images of Dengue Virus

Goal: To study the viral particle surface characteristics and size of the particle

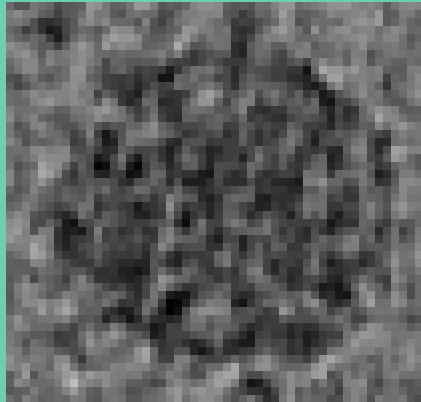
- Cryo EM images of Dengue viral particles
- Quantitative analysis of mature, immature and partially immature particles

Cryo Electron Microscopy Images of Dengue Virus

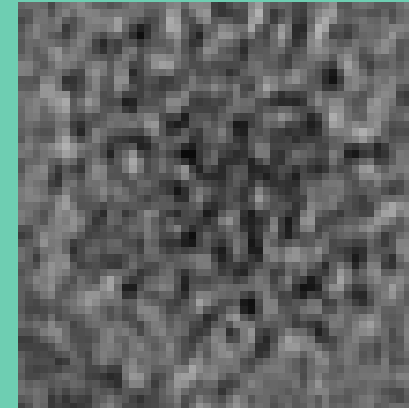
Mature



Partially Mature



Immature



- The Dengue virus particles had dense interiors and were roughly round
 - **Mature** particles had a dense interior and a surface that was smooth
 - **Partially mature** particles had a dense interior and a surface that had both smooth and spiky regions
 - **Immature** particles had a dense interior and a surface that was spiky.

Cryo EM Particle Size Analysis of Denv1-4

Particle Type:	Particle Diameter Avg (nm)	Spikes Diameter Avg (nm)
Denv1	43-61	5-6
Denv2	40-54	4-5
Denv3	40-57	4-6
Denv4	43-52	4-5

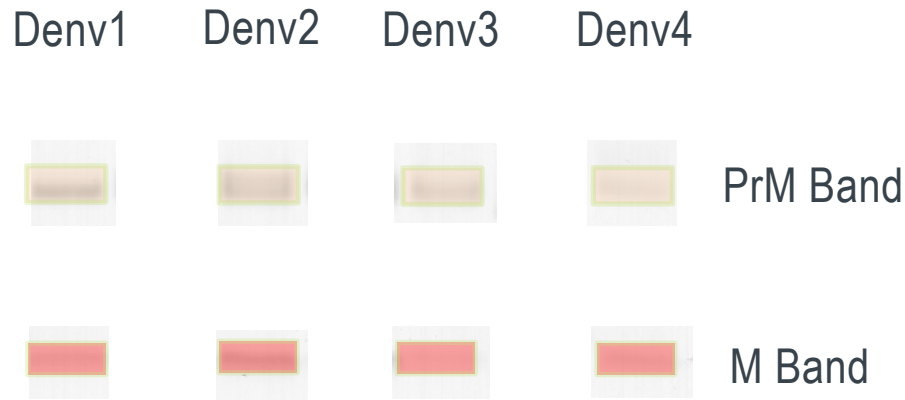
Size order: Immature > Partially Immature > Mature

SDS-PAGE of Dengue Virus proteins

Goal: To study the viral proteins and quantify the M and prM proteins

- Identify structural proteins
- Quantitative analysis of M and prM proteins

SDS-PAGE Separation of Dengue Proteins for Serotype 1-4



- SDS-PAGE analysis shows the separation of structural proteins
- prM and M protein bands were excised, digested and confirmed with MS
- PrM to M protein ration can be obtained

Conclusions & Future Directions

- CE can be used as a screening technique to characterize Dengue DS
- Quantitative analysis of mature and immature particles can be done by Cryo-EM and other techniques
- SDS-PAGE analysis can be used to quantitate protein composition and maturity
- Antibody based differentiation is possible if mAb were used and they bind to different proteins with same degree

Future Directions:

- New analytical approaches to characterize particle are underway using analytical ultracentrifuge, HPLC-MS (peptide analysis) and other flow cytometry techniques

Acknowledgements:

Biologics & Vaccine Analytics

- Pamela Mathis
- Luca Benetti
- Takle Garry Brian
- Anastasija Jancina
- Cara Groller
- Kevin Ludwig
- Luke Serensits
- Natalie Dominique

Analytical R&D

- Richard Rustandi
- Josef Vlasak
- Dawod Mohammed
- Wang Shiyi
- Price Colleen
- Rolling Judith
- Ashley Prout
- Damon Barbacci
- Dave Foreman

Bio-Process

- Michael Winters
- Laura Kuczynski

Merck Post Doc Association

