

**510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION
DECISION SUMMARY**

A. 510(k) Number: K113336

B. Purpose for Submission: New device

C. Measurand: Dengue Virus (DENV) RNA target sequence

D. Type of Test: An *in vitro* molecular diagnostic test that consists of a panel of oligonucleotide primers and dual-labeled hydrolysis (TaqMan®) probes for the qualitative detection of dengue virus target sequences in serum and plasma using nucleic acid isolation, amplification, and detection on the ABI 7500 Fast Dx Real-Time PCR instrument.

E. Applicant: Centers for Disease Control and Prevention (CDC)

F. Proprietary and Established Names: CDC DENV-1-4 Real-Time RT-PCR Assay. Common Name: Dengue Real-Time RT-PCR

G. Regulatory Information:

1. Regulation section: 21 CFR 866.3946: Dengue Virus Nucleic Acid Amplification Test Reagents
2. Classification: Class II (de novo)
3. Product code: OZB, NSU
4. Panel: Microbiology (83)

H. Intended Use:

1. Intended use(s):

The CDC DENV-1-4 Real-Time RT-PCR Assay is intended for use on an Applied Biosystems (ABI) 7500 Fast Dx Real-Time PCR Instrument:

- For the diagnosis of dengue in serum or plasma collected from patients with signs and symptoms consistent with dengue (mild or severe) during the acute phase;
- For the identification of dengue virus serotypes 1, 2, 3 or 4 from viral RNA in serum or plasma (sodium citrate) collected from human patients with dengue during the acute phase;
- To provide epidemiologic information for surveillance of circulating dengue viruses.

Testing of clinical blood specimens (serum or plasma) with the CDC DENV-1-4 Real-Time RT-PCR Assay should not be performed unless the patient clinical and/or epidemiologic criteria for testing suspect dengue cases.

The CDC DENV-1-4 Real-Time RT-PCR Assay is not FDA cleared or approved for the screening of blood or plasma donors.

Negative results obtained with this test do not preclude the diagnosis of dengue and should not be used as the sole basis for treatment or other patient management decisions.

This device is for distribution to laboratories with personnel who have training and experience in standardized molecular diagnostic testing procedures and viral diagnosis, and appropriate biosafety equipment and containment

2. Indication(s) for use:

Same as Intended Use

3. Special conditions for use statement(s):

For *in vitro* diagnostic use

4. Special instrument requirements:

Extraction:

- a. Roche MagNA Pure LC Total NA Kit and Magna Pure Instrument.
- b. Qiagen QIAamp® DSP Viral RNA Mini Kit (Manually) and/or in combination with the QIAcube Instrument (small centrifuge)

PCR: Applied Biosystems® 7500 Fast Dx Real-Time PCR Assay instrument with System Sequence Detection 1.4 Software provided by manufacturer.

I. Device Description:

The CDC DENV-1-4 Real-Time RT-PCR Assay is a panel of oligonucleotide primers and dual-labeled hydrolysis (TaqMan®) probes designed to be utilized in real-time RT-PCR assays using the ABI 7500 Fast Dx Real-Time PCR instrument for detection of DENV-1, -2, -3 and -4 viral RNA in serum or plasma from human patients with dengue.

The CDC DENV-1-4 Real-Time RT-PCR Assay includes control materials as described below:

- Positive control materials, which consist of heat-inactivated DENV-1 Haw, DENV-2 NGC, DENV-3 H87, and DENV-4 H241 viruses.
- A Human Specimen Control (HSC), consisting of noninfectious (beta propiolactone inactivated) cultured human cell material that provides a positive signal in the assay and demonstrates successful recovery of RNA as well as the integrity of the RNA extraction reagents.
- Internal positive control, the human RNase P (RP) primer and probe set detects human RP and is used with each clinical specimen to indicate that adequate isolation of nucleic acid resulted from the extraction of the specimen. A positive RP assay result also ensures that common reagents and equipment are functioning properly and demonstrates the absence of inhibitory substances.

RNA extractions can be done using the Qiagen QIAamp® DSP Viral RNA Mini Kit either manually or in combination with the QIAcube Instrument (small centrifuge) and/or Roche MagNA Pure LC total nucleic acid isolation kit and Magna Pure Instrument.

The RT-PCR assay is run on the Applied Biosystems® 7500 Fast Dx Real-Time PCR instrument using Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System. The assay can be run in singleplex (each DENV serotype detected in a separate reaction) or in multiplex (four DENV serotypes are run in the same reaction). The two formats provide equal sensitivity.

Device Components (Provided)

Box 1: Detection Kit (Primer and Probe Sets) and Package Insert/Instructions for Use

Box 2: Positive Control Kit (A mix of heat inactivated DENV-1, -2, -3 and -4 standards)

Box 3: Human Specimen Extraction Control (HSC)

Ancillary Reagents (Not provided): The following is a list of ancillary reagents that are not supplied with the CDC DENV-1-4 Real-Time RT-PCR Assay. The Invitrogen and Roche products are included in CDC's reagent qualification program.

	Reagents	Quantity	Catalog No
rRT-PCR Enzyme Mastermix Options	Invitrogen SuperScript™ III Platinum® reactions One-Step Quantitative RT-PCR System (without Rox)*	100 reactions	11732-020
		500 reactions	11732-088
Nucleic Acid Purification Kit Options	Qiagen QIAamp® DSP Viral RNA Mini Kit and/or Qiagen QIAcube **	50 Extractions	61904 or 9001292
	MagNA Pure LC total Nucleic Acid Isolation Kit on the MagNA Pure LC 2.0 instrument*	192 Extractions	03 038 505 001

*The CDC DENV-1-4 Real-Time RT-PCR Assay test performance requires that only qualified ancillary reagent lots be used with the device. Any lots not specifically qualified by the CDC-Dengue Branch for use with the CDC DENV-1-4 Real-Time RT-PCR Assay are not valid for use with this device, and may affect device performance.

**Qiagen QIAamp® DSP Viral RNA Mini Kit and Qiagen QIAcube are produced under Good Manufacturing Practices (GMP).

Lots which are qualified under this process are communicated through a Qualified Reagent Lot List. This list is maintained by CDC and made available to the end users of the CDC DENV-1-4 Real-Time RT-PCR by including it with the Product Insert as well as through the Product Support mechanism identified in the Product Insert.

J. Substantial Equivalence Information:

1. Predicate device name(s): Not applicable
2. Predicate Numbers (s): Not applicable
3. Comparison with predicate: Not applicable

K. Standard/Guidance Documents Referenced (if applicable):

1. CLSI EP5: Evaluation of Precision Performance of Clinical Chemistry Devices- Second Edition, Villanova PA
2. CLSI EP7-A2: Interference Testing in Clinical Chemistry; Approved Guideline, 2nd Ed.
3. CLSI EP17-A: Protocols for Determination of Limits of Detection (2004).

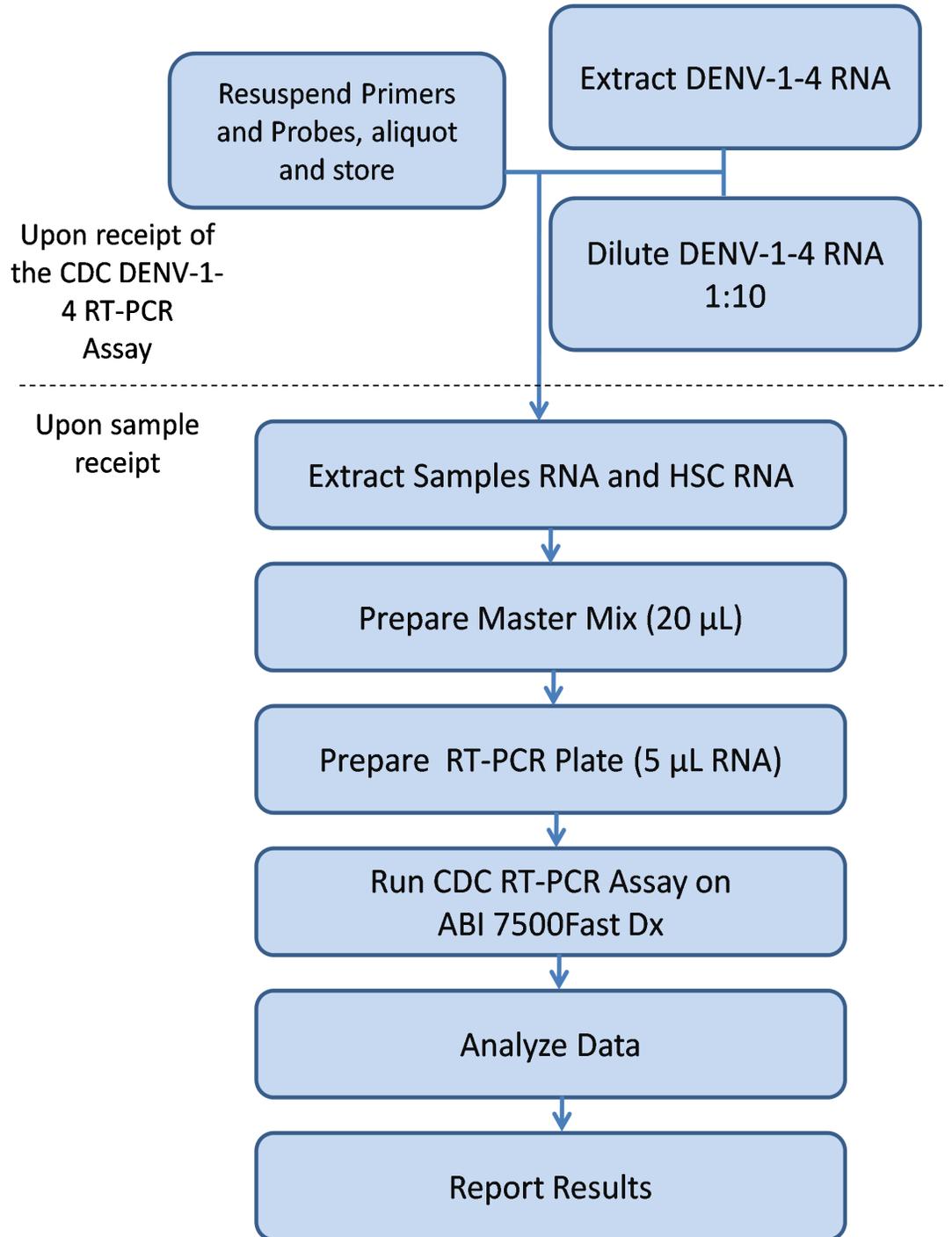
Note: A Special Controls Guidance Document will be promulgated.

L. Test Principle:

The CDC DENV-1-4 Real-Time RT-PCR Assay is used in rRT-PCR on an ABI 7500 Fast Dx Real-Time PCR Instrument. The CDC DENV-1-4 Real-Time RT-PCR assay includes a set of oligonucleotide primers and dual-labeled hydrolysis (Taqman®) probes for in vitro qualitative detection of dengue virus serotypes 1, 2, 3 or 4 from serum or plasma collected from human patients with signs and symptoms consistent with dengue (mild or severe). The targeted regions of viral RNA are transcribed into complementary DNA (cDNA) and amplified by the polymerase chain reaction (PCR). The fluorescently labeled probes anneal to amplified DNA fragments and the fluorescent signal intensity is monitored by the ABI 7500 Fast Dx instrument during each PCR cycle. Amplification of target is recorded as increase of fluorescence over time in comparison to background signal.

A positive control virus mix is also included, which consists of heat-inactivated DENV-1 Haw, DENV-2 NGC, DENV-3 H87, and DENV-4 H241 viruses. A Human Specimen Control (HSC) is a noninfectious cultured human cell material that provides a positive signal in the assay and demonstrates successful recovery of RNA as well as the integrity of the RNA extraction reagent. The human RNase P RNA (RP) is present in cultured cell material and in most clinical samples and detectable by RT-PCR using the primers and probes provided. The CDC DENV-1-4 Real-Time RT-PCR Assay can be run in singleplex (each DENV serotype detected in a separate reaction) or in multiplex (four DENV serotypes are run in the same reaction). These two formats provide equal sensitivity.

Summary of Dengue Testing Process



Recording and interpretation of the assay results:

CDC DENV-1-4 Real-Time RT-PCR Assay Users Guide for Interpretation of Results – Quick Reference and Reporting

DENV-1	DENV-2	DENV-3	DENV-4	RP Target	Report
+	-	-	-	±	Positive DENV-1 detection*
-	+	-	-	±	Positive DENV-2 detection*
-	-	+	-	±	Positive DENV-3 detection*
-	-	-	+	±	Positive DENV-4 detection*
-	-	-	-	+	Negative for DENV, result does not preclude infection
-	-	-	-	-	Inconclusive test, likely poor extraction or sample quality**

* If sample is positive for 2 serotypes, repeat the test. If sample is repetitively reactive for both serotypes the result is indicative of a dual infection and should be confirmed by the CDC-Dengue Branch.

** When an inconclusive result is obtained, re-extract the specimen and test the newly extracted RNA (recommended) or re-test the extracted RNA. If the re-tested sample is negative for all markers and all controls exhibit the expected performance, the result is “Inconclusive” and a new specimen should be collected if possible.

RNase P (RP)

All clinical samples should exhibit fluorescence amplification curves in the RNase P reaction that cross the threshold line within 37 cycles (< 37 Ct), thus indicating the presence of the human RNase P gene. Failure to detect RP in any clinical specimens may indicate:

- Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation
- Absence of sufficient human cellular material due to poor collection or loss of specimen integrity
- Improper assay set up and execution
- Reagent or equipment malfunction

If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:

- If the DENV-1, -2, -3 and -4 are positive, even in the presence of a negative RP, the dengue result should be considered valid. It is possible, that some samples may fail to exhibit RP amplification curves due to low levels in the original clinical sample. A negative RP signal does not preclude the presence of dengue virus in a clinical specimen.
- If all dengue markers and RP are negative for the specimen, the assay is “inconclusive” for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after retest, report the results as “inconclusive” and a new specimen should be collected if possible.

The RP assay may be negative when testing virus culture samples.

M. Performance Characteristics (if/when applicable):

1. Analytical performance:

a. Precision/Reproducibility:

The reproducibility of the CDC DENV-1-4 Real-Time RT-PCR Assay was evaluated at three testing sites (CDC Dengue Branch Lab and two external independent sites). Operator to operator, run to run and site to site reproducibility were evaluated using four test panels which included negative (unspiked sample), high negative (1:10 dilution below the LoD), low positive (equal to the LoD) and moderate positive (2-3x higher than the LoD) samples. In order to develop these panels, cultured, quantified (pfu/mL) stocks of whole virus (strains DENV-1 Haw, DENV-2 NGC, DENV-3 H87 and DENV-4 H241) were used to spike into dengue negative human serum. Each sample of the panel was tested twice a day for at least 5 days by two operators with each operator running the panel members two times a day. Test operators were blinded with respect to test panel members for each run and device lot to ensure that panel results could not be predicted by the operator.

Two RNA extraction methods validated for the CDC DENV-1-4 Real-Time RT-PCR were used in the reproducibility study. The CDC Dengue Branch Lab and one external site used the Qiagen QIAamp® DSP Viral RNA Mini Kit and second external site used the MagNA Pure LC total Nucleic Acid Isolation Kit. The manufacturer’s instructions for use provided in the package insert were followed. Results generated for each of the extraction methods are summarized in the Table below.

Reproducibility Studies for the CDC DENV-1-4 Real-Time RT-PCR Assay on the ABI 7500 Fast Dx Real-Time PCR Instrument and SSD version 1.4 Software at three sites

		Site 1			Site 2			Site 3		
		AVG CT	% CV	Agreement with Expected Result	AVG CT	% CV	Agreement with Expected Result	AVG CT	% CV	Agreement with Expected Result
DENV-1	Moderate Positive	24.23	5.24	20/20	25.83	2.40	20/20	17.91	3.96	20/20
	Low Positive	30.45	3.68	19/20	31.15	1.32	19/20	21.28	3.34	20/20
	High Negative	38.75	3.51	16/20	38.70	4.16	15/20	19.41	5.87	16/20
	Negative	NA	NA	20/20	NA	NA	20/20	38.54*	NA	19/20
DENV-2	Moderate Positive	26.89	5.09	20/20	24.97	2.76	20/20	18.65	5.52	20/20
	Low Positive	32.02	3.94	18/20	30.80	3.21	19/20	22.27	4.63	20/20
	High Negative	40.24	2.91	14/20	38.08	2.42	17/20	19.72	8.01	15/10
	Negative	NA	NA	20/20	39.22	NA	20/20	41.72*	NA	19/20
DENV-3	Moderate Positive	25.53	4.97	20/20	25.25	4.32	20/20	18.51	4.16	20/20
	Low Positive	30.62	3.66	20/20	30.88	2.33	20/20	21.56	3.57	20/20
	High Negative	38.91	3.16	15/20	38.49	4.65	16/20	21.10	5.73	16/20
	Negative	NA	NA	20/20	39.71	NA	20/20	38.63*	NA	19/20
DENV-4	Moderate Positive	24.83	4.27	20/20	25.46	3.61	20/20	17.78	6.13	20/20
	Low Positive	30.10	2.82	19/20	30.40	6.09	19/20	21.30	3.62	19/20
	High Negative	39.14	3.68	14/20	38.02	3.10	15/20	26.90	8.18	17/20
	Negative	NA	NA	20/20	NA	NA	20/20	NA	NA	20/20
Negative Control		NA	NA	20/20	NA	NA	20/20	NA	NA	20/20

NA: Not applicable (samples did not obtain a CT value).

*Only one specimen obtained a CT value.

Reproducibility Study Summary for the CDC DENV-1-4 Real-Time RT-PCR Assay on the ABI 7500 Fast Dx Real-Time PCR Instrument and SSD version 1.4 Software

		AVG CT	% CV	Agreement with expected Result	95% CI
DENV-1	Moderate Positive	25.52	3.41	60/60 (100 %)	94.0 - 100
	Low Positive	31.03	2.42	58/60 (96 %)	88.6 - 99.11
	High Negative	38.60	3.55	47/60 (21 %)	15.9 - 39.6
	Negative	38.54*	NA	60/60 (100 %)	NA
DENV-2	Moderate Positive	26.32	3.91	60/60 (100 %)	94.0 - 100
	Low Positive	31.77	3.43	57/60 (95 %)	86.3 - 98.29
	High Negative	38.91	3.14	46/60 (23%)	14.4 - 35.4
	Negative	40.32*	NA	60/60 (100 %)	NA
DENV-3	Moderate Positive	26.01	5.96	60/60 (100 %)	94.0 - 100
	Low Positive	31.10	4.57	60/60 (100 %)	94.0 - 100
	High Negative	39.25	3.59	45/60 (25%)	15.8 - 37.23
	Negative	39.17*	NA	60/60 (100 %)	NA
DENV-4	Moderate Positive	25.47	4.00	60/60 (100 %)	94.0 - 100
	Low Positive	30.62	3.89	57/60 (95 %)	86.3 - 98.29
	High Negative	38.62	4.17	46/60 (23%)	14.4 - 35.4
	Negative	NA	NA	60/60 (100 %)	NA
Negative Control		NA	NA	10/10 (100 %)	NA

*AVG CT value is based on one or two samples.

b. Linearity/assay reportable range: Not applicable, qualitative assay

c. Traceability, Stability, Expected values (controls, calibrators, or methods):

Before analyzing the samples prepared using the CDC DENV-1-4 Real-Time RT-PCR Assay on the ABI 7500 Fast Dx Real-Time PCR instrument, the ABI instrument must be prepared following the procedures described in the Package Insert for the CDC DENV-1-4 Real-Time RT-PCR Assay and the ABI 7500 Fast Dx Real-Time PCR instrument User Manual.

Assay Quality Control

Quality control in laboratories performing the CDC DENV-1-4 Real Time RT-PCR Assay must be performed in conformance with local, state, and federal regulations or accreditation requirements and the user's laboratory's standard quality control procedures. It is recommended that the user refer to

CLSI document C24-A2 - Statistical Quality Control for Quantitative Measurements.

No Template Control (NTC)

The NTC consists of using nuclease-free water in the rRT-PCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence amplification curves that cross the threshold line. If any of the NTC reactions exhibit an amplification curve that crosses the cycle threshold, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

DENV-1- 4 Positive Controls

The positive control consists of a mix of DENV-1, -2, -3, and -4 dengue virus from C636 cell culture supernatant. Purified RNA from the positive control will yield a positive result with the primer and probe sets designed for DENV-1, DENV-2, DENV-3 and DENV-4.

Extraction Control - Human Specimen Control (HSC)

The HSC control consists of noninfectious cultured human cell (A549) material. The HSC is used as a RNA extraction procedural control to demonstrate successful recovery of RNA as well as extraction reagent integrity. Purified RNA from the HSC should yield a positive result with the RP primer and probe set and negative results with all dengue specific markers.

A negative amplification/detection control (NTC) and a positive control should be included in each run. An HSC extraction control must proceed through nucleic acid isolation for each batch of specimens to be tested.

d. Limit of Detection (LoD):

i) LoD of CDC DENV-1-4 Real-Time RT-PCR Assay (Singleplex) using a manual RNA extraction method (Qiagen QIAamp[®] DSP Viral RNA Mini Kit):

The LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Singleplex) was determined using a 5-replica panel of viruses serially diluted into dengue negative human serum or plasma at 1:10 dilutions. The panels were prepared from cultured, quantified (pfu/mL) stocks of whole virus (strains DENV-1 Haw, DENV-2 NGC, DENV-3 H87, DENV-4 H241). Viral RNA from all replica samples was extracted using the Qiagen QIAamp[®] DSP Viral RNA Mini Kit (cat# 61904). Each viral RNA elution was tested following the procedure described for the CDC DENV-1-4 Real-Time RT-PCR Assay using the Invitrogen SuperScript[®] III Platinum One Step Quantitative RT-PCR System (cat# 11732-088) on the Applied Biosystems[®] 7500 Fast Dx thermocycler. The table below shows the Average CT value for the 5 replicas

of each dilution and the corresponding standard deviation, number of positive samples over the total number of replicas and the positive rate.

Limit of Detection of the CDC DENV-1-4 Real-Time RT-PCR Assay (Singleplex) in Serum and Plasma

Viruses	Virus Concent. (pfu/ml)	Serum				Plasma			
		Avg CT	STD	Positive/ Total	Positivity Rate	Avg CT	STD	Positive/ Total	Positivity Rate
DENV-1	10 ⁷	21.67	0.29	5/5	100%	22.46	0.32	5/5	100%
	10 ⁶	26.15	0.23	5/5	100%	26.82	0.23	5/5	100%
	10 ⁵	30.90	0.73	5/5	100%	31.47	0.76	5/5	100%
	10 ⁴	34.65	2.14	5/5	100%	35.40	0.89	5/5	100%
	10 ³	36.36	1.73	5/5	100%	35.82	3.20	5/5	100%
	10 ²	N/A	N/A	0/5	0	N/A	N/A	0/5	0
	10 ¹	N/A	N/A	0/5	0	N/A	N/A	0/5	0
	10 ⁰	N/A	N/A	0/5	0	N/A	N/A	0/5	0
DENV-2	10 ⁷	21.65	0.23	5/5	100%	21.12	0.26	5/5	100%
	10 ⁶	24.74	0.31	5/5	100%	24.36	0.28	5/5	100%
	10 ⁵	28.83	0.27	5/5	100%	27.15	0.26	5/5	100%
	10 ⁴	31.75	0.52	5/5	100%	32.39	0.84	5/5	100%
	10 ³	35.78	0.23	5/5	100%	36.56	0.67	5/5	100%
	10 ²	39.67	N/A	0/5	0	39.6	N/A	0/5	0
	10 ¹	41.9	N/A	0/5	0	42.5	N/A	0/5	0
	10 ⁰	N/A	N/A	0/5	0	N/A	N/A	0/5	0
DENV-3	10 ⁷	20.67	0.25	5/5	100%	22.21	0.38	5/5	100%
	10 ⁶	24.18	0.77	5/5	100%	25.34	0.27	5/5	100%
	10 ⁵	28.48	0.55	5/5	100%	29.39	0.14	5/5	100%
	10 ⁴	32.27	0.73	5/5	100%	32.85	0.18	5/5	100%
	10 ³	36.58	1.32	4/5	80%	35.36	1.03	5/5	100%
	10 ²	39.71	N/A	0/5	0	38.36	0.06	3/5	60%
	10 ¹	42.01	N/A	0/5	0	41.87	N/A	0/5	0
	10 ⁰	N/A	N/A	0/5	0	N/A	N/A	0/5	0
DENV-4	10 ⁷	22.46	0.13	5/5	100%	24.75	0.18	5/5	100%
	10 ⁶	26.33	0.48	5/5	100%	28.20	0.12	5/5	100%
	10 ⁵	29.64	0.83	5/5	100%	31.29	0.40	5/5	100%
	10 ⁴	32.61	0.49	5/5	100%	34.05	1.33	5/5	100%
	10 ³	36.68	0.46	5/5	100%	36.72	1.18	5/5	100%
	10 ²	39.55	N/A	0/5	0	38.73	2.34	1/5	20%
	10 ¹	N/A	N/A	0/5	0	N/A	N/A	0/5	0
	10 ⁰	N/A	N/A	0/5	0	N/A	N/A	0/5	0

The observed LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Singleplex) in serum and plasma was 1×10^3 pfu/mL.

The LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) was also determined using a 5-replica panel of viruses serially diluted into dengue negative human serum or plasma as described for the CDC DENV-1-4 Real-Time RT-PCR Assay (Singleplex). The LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) in serum and plasma was shown similar (1×10^3 pfu/mL) to the CDC DENV-1-4 Real-Time RT-PCR Assay (Singleplex) using a manual RNA extraction method (Qiagen QIAamp® DSP Viral RNA Mini Kit).

ii) LoD of CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) using an automated RNA extraction method (Qiagen QIAamp® DSP Viral RNA Mini Kit and Qiagen QIAcube):

The LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) was established using a 5-replica panel of viruses sequentially diluted in human serum or plasma at 1:10 dilutions. In order to develop these panels, cultured, quantified (pfu/mL) stocks of whole virus (strains DENV-1 Haw, DENV-2 NGC, DENV-3 H87, DENV-4 H241) were used. Viral RNA from all replica samples was extracted using the Qiagen QIAamp® DSP Viral RNA Mini Kit (cat# 61904) on the Qiagen QIAcube (cat# 9001292) following manufacturer's protocol. Each viral RNA elution was tested following the procedure described for the CDC DENV-1-4 Real-Time RT-PCR Assay using the Invitrogen SuperScript® III Platinum OneStep Quantitative RT-PCR System (cat# 11732-088) on the Applied Biosystems® 7500 Fast Dx thermocycler.

The LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) in serum and plasma was the same (1×10^3 pfu/mL) for both RNA extraction methods, using the automated (Qiagen QIAamp® DSP Viral RNA Mini Kit and Qiagen QIAcube) and the manual procedure (Qiagen QIAamp® DSP Viral RNA Mini Kit).

iii) LoD of CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) using an automated RNA extraction method (MagNA Pure LC Total Nucleic Acid Isolation Kit on the MagNA Pure LC 2.0 Instrument):

The LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) was established using a 5-replica panel of viruses sequentially diluted in human serum or plasma at 1:10 dilutions. In order to develop these panels, cultured, quantified (pfu/mL) stocks of whole virus (strains DENV-1 Haw, DENV-2 NGC, DENV-3 H87, DENV-4 H241) were used. Viral RNA from all replica samples was extracted using Roche MagNA Pure LC 2.0 total Nucleic Acid

Isolation Kit (03 038 505.001) on the Roche Magna Pure LC 2.0 instrument following manufacturer's protocol. Each viral RNA elution was tested following the procedure described for the CDC DENV-1-4 Real-Time RT-PCR Assay using the Invitrogen SuperScript[®] III Platinum OneStep Quantitative RT-PCR System (cat# 11732-088) on the Applied Biosystems[®] 7500 Fast Dx thermocycler.

The LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) in serum and plasma using an automated viral RNA extraction method (MagNA Pure LC total Nucleic Acid Isolation Kit on the MagNA Pure LC 2.0 instrument) was 1×10^3 pfu/mL.

There was no difference in the LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) using the described three viral extraction methods - an automated MagNA Pure LC total Nucleic Acid Isolation Kit on the MagNA Pure LC 2.0 instrument, an automated Qiagen QIAamp[®] DSP Viral RNA Mini Kit and Qiagen QIAcube, or a manual procedure (Qiagen QIAamp[®] DSP Viral RNA Mini Kit).

iv) Analytical Reactivity

In order to assess if the CDC DENV-1-4 Real-Time RT-PCR Assay detects a variety of currently circulating strains, the observed LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay was further confirmed using the additional Dengue-1 – 4 strains. Twenty nine Dengue-1 – 4 isolates obtained from patients from different countries were cultured and quantified. The quantified stocks were serially diluted in human serum at 1:10 dilutions to 10^3 and 10^2 pfu/mL and were tested. Triplicate samples of each dilution were tested by the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex).

The results showed that the observed LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay was similar in cultured isolates obtained from patients from different countries. Results are shown in the table below.

DENV isolates tested with the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex)

YEAR	Country	Genotype	10 ³ pfu/ml				10 ² pfu/ml			
			AVG CT	STD	Pos/Total	GCE/ml	AVG CT	STD	Pos/Total	GCE/ml
DENV-1 Strains										
2003	Brazil	African/American	31.99	1.74	3/3	1.89 x 10 ⁴	NA	NA	0/3	N/A
2007	Mexico	African/American	34.45	0.47	2/3	9.87 x 10 ⁴	NA	NA	0/3	N/A
2007	Venezuela	African/American	32.32	1.08	3/3	1.58 x 10 ⁴	NA	NA	0/3	N/A
1994	Sri Lanka	Asian	32.88	0.18	3/3	8.99 x 10 ³	NA	NA	0/3	N/A
2004	Philippines	South Pacific	33.29	0.71	3/3	7.23 x 10 ⁴	NA	NA	0/3	N/A
2004	Bangkok	Asian	36.21	1.42	2/3	2.75 x 10 ⁴	NA	NA	0/3	N/A
DENV-2 Strains										
2003	Brazil	SE Asian/American	32.39	0.32	3/3	8.11 x 10 ⁴	NA	NA	0/3	N/A
2005	Colombia	SE Asian/American	31.69	0.34	3/3	7.66 x 10 ⁴	NA	NA	0/3	N/A
2007	Ivory Coast	SE Asian	31.67	0.34	3/3	2.46 x 10 ⁴	36.6	1.37	2/3	7.22 x 10 ³
2003	Viet Nam	Asian I	32.18	0.40	3/3	2.17 x 10 ⁴	36.11	NA	1/3	6.89 x 10 ¹
2006	Thailand	SE Asian	32.14	0.87	3/3	9.56 x 10 ³	NA	NA	0/3	N/A
2007	Dominican R.	SE Asian/American	32.78	0.32	3/3	7.24 x 10 ⁴	35.87	NA	1/3	3.44 x 10 ³
2001	Costa Rica	SE Asian/American	32.04	0.14	3/3	3.15 x 10 ⁴	NA	NA	0/3	N/A
2003	Peru	SE Asian/American	32.39	0.32	3/3	5.99 x 10 ²	37.12	1.26	1/3	1.88 x 10 ³
2006	Burkina Faso	Cosmopolitan	32.86	1.05	3/3	2.12 x 10 ⁴	37.44	0.86	1/3	7.98 x 10 ³
DENV-3 Strains										
2006	Puerto Rico	Indian Subcont.	32.04	0.42	3/3	6.99 x 10 ⁴	NA	NA	0/3	N/A
2003	Brazil	Indian Subcont.	31.99	1.74	3/3	1.39 x 10 ⁴	36.99	1.27	1/3	8.9 x 10 ³
1995	Samoa	Indian Subcont.	36.69	1.47	2/3	5.12 x 10 ⁴	NA	NA	0/3	NA
2003	Bangkok	Indian Subcont.	32.16	0.81	3/3	2.10 x 10 ⁴	NA	NA	0/3	N/A
2000	Ecuador	Indian Subcont.	32.88	0.18	3/3	1.87 x 10 ⁴	36.41	0.91	1/3	6.34 x 10 ³
1991	Cook Island	SE Asian/S. Pacific	33.29	0.71	3/3	2.11 x 10 ⁴	NA	NA	0/3	N/A
DENV-4 Strains										
2006	Colombia	Indonesian	36.60	1.30	2/3	2.23 x 10 ⁴	NA	NA	0/3	N/A
2006	Mexico	Indonesian	32.39	0.32	3/3	9.15 x 10 ⁴	NA	NA	0/3	N/A
1992	Sri Lanka	SE Asian	31.70	0.33	3/3	2.44 x 10 ⁴	36.59	0.76	1/3	3.65 x 10 ³
2006	Thailand	Indonesian	35.78	0.40	2/3	1.45 x 10 ⁴	NA	NA	0/3	N/A
1994	St. Croix	Indonesian	32.18	0.40	3/3	2.89 x 10 ⁴	NA	NA	0/3	N/A
1999	Ecuador	Indonesian	32.14	0.87	3/3	5.35 x 10 ⁴	36.56	NA	1/3	7.98 x 10 ³
1995	Micronesia	Asian	32.78	0.32	3/3	2.13 x 10 ⁴	NA	NA	0/3	2.77 x 10 ³

GCE/mL- genome copy equivalent/mL

e. Analytical specificity:

Cross Reactivity: The analytical specificity of the CDC DENV-1-4 Real-Time RT-PCR Assay was evaluated by testing the device with nucleic acids extracted from 12 organisms representing common pathogens present in blood, serum or plasma samples of patients with febrile illness, included in the differential diagnosis of dengue. These pathogens were obtained from CDC repositories. Ten of these pathogens were used to spike human serum (confirmed negative for dengue virus) at the clinically significant concentrations. These 10 organisms included four RNA arboviruses (West Nile virus [WNV], Yellow Fever virus [YFV], Saint Louis Encephalitis virus [SLEV], and Chikungunya virus [CHIKV]). WNV, YFV and SLEV are

flaviviruses related to DENV. Herpes simplex virus 1 and 2 (HSV-1 and -2), cytomegalovirus (CMV), and varicella zoster virus (VZV) are DNA viruses selected for this study. Two bacterial organisms, *Leptospira* and *Borrellia*, were also spiked in serum for cross reactivity studies.

The CDC DENV-1-4 Real Time RT-PCR Assay was performed on all 12 samples in triplicate. RNA was extracted using the Qiagen QIAamp[®] DSP Viral RNA Mini Kit and was tested with the CDC DENV-1-4 Real-Time RT-PCR Assay using the Invitrogen SuperScript[®] III Platinum OneStep Quantitative RT-PCR System on the Applied Biosystems[®] 7500 Fast Dx thermocycler. Negative results were obtained with the CDC DENV-1-4 Real Time RT-PCR Assay in all triplicate samples for all 12 tested organisms. No cross reactivity was observed with any panel member tested at clinically significant concentrations.

Cross Reactivity Panel

Pathogen	Sample type	Concentration	DENV RT-PCR
Virus		pfu/ml	Rate positive
WNV	spiked serum	6.9×10^7	0/3
YFV	spiked serum	3.7×10^6	0/3
SLEV	spiked serum	3.7×10^6	0/3
CHIKV	spiked serum	4.0×10^6	0/3
HCV	clinical serum	unknown*	0/3
HAV	clinical serum	unknown*	0/3
HSV-1	spiked serum	1.0×10^5	0/3
HSV-2	spiked serum	1.0×10^5	0/3
CMV	spiked serum	1.0×10^5	0/3
VZV	spiked serum	1.0×10^5	0/3
Bacteria		bacteria/ml	
<i>Leptospira</i>	spiked serum	2.5×10^5	0/3
<i>Borrelia burgdorferi</i>	spiked serum	1.0×10^6	0/3

*Presence of these pathogens in clinical samples was established by non-quantitative RT-PCR assays (concentration of pathogen is unknown)

f. Interference studies:

Performance of the CDC DENV-1-4 Real-Time RT-PCR Assay was evaluated in the presence of potential interfering substances which could reasonably be expected to be present in serum and plasma specimens. All interference studies were carried out in the presence of cultured, quantified (pfu/mL) stocks of whole virus (strains DENV-1 Haw, DENV-2 NGC, DENV-3 H87, DENV-4 H241) diluted to concentrations 1:10 higher than the LoD dilution, equal to the LoD dilution, and the 1:10 dilution below the LoD. Each viral dilution was tested three times in normal human serum (NHS) or in NHS containing bilirubin (342 $\mu\text{mol/L}$), cholesterol (13 mmol/L), hemoglobin (2 g/L), triglycerides (37 mmol/L) and genomic DNA (400 $\mu\text{g}/100 \text{ mL}$). The levels tested for each endogenous substance were based on the Clinical

Laboratories Institute (CLSI) Standard, EP7-A2 (2005).

Viral RNA from every sample was extracted using the Qiagen QIAamp® DSP Viral RNA Mini Kit. Extracted viral RNAs were tested following the procedure described for the CDC DENV-1-4 Real-Time RT-PCR Assay using the Invitrogen SuperScript® III Platinum OneStep Quantitative RT-PCR System on the Applied Biosystems® 7500 Fast Dx thermocycler.

No significant interference was observed in the presence of the described potential interfering substances at the tested levels.

g. Carry-Over/Cross Contamination:

To assess possible cross contamination of samples in the CDC DENV-1-4 Real-Time RT-PCR Assay, 8 replica sets of DENV-1 Haw, DENV-2 NGC, DENV-3 H87, DENV-4 H241 were tested in an alternating series. Cultured, quantified (pfu/mL) stocks of DENV-1, -2, -3 and 4 were diluted to high-positive (10^7 pfu/ml) and high-negative (5×10^2 pfu/ml) concentrations. Eight high-positive and 8 high-negative replicas were tested in an alternating series by the CDC DENV-1-4 Real-Time RT-PCR Assay. Viral RNA from all replica samples was extracted using the Qiagen QIAamp® DSP Viral RNA Mini Kit. Each viral RNA elution was tested following the procedure described for the CDC DENV-1-4 Real-Time RT-PCR Assay using the Invitrogen SuperScript® III Platinum OneStep Quantitative RT-PCR System on the Applied Biosystems® 7500 Fast Dx thermocycler. All results were as expected. All negative samples tested negative (32/32) and all positive samples tested positive (32/32).

h. Fresh vs. Frozen Specimens:

To determine the effect of freezing and the effect of freeze/thaw cycles on the CDC DENV-1-4 Real-Time RT-PCR Assay results, a comparison study was performed using spiked human serum samples. Based upon the initial CDC DENV-1-4 Real-Time RT-PCR Assay results obtained with serially diluted virus in serum, CDC chose moderate and low positive dilutions and prepared them in triplicate sets. These sets were frozen at -80°C for 24 hours and subject to five consecutive freeze/thaw cycles. Frozen aliquots (-80°C for 24h.) of these specimens were thawed, extracted using the Qiagen QIAamp® DSP Viral RNA Mini Kit (cat# 61904) following manufacturer's protocol, and compared to the original fresh sample test results. Each nucleic acid sample was tested following the procedure described for the CDC DENV-1-4 Real-Time RT-PCR Assay using the Invitrogen SuperScript® III Platinum OneStep Quantitative RT-PCR System (cat# 11732-088) on the Applied Biosystems® 7500 Fast Dx thermocycler. The study showed 100% agreement (qualitative) between the initial CDC DENV-1-4 Real-Time RT-PCR Assay (0) result and the result after each freeze/thaw cycle (1, 2, 3, 4 and 5).

2. Comparison studies:

a. *Method comparison with predicate device:*

There is no predicate device for this assay. However, the clinical performance of the CDC DENV-1-4 Real-Time RT-PCR Assay was evaluated against two laboratory developed assays: 1) CDC IgM anti-DENV Capture Enzyme Linked Immunosorbent Assay (MAC-ELISA) and 2) CDC molecular detection method (run at CDC) followed by bi-directional sequencing of the DENV E gene (1485 bp). Positive and negative percent agreement for method comparison studies were calculated in comparison to these two different methods.

For additional details please see section 3 Clinical studies subsection c.

b. *Matrix Comparison:*

Matrix comparison was not required because the LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay in serum and plasma showed no difference.

3. Clinical studies:

a. *Clinical Sensitivity:* N/A

b. *Clinical specificity:* N/A

c. *Other clinical supportive data* (when a. and b. are not applicable):

Clinical Performance:

Prospective Studies: Performance characteristics of the CDC DENV-1-4 RT-PCR Assay were established during a prospective study at 3 public health laboratories (2009-2011). A total of 86 serum samples were prospectively collected from dengue-suspected, febrile patients during the first 5 days of symptoms and were tested at the three sites. There were twenty five (25) and thirty six (36) serum samples tested at Site 1 and 3 respectively using the Qiagen QIAamp[®] DSP Viral RNA Mini Kit (cat# 61904) extraction procedure following the manufacturer's protocol. Twenty five (25) serum samples were tested at Site 2 using the Roche MagNA Pure LC Total Nucleic Acid Isolation Kit (03 038 505 001). The eluted viral RNA was tested using the Invitrogen SuperScript[®] III Platinum OneStep Quantitative RT-PCR System (cat# 11732-088) on the Applied Biosystems[®] 7500 Fast Dx thermocycler. All 86 samples subsequently underwent bi-directional sequencing of the DENV E gene (1485 bp). The prospectively collected samples were not accompanied by a second, convalescent specimen. Percent agreement was determined in

comparison to the CDC molecular detection method followed by sequencing. Overall prospective comparison results are presented in the table below.

Overall Prospective Comparison Results

Multiplex CDC DENV-1-4 Real-Time RT-PCR Assay Comparison Results				
		Reference Method (Sequencing)		Total
		Positive	Negative	
CDC DENV-1-4 Real-Time RT-PCR Assay	Positive	47	0	47
	Negative	1*	38	39
	Total	48	38	86
Agreement Statistics				
		Value	95% Confidence Interval	
Positive Percent Agreement		97.92% (47/48)	89.10% – 99.63%	
Negative Percent Agreement		100% (38/38)	90.82% – 100%	

*One sample was negative on the CDC DENV-1-4 Real-Time RT-PCR Assay which was positive for DENV-3 by bi-directional sequencing. All other negative RT-PCR samples were also negative by E gene bi-directional sequencing.

Retrospective Studies: Performance characteristics of the CDC DENV-1-4 Real-Time RT-PCR Assay were established during a retrospective study at the CDC Dengue Branch. Three hundred seventy one serum samples were obtained from the archived CDC routine dengue surveillance specimens collected in pairs during 2007-2011. The first sample (acute) was collected during the first five days of illness, and the second sample (convalescent) was obtained at least 6 days after the onset of symptoms. These samples were tested with the IgM anti-DENV Capture Enzyme Linked Immunosorbent Assay (CDC MAC-ELISA – validated in-house) in order to establish seroconversion. The results of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) obtained for the acute samples were compared to the IgM anti-DENV seroconversion results in the paired samples.

Nucleic acid from all acute samples was extracted using the Qiagen QIAamp® DSP Viral RNA Mini Kit (cat# 61904) following manufacturer’s protocol. Each nucleic acid sample was tested following the procedure described for the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) using the Invitrogen SuperScript®III Platinum OneStep Quantitative RT-PCR System (cat# 11732-088) on the Applied Biosystems® 7500 Fast Dx thermocycler. The percent agreement is calculated for the number of samples that received positive or negative results in the CDC DENV-1-4 RT-PCR assay and using IgM anti-DENV seroconversion as a comparator. In addition, bi-directional sequencing of the DENV E gene (1485 bp) was obtained, and corroborated the results of, the CDC

DENV-1-4 Real-Time RT-PCR Assay on all positive samples except for one. The following table summarizes the results obtained on the 371 specimens that originated from the following sources: non-United States dengue cases (n=39); Puerto Rico dengue cases (n=82); 250 negative cases from Puerto Rico (no IgM anti-DENV conversion). Overall retrospective comparison results are presented in the table below.

Overall Retrospective Comparison Results

Multiplex CDC DENV-1-4 Real-Time RT-PCR Assay Comparison Results				
		Reference Method (IgM anti-DENV Conversion)		
		Positive	Negative	Total
CDC DENV-1-4 Real-Time RT- PCR Assay	Positive	100*	4***	104
	Negative	2**	265	267
Total		102	269	371
		Value	95% Confidence Interval	
Positive Percent Agreement		98.04% (100/102)	93.13% - 99.46%	
Negative Percent Agreement		98.51% (265/269)	96.24% - 99.42%	

*One DENV-1 case was positive on the CDC DENV-1-4 Real-Time RT-PCR Assay (multiplex) and confirmed positive by IgM anti-DENV conversion, but not sequenced effectively enough to produce an interpretable result. **Two samples were negative by the CDC-DENV-1-4 Real-Time RT-PCR Assay (multiplex). One of these samples was DENV-3 positive by the CDC-DENV-1-4 Real-Time RT-PCR Assay (singleplex) and was further confirmed DENV-3 positive by sequencing. The other sample was confirmed DENV-3 positive by sequencing. *** Four samples showed positive RT-PCR results and were not confirmed by IgM anti-DENV seroconversion. Two of these samples were positive results for DENV-3, and the other two samples were DENV-4 positive. These results were confirmed by E gene bi-directional sequencing.

4. Clinical cut-off: N/A
5. Expected values/Reference range:

The percent of positive cases identified by the CDC DENV-1-4 Real-Time RT-PCR Assay will vary depending on the nature of the surveillance process, given that an unknown proportion of febrile cases reported may not be dengue. It is expected that the proportion of serotypes identified will vary with time, location and patient population. In dengue endemic areas, usually one serotype may be more prevalent than the others. A serotype may not be detected for long periods and then re-emerge and become the predominant serotype. Different countries may have different serotype predominance; therefore travelers returning to the United States

from dengue-endemic countries may have been infected with different serotypes. Puerto Rico has had the four DENV serotypes circulating at different times. During the 2007 epidemic, DENV-2, and DENV-3 were the predominant serotypes and during the 2010 epidemic, DENV-1 and DENV-4 were predominant. Florida has experienced autochthonous DENV-1 transmission in 2009 and 2010; and travel-associated cases (Florida residents travelling internationally) have been identified with DENV-1, DENV-2 and DENV-4.

In serologically confirmed cases, the CDC DENV-1-4 Real-Time RT-PCR Assay positively identified 93% of cases. A 97% agreement between positive results on the CDC DENV-1-4 Real-Time RT-PCR Assay and sequencing was obtained.

N. Proposed Labeling:

The labeling is sufficient and it satisfies the requirements of 21 CFR Part 809.10.

O. Conclusion:

The petition for Evaluation of Automatic Class III Designation for this device is accepted. The device is classified as Class II under regulation 21 CFR 866.3946 with special controls. The special controls guidance document "*Class II Special Controls Guidance Document: Dengue virus Nucleic Acid Amplification Test Reagents*" will shortly be available.