



CDC DENV-1-4 Real-Time RT-PCR Assay

for Detection and Serotype Identification of
Dengue Virus

Instructions for Use Package Insert

Catalog No. KK0128
200 reactions

For In-vitro Diagnostic Use
(IVD)

Performance Characteristics Have Been Determined

04-12-2013

Centers for Disease Control and Prevention
National Center for Emerging and Zoonotic Infectious Diseases
Division of Vector-Borne Diseases
Dengue Branch
1324 Canada Street, San Juan, PR 00920



TABLE OF CONTENTS

1. INTENDED USE	4
2. SUMMARY AND EXPLANATION.....	5
2.1 Principles of the Procedure	6
3. SUMMARY OF DENGUE TESTING PROCESS	7
4. MATERIALS PROVIDED	8
5. MATERIALS REQUIRED (BUT NOT PROVIDED)	10
5.1 Reagents	10
5.2 Equipment and Consumables Required (But Not Provided)	10
6. IMPORTANT PUBLIC HEALTH SURVEILLANCE INFORMATION	11
7. REAGENT STORAGE, HANDLING, AND STABILITY	11
8. SPECIMEN COLLECTION, HANDLING AND STORAGE	12
8.1 Collecting the Specimen	12
8.2 Transporting the Specimen	12
8.3 Storing Specimens	12
9. SPECIMEN REFERRAL TO CDC	13
10. REAGENTS AND CONTROLS PREPARATION	13
10.1 Primer and Probe Preparation.....	13
10.2 Human Specimen Control (HSC) Preparation.....	14
10.3. DENV-1-4 Positive Control Mix Preparation.....	14
10.4 General Preparation	14
11. WARNINGS AND PRECAUTIONS.....	15
12. NUCLEIC ACID (RNA) EXTRACTION.....	16
13. ASSAY SETUP	16
13.1 Master Mix Preparation/Plate Setup	16
14. CREATE A RUN TEMPLATE ON THE ABI 7500 FAST DX REAL-TIME PCR INSTRUMENT	18
14.1 Singleplex Assay.....	20
14.2 Multiplex Assay.....	25
15. DEFINING THE INSTRUMENT SETTINGS.....	29
16. RUNNING A TEST.....	30
17. DATA ANALYSIS.....	34
18. INTERPRETATION OF RESULTS	36
18.1 Extraction and Control Results and Interpretation	36
19. STANDARD-BASED ELECTRONIC LABORATORY REPORTING FOR DENGUE	37
19.1 Recommendations For Uniform Coding And Vocabulary For Diagnostic Testing.....	37
19.2 Process For Achieving Uniformity In Laboratory Test Results.....	38
20. CDC DENV-1-4 REAL-TIME RT-PCR ASSAY USERS GUIDE FOR INTERPRETATION OF RESULTS	39
21. QUALITY CONTROL.....	40
22. LIMITATIONS	40
23. EXPECTED VAUES	41
24. PERFORMANCE CHARACTERISTICS	41
24.1 Clinical Performance.....	41
24.2 Reproducibility.....	43
24.3 Analytical Sensitivity.....	45



24.4 Analytical Specificity	49
24.5 Carry Over/Cross Contamination	50
25. REFERENCES	51
26. ADDITIONAL RESOURCES	52
27. CONTACT INFORMATION, ORDERING, AND PRODUCT SUPPORT	53





CDC DENV-1-4 Real-Time RT-PCR Assay

for Detection and Serotype Identification of
Dengue Virus

I. INTENDED USE

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 meets 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. SUMMARY AND EXPLANATION

Dengue is an illness caused by infection with any one of four related dengue virus (DENV) serotypes (DENV-1, -2, -3 and -4) which are transmitted by *Aedes* sp mosquitoes, and affects an estimated 50 million people in approximately 100 countries annually (1). Infection by one DENV serotype confers long-term immunity to that serotype but not to the other three. Therefore, in dengue endemic countries, people are likely to be infected more than once over their lifetime. In the United States, dengue is endemic in Puerto Rico (2-4) the Virgin Islands (5, 6), and American Samoa and other U.S.-affiliated Pacific Islands. In non-endemic areas of the United States, dengue is the most frequent cause of febrile illness among travelers returning from tropical or subtropical areas of the Caribbean, Latin America and Asia (7). In addition, occasional outbreaks occur in areas of the United States where the vector mosquito is present, such as along the U.S.-Mexico border (8, 9), Florida (10, 11), and Hawaii (12). In the United States dengue is a nationally notifiable disease.

The majority (~75%) of DENV infections are asymptomatic. Among persons with symptomatic DENV infection (dengue), the illness occurs in three phases (1). During the acute phase, the principal symptom is 2–7 days of fever, which is often accompanied by one or more of the following: headache, retro-orbital eye pain, joint pain, muscle and/or bone pain, rash, mild bleeding manifestations (e.g., nose or gum bleed, petechiae, or easy bruising) and low white cell count. The critical phase of dengue begins at defervescence which marks a 24 to 48 hour period in which compensated or decompensated shock may occur due to increased capillary permeability with plasma leakage that produces ascites, pleural effusions and “third spacing” of fluids. The presence of these signs and/or symptoms is now called severe dengue rather than dengue hemorrhagic fever or dengue shock syndrome. Without appropriate treatment, patients with severe dengue are at risk of death. Other warning signs of severe dengue include abdominal pain, vomiting, thrombocytopenia and mild to severe hemorrhagic manifestations, including tendency to bruise easily, petechiae, menorrhagia and mucous membrane bleeding of the nose or gums. The convalescent phase of dengue lasts for 4-7 days (1).

Laboratory diagnosis of dengue is best made during the acute phase of the illness when DENV circulates in the blood and can be detected by assays to detect the viral RNA genome (13-15) or soluble antigens (i.e., NS1 antigen) (16). Anti-DENV IgM antibody to DENV is also produced during the acute phase of the illness and becomes detectable by ELISA at days 3-5 after onset of fever (17, 18). At this point the optimum testing algorithm for dengue has not yet been determined. If results of the DENV detection test (e.g., RT-PCR) are negative (days 1-5 after fever onset), anti-DENV IgM testing should be considered. If the patient first presents during the critical or convalescent phases of the illness, laboratory diagnosis is best made using a test for IgM antibody to DENV.

The CDC DENV-1-4 Real-Time RT-PCR Assay is a nucleic acid amplification assay that detects DENV serotypes 1, 2, 3 or 4 RNA from human serum or plasma collected from human patients with signs and symptoms consistent with dengue infection.



2.1 Principles of the Procedure

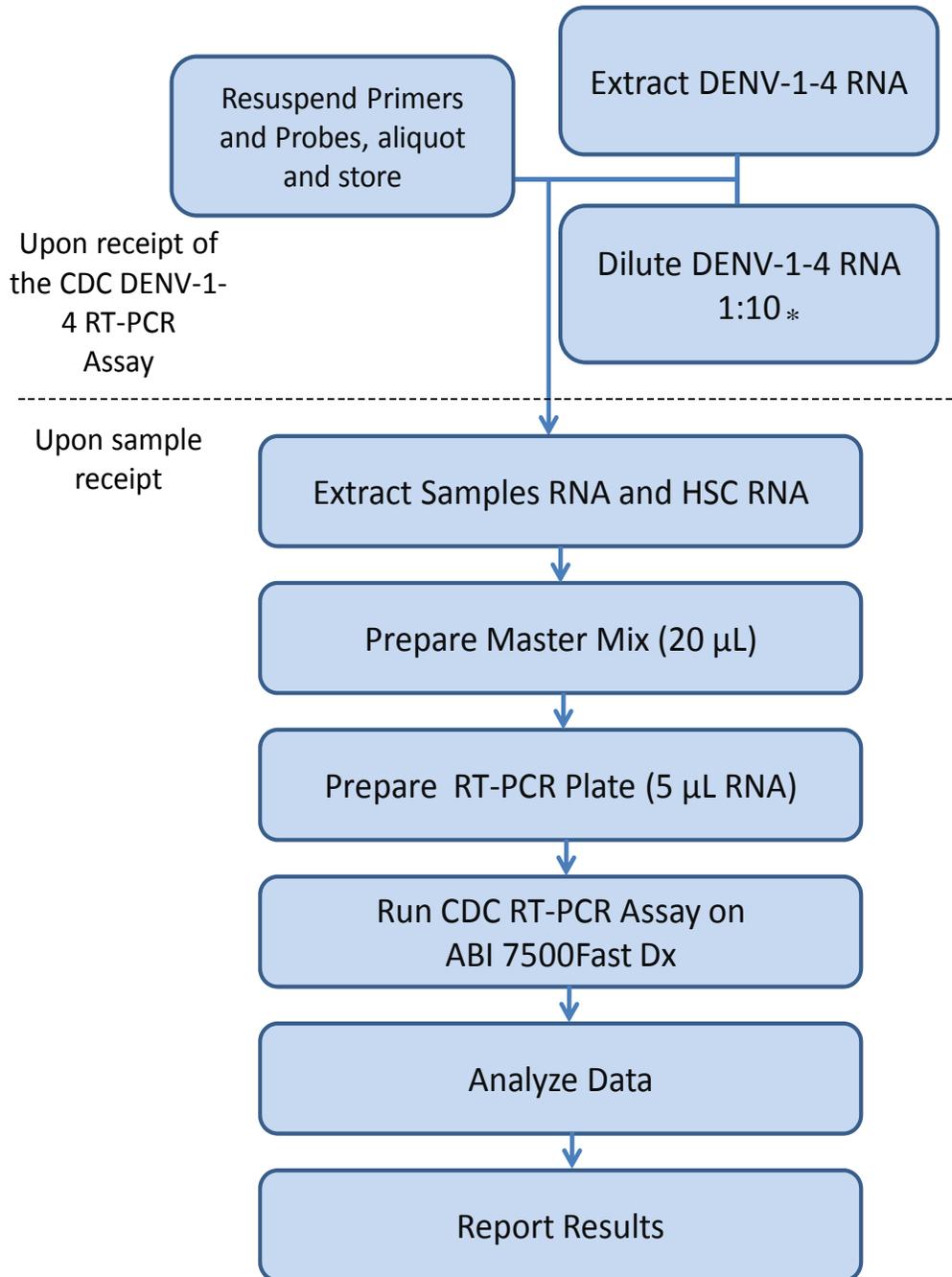
The CDC DENV-1-4 Real-Time RT-PCR Assay is used in rRT-PCR on an ABI 7500 Fast Dx Real-Time PCR Instrument. The CDC DENV-1-4 Real-Time RT-PCR Assay includes a set of oligonucleotide primers and dual-labeled hydrolysis (Taqman®) probes for in vitro qualitative detection of DENV serotypes 1, 2, 3 or 4 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 (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. 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 (the four DENV serotypes are run in the same reaction). These two formats provide equal sensitivity.



3.

Summary of Dengue Testing Process



* Diluted RNA is optional. Always include undiluted RNA control.



4. MATERIALS PROVIDED

CDC DENV-1-4 Real-Time RT-PCR Assay:

- 1- Package Insert/ Instructions for Use (this brochure)
- 2- Box 1: Detection Kit (Primer and Probe Sets)
- 3- Box 2: Positive Control Kit (a mix of heat inactivated DENV-1, -2, -3 and -4 standards)
- 4- Box 3: Human Specimen Extraction Control (HSC)

Box 1: Detection Kit (Primer and Probe Sets)
(Store at 2-8 °C in PCR Reagent Preparation Area)

Label	Part #	Description	Quantity/ Tube	Reactions/ Tube
D1-F	SO3504	DENV-1 Forward Primer	5 nmol	200
D1-R	SO3505	DENV-1 Reverse Primer	5 nmol	200
D2-F	SO3507	DENV-2 Forward Primer	5 nmol	200
D2-R	SO3508	DENV-2 Reverse Primer	5 nmol	200
D3-F	SO3510	DENV-3 Forward Primer	5 nmol	200
D3-R	SO3511	DENV-3 Reverse Primer	5 nmol	200
D4-F	SO3513	DENV-4 Forward Primer	5 nmol	200
D4 R	SO3514	DENV-4 Reverse Primer	5 nmol	200
RP-F	SO2669	RNase P Forward Primer	5 nmol	200
RP-R	SO2670	RNase P Reverse Primer	5 nmol	200
D1-Probe	SO3506	DENV-1 Probe	1 nmol	200
D2-Probe	SO3509	DENV-2 Probe	1 nmol	200
D3-Probe	SO3512	DENV-3 Probe	1 nmol	200
D4-Probe	SO3515	DENV-4 Probe	1 nmol	200
RP-Probe	SO3516	RNase P Probe	1 nmol	200



**Box 2: Positive Control Kit (a mix of heat inactivated
DENV-1, -2, -3 and -4 standards)
(Store at -20 °C in RNA Handling Area)**

Reagent Label	Part #	Description	Qty/ Tube	# of Tubes
DENV-1-4 Control	SO3517	Dengue virus serotypes 1-4 mix for use as a positive control in the CDC DENV-1-4 Real-Time RT-PCR Assay procedure to ensure the detection of DENV-1, DENV-2, DENV-3 and DENV-4. Dengue virus serotypes 1-4 mix contains heat-inactivated DENV-1 Haw, DENV-2 NGC, DENV-3 H87 and DENV-4 H241.	1 mL	4

**Box 3: Human Specimen Extraction Control
(Store at -20 °C in Nucleic Acid Extraction Room)**

Reagent Label	Part #	Description	Qty/ Tube	# of Tubes
HSC	HS0096	Human Specimen Control: For use as an RNA extraction procedural control with the CDC DENV-1-4 Real-Time RT-PCR Assay to demonstrate successful recovery of RNA from human serum or plasma samples. Purified RNA from Human Specimen Control material should yield a positive result with the RP primer and probe set and negative result with all DENV specific markers. The HSC consists of non-infectious (beta propiolactone inactivated) cultured human cell material supplied as a liquid suspended in 0.01 M PBS at pH 7.2–7.4.	1 mL	4



5. MATERIALS REQUIRED (BUT NOT PROVIDED)

5.1 Reagents

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 #
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 Qiagen QIAcube Instrument**	50 Extractions	61904 and 9001292
	Roche MagNA Pure LC total Nucleic Acid Isolation Kit *	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 (Cat # 61904) is produced under Good Manufacturing Practices (GMP). These RNA extraction kits can be used manually or in combination with the QIAcube Instrument (Cat # 9001292).

5.2 Equipment and Consumables Required (But Not Provided)

- RNase/DNase-free 1.5 ml polypropylene microcentrifuge tubes
- Molecular grade water (RNase/DNase Free)
- Sterile nuclease-free filtered pipette tips
- Micropipettors (1, 1 - 10, 10 - 200 and 100 - 1000 µL)
- 96-well cold block
- Benchtop Microcentrifuge
- Personnel Protective Equipment (PPE)
- -70° C and -20 °C Freezer(s)
- +4° C Refrigerator
- Plasticware and consumables
- 100% Ethanol (EtOH)
- 10% bleach
- Disposable gloves
- DNAzap™
- RNase AWAY®
- MagNA Pure LC 2.0 instrument (05 197 686 001) for automated RNA extractions using MagNA Pure LC total Nucleic Acid Isolation Kit
- MagNAPure LC 2.0 system consumables
- QIAcube Purification instrument (9001292), optional for RNA extractions using Qiagen RNA extraction kits
- QIAcube Purification system consumables



- Applied Biosystems 7500 Fast Dx Real-time PCR instrument (4406984) with System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, CA)
- Applied Biosystems 7500 Fast Sequence Detection Consumables (Applied Biosystems, Foster City, CA)
- ABI MicroAmp™ Fast 8-tube strip 0.1 mL, cat#4358293 (required), or ABI MicroAmp™ Optical 8-cap strip, cat#4323032 (required)
- ABI MicroAmp™ Fast Optical 96-Well Reaction Plate with (0.1 mL), part #4346906 (with barcode) or #4346907 (without barcode), or part 4366932 (alternate to 8-strip tubes)

6. IMPORTANT PUBLIC HEALTH SURVEILLANCE INFORMATION

Dengue is a nationally notifiable disease in the United States and patients testing positive with CDC DENV-1-4 Real-Time RT-PCR Assay or for IgM anti-DENV should be reported to state, local or territorial health departments. Clinicians should have a high level of suspicion and consider dengue diagnostic testing among patients with acute febrile illness in the following settings:

1. In dengue endemic areas of the United States (e.g., Puerto Rico, Virgin Islands, U.S.-affiliated Pacific Islands) in patients with acute febrile illness of 1-8 days duration with or without signs and symptoms of mild or severe dengue;
2. Among recently returning travelers from tropical areas with an acute febrile illness of 1-8 days duration with or without symptoms of mild or severe dengue;
3. Patients in areas of the United States that have previously experienced dengue outbreaks or have DENV competent vectors (e.g., U.S.-Mexico border, Florida) with an acute febrile illness with or without symptoms of mild or severe dengue.

CDC offers reference diagnostic testing and consultation for suspect dengue cases. Please visit <http://www.cdc.gov/dengue> for clinical and laboratory guidelines.

CDC and WHO References:

<http://www.cdc.gov/dengue/>

<http://www.cdc.gov/dengue/clinicalLab/index.html>

<http://www.healthmap.org/dengue/index.php>

<http://www.who.int/csr/disease/dengue/en/>

7. REAGENT STORAGE, HANDLING, AND STABILITY

- Store all primers and probes at 2–8°C until re-hydrated for use; store all control materials at -20°C.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on ice or cold block at all times during preparation and use.
- Controls must be thawed and kept on ice at all times during preparation and use.



8. SPECIMEN COLLECTION, HANDLING AND STORAGE

Inadequate or inappropriate specimen collection, storage, and transport are likely to yield false negative test results. Training in specimen collection is highly recommended due to the importance of specimen quality.

To diagnose dengue, the laboratory requires a blood sample taken during the acute period of the disease (first 7 days of symptoms). If the patient makes the first visit to the physician on or after day 7 of onset of the symptoms, that sample is likely to not render a positive RT-PCR result.

8.1 Collecting the Specimen

- Once there is a clinical diagnosis of suspected dengue, take a venous, whole blood sample.
- Follow serum or plasma specimen collection devices manufacturer instructions for proper collection, separation and storage methods. We recommend that separated serum or plasma samples are frozen at -20 °C and sent or shipped in dry ice to the testing laboratories. If dry ice is not available, we recommend that separated serum or plasma is maintained on ice or in a refrigerator for no longer than 2 hours before it is either frozen at -20 °C or tested.
- All serum samples used for the validation of the CDC DENV-1-4 RT-PCR assay were derived from blood samples collected in red-top or in serum separator tubes (red marble or tiger-top). Sodium Citrate plasma was used for analytical sensitivity studies.

8.2 Transporting the Specimen

- Ensure that when transporting human blood, plasma or serum specimens, all applicable regulations for transport of potentially infectious biological specimens are met.
- Transport/ship human serum or plasma samples in dry ice.

8.3 Storing Specimens

- Store specimens at -20 °C upon receipt. Thaw sample and keep on ice during sample processing. Store remaining of sample at -70 °C for long-term keeping.



9. SPECIMEN REFERRAL TO CDC

- Ship all specimens and related RNA overnight to the CDC Dengue Branch.
- Ship frozen specimens on dry ice and non-frozen specimens on cold packs. Ship extracted RNA on dry ice.
- Refer to the International Air Transport Association (IATA – www.iata.org) for requirements for shipment of human or potentially infectious biological specimens).
- For more information about specimen referral, please contact ckq2@cdc.gov. Sample management and shipping can be found at <http://www.cdc.gov/dengue>.

Send samples to the following recipient:

Chief of Molecular Diagnostics
Dengue Branch
Centers for Disease Control and Prevention
Att. Dr. Jorge L. Munoz-Jordan
1324 Cañada Street
San Juan, PR 00920

The emergency contact number for CDC Emerging Operation Center (EOC) is 770-488-7100.

10. REAGENTS AND CONTROLS PREPARATION

10.1 Primer and Probe Preparation

1. Upon receipt, store lyophilized primers and probes at 2-8 °C.
2. Rehydration
 - a. Remove primers and probes from 2-8 °C.
 - b. Pipette 0.1 ml (100 µL) of 10 mM Tris, pH 7.4 - 8.2 or PCR-grade water into each dried PCR primer or probe.
 - c. Allow primers and probes to fully rehydrate for 1 hour at room temperature.
 - d. After primers and probes are fully rehydrated, pulse vortex to ensure a homogeneous solution.
3. Aliquot
 - a. Label one (1) nuclease-free, sterile, tube for each primer and probe with the following information:
 - i. Primer or Probe name
 - ii. Kit Lot #
 - iii. Expiration date
4. Storage
 - a. After rehydration
 - i. Primers
 1. Store aliquots at -20°C or below until expiration date as long as QC requirements are met.



2. Thawed aliquots of primers may be stored at 2-8 °C for up to 3 months.
- ii. Probes
 1. Aliquots of probes are stored at -20°C or below until expiration date as long as QC requirements are met.
 2. Thawed aliquots of probes may be stored at 2-8 °C in the dark for up to 3 months.

10.2 Human Specimen Control (HSC) Preparation

1. Reagent
 - a. Noninfectious cultured human cell material supplied as a liquid suspended in 0.01 M PBS.
 - b. Volume: 1 mL per vial.
2. Storage
 - a. Store at -20 °C or below upon receipt. DO NOT DILUTE.

10.3. DENV-1-4 Positive Control Mix Preparation

1. Reagent
 - a. Inactivated, noninfectious DENV-1-4 Mix is supplied as a liquid suspended in normal human serum in four separate tubes.
 - b. Each tube contains enough DENV-1-4 Mix for approximately 7 extractions.
2. Storage
 - a. Store at -20 °C or below upon receipt. Do not dilute.

10.4 General Preparation

Equipment Preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use. Decontamination agents should be used such as 5% bleach, 70% ethanol, *DNAzap*[™] or *RNase AWAY*[®] to minimize the risk of nucleic acid contamination.

Real-time RT-PCR Reagents

- Place Invitrogen 2X PCR Master Mix and Superscript III RT / Platinum Taq enzyme mix in a cold rack at 4-8°C.
- Completely thaw the 2X PCR Master Mix vial.
- Mix the 2X PCR Master Mix by inversion 10 times.
- Briefly centrifuge 2X PCR Master Mix and Superscript III RT / Platinum Taq enzyme mix, then place in cold rack.



II. WARNINGS AND PRECAUTIONS

- For *in-vitro* diagnostic use (IVD).
- Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in areas where reagents and human specimens are handled.
- Handle all specimens as if infectious using safe laboratory procedures. Refer to Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition BMBL. (<http://www.cdc.gov/biosafety/>) for standard biological safety guidelines for all procedures.
- Specimen processing should be performed in accordance with national biological safety regulations.
- Use personal protective equipment such as (but not limited to) gloves and lab coats when handling kit reagents while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.
- Due to the sensitivity of Real-time RT-PCR assays, special precautions must be followed to avoid false positive amplifications. The following precautionary steps are recommended.
 - Maintain separate areas for assay setup and handling of nucleic acids.
 - Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
 - Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
 - Change gloves between samples and whenever contamination is suspected.
 - Keep reagent and reaction tubes capped or covered as much as possible.
 - Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 5% bleach, **DNAzap™** or **RNase AWAY** to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
- Reagents, master mix, and RNA should be maintained on cold block during preparation and/or use to ensure stability.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and maintained on cold block at all times during preparation and use.
- Dispose of unused kit reagents and human specimens according to local, state, and federal regulations.



12. NUCLEIC ACID (RNA) EXTRACTION

Performance of the CDC DENV-1-4 Real-Time RT-PCR Detection Assay is dependent on the amount and quality of template RNA purified from human specimens. The following commercially available RNA extraction kits and procedures have been qualified and validated for recovery and purity of DENV RNA for use with the Assay:

1. QIAamp® DSP Viral RNA Mini Kit (Qiagen, CAT# 61904). This kit is produced under GMP; therefore, CDC does not monitor stability of this kit. These RNA extraction procedures can be performed manually or in the QIAcube Instrument (9001292).
2. Roche MagNA Pure LC total Nucleic Acid Isolation Kit (03-038 505 001) for viral RNA extraction on The Roche MagNA Pure LC 2.0 (05 197 686 001). This procedure is to be run using Roche MagNA Pure system products only.
3. Store extracted RNA at -20 °C if PCR is to be done within 24 hours; otherwise, keep remaining RNA at -80 °C.

Disclaimer: Manufacturer's recommended procedures are to be used for sample extraction with any of the 2 above mentioned RNA extraction procedures. Names of vendors or manufacturers are provided as examples of suitable product sources. Inclusion does not imply endorsement by the Centers for Disease Control and Prevention.

13. ASSAY SETUP

13.1 Master Mix Preparation/Plate Setup

Prepare Master Mix according to the following tables.

Note: It is necessary to make excess reaction master mix to allow for pipetting error.

Example: *if number of samples (n) including controls = 1-14, the $N = n+1$*

if number of samples (n) including controls >15, then $N = n+2$



Example of Singleplex Reaction (any serotype)*

Reagent	Volume	Volume/rx	Total Number Reactions (N=10+1)	Total Volume
Nuclease-free Water	5.55 µL	N x 5.55 µL	N = 10 + 1 = 11	61.05µL
2X PCR Master Mix	12.5 µL	N x 12.5 µL	N = 10 + 1 = 11	137.5 µL
Forward Primer	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Reverse Primer	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
DENV Probe	0.45 µL	N x 0.45 µL	N = 10 + 1 = 11	4.95 µL
SuperScript™ III RT/Platinum®Taq Mix	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Total Volume	20 µL	N x 20 µL		220 µL

*To complete the CDC DENV-1-4 RT-PCR Assay in singleplex mode, four separate reactions, one for each serotype, must be performed.

Example of Multiplex DENV-1, -2, -3,-4 Reactions

Reagent	Volume	Volume/rx	Total Number Reactions (N)	Total Volume
Nuclease-free Water	2.2 µL	N x 2.2 µL	N = 10 + 1 = 11	24.2 µL
2X premix	12.5 µL	N x 12.5 µL	N = 10 + 1 = 11	137.50 µL
Primer D1-F	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer D1-R	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer D2-F	0.25 µL	N x 0.25 µL	N = 10 + 1 = 11	2.75 µL
Primer D2-R	0.25 µL	N x 0.25 µL	N = 10 + 1 = 11	2.75 µL
Primer D3-F	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer D3-R	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer D4-F	0.25 µL	N x 0.25 µL	N = 10 + 1 = 11	2.75 µL
Primer D4-R	0.25 µL	N x 0.25 µL	N = 10 + 1 = 11	2.75 µL
Probes (DENV-1-4)	0.45 µL	N x 0.45 µL	N = 10 + 1 = 11	4.95 µL
SuperScript™ III RT/Platinum®Taq Mix	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Total Volume	20 µL	N x 20 µL		220 µL



Example of HSC Reaction

(This reaction is run separate from either singleplex or multiplex reactions)

Reagent	Volume	Volume/rx	Total Number Reactions (N=10+1)	Total Volume
Nuclease-free Water	5.5 µL	N x 5.5 µL	N = 10 + 1 = 11	60.5 µL
2X PCR Master Mix	12.5 µL	N x 12.5 µL	N = 10 + 1 = 11	137.5 µL
Primer RP-F	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer RP-R	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
RP Probe	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
SuperScript™ III RT/Platinum®Taq Mix	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Total Volume	20 µL	N x 20 µL		220 µL

Prepare Mix on ice and add template.

- a. Place PCR plate on ice.
- b. Add 20 µL of Master Mix to each well.
- c. Add 5 µL of extracted sample, including HSC samples.
- d. Seal with optical 8-cap strip and place plate in Applied Biosystems 7500 Fast DX Real-time PCR System.

14. CREATE A RUN TEMPLATE ON THE ABI 7500 FAST DX REAL-TIME PCR INSTRUMENT (REQUIRED IF NO TEMPLATES EXIST)

If the template already exists on your instrument, please proceed to the **RUNNING A TEST** section.

Otherwise, follow the process below:

1. Launch the Applied Biosystems 7500 Fast Dx Real-time PCR System by double clicking on the Applied Biosystems 7500 Fast Dx System icon on the desktop.
2. A new window should appear, select **Create New Document** from the menu.

Figure 1. New Document Wizard Window

New Document Wizard

Define Document
Select the assay, container, and template for the document, and enter the operator name and comments.

Assay: Standard Curve (Absolute Quantitation)

Container: 96-Well Clear

Template: Blank Document Browse...

Run Mode: Standard 7500

Operator: EDGARDU

Comments: SDS v1.4

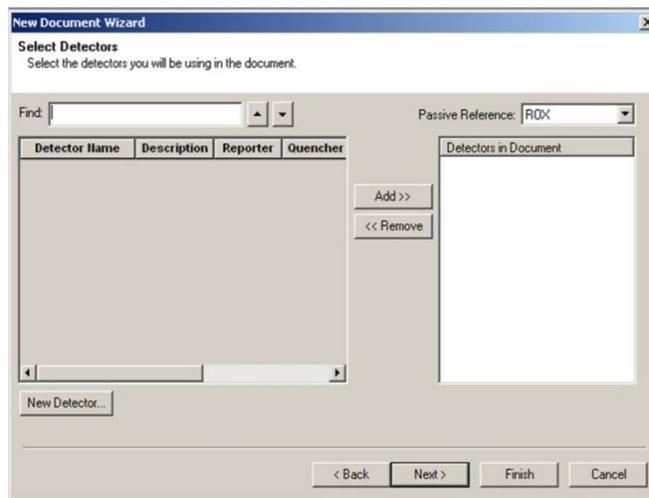
Plate Name: Dengue

< Back Next > Finish Cancel



3. The **New Document Wizard** screen in **Figure 1** will appear. Select:
 - a. Assay: **Standard Curve (Absolute Quantitation)**
 - b. Container: **96-Well Clear**
 - c. Template: **Blank Document**
 - d. Run Mode: **Standard 7500**
 - e. Operator: ***Your name***
 - f. Comments: **SSD v1.4**
 - g. Plate Name: ***Your choice***
4. After making selections, click **Next** at the bottom of the window.
5. After selecting next, the **Select Detectors** screen (**Figure 2**) will appear.
6. Click the **New Detector** button (see **Figure 2**).

Figure 2. Creating New Detectors



If you are running the Singleplex Assay, continue with step 7 on the next page.

If you are running the Multiplex Assay, continue on page 25.

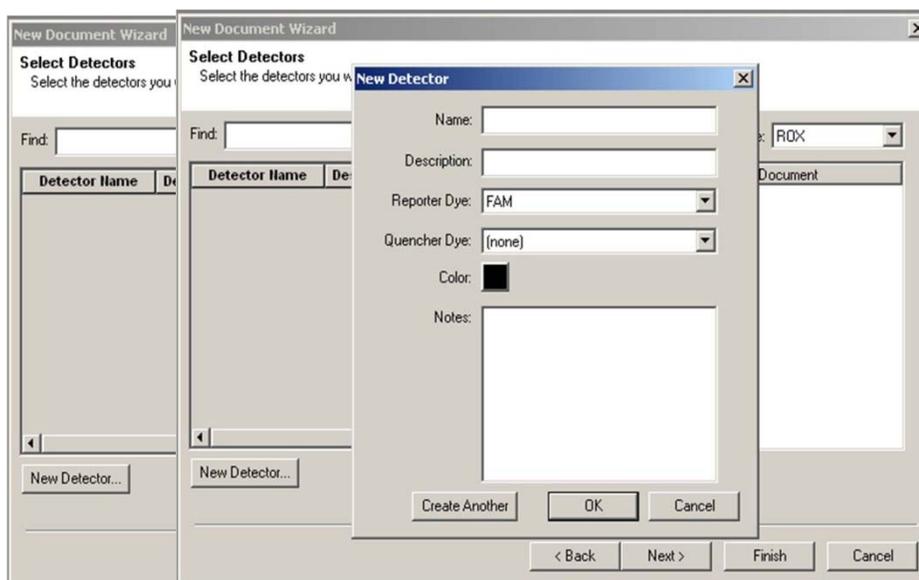


14.1 Singleplex Assay

THE FOLLOWING STEPS ARE SPECIFIC FOR CREATING RUN TEMPLATES FOR SINGLEPLEX ASSAY

7. The New Detector window will appear (**Figure 3**). A new detector will need to be defined for each dengue primer and probe set. Creating these detectors will enable you to analyze each primer and probe set individually at the end of the reaction.

Figure 3. Creating New Detector



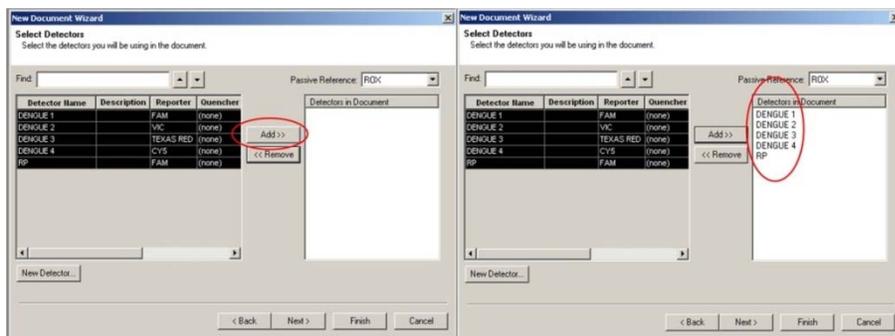
8. Start by creating the DENV-1 Detector. Include the following:
 - a. Name: **DENGUE 1**
 - b. Description: *leave blank*
 - c. Reporter Dye: **FAM**
 - d. Quencher Dye: **(none)**
 - e. Color: *to change the color of the detector indicator do the following:*
 - Click on the color square to reveal the color chart.
 - Select black as the color by clicking on the black square.
 - After selecting color click **OK** to return to the New Detector screen.
 - f. Click the **New Detector** button of the New Detector screen to return to the screen shown in **Figure 3**. Repeat steps 6-8 for each DENGUE target in the Assay. Please select the colors as indicated in the following chart.



Name	Reporter Dye	Quencher Dye	Color
DENGUE 1	FAM	(NONE)	BLACK
DENGUE 2	VIC	(NONE)	BLUE
DENGUE 3	TEXAS RED	(NONE)	RED
DENGUE 4	CY5	(NONE)	GREEN
RP	FAM	(NONE)	BROWN

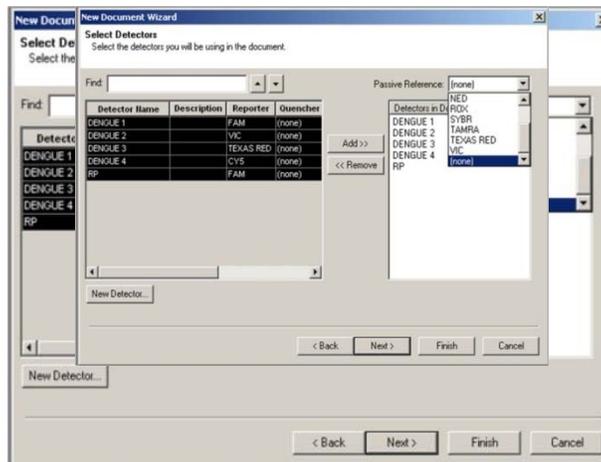
- After each Detector is added, the **Detector Name**, **Description**, **Reporter**, and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 4**).
- Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (see **Figure 4**). Detector names will appear on the right hand side of the **Select Detectors** window (**Figure 4**).

Figure 4. Adding New Detectors to Document



- Once all detectors have been added, select (none) for Passive Reference at the top right hand drop down menu (**Figure 5**).

Figure 5. Select Passive Reference



12. Click **Next** at the bottom of the **Select Detectors** window to proceed to the **Set Up Sample Plate** window (**Figure 6**).
13. In the **Set Up Sample Plate** window (**Figure 6**), use your mouse to select row A from the lower portion of the window, in the spreadsheet (see **Figure 6**).
14. Then in the top portion of the window, select detector **DENGUE 1**. A check will appear next to the detector you have selected (**Figure 6**). You will also notice the column in the spreadsheet will be populated with a colored “U” icon to indicate which detector you have selected.
15. Repeat steps 14-15 for each detector that will be used in the assay.

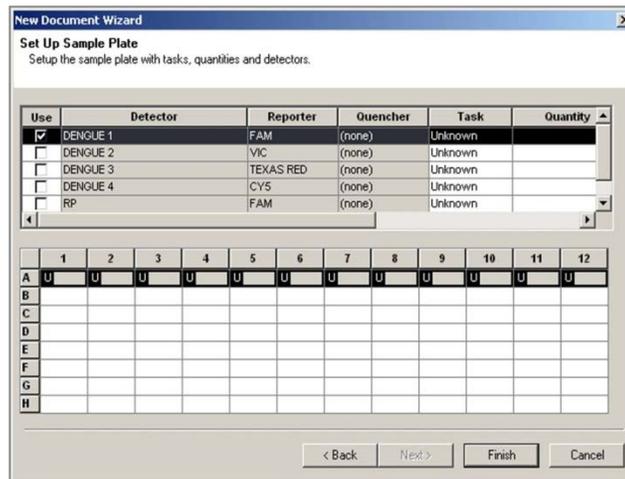
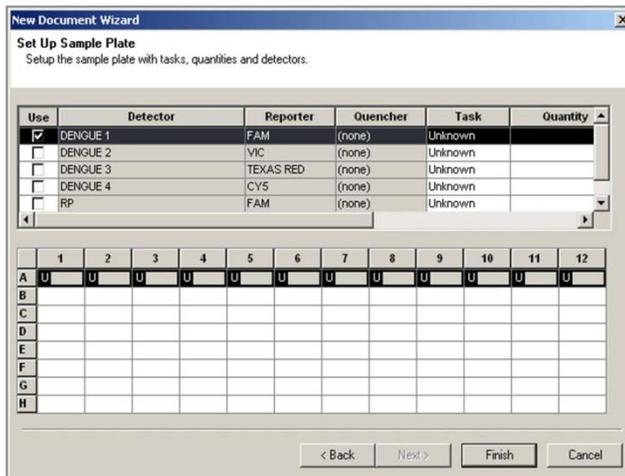


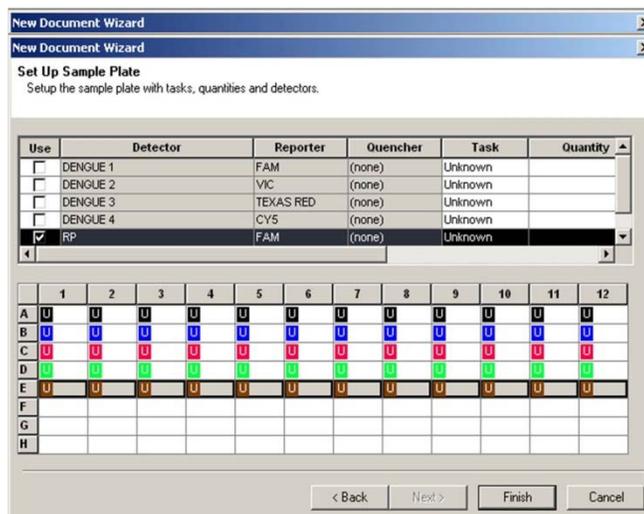
Figure 6. Sample Plate Setup



16. Select **Finish** after detectors have been assigned to their respective columns (**Figure 7**).

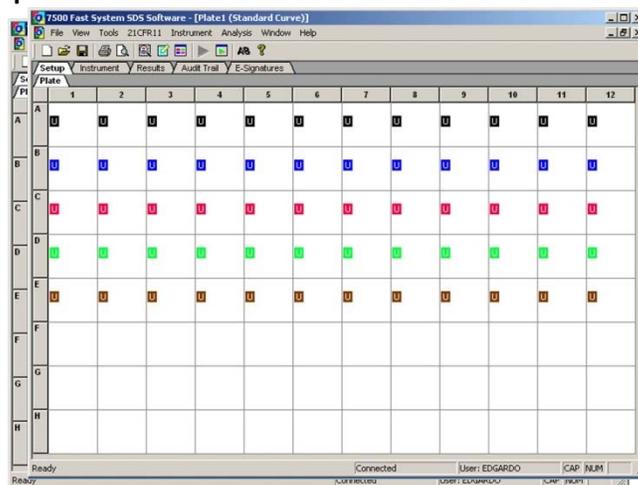


Figure 7. Sample Plate Setup



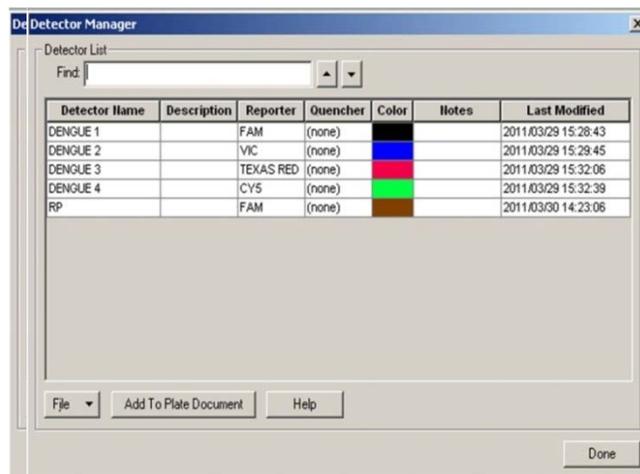
17. After clicking **Finish**, there will be a brief pause allowing the Applied Biosystems 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. **Note: The machine must be turned on for initialization.**
18. After initialization, the **Plate** tab of the **Set up (Figure 8)** will appear.
19. Each well of the plate should contain colored U icons that correspond with the detector labels that were previously chosen. To confirm detector assignments, select **Tools** from the file menu, then select **Detector Manager**.

Figure 8. Plate Set up Window



20. The Detector Manager window will appear (**Figure 9**).

Figure 9. Detector Manager Window



21. Confirm all dengue detectors are included and that each dengue target has a **Reporter** set to respective reporter dye and the **Quencher** is set to **(none)**.
22. If all detectors are present, select **Done**. The detector information has been created and assigned to wells on the plate.

[Continue on page 29.](#)

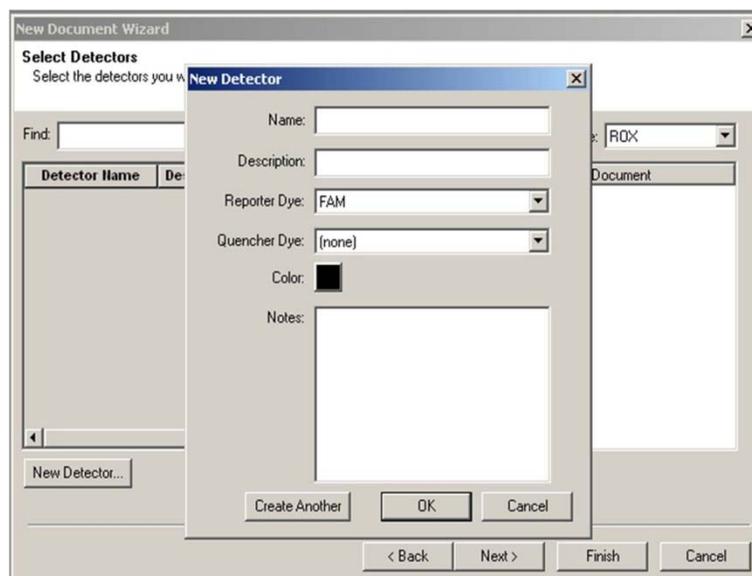


14.2 Multiplex Assay

THE FOLLOWING STEPS ARE SPECIFIC FOR CREATING RUN TEMPLATES FOR MULTIPLEX ASSAY

- The New Detector window will appear (**Figure 10**). A new detector will need to be defined for each dengue primer and probe set. Creating these detectors will enable you to analyze each primer and probe set individually at the end of the reaction.

Figure 10. Creating New Detector



- Start by creating the DENV-1 Detector. Include the following:
 - Name: **DENGUE 1**
 - Description: *leave blank*
 - Reporter Dye: **FAM**
 - Quencher Dye: **(none)**
 - Color: *to change the color of the detector indicator do the following:*
 - Click on the color square to reveal the color chart
 - Select black as the color by clicking on the black square
 - After selecting color click **OK** to return to the New Detector screen
 - Click the **New Detector** button of the New Detector screen to return to the screen shown in **Figure 10**
- Repeat step 6-8 for each DENGUE target in the Assay. Please select the colors as indicated in the following chart.

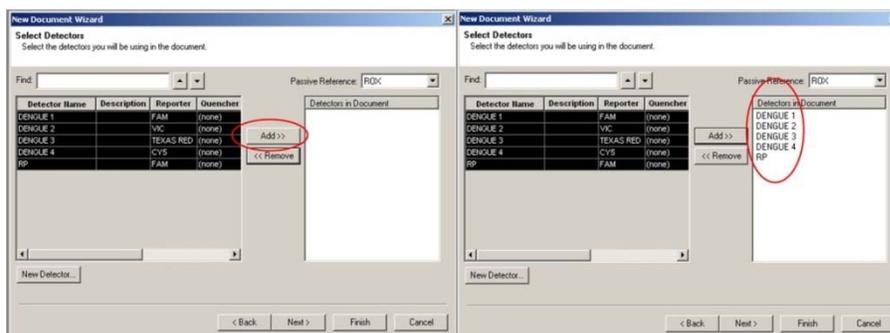


Name	Reporter Dye	Quencher Dye	Color
DENGUE 1	FAM	(NONE)	BLACK
DENGUE 2	VIC	(NONE)	BLUE
DENGUE 3	TEXAS RED	(NONE)	RED
DENGUE 4	CY5	(NONE)	GREEN
RP	FAM	(NONE)	BROWN

10. After each Detector is added, the **Detector Name**, **Description**, **Reporter**, and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 11**).

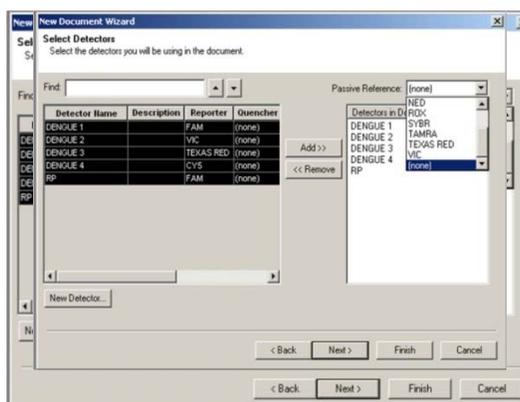
11. Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (see **Figure 11**). Detector names will appear on the right hand side of the **Select Detectors** window (**Figure 11**).

Figure 11. Adding New Detectors to Document



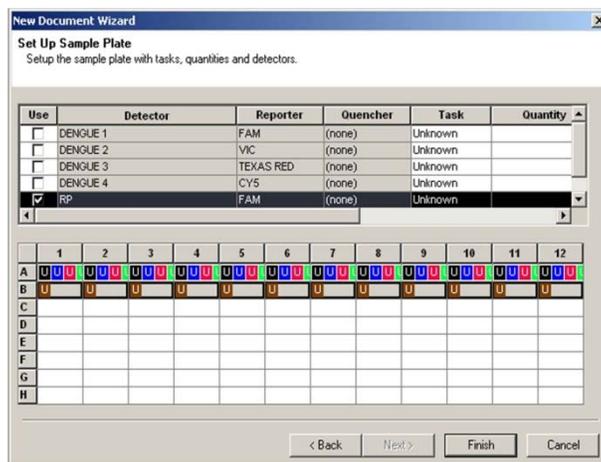
12. Once all detectors have been added, select (none) for Passive Reference at the top right hand drop down menu (**Figure 12**).

Figure 12. Select Passive Reference



13. Click **Next** at the bottom of the **Select Detectors (Figure 12)** window to proceed to the **Set Up Sample Plate** window.
14. In the **Set Up Sample Plate** window (**Figure 13**), use your mouse to select row A from the lower portion of the window, in the spreadsheet (see **Figure 13**).
15. Then in the top portion of the window, select detector **DENGUE 1, DENGUE 2, DENGUE 3 AND DENGUE 4**. A check will appear next to each detector you have selected (**Figure 13**). You will also notice the column in the spreadsheet will be populated with a colored “U” icon to indicate each detector you have selected.
16. Select row B from the lower portion of the window, in the spreadsheet.
17. The in the top portion of the window, select detector **RP**. A check will appear next to the detector you have selected. You will also notice the column in the spreadsheet will be populated with a colored “U” icon to indicate the RP detector you have selected.
18. Repeat steps 14-16 for each corresponding row where samples will be located in the plate.

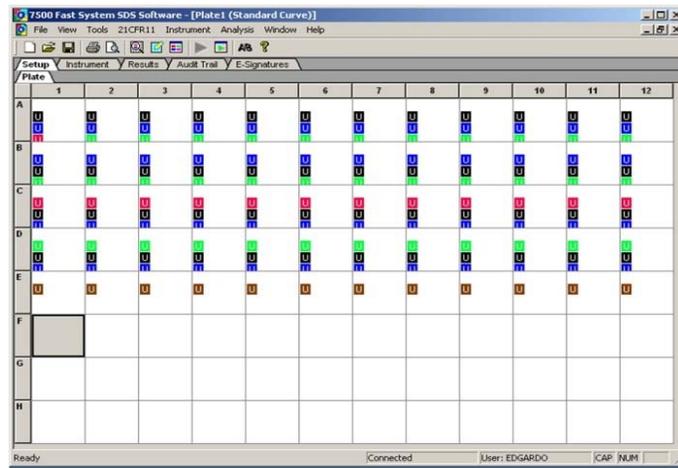
Figure 13. Sample Plate Setup



19. Select **Finish** after detectors have been assigned to their respective columns.
20. After clicking Finish, there will be a brief pause allowing the Applied Biosystems 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. **Note: The machine must be turned on for initialization.**
21. After initialization, the **Plate** tab of the **Set up (Figure 14)** will appear.
22. Each well of the plate should contain colored U icons that correspond with the detector labels that were previously chosen. To confirm detector assignments, select **Tools** from the file menu, then select **Detector Manager**.

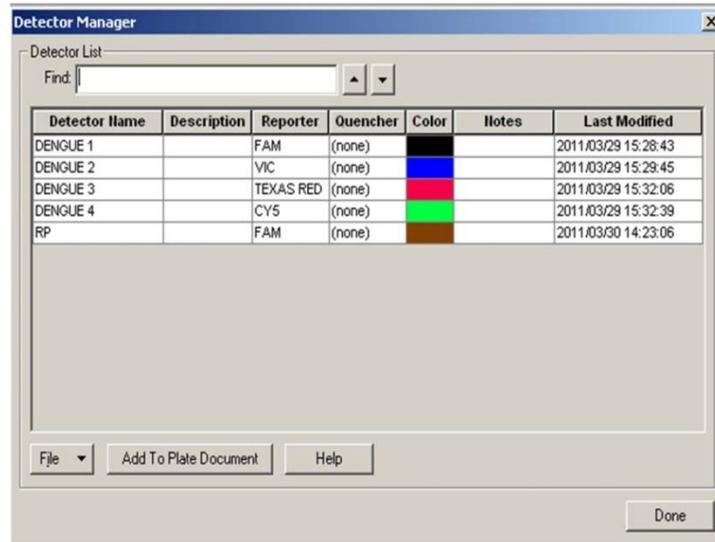


Figure 14. Plate Set up Window



23. The Detector Manager window will appear (Figure 15).

Figure 15. Detector Manager Window



24. Confirm all dengue detectors are included and that each dengue target has a **Reporter** set to respective reporter dye and the **Quencher** is set to **(none)**.

25. If all detectors are present, select **Done**. The detector information has been created and assigned to wells on the plate.

Continue on next page.



15. DEFINING THE INSTRUMENT SETTINGS

1. After detectors have been created and assigned, proceed to instrument set up.
2. Select the **Instrument** tab to define thermal cycling conditions.
3. Modify the thermal cycling conditions as follows (**Figure 16**):
 - a. In Stage 1, Set to **30 min at 50°C; 1 Rep**
 - b. In Stage 2, Set to **2.0 min at 95°C; 1 Rep**
 - c. In Stage 3, Step 1 set to **15 sec at 95°C**
 - d. In Stage 3, Step 2 set to **1 min at 60.0°C**
 - e. In Stage 3 Reps should be changed to **45**
 - f. Under **Settings** (**Figure 16**), bottom left-hand box, change volume to 25 μ L
 - g. Under **Settings**, **Run Mode** selection should be **Standard 7500**
 - h. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 16**)

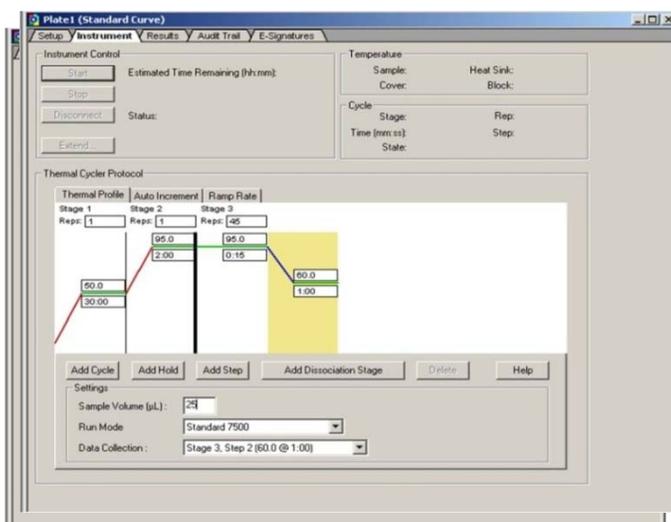
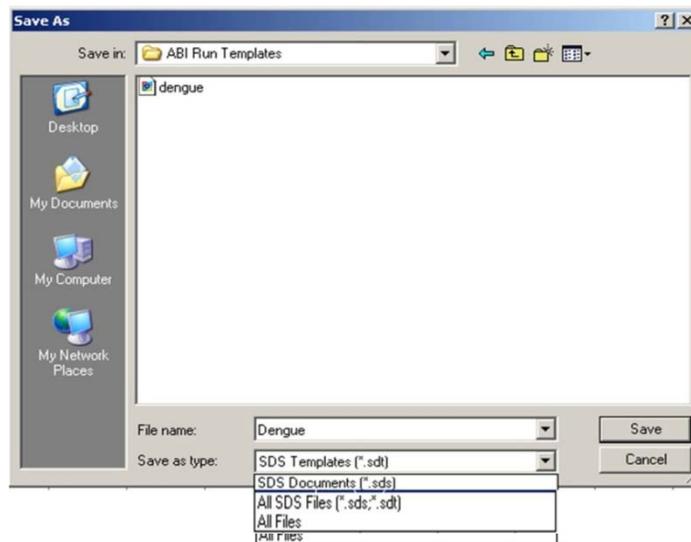


Figure 16. Instrument Window

4. After making changes to the **Instrument** tab, the template file is ready to be saved. To save the template, select **File** from the top menu, then select **Save As**.
5. Save the template as **Dengue** in desktop folder labeled **"ABI Run Templates"** (you must create this folder). Save as type should be SDS Templates (*.sdt). (**Figure 17**).



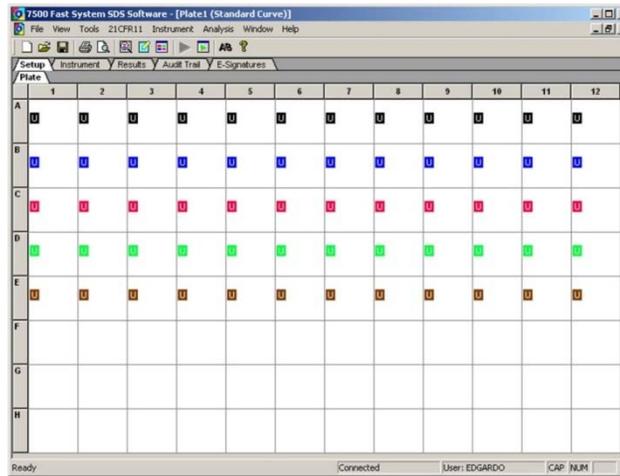
Figure 17. Saving Template



16. RUNNING A TEST

1. Turn on the ABI 7500 Fast Dx Real-time PCR instrument.
2. Launch the Applied Biosystems 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
3. A new window should appear, select **Open Existing Document** from the menu.
4. Navigate to select your ABI Run Template folder from the desktop.
5. Double click on the **Dengue** template file.
6. There will be a brief pause allowing the Applied Biosystems 7500 Fast Dx Real-time PCR system to initialize. This initialization is followed by a clicking noise. **Note: The machine must be turned on for initialization.**

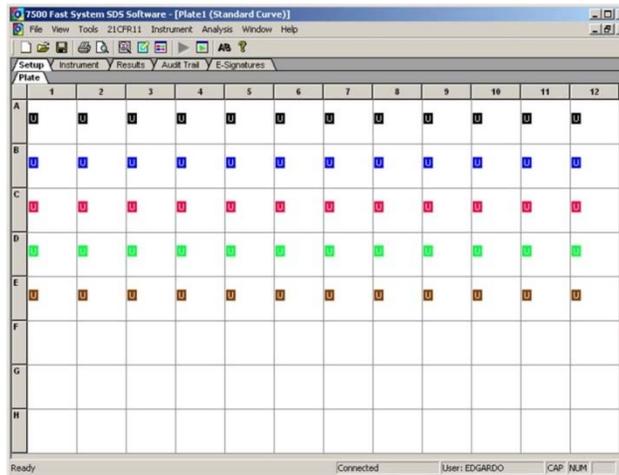




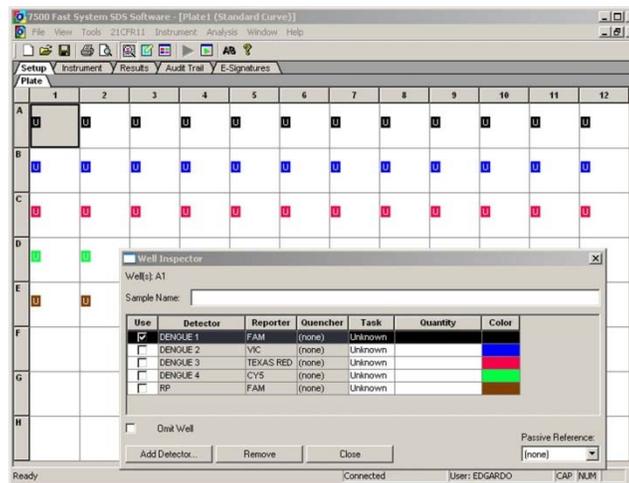
7. After the instrument initializes, a plate map will appear (**Figure 18**). The detectors and controls should already be labeled as they were assigned in the original template.



Figure 18. Plate Set up Window



8. Click the **Well Inspector** icon  from the top menu.
9. Highlight specimen wells of interest on the plate map.
- 10.

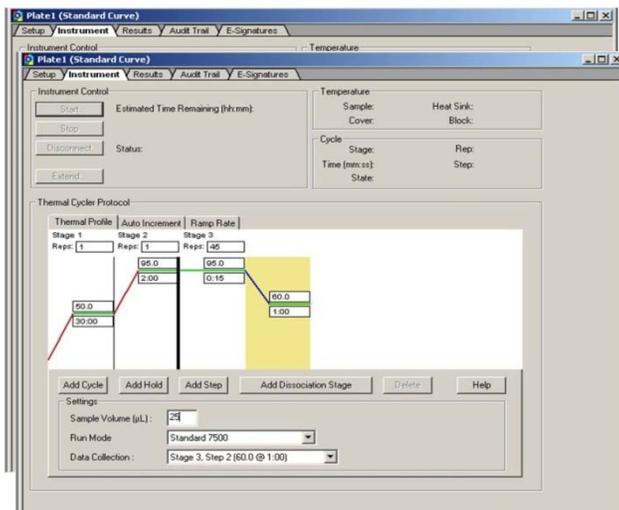


11. Repeat steps 9-10 until all sample identifiers are added to the plate setup.
12. Once all specimen and control identifiers are added click the **Close** button on the **Well Inspector** window to return to the **Plate** set up tab.
13. Click the **Instrument** tab at the upper left corner.



14. The reaction conditions, volumes, and type of 7500 reaction should already be loaded (**Figure 20**).

Figure 20. Instrument Settings



15. Ensure settings are correct (refer to the *Defining Instrument Settings*).

16. Before proceeding, the run file must be saved; from the main menu, select **File**, then **Save As**. Save in appropriate run folder designation.

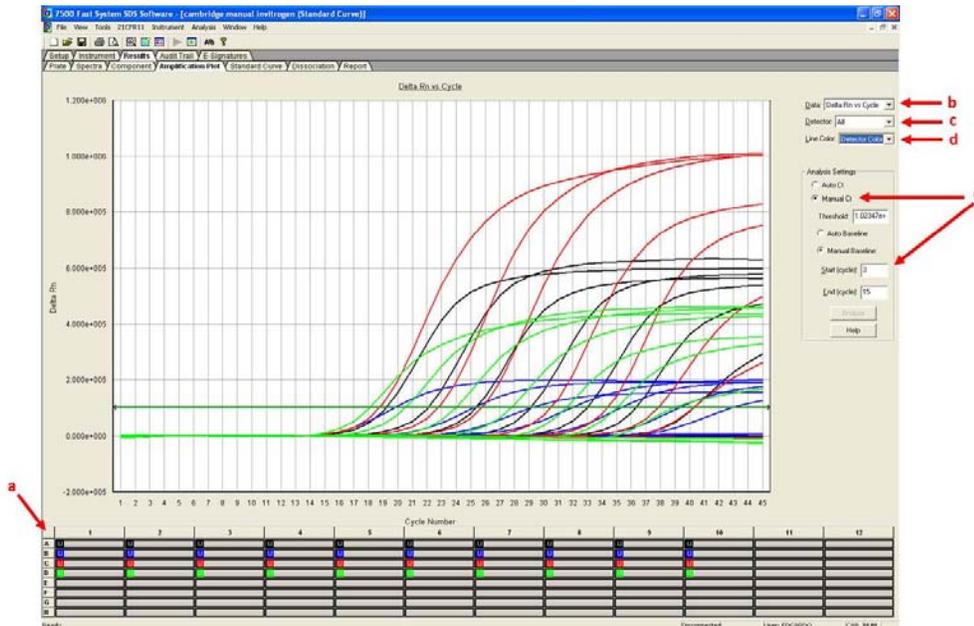
17. Once run file is saved, click the **Start** button. *Note: The run should take approximately 2 hrs to complete.*



17. DATA ANALYSIS

1. After the run has completed, select the Results tab at the upper left corner of the software.
2. Select the Amplification Plot tab to view the raw data (**Figure 21**).

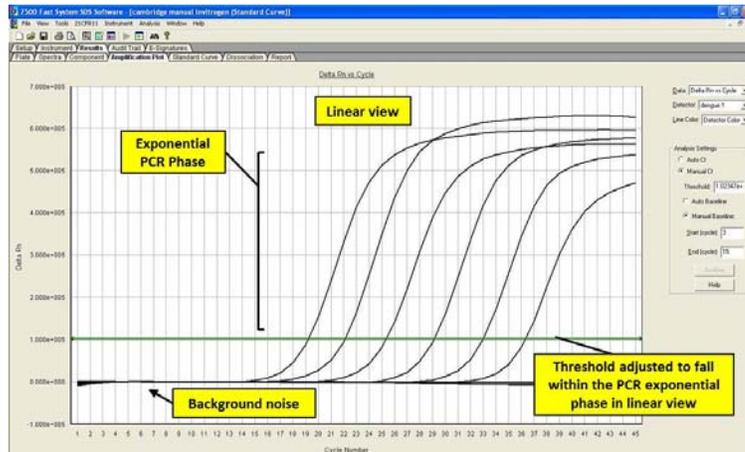
Figure 21. Amplification Plot Window



3. Start by highlighting all the samples from the run; to do this, click on the upper left hand box (**a**) of the sample wells (**Figure 21**). All the growth curves should appear on the graph.
4. On the right hand side of the window, (**b**) the **Data** drop down selection should be set to **Delta Rn vs Cycle**.
5. Select **DENGUE 1** from the (**c**) the **Detector** drop down menu using the downward arrow.
 - a. Please note that each detector is analyzed individually to reflect different performance profiles of each primer and probe set.
6. In the **Line Color** drop down, (**d**) **Detector Color** should be selected.
7. Under **Analysis Settings** select **Manual Ct** (**e**).
 - a. Do not change the **Manual Baseline** default numbers. Using the mouse, click and drag the red threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal (**Figure 22**).



Figure 22. Amplification Plot



8. Click the **Analyze** button in the lower right corner of the window. The red threshold line will turn green, indicating the data has been analyzed.
9. Repeat steps 5-9 to analyze results generated for each set of markers (i.e. DENGUE 1, DENGUE 2, DENGUE 3, DENGUE 4, RP).
10. Save analysis file by selecting **File** then **Save As** from the main menu.
11. After completing analysis for each of the markers, select the **Report** tab above the graph to display the Ct values. To filter report by sample name in ascending or descending order, simply click on **Sample Name** on the table (Figure 23).

Figure 23. Report



18. INTERPRETATION OF RESULTS

18.1 Extraction and Control Results and Interpretation

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 Control

The positive control consists of a mix of DENV-1, -2, -3, and -4 dengue virus from C6/36 cell culture supernatant. Purified RNA from the positive control will yield a positive result with the following primer and probe sets: DENV-1, DENV-2, DENV-3 and DENV-4.

Human Specimen Control (HSC) (Extraction Control)

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.

RNase P (RP)

- All clinical samples should exhibit fluorescence amplification curves in the RP 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 all 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.

DENV Markers (DENV-1, -2, -3 and -4)

- When all controls exhibit the expected performance, a specimen is considered negative if DENV marker amplification curves DO NOT cross the threshold line within 37 cycles (< 37 Ct) and RP amplification curve does cross the threshold line within 37cycles (< 37 Ct).
- When all controls exhibit the expected performance, a specimen is considered positive for either DENV-1, -2, -3 or -4 if the DENV marker (e. g., DENV-1, -2, -3 or -4) amplification curve crosses the threshold line within 37 cycles (<37 Ct). If more than one DENV marker crosses the threshold line within cycle 37 (<37 Ct), the extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the re-tested sample is still positive for two DENV markers and all controls exhibit the expected performance, the result may indicate “dual DENV infection.” Dual infections have been rarely reported. If there is an indication of dual infection, sample should be sent to the CDC-Dengue Branch for confirmatory testing (Email: ckq2@cdc.gov).
- When all controls exhibit the expected performance and none of the amplification curves for DENV markers or the RP marker cross the threshold line within 37 cycles (<37 Ct), the result is inconclusive. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. 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.

19. STANDARD-BASED ELECTRONIC LABORATORY REPORTING FOR DENGUE

19.1 Recommendations for Uniform Coding And Vocabulary For Diagnostic Testing

The following information is provided to assist the performing laboratory in complying with new federal guidelines for the meaningful use of health information. The standardization of test information as well as the method for achieving electronic data exchange has become a priority for the federal government and healthcare systems. It is expected that a uniform approach to the terminology and exchange format for diagnostic assays used for the identification and characterization of pathogens that humans can acquire will accelerate this process.

To achieve this goal it is necessary to harmonize human readable language and machine readable language through the use of common exchange protocols as well as standard vocabulary and codes. The implementation of adopted standards must be harmonized across all performing laboratories to ensure interoperability. Harmonization refers to the process of insuring that all users interpret and use the standards in the same way. Without this harmonization process, it is possible for different laboratories to implement the standards differently and thus result in the generation of data that cannot be shared or used for its intended purposes.



This interoperability is especially important for laboratory tests associated with diseases of public health importance. The ability of the local and state officials as well as the federal government to receive tests results associated with human case definition is critical for identifying new outbreaks and for epidemiological investigations needed to limit the spread of disease.

19.2 Process for Achieving Uniformity In Laboratory Test Results

The laboratory performing diagnostic assays may utilize a Laboratory Information Management Systems (LIMS) with connections to a hospital or medical system Electronic Health Record (EHR). The U.S. Department of Health and Human Services recommends the use of specific coding systems for achieving uniformity of test results incorporated into electronic messages. These coding systems include LOINC Logical Observation Identifiers Names and Codes (LOINC® -- (<http://www.loinc.org>) and SNOMED CT--Systematic Nomenclature of Medicine--Clinical Terms (<http://www.ihtsdo.org/>). These coding systems have specific capabilities that are essential for achieving uniformity.

Standard English terminology for the test name and text results have been established by the CDC developer of this assay through collaboration with the testing community and expert knowledge of the processes involved. It is recognized that this terminology will differ in countries outside the United States. However, through the use of national and international agreements, it is possible to establish a universal set of codes and terms to accurately characterize laboratory observations. At this time, there are no recommendations that apply to the reporting of results of this assay within the United States, but they may be available in the future.

LOINC provides for a common understanding of the medical procedure or process related to the specific assay, in this case the process of detecting the presence of dengue virus serotypes 1-4. The LOINC codes describe the specific molecular methodology employed by the assay; recovery and amplification of an RNA target and that multiple targets are employed. This approach unambiguously distinguishes the test from other molecular assays so that the manufacturer and the FDA can track its performance in comparison with other assays in the future.

SNOMED CT codes provide for unambiguous representation of the test results and allow the application of specific concepts such as “detected” or “positive”. These codes provide for description of the source or location of the specimen being tested. Also specified are terms for conveying information about failures of the test procedure or the lack of adequate specimen.

It is recommended that the LOINC test code and the test results, represented by SNOMED codes, be incorporated into a Health Level 7 (HL7) standardized laboratory test message format for electronic routing of results to and from HL7 compliant systems. This would achieve the goal of compliance with new federal guidelines for the meaningful use of health information and



harmonized human readable language and machine readable language through the use of common exchange protocols as well as standard vocabulary and codes.

The test result message for this assay includes results from all component tests and a conclusion or interpretation. The results are to be incorporated into a standard Health Level 7 (HL7) electronic format for laboratory test messaging. More information about HL7 can be found at <http://www.hl7.org>. Several versions of HL7 exist and there are several implementations in use for each version. Examples of public health messaging systems currently using a constrained version of HL7 v2.3.1 ORU^R01 message to report results can be viewed at PHINVADS: <http://phinivads.cdc.gov/vads>. All related vocabulary can be found as a view and downloaded from: <https://phinivads.cdc.gov/vads/ViewCodeSystem.action?id=2.16.840.1.114222.4.5.277>.

20. 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 test as indicated above. If sample is repetitively reactive for both serotypes the result may be 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.



21. QUALITY CONTROL

- Quality control requirements must be performed in conformance with local, state, and federal regulations or accreditation requirements and the user laboratory's standard quality control procedures. It is recommended that the user refer to CLSI document C24-A2, Statistical Quality Control for Quantitative Measurements: Principles and Definitions: [Approved Guideline-Second Edition] or other published guidelines for general quality control recommendations. For further guidance on appropriate quality control practices, refer to 42CFR 493.1202(c).
- Quality control procedures are intended to monitor reagent and assay performance.
- Test all positive controls prior to running diagnostic samples with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice (cGLP) recommends including a positive extraction control in each nucleic acid isolation batch.
- HSC extraction control must proceed through nucleic acid isolation per batch of specimens to be tested.
- Always include a negative control (NTC), and the appropriate positive control (DENV-1 – 4) in each amplification and detection run.

22. LIMITATIONS

- This device is subject to a special control requiring that distribution be limited to laboratories with (i) experienced personnel who have training in standardized molecular testing procedures and expertise in viral diagnosis, and (ii) appropriate biosafety equipment and containment (21CFR866.3332(b)(2)).
- Negative results do not preclude dengue virus infection and should not be used as the sole basis for treatment or other patient management decisions. A negative specimen collected between days 3-7 after onset of the febrile illness should be retested with an anti-DENV IgM test to increase the likelihood of making the diagnosis of dengue.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- Do not use any reagent past the expiration date.
- The performance of this test has not been established for monitoring treatment of dengue.
- The performance of this test has not been established for screening of blood or blood product for the presence of DENV infection.
- Detection of viral RNA may not indicate the presence of infectious virus or that dengue is the causative agent for clinical symptoms.
- This test cannot rule out diseases caused by other bacterial, viral or parasitic pathogens.
- Assay performance characteristics have not been established for prenatal screening, or for general population screening without symptoms consistent with Dengue Fever. The test is not FDA cleared for the screening of blood or plasma donors.



23. EXPECTED VALUES

The percent of positive cases identified by the CDC DENV-1-4 Real-Time RT-PCR Assay will vary depending on 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.

24. PERFORMANCE CHARACTERISTICS

24.1 Clinical Performance

Prospective Studies

Performance characteristics of the CDC DENV-1-4 Real-Time 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).



Overall Prospective Comparison Results

Multiplex CDC DENV-1-4 Real-Time RT-PCR Assay Comparison Results				
		Reference Method (Sequencing)		
		Positive	Negative	Total
CDC DENV-1-4 Real-Time RT-PCR Assay	Positive	47	0	47
	Negative	1*	38	39
	Total	48	38	86
		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 RT-PCR assay which was positive for DENV-3 by E gene bi-directional sequencing. All other negative RT-PCR samples were also negative by E gene bi-directional sequencing.

Retrospective Studies

The 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

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 had positive RT-PCR results but were not confirmed by IgM anti-DENV seroconversion. Two of these samples were positive for DENV-3 and the other two samples were positive for DENV-4 positive. These results were confirmed by E gene bi-directional sequencing.

24.2 Reproducibility

The reproducibility of the CDC DENV-1-4 Real-Time RT-PCR Assay was evaluated at three testing sites (the CDC Dengue Branch Lab and two external independent sites). Operator to operator, run to run and site to site reproducibility were evaluated using five test panels which include negative, high negative, low positive, and moderate positive 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. 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 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 a 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 following table.



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

		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
Neg. 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
Neg. Control		NA	NA	10/10 (100 %)	NA

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

24.3 Analytical Sensitivity

Limit of Detection

The limit of detection (LoD) of the CDC DENV-1-4 Real-Time RT-PCR Assay (Singleplex) was determined using a 5-replica panel of viruses serially diluted in 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 on the Qiagen QIAcube. 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 observed LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Singleplex) in serum and plasma was 1×10^3 pfu/mL and is shown in the following table.



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 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).

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 similar (1×10^3 pfu/mL) for both RNA extraction methods: using an automated (Qiagen QIAamp[®] DSP Viral RNA Mini Kit and Qiagen QIAcube) or a manual procedure (Qiagen QIAamp[®] DSP Viral RNA Mini Kit).

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 was observed similar (1×10^3 pfu/mL) using an automated viral RNA extraction method (MagNA Pure LC total Nucleic Acid Isolation Kit on the MagNA Pure LC 2.0 instrument).

There was no difference in the LoD of the CDC DENV-1-4 Real-Time RT-PCR Assay (Multiplex) using 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).



Analytical Reactivity

In order to assess if the CDC DENV-1-4 Real Time RT-PCR 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 additional DENV 1 – 4 strains. Twenty nine DENV 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 observed LoD of the CDC DENV-1-4 RT-PCR Assay was similar in cultured isolates. 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	South Pacific	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	Thailand	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
2007	Colombia	SE Asian/American	31.69	0.34	3/3	7.66 x 10 ⁴	NA	NA	0/3	N/A
1980	Ivory Cost	Sylvatic	31.67	0.34	3/3	2.46 x 10 ⁴	36.6	1.37	2/3	7.22 x 10 ³
1988	Vietnam	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	Asian I	32.14	0.87	3/3	9.56 x 10 ³	NA	NA	0/3	N/A
2003	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
2003	Costa Rica	SE Asian/American	32.04	0.14	3/3	3.15 x 10 ⁴	NA	NA	0/3	N/A
1996	Peru	American	32.39	0.32	3/3	5.99 x 10 ²	37.12	1.26	1/3	1.88 x 10 ³
1982	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	South Pacific	36.69	1.47	2/3	5.12 x 10 ⁴	NA	NA	0/3	NA
2006	Thailand	Thailand	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	South 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	SE Asian	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	SE Asian	32.78	0.32	3/3	2.13 x 10 ⁴	NA	NA	0/3	2.77 x 10 ³



24.4 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]), of which 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 *Borrelia*, were also spiked in serum for cross reactivity studies. The CDC DENV-1-4 Real Time RT-PCR Assay was performed on all 12 sample triplicates. 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.

Pathogen	Sample type	Concentration	DENV RT-PCR
			Rate positive
Virus		pfu/ml	
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



Interference

Performance of the CDC DENV-1-4 Real-Time RT-PCR Assay was characterized 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 µmol/L), cholesterol (13 mmol/L), hemoglobin (2 g/L), triglycerides (37 mmol/L) and genomic DNA (400 µg/100 mL). The levels tested for each endogenous substance were based on the Clinical Laboratories institute (NCCLS) 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 interference was observed in the presence of the described potential interfering substances tested at the concentrations specified above.

24.5 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. Negative samples tested were negative (32/32) and positive samples were positive (32/32) 100% of the time.



25. REFERENCES

1. World-Health-Organization. 2009. *Dengue: guidelines for diagnosis, treatment, prevention and control*.
2. Rigau-Perez, J. G., A. V. Vorndam, and G. G. Clark. 2001. *The dengue and dengue hemorrhagic fever epidemic in Puerto Rico, 1994-1995*. *Am J Trop Med Hyg* 64:67-74.
3. Tomashek, K. M., A. Rivera, J. L. Munoz-Jordan, E. Hunsperger, L. Santiago, O. Padro, E. Garcia, and W. Sun. 2009. *Description of a large island-wide outbreak of dengue in Puerto Rico, 2007*. *Am J Trop Med Hyg* 81:467-474.
4. McElroy, K. L., G. A. Santiago, N. J. Lennon, B. W. Birren, M. R. Henn, and J. L. Munoz-Jordan. 2011. *Endurance, refuge, and reemergence of dengue virus type 2, Puerto Rico, 1986-2007*. *Emerg Infect Dis* 17:64-71.
5. Prince, H. E., J. L. Matud, and J. M. Lieberman. 2011. *Dengue virus immunoglobulin M detection in a reference laboratory setting during the 2010 dengue virus outbreak on Caribbean islands*. *Clin Vaccine Immunol* 18:1104-1107.
6. Mohammed, H., M. Ramos, J. Armstrong, J. Munoz-Jordan, K. O. Arnold-Lewis, A. Ayala, G. G. Clark, E. S. Tull, and M. E. Beatty. 2010. *An outbreak of dengue fever in St. Croix (US Virgin Islands), 2005*. *PLoS ONE* 5:e13729.
7. Centers for Disease, C., and Prevention. 2010. *Travel-associated Dengue surveillance - United States, 2006-2008*. *MMWR. Morbidity and mortality weekly report* 59:715-719.
8. Brunkard, J. M., J. L. Robles Lopez, J. Ramirez, E. Cifuentes, S. J. Rothenberg, E. A. Hunsperger, C. G. Moore, R. M. Brussolo, N. A. Villarreal, and B. M. Haddad. 2007. *Dengue Fever Seroprevalence and Risk Factors, Texas-Mexico Border, 2004*. *Emerg Infect Dis* 13:1477-1483.
9. Ramos, M. M., H. Mohammed, E. Zielinski-Gutierrez, M. H. Hayden, J. L. Lopez, M. Fournier, A. R. Trujillo, R. Burton, J. M. Brunkard, L. Anaya-Lopez, A. A. Banicki, P. K. Morales, B. Smith, J. L. Munoz-Jordan, and S. H. Waterman. 2008. *Epidemic Dengue and Dengue Hemorrhagic Fever at the Texas-Mexico Border: Results of a Household-based Seroepidemiologic Survey, December 2005*. *Am J Trop Med Hyg* 78:364-369.
10. Centers for Disease, C., and Prevention. 2010. *Locally acquired Dengue--Key West, Florida, 2009-2010*. *MMWR. Morbidity and mortality weekly report* 59:577-581.
11. Radke, E. G., C. J. Gregory, K. W. Kintziger, E. K. Sauber-Schatz, E. A. Hunsperger, G. R. Gallagher, J. M. Barber, B. J. Biggerstaff, D. R. Stanek, K. M. Tomashek, and C. G. Blackmore. 2012. *Dengue outbreak in Key West, Florida, USA, 2009*. *Emerg Infect Dis* 18:135-137.
12. Effler, P. V., L. Pang, P. Kitsutani, V. Vorndam, M. Nakata, T. Ayers, J. Elm, T. Tom, P. Reiter, J. G. Rigau-Perez, J. M. Hayes, K. Mills, M. Napier, G. G. Clark, and D. J. Gubler. 2005. *Dengue fever, Hawaii, 2001-2002*. *Emerg Infect Dis* 11:742-749.
13. Grobusch, M. P., M. Niedrig, K. Gobels, K. Klipstein-Grobusch, and D. Teichmann. 2006. *Evaluation of the use of RT-PCR for the early diagnosis of dengue fever*. *Clin Microbiol Infect* 12:395-397.
14. Johnson, B. W., B. J. Russell, and R. S. Lanciotti. 2005. *Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay*. *J Clin Microbiol* 43:4977-4983.



15. Lanciotti, R. S. 2003. *Molecular amplification assays for the detection of flaviviruses*. *Adv Virus Res* 61:67-99.
16. Bessoff, K., E. Phoutrides, M. Delorey, L. N. Acosta, and E. Hunsperger. 2010. *Utility of a commercial nonstructural protein 1 antigen capture kit as a dengue virus diagnostic tool*. *Clin Vaccine Immunol* 17:949-953.
17. Peeling, R. W., H. Artsob, J. L. Pelegrino, P. Buchy, M. J. Cardoso, S. Devi, D. A. Enria, J. Farrar, D. J. Gubler, M. G. Guzman, S. B. Halstead, E. Hunsperger, S. Kliks, H. S. Margolis, C. M. Nathanson, V. C. Nguyen, N. Rizzo, S. Vazquez, and S. Yoksan. 2010. *Evaluation of diagnostic tests: dengue*. *Nat Rev Microbiol* 8:S30-38.
18. Hunsperger, E. A., S. Yoksan, P. Buchy, V. C. Nguyen, S. D. Sekaran, D. A. Enria, J. L. Pelegrino, S. Vazquez, H. Artsob, M. Drebot, D. J. Gubler, S. B. Halstead, M. G. Guzman, H. S. Margolis, C. M. Nathanson, N. R. Rizzo Lic, K. E. Bessoff, S. Kliks, and R. W. Peeling. 2009. *Evaluation of commercially available anti-dengue virus immunoglobulin M tests*. *Emerg Infect Dis* 15:436-440.

26. ADDITIONAL RESOURCES

1. <http://www.cdc.gov/dengue/>
2. <http://www.cdc.gov/dengue/clinicalLab/index.html>
3. <http://www.healthmap.org/dengue/index.php>
4. <http://www.who.int/csr/disease/dengue/en/>



27. CONTACT INFORMATION, ORDERING, AND PRODUCT SUPPORT

FOR TECHNICAL AND PRODUCT SUPPORT, CONTACT THE CDC DENGUE BRANCH SUPPORT TEAM DIRECTLY:

For Technical Support

Send Email to: DenguePCRSupport@cdc.gov

For Ordering:

Send Email to: DenguePCROrdering@cdc.gov

Include the following information in your message:

- Laboratory name and address
- Qualified contact person
- Phone number
- Email address
- Shipping address

