Rapid real-time RT-PCR reagents for molecular serotyping of dengue viruses using the 3M Integrated Cycler

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Modified Abstract

Introduction: Different serotypes of dengue viruses infect humans in tropical and subtropical regions of the world where roughly one third of the world's population resides. Molecular tests have proven to be effective for detecting dengue viruses and for dengue serotyping; however, these tests are not widely available. We have developed real-time RT-PCR reagents for detection and molecular serotyping of dengue viruses and evaluated the assay performance for 4 dengue serotypes.

Materials and Methods: For detection of individual serotypes, RNA was extracted from the following strains: serotype 1 (TH-S-man, WHO West Pac 74), serotype 2 (TH-36, WHO-S16803), serotype 3 (H87, WHO-CH53489), and serotype 4 (H241, TVP-376). Contrived samples were generated by spiking individual strains of virus into human serum or whole blood. RNA was extracted from these samples and from a dengue CDC proficiency panel (CDC Division of Vector Borne Infectious Diseases, Dengue Branch) using a MagNA Pure LC and Total Nucleic Acid Isolation Kit (Roche). Real-time RT-PCR was performed on RNA samples.

Results: The results obtained after assaying the CDC proficiency panel showed that individual dengue serotypes were correctly detected with no cross-reactivity between strains. In addition, no reactivity was observed with non-dengue viruses including: West Nile virus, St. Louis encephalitis virus, yellow fever virus, hepatitis C virus, hepatitis D virus, and Chikungunya virus.

Conclusion: The Focus Diagnostics reagents accurately detected the individual serotypes of dengue virus and did not cross-react with the other flaviviruses tested. Amplification and detection were completed in approximately 1 hour following nucleic acid extraction steps.

Methods

Virus strains: The following dengue serotypes were used in the study: serotype 1 (TH-S-man, WHO West Pac 74), serotype 2 (TH-36, WHO-S16803), serotype 3 (H87, WHO-CH53489), and serotype 4 (H241, TVP-376).

Nucleic acid preparation: For contrived specimens, 200 µL of each sample was extracted using the Roche MagNA Pure LC automated system with a Total Nucleic Acid Isolation Kit (Roche Diagnostics, Indianapolis, IN), and eluted in 50 µL of elution buffer. An RNA internal control was added to each specimen prior to extraction to monitor the extraction process and PCR inhibition.

Real-time RT-PCR amplification and detection: Real-time RT-PCR was performed with 5 µL of extracted RNA in a total reaction volume of 10 µL using the 3M Integrated Cycler. Each of the Focus Diagnostics primer sets targets one of the 4 dengue virus serotypes. Fluorescent signal for target-specific PCR products was detected at 58°C.

Detection of Serotypes: Dengue viruses were diluted in either human serum or whole blood and extracted. Each extracted sample was tested in duplicate reactions, and Ct values were averaged.

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Methods (Cont.)

Cross Reactivity: Genomic DNA or RNA from a variety of viral (≥10^5 $TCID_{50}$/mL) and bacterial (≥10^6 CFU/mL) pathogens, or positive clinical specimens (with target Ct<30) were tested to verify lack of cross-reactivity. Each organism was spiked into a 200 µL aliquot of transport medium to generate contrived samples, and extracted nucleic acids were tested.

CDC Proficiency Panel: A study was performed with a CDC dengue proficiency panel. The blind panel, consisting of contrived samples for all 4 serotypes and negative samples, was extracted and tested using the Focus Diagnostics reagents. The data was then sent to CDC Dengue Branch, Puerto Rico. CDC provided the answer key to the panel along with the expected results.

Results

Serotype Coverage: The Focus Diagnostics reagents accurately detected and identified each of the 2 strains tested for each serotype (Table 1).

Table 1. Analytical reactivity for each dengue serotype. Threshold cycle (Ct) values were generated using 1:10,000 dilution of each virus in whole blood.

Dengue		Focus Diagnostics Reagents			
Serotype	Strain	DENV1	DENV2	DENV3	DENV4
1	TH-S-man	30.2	0	0	0
1	WHO West Pac 74	30.3	0	0	0
2	TH-36	0	30.0	0	0
2	WHO-S16803	0	29.4	0	0
3	H87	0	0	29.3	0
3	WHO-CH53489	0	0	30.2	0
4	H241	0	0	0	26.2
4	TVP-376	0	0	0	27.7

Note: No amplification signal was detected for the reactions with Ct =0.

Cross Reactivity: There was no cross-reactivity with the pathogens tested (Table 2). Reactivity was seen only with intended target serotypes (Figure 1).

Table 2. Organisms tested for cross reactivity

Adenovirus 1	Hepatitis D Virus	*Mycobacterium tuberculosis*
Adenovirus 7A	Herpes Virus -6	Parechovirus
Aspergillus	Herpes Virus -7	Parvovirus
BK Virus	Herpes Virus -8	Rubella
Chikungunya Virus	HIV-1	St Louis Encephalitis Virus
Cytomegalovirus	HIV-2	*Toxoplasma gondii*
Enterovirus 71	HSV-1	Varicella Zoster Virus
Epstein Barr Virus	HSV-2	West Nile Virus
Hepatitis B Virus	HTLV-1	Yellow Fever Virus
Hepatitis C Virus		

Results (Cont.)

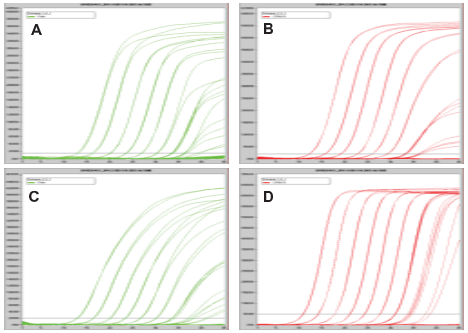

Figure 1. Amplification curves of dengue serotypes 1, 2, 3 and 4 with the Focus Diagnostics reagents. A set of serial dilutions for serotype 1 (A), serotype 2 (B), serotype 3 (C), and serotype 4 (D) are shown.

CDC Proficiency Panel: The results of the CDC proficiency panel showed a similar dynamic range of detection with the results provided by CDC Dengue Branch (Table 3). Focus Diagnostics primer sets reacted only with intended serotype, and gave Ct = 0 (not detected) for other serotypes.

Table 3. Results for the CDC dengue proficiency panel.

Viral Load (copy/mL)*	Serotype*	CDC Assay Results*		Focus Diagnostics Results	
		Ct	Serotype	Ct	Serotype
89,800,000	DENV-1	18.2	DENV-1	21.5	DENV-1
8,980,000	DENV-1	22.5	DENV-1	25.1	DENV-1
898,000	DENV-1	25.0	DENV-1	28.9	DENV-1
89,800	DENV-1	29.6	DENV-1	33.0	DENV-1
8,980	DENV-1	32.2	DENV-1	34.9	DENV-1
898	DENV-1	38.5	DENV-1	39.2	DENV-1
89	DENV-1	NEG	NEG	NEG	NEG
9	DENV-1	NEG	NEG	NEG	NEG
375,000,000	DENV-2	19.1	DENV-2	17.0	DENV-2
37,500,000	DENV-2	22.0	DENV-2	20.1	DENV-2
3,750,000	DENV-2	25.4	DENV-2	23.3	DENV-2
375,000	DENV-2	28.7	DENV-2	27.0	DENV-2
37,500	DENV-2	31.8	DENV-2	30.5	DENV-2
3,750	DENV-2	36.7	DENV-2	33.8	DENV-2
375	DENV-2	NEG	NEG	NEG	NEG
38	DENV-2	NEG	NEG	NEG	NEG

Results (Cont.)

Table 3 (Continued). Results for the CDC dengue proficiency panel.

Viral Load (copy/mL)*	Serotype*	CDC Assay Results*		Focus Diagnostics Results	
		Ct	Serotype	Ct	Serotype
266,000,000	DENV-3	18.6	DENV-3	19.6	DENV-3
26,600,000	DENV-3	21.6	DENV-3	23.3	DENV-3
2,660,000	DENV-3	24.8	DENV-3	26.8	DENV-3
266,000	DENV-3	28.2	DENV-3	30.0	DENV-3
26,600	DENV-3	31.9	DENV-3	33.5	DENV-3
2,660	DENV-3	34.3	DENV-3	37.1	DENV-3
266	DENV-3	NEG	NEG	NEG	NEG
26	DENV-3	NEG	NEG	NEG	NEG
331,000,000	DENV-4	15.8	DENV-4	18.3	DENV-4
33,100,000	DENV-4	18.8	DENV-4	21.7	DENV-4
3,310,000	DENV-4	22.2	DENV-4	25.0	DENV-4
331,000	DENV-4	25.3	DENV-4	28.3	DENV-4
33,100	DENV-4	29.0	DENV-4	31.7	DENV-4
3,310	DENV-4	31.6	DENV-4	35.5	DENV-4
331	DENV-4	33.9	DENV-4	38.4	DENV-4
33	DENV-4	NEG	NEG	40.4	DENV-4
NEG	NEGATIVE	NEG	NEG	NEG	NEG
NEG	NEGATIVE	NEG	NEG	NEG	NEG

*Viral load, serotype and CDC assay results were kindly provided by CDC Dengue Branch.

Conclusions

- Focus Diagnostics reagents correctly detected and differentiated different serotypes of dengue virus using the 3M Integrated Cycler.
- No cross-reactivity was observed with 25 viruses, 1 bacterium, 1 fungus and 1 Protozoan parasite.
- Focus Diagnostics reagents showed similar dynamic range of detection relative to the CDC assay.

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These reagents are for research use only, and not for sale in the US.

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