



EVALUATION OF THE ERAGEN® MULTICODE®-RTx ENTEROVIRUS PROTOTYPE TEST COMPARED TO THE CEPHEID® ENTEROVIRUS ASR AND A LABORATORY-DEVELOPED REAL-TIME PCR ASSAY

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ABSTRACT

Introduction

The enteroviruses are a diverse group of viruses which are responsible for a broad range of diseases, including the majority of reported aseptic meningitis cases in the United States. Prompt and accurate diagnosis of enteroviral meningitis is necessary for the most effective patient management (1). The increased sensitivity and decreased turn-around time provided by real-time PCR has made it the preferred method for detection of enterovirus from various sample types, including cerebral spinal fluid (CSF). Although there is a significant degree of sequence variation among the greater than 70 distinct serotypes of enterovirus, the 5' untranslated region (5' UTR) is relatively conserved and has been a useful target for molecular tests. The objective of the current study was to assess the sensitivity, specificity and accuracy of the EraGen Biosciences (Madison, WI) MultiCode-RTx Enterovirus prototype test compared to the Cepheid (Sunnyvale, CA) Enterovirus ASR and a laboratory-developed real-time PCR assay.

Materials and Methods

A total of 86 clinical CSF specimens were tested using three real-time PCR assays. The MultiCode-RTx Enterovirus prototype test was performed on the ABI 7500 Fast. The Cepheid Enterovirus ASR was performed on the Cepheid SmartCycler II according to the manufacturer's instructions. The laboratory-developed assay was performed on the ABI 7000 and 7500 instruments. In addition, analytical sensitivity of the MultiCode-RTx Enterovirus prototype was evaluated on three different real-time PCR instrument platforms (ABI 7500 Fast, Roche LightCycler 1.2, and Cepheid SmartCycler II).

Results

Thirty-seven samples tested positive for enterovirus in at least two of the assays. The performance characteristics of the three tests for classification of enterovirus cases are shown in the table below.

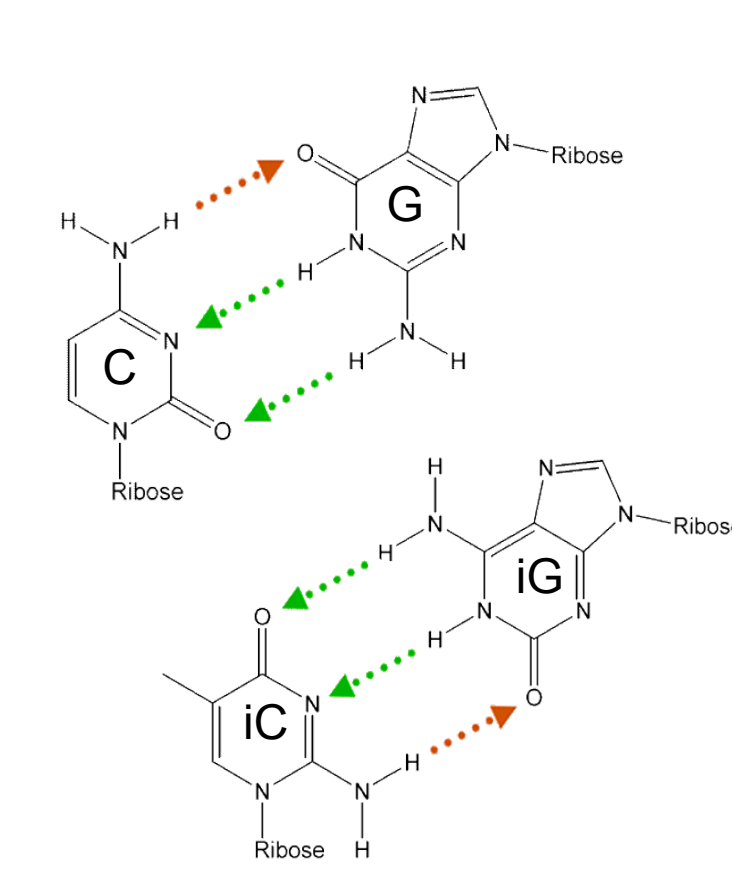
Test	Sensitivity	Specificity	Accuracy
EraGen	97.3%	98.0%	97.7%
Lab-developed	97.3%	100%	98.8%
Cepheid	94.6%	100%	97.7%

The analytical limit of detection for the MultiCode-RTx Enterovirus prototype was determined to be equivalent across each real-time PCR instrument tested.

Conclusions

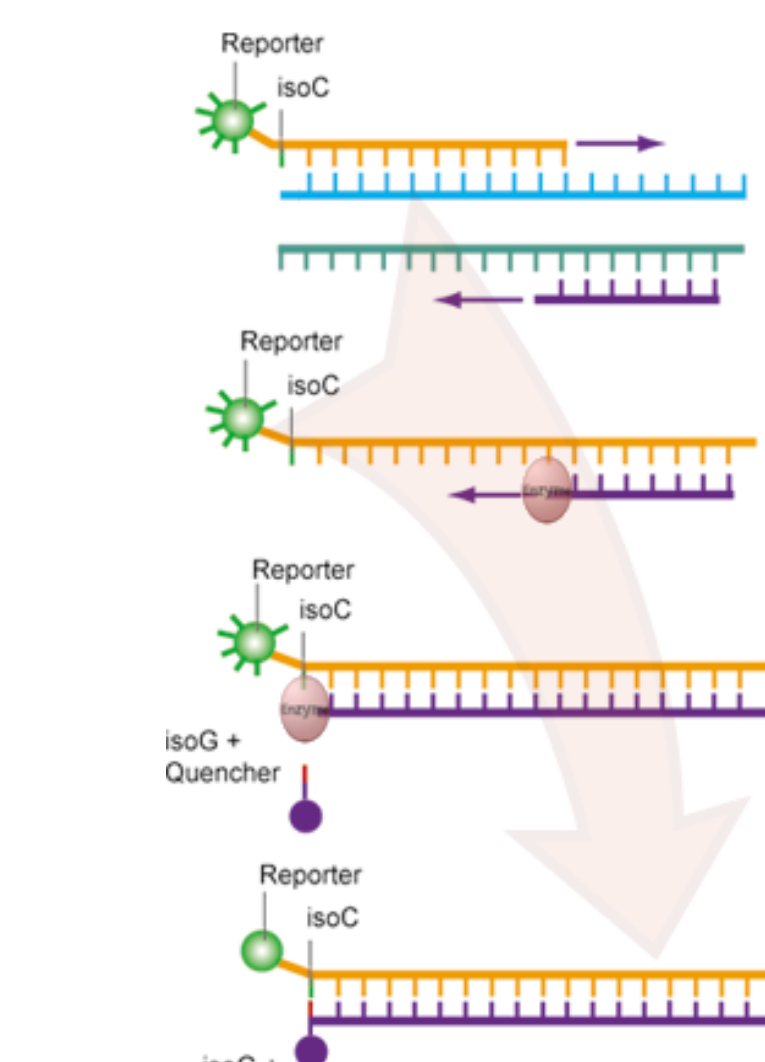
The MultiCode-RTx Enterovirus prototype, the Cepheid Enterovirus ASR, and a home-brew real-time PCR test demonstrate equivalent performance in the detection of enterovirus from clinical CSF specimens. In addition, the MultiCode-RTx Enterovirus prototype demonstrates equivalent sensitivity on three commonly used real-time PCR instruments.

MultiCode-RTx Technology



MultiCode bases have altered base pairing specificity.

The pattern of hydrogen bond donors and acceptors is rearranged for the MultiCode base pair between 5-methyl-isocytosine (iC) and isoguanosine (iG) compared to a standard cytosine (C) guanosine (G) base pair.



MultiCode-RTx system schematic.

Targets are PCR amplified with one standard primer and one primer that contains a single iC nucleotide adjacent to a fluorescent reporter. Amplification is performed in the presence of dabcyl-diGTP. Site-specific incorporation places the quencher in close proximity to the reporter resulting in a decrease in fluorescence (2).

MATERIALS AND METHODS

Specimen Acquisition and Processing

A total of 86 CSF specimens that were submitted to the Molecular Diagnostics Laboratory, Emory Medical Laboratories, for detection of enterovirus RNA were included in the study. These specimens were stored at -70°C prior to testing with MultiCode-RTx and Cepheid ASRs. The specimens were extracted using the Roche MagNA Pure LC Total Nucleic Acid Isolation Kit for analysis with the laboratory-developed real-time PCR assay. Each specimen was re-extracted using the Qiagen QIAamp Viral RNA Mini Kit for analysis with EraGen's MultiCode-RTx Enterovirus Prototype Test and Cepheid's Enterovirus ASR. Briefly, 140 µL primary specimen was extracted according to the manufacturer's Spin Protocol and eluted into 70 µL elution buffer.

Control Template Processing

Cultured Coxsackie A24 virus was used as control material for determining the analytical limit of detection of the MultiCode-RTx Enterovirus Prototype test and the Cepheid Enterovirus ASR. The Cox A24 control material was extracted using the Qiagen QIAamp Viral RNA Mini Kit. Quantitation of the cultured virus was performed by ViroMed Laboratories and values presented assume 100% recovery during extraction.

MultiCode-RTx Amplification

For each MultiCode-RTx reaction the following 25 µL reaction was performed:

5.0 µL	EraGen ISolution™ (5X)
1.0 µL	Enterovirus Primer Mix
1.0 µL	RNA Universal Reference (25X)
0.5 µL	Titanium Taq (50X)
0.5 µL	MMLV-RT (25 U/µL)
12.0 µL	Nuclease-Free Water
5.0 µL	Sample

Step	Time	Temperature	Repeat
Reverse Transcription	15 min	50°C	1
Hot Start	2 min	95°C	1
Denature	5 sec	95°C	50
Anneal	10 sec	58°C	
Extend	20 sec	72°C	
Melt		60°C -95°C	1

All clinical specimens were tested on the ABI 7500 Fast Real Time PCR System. Cox A24 control material was tested using the same reaction conditions on the ABI 7500 Fast, the Roche LightCycler 1.2, and the Cepheid SmartCycler II.

MultiCode-RTx Data Analysis

Raw data was exported from each real-time PCR instrument and imported into EraGen's MultiCode-RTx Analysis Software. Enterovirus positive samples were determined using the following criteria: real-time amplification curves in the FAM channel crossing the amplification threshold set at 10 SD from the baseline and melt peaks between 81.0°C – 86.5°C. Negative samples were verified by amplification of the RNA Universal Reference, an amplification control, in the HEX channel.

Cepheid Enterovirus ASR Amplification

For every 2 Cepheid reactions the following 50 µL reaction mix was prepared and reactions were performed with the following cycling conditions on the SmartCycler II:

25.0 µL	Invitrogen Reaction Mix (2X)
1 each	Cepheid ASR Bead
2.8 µL	Magnesium Sulfate (50 mM)
2.0 µL	RT/Platinum Taq Mix (50X)
10.2 µL	Nuclease-Free Water
10.0 µL	Sample

Step	Time	Temperature	Repeat
Reverse Transcription	30 min	55°C	1
Hot Start	2 min	95°C	1
Denature	15 sec	95°C	50
Anneal	15 sec	55°C	
Extend	15 sec	72°C	

Cepheid Data Analysis

Enterovirus positive samples were identified by a real-time amplification curve in the FAM channel or the Alexa® 532 channel that crosses the amplification threshold set at 30 RFU.

Laboratory-developed Real-Time PCR Assay Amplification

For the Laboratory-developed Assay, a reverse transcriptase reaction was first performed with the following setup.

1.0 µL	RT Buffer (10X)
2.2 µL	MgCl ₂ (25 mM)
2.0 µL	dNTPs (10 mM)
0.5 µL	Random Hexamers (50 µM)
0.2 µL	RNase Inhibitor (20 U/µL)
0.25 µL	MultiScribe Reverse Transcriptase (50 U/µL)
4.0 µL	Sample

Time	Temperature
10 min	25°C
30 min	48°C
5 min	95°C

Following reverse transcription, 40 µL reaction mix was added to the RT reaction and PCR cycling was performed on the ABI 7000 or the ABI 7500 Real Time PCR System.

Step	Time	Temperature	Repeat
Hot Start	2 min	50°C	1
	10 min	95°C	1
Denature	15 sec	95°C	40
Annual/Extend	1 min	60°C	

Laboratory-developed Assay Data Analysis

Instruments were set to auto baseline and auto Ct for analysis of amplification curves. Any samples with Ct ≤ 40 were considered positive.

Performance of MultiCode-RTx Enterovirus Prototype Test

	Reference Positive	Reference Negative
MultiCode-RTx Positive	36	1
MultiCode-RTx Negative	1	48

Measure	Value	95% Confidence Interval
Sensitivity	97.3%	86.2 – 99.5%
Specificity	98.0%	89.3 – 99.4%
Accuracy	97.7%	91.9 – 99.6%

Experimental Details

• 86 CSF Specimens were extracted and tested using the MultiCode-RTx Enterovirus Prototype Test on the ABI 7500 Fast. Results were compared to results obtained with the Cepheid Enterovirus ASR and the Laboratory-developed Real-Time PCR Assay.

Results

• One specimen tested positive for Enterovirus with the MultiCode-RTx Test but negative by the Cepheid ASR and the Laboratory-developed Assay.
 • One specimen tested negative for Enterovirus with the MultiCode-RTx Test but positive by the Cepheid ASR and the Laboratory-developed Assay.

Performance of Cepheid Enterovirus ASR

	Reference Positive	Reference Negative
Cepheid Positive	35	0
Cepheid Negative	2	49

Measure	Value	95% Confidence Interval
Sensitivity	94.6%	82.3 – 98.5%
Specificity	100.0%	92.7 – 100.0%
Accuracy	97.7%	91.9 – 99.4%

Experimental Details

• 86 CSF Specimens were extracted and tested using the Cepheid Enterovirus ASR on the Cepheid SmartCycler II. Results were compared to results obtained with the MultiCode-RTx Test and the Laboratory-developed Assay.

Results

• Two specimens tested negative for Enterovirus with the Cepheid ASR but positive by the MultiCode-RTx Test and the Laboratory-developed Assay.

Performance of Laboratory-Developed Real-Time PCR Assay

	Reference Positive	Reference Negative
Lab-Developed Positive	36	0
Lab-Developed Negative	1	49

Measure	Value	95% Confidence Interval
Sensitivity	97.3%	86.2 – 99.5%
Specificity	100.0%	92.7 – 100.0%
Accuracy	98.8%	93.7 – 99.8%

Experimental Details

• 86 CSF Specimens were extracted and tested using the Laboratory Developed Real-Time PCR Assay. Results were compared to results obtained with the Cepheid ASR and the MultiCode-RTx Test.

Results

• One specimen tested negative for Enterovirus with the Laboratory-developed Assay but positive by the Cepheid ASR and the MultiCode-RTx Test.

Analytical Limit of Detection of Cox A24 with EraGen and Cepheid Tests

Test	TCID ₅₀ /rxn	Avg Ct	Avg Tm	Positivity
MultiCode-RTx	2.3E-05	33.6 ± 0.3	81.5 ± 0.3	100%
	4.6E-06	36.5 ± 0.7	81.7 ± 0.3	100%
	9.4E-07	38.5 ± 0.9	81.8 ± 0.2	100%
	1.9E-07	47.2 ± 1.9	81.5 ± 0.2	16%
Cepheid ASR	2.3E-05	33.3 ± 0.2	NA	100%
	4.6E-06	35.6 ± 0.2	NA	100%
	9.4E-07	38.0 ± 0.7	NA	100%
	1.9E-07	40.9 ± 0.3	NA	37%

Experimental Details

• 5-fold dilutions of Coxsackie A24 Viral RNA were tested in 16 replicates at each concentration.
 • MultiCode-RTx Enterovirus Prototype Test was performed on the ABI 7500 Fast.
 • Cepheid Enterovirus ASR was performed on the Cepheid SmartCycler II.

Results

• The limit of detection for Cox A24 with the MultiCode-RTx Test is 9.4E-7 TCID₅₀/reaction.
 • The limit of detection for Cox A24 with the Cepheid ASR is 9.4E-7 TCID₅₀/reaction.

MultiCode-RTx Instrument Equivalence

Instrument	TCID ₅₀ /rxn	Avg Ct	Avg Tm	Positivity
ABI 7500	4.6E-06	34.7 ± 0.8	81.3 ± 0.1	100%
	9.4E-07	36.8 ± 0.8	81.3 ± 0.3	100%
Roche LightCycler 1.2	4.6E-06	39.6 ± 0.5	82.1 ± 0.1	100%
	9.4E-07	42.5 ± 0.8	81.7 ± 0.3	100%
Cepheid SmartCycler II	4.6E-06	36.2 ± 0.6	82.9 ± 0.1	100%
	9.4E-07	38.9 ± 1.1	82.8 ± 0.1	100%

Experimental Details

• Identical reaction conditions were used for the MultiCode-RTx Enterovirus Prototype Test on the ABI 7500 Fast, the Roche LightCycler 1.2 and the Cepheid SmartCycler II.

• 8 replicates of Coxsackie A24 Viral RNA were tested at the limit of detection and at 5X the limit of detection.
 • 100% of replicates at the limit of detection for Cox A24 were detected across all three instrument platforms.

CONCLUSIONS

Three real-time PCR assays for the detection of enteroviruses were compared and found to give equivalent performance on a set of 86 CSF specimens. Both the MultiCode-RTx Enterovirus Prototype Test and the Laboratory-developed Assay gave a sensitivity value of 97.3% (missing one positive sample each). The Cepheid Enterovirus ASR gave 94.6% sensitivity (missing two positive samples). All three tests gave specificity and accuracy values of at least 97%.

The MultiCode-RTx Test and the Cepheid ASR gave equivalent sensitivities on Coxsackie A24 virus at 9.4E-7 TCID₅₀/reaction. An additional benefit of the MultiCode-RTx Test is its broad instrument compatibility that makes use of a universal protocol for reaction setup and PCR cycling. Equivalent sensitivity was observed for Cox A24 at the limit of detection on the ABI 7500 Fast, the Roche LightCycler 1.2 and the Cepheid SmartCycler II.

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