Enterovirus D68 (EV-D68) 2014 Outbreak Strain-Specific Real-Time Reverse Transcription / Polymerase Chain Reaction (rRT-PCR) Assay Instructions-Version 10/14/2014

The CDC Picornavirus Laboratory from mid-September to mid-October developed and evaluated the EV-D68-specific rRT-PCR assay. The assay and protocol are primarily focused on evaluating respiratory disease due to EV-D68. Some developmental and validation information can be found in Appendix B. The protocol that follows has worked reliably in our hands with little difficulty in any phase of the testing process, including interpretation of results.

Acceptable Specimens

- Nasopharyngeal and/or Oropharyngeal swabs (NP/OP): high diagnostic yield
- Other respiratory specimens, nasal wash, aspirates: high diagnostic yield
- Serum: few specimens tested—expected diagnostic yield low, but EV-D68 has been detected in serum from a few patients aged 2 years to 10 years

Specimen Handling and Storage

- Specimens should be kept frozen at -20°C or lower
- If a delay in extraction is expected, store specimens at -70°C or lower
- Extracted nucleic acids should be stored at -70°C or lower

Materials:

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources. Use of trade names is for identification purposes only and does not constitute endorsement by CDC or the Department of Health and Human Services.

Assay reagents:

- SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen, catalog #11732-088)
- Molecular grade water, nuclease-free
- Primers and probe
    - AN887 5’ CAA ACT CGC ACA GTG ATA AAY CAR CA 3’ Sense primer
    - AN893 5’ GTA TTA TTA CTA CTA CCA TTC ACN GCN AC 3’ Antisense primer
    - AN890 5’ **FAM** GTC CAT TTG AAA AAG TTC TTG TC **BHQ1** 3’ Probe (antisense)
        - FAM = 6-carboxyfluorescein
        - BHQ1 = Black Hole Quencher®-1 (Biosearch Technologies, Novato, CA)

Extraction reagents:

- QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA; catalog #: 52904 or 52906)

Equipment and Consumables

- 10% bleach solution (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach), made fresh daily
- Disposable, powder-free gloves
- Laboratory marking pen
- P2/P10, P200, and P1000 aerosol barrier pipette tips
- 1.5 mL microcentrifuge tubes
- 0.2 mL PCR reaction strip tubes (Applied Biosystems; 4316567)
- 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)
- MicroAmp Optical 8-cap Strips (Applied Biosystems; catalog #4323032)
- Vortex mixer
- Microcentrifuge
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- Racks for 1.5 mL microcentrifuge tubes
- 2 x 96-well -20 °C cold blocks
- AB 7500 Real-Time PCR Systems (Applied Biosystems; catalog #4406985 or #4406984)

Quality Control

rRT-PCR is an exquisitely sensitive test method and should be conducted following strict quality control and quality assurance procedures. Following these guidelines will help minimize chances of false-positive and false-negative results.

General Considerations:

- Personnel must be familiar with the protocol and instruments used.
- Maintain separate areas and dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips, gowns and gloves) for assay reagent setup, handling of extracted nucleic acids, and rRT-PCR amplification.
- Work flow must always be from the clean area to the dirty area.
- Wear clean, previously unworn, disposable lab coats and new, powder-free gloves during assay reagent setup and handling of extracted nucleic acids. Change gloves whenever you suspect they may be contaminated.
- Store primers/probe and enzyme master mix at appropriate temperatures (see product inserts). Do not use reagents beyond their expiration dates.
- Keep reagent tubes and reactions capped as much as possible.
- Clean surfaces using an acceptable surface decontaminant (see above).
- Do not bring extracted nucleic acid or PCR amplicons into the mastermix setup area.
- Use aerosol barrier (filter) pipette tips only.
- Use optical strip 8-cap strips only. Do not use PCR plate sealing film.

Assay Controls

- Assay Controls should be run concurrently with all test samples.
    - Two positive controls (PC): 2014 outbreak strain EV-D68 viral RNAs, concentration adjusted to yield a Ct value of 30-32 (low but consistent positive)
    - Two no template controls (NTC): nuclease free H_2O

Instructions for Use

- Respiratory specimens and sera should be extracted with Qiagen Viral RNA Mini Kit, following the manufacturer's spin column protocol.
- Sample extractions must yield RNA or total nucleic acid of sufficient volume to cover any subsequent testing requirements (a minimum of 60 µL is recommended).
- Retain specimen extracts in cold block or on ice until testing. If testing will be delayed, freeze immediately at ≤ -20°C (preferably -70°C). Thaw only the number of extracts that will be tested in a single day. Ideally, do not freeze or thaw extracts more than once before testing.

Stock Reagent Preparation: Real-time Primers/Probes

Precautions: These reagents should be handled only in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.

- Concentrated primer and probe stocks from the synthesis facility must be diluted to the working concentrations in nuclease free H_2O. Primers AN887 and AN893 should be diluted to 10 µM (10 pmol/µl). Probe AN890 should be diluted to 5 µM (5 pmol/µl).
- Mix diluted working stocks and aliquot primers/probe in 100 µL (enough volume for a single 96 well reaction plate) or smaller volumes, depending on the specimen load in your laboratory. Store a single working aliquot of primers/probe at 2-8°C in the dark. Do not refreeze (stability unknown due to manufacturing variation—primers/probes from our onsite core facility are generally stable for > month). Store remaining aliquots at ≤-20°C in a non-frost-free freezer.

Equipment Preparation

1. Turn on AB 7500 and start the 7500 software.
2. Perform plate set up and select cycling protocol on the instrument.

Table 1. EV-D68 RT-rPCR Cycling Conditions

STEP	CYCLES	TEMPERATURE	TIME
REVERSE TRANSCRIPTION	1	50°C	30 min
TAQ POLYMERASE ACTIVATION	1	95°C	2 min
PCR AMPLIFICATION	45	95°C	15 sec
		55°C	1 min
		72°C	5 sec

- Fluorescent detection at the 55°C annealing step.

Instrument Settings

- Reporter: FAM, Quencher: None
- Passive Reference Dye: None
- Run Mode: Standard
- Sample Volume: 25 µL

Master Mix and Plate Set-Up

Note: Plate set-up or strip tube configuration can vary with the number of specimens and workday organization. NTCs and PCs must be included in each run.

1. In the reagent set-up room clean hood, place rRT-PCR buffer, enzyme, and primers/probe on ice or cold-block. Keep cold during preparation and use.

2. Thaw 2X Reaction Mix prior to use. Once thawed, the 2X reaction buffer can be aliquoted in volumes appropriate for the laboratory testing volume or work flow.

3. Mix buffer, enzyme, and primers/probe by inversion.

4. Pulse centrifuge buffer and primers/probe and return to ice.

5. Label one 1.5 ml microcentrifuge tube for the master mix.

6. Determine the number of reactions (N) to set up. It is necessary to make excess reaction mix for the NTC and PC reactions and to account for pipetting error. Use the following guide to determine N:

- If the number of samples (n) including controls equals 1 through 14, then N = n + 1
- If the number of samples (n) including controls is 15-40, then N = n + 3
- If the number of samples (n) including controls is >40, then N = n + 5

7. rRT-PCR Reaction Mix:

- For the EV-D68 primer/probe set, calculate the amount of each reagent to be added for each reaction mixture (N = # of reactions).

Table 2. rRT-PCR Reaction Master Mix

SuperScript® III Platinum® One-Step qRT-PCR Kit

Component	Volume (µl)
2X Reaction Buffer	N x 12.5
SS III RT/Platinum Taq Mix	N x 0.50
AN887 (10 µM)	N x 0.80
AN893 (10 µM)	N x 0.80
AN890 (5 µM)	N x 0.80
$MgSO_4$ (50 mM)	N x 0.50
H_2O, nuclease free	N x 4.10
Total Volume	N x 20.0

8. Mix reaction components by pipetting slowly up and down (avoid bubbles).

9. Add 20 µl of master mix into each well of a chilled optical plate or strip tube(s).

10. Before moving the plate to the nucleic acid handling area, add 5 µl of nuclease-free water to the NTC well(s).

11. Loosely apply optical strip caps to the tops of the reaction wells and move plate/strip tubes to the nucleic acid handling area on cold block.

12. Gently mix specimen RNA extracts and PC and pulse centrifuge.

13. Pipette 5 µl of the first specimen RNA into the well designated for that sample. Follow in sequential order according to your plate/strip tube template. Change tips after each sample addition.

14. Cap the column/strip to which the RNA has been added. This will enable you to keep track of where you are on the plate/strip tubes.

15. Continue with the remaining samples. Change gloves between samples if you suspect they have become contaminated.

16. Pipette 5 µl of the positive control into designated well(s) and cap. Secure all strip caps with capping tool.

17. Transport the plate to the amplification area on cold block.

18. Centrifuge the plate at 500 x *g* for 1 min to remove bubbles or drops that may be present in the wells. Strip tubes may be spun 10-30 seconds in a strip microcentrifuge.

19. Place plate/strip tubes on pre-programed AB 7500 and start run.

Data Analysis

After completion of the run, save and analyze the data following the instrument manufacturer's instructions. Analysis should be done using a manual threshold setting. The threshold should be adjusted to fall within the exponential phase of the fluorescence curves and above any background signal. The procedure chosen for setting the threshold should be used consistently.

Accurate interpretation of rRT-PCR results requires careful consideration of several assay parameters. The following are general guidelines for advanced users (detailed analysis protocol in Appendix A):

1. The PC(s) should be **positive** and with Ct values within 35 cycles.

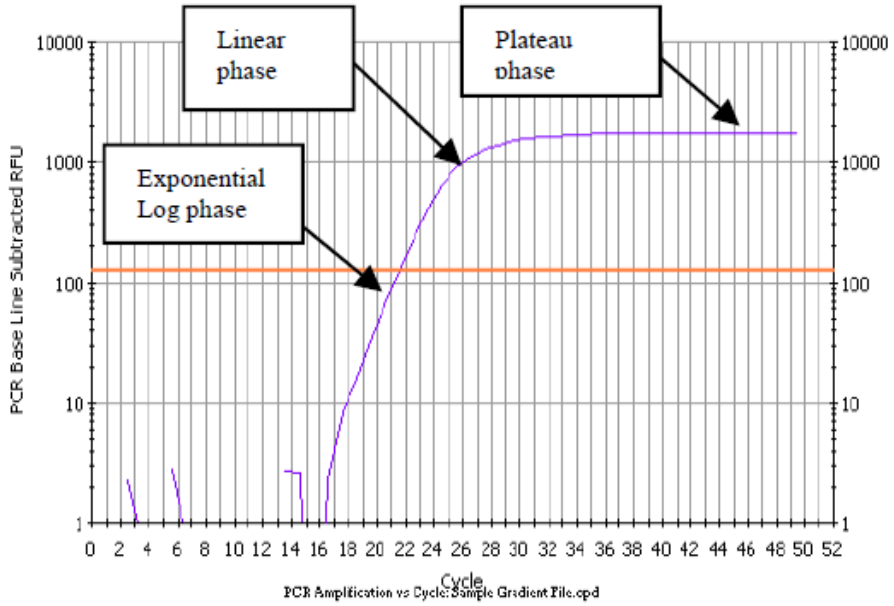
- If PC(s) are *negative*, the testing results for that plate are invalid.
    - Repeat rRT-PCR test.
        - If the PC is negative, but test samples are positive, use a new PC aliquot. If the PC and all test samples are negative, consider using a new enzyme mastermix.
    - If repeat testing generates negative PC results, send the experiment run file (extension .eds) to wnix@cdc.gov for consultation.

2. NTCs should be **negative**.

- If NTCs are *positive,* the testing results for that plate are invalid.
    - Clean potential DNA contamination from bench surfaces and pipettes in the reagent setup and template addition work areas.
    - Discard working reagent dilutions and remake from fresh stocks.
    - Repeat extraction and test multiple NTCs during rRT-PCR run.
    - Repeat rRT-PCR test.

Appendix A

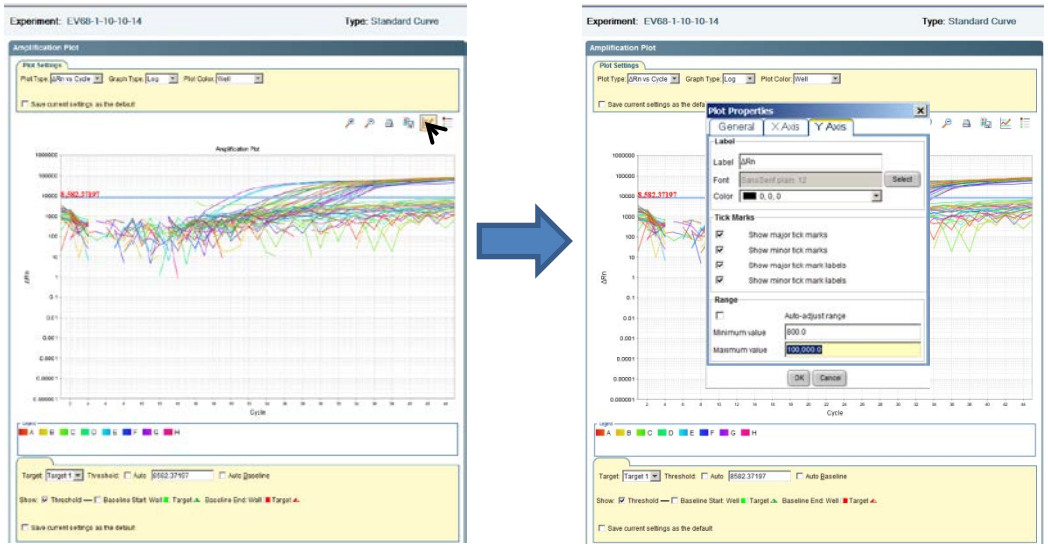
True positive EV-D68 real-time RT-PCR specimens should produce exponential curves with logarithmic, linear, and plateau phases. Below is a log view of an ideal PCR curve noting each stage of the amplification plot.



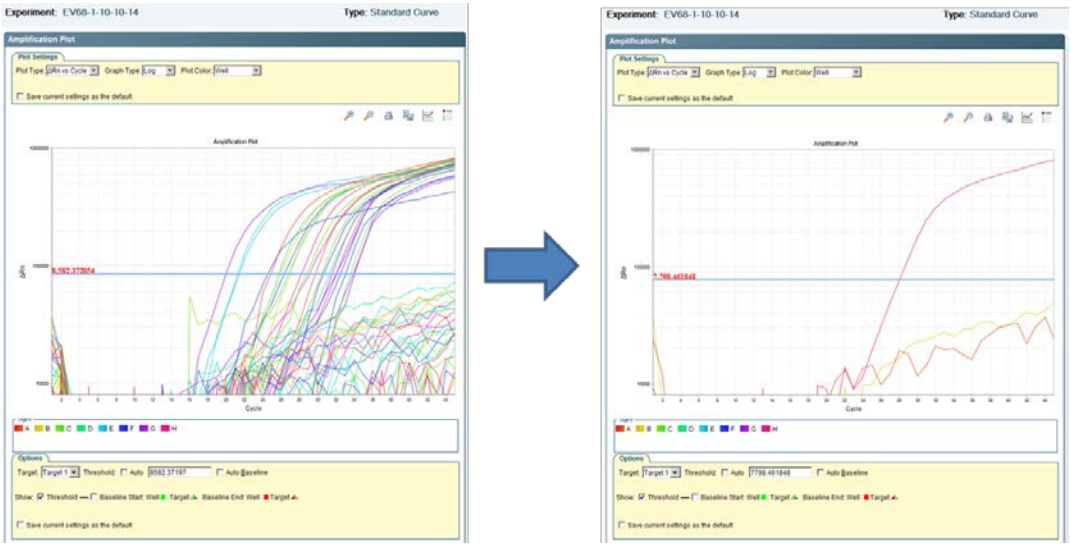
An example of an EV-D68-specific real-time RT-PCR is shown below.

Analyze the experiments in the default window “Log” view.

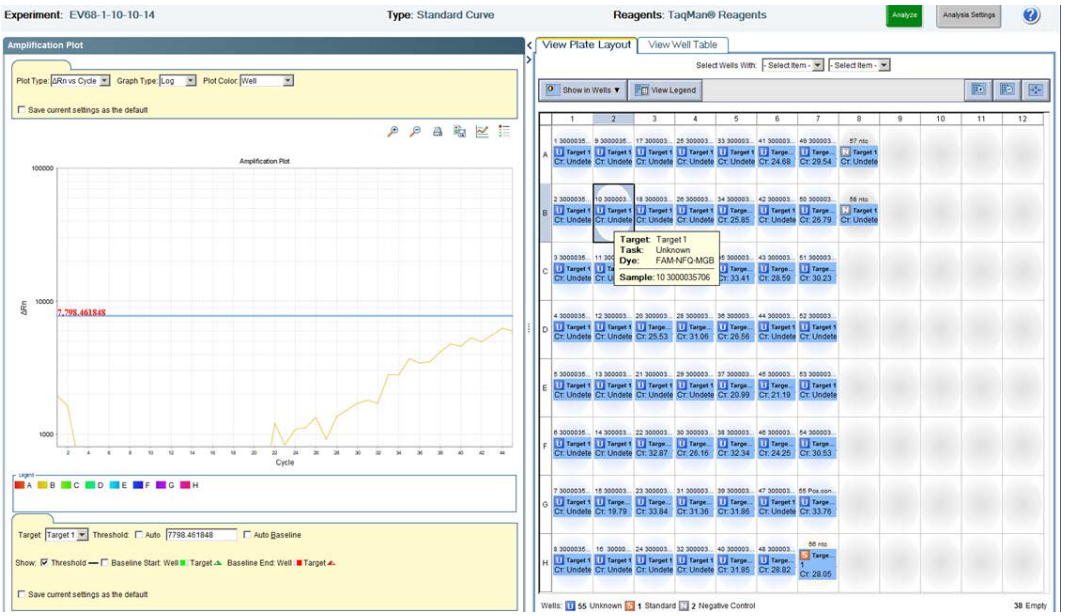
Above. Uncheck the “Auto” threshold and “Auto Baseline” boxes, so the threshold can be adjusted manually. Move the threshold to the linear portion of the sigmoid curves.



Above. Click the "Plot Properties" icon. Click the "Y Axis" tab and change the "Minimum value" to 800 and the "Maximum value" to 100,000. Click "OK." Minimum and maximum y-axis values may vary with different manufacturer's primer/probe sets and different AB 7500 machines.



Above. The threshold can be precisely adjusted in this view if needed. Validate the run by assessing the positive (PC) and negative controls (NTC).



Above. Assess the results one-by-one and record the results. This specimen is negative for EV-D68.

Above. This specimen is positive for EV-D68.

Above. A negative specimen crossing the threshold with a Ct of 39.7. Note the lack of sigmoid curve shape and note the difference with two late Ct positive specimens in the second panel.

Assay Limitations:

Interpretation of rRT-PCR test results must account for the possibility of false-negative and false-positive results. False-negative results can arise from poor sample collection or degradation of the viral RNA during shipping or storage. Low-titer virus specimens collected too late in illness may also make it difficult to confirm a diagnosis. The most common cause of false-positive results is contamination with previously amplified DNA. The use of rRT-PCR helps mitigate this problem by operating as a contained system. A more difficult problem is the cross-contamination that can occur between specimens during collection, shipping, and aliquoting in the laboratory. Liberal use of negative control samples in each assay and a well-designed plan for confirmatory testing can help ensure that laboratory contamination is detected and that false positive test results are not reported

Our experience with the EV-D68-specific test interpretation has been without serious issues to date. With the deployment of the test in many labs across the US, issues will inevitably arise. Different sources of primers and probe, and variable laboratory expertise with real-time RT-PCR are just two of the factors that will lead to questions. When questions arise in the real-time testing process, you may consult us via email. Send the AB 7500 experiment file as an attachment to wnix@cdc.gov with an explanation of what the issue is and we will get back to you with comments and suggestions.

Appendix B

Primer and Probe Design:

More than 1000 respiratory specimens have been processed at CDC since the beginning of the EV-D68 outbreak. Around 50% of these specimens have been identified as EV-D68. The gold standard testing process provides a partial viral protein 1 (VP1) gene sequence of 340 nucleotides (NT) from the first half of the gene1. The EV-D68-specific assay primers and probe were designed from an alignment of 576 EV-D68 partial VP1 sequences from the 2014 US outbreak and recent EV-D68 phylogenetic ancestors from the United States (2013 viruses; CDC database), Spain (2012 virus; KF254918), Italy (2012 viruses; KC763167, KC763162), China (2012 viruses; JX898785, JQ924865), and Thailand (2011 virus; JQ411807). Coordinates for the primers and probe relative to the 2014 outbreak strain US/MO/14-18947 (KM851225) are as follows:

Identification #	Orientation	Gene	Location (5'-3' Sense Strand)	Amino-Acid Motif (NH-COOH)
AN887	Sense	VP1	2518-2543	QTRTVINQH
AN893	Antisense	VP1	2761-2789	VAVNGSSNNT
AN890 (Probe)	Antisense	VP1	2647-2669	DKNFFKWT

The primers and probe sequences are reiterated below:

AN887 5' CAA ACT CGC ACA GTG ATA AAY CAR CA 3'

AN893 5' GTA TTA TTA CTA CTA CCA TTC ACN GCN AC 3'

AN890 5' **FAM** GTC CAT TTG AAA AAG TTC TTG TC **BHQ1** 3'

Primer and probe sites were first visualized in an amino-acid alignment of all the contemporary EV species D (EV-D) identified to date (EV-D68, ABL61317; EV-D70, BAA18891; EV-D94, ABK88241, ABL61316; EV-D111, ADY76973; EV-D120, AGU46444, AGU46445) and 2014 US EV-D68 strains plus the older prototype strain Fermon (1962; AAR98503). The amino-acid motif for primer AN887 is largely conserved in EV-D, except for the carboxyl Q residue that is shared by EV-D68 and EV-D94 only. Conservation of the probe motif is high for all EV-D. US 2014 EV-D68 strains and other contemporary EV-D68 strains in GenBank all have a unique D residue and a carboxyl K residue shared with EV-D94 only. The amino-acid motif for primer AN893 is specific for contemporary EV-D68s and is somewhat different in the Fermon strain, but significantly different from all other EV-D.

Degeneracy in the sense and antisense primers was kept to a minimum in the design. Extension end (3' end) degeneracy for AN887 is four-fold, while for AN893 degeneracy is sixteen-fold. The probe AN890 is non-degenerate. From the large NT alignment of 576 recent EV-D68s from the US, Asia, and Europe, codon usage was inferred for EV-D68-specific primer and probe design. As designed, the EV-D68 assay will detect all the EV-D68s in the alignment (*in silico* analysis).

The amplicon size of 272 NT is larger than ideal for a real-time RT-PCR assay and the Taqman® probe has a G at the 5' end linked to the fluorophore FAM. However, neither of these design issues has any significant impact on the performance of the assay. Both compromises were made due to the limited VP1 sequence span available, in the interest of assuring a highly specific assay with no cross-reactivity with other EV-Ds.

Analytical Specificity:

EV-D cell culture isolates from the laboratory collection were tested with the EV-D68-specific assay. Undiluted RNA extracted from cell culture supernatants was tested.

EV-D Cell Culture Isolate	EV-D68-Specific Assay Result
EV-D70, Prototype J670/1971	Negative
EV-D94, Nigeria 2010, a	Negative
EV-D94, Nigeria 2010, b	Negative
EV-D94, Angola 2012	Negative
EV-D111, Angola 2012, a	Negative
EV-D111, Angola 2012, b	Negative
EV-D68, Prototype Fermon 1962	Negative
EV-D68, USA/MO/14-18947	Positive

EV-D120, isolated from gorillas and a chimpanzee, in Cameroon and the Democratic Republic of Congo, respectively, was not available for testing. To date this virus has not been detected in humans. EV-D111 was first detected in chimpanzees in Cameroon, but was later identified in human stool specimens.

Other common respiratory viruses circulating during EV season were tested. One-hundred rhinovirus (RV) species A and B (RV-A, RV-B) were tested with the EV-D68-specific assay. Undiluted RNA extracted from cell culture supernatants was tested. All EV-D68 specific assay results were negative.

Diagnostic Sensitivity and Specificity / Predictive Values:

134 respiratory specimens tested with the EV VP1 sequencing assay[1] were used as the "gold standard" results set to evaluate the EV-D68-specific assay for diagnostic sensitivity and specificity. Absolute sensitivity (limit of detection) for the EV VP1 protocol is less than ten EV genome copies.

		EV VP1 Standard Typing Assay[1]		
		+	**-**	
EV-D68-Specific Assay	**+**	**(True Positive)** 69	**(False Positive)** 3	Positive Predictive Value 96%
	-	**(False Negative)** 0	**(True Negative)** 65	Negative Predictive Value 100%
		Sensitivity 100%	Specificity 96%	

There is good agreement between the two assays. Two false positives had high EV-D68-specific assay Ct values (40.1 and 40.9). Assuming that the analytical sensitivity of the two assays is about equal, both of these specimens, though determined to be false positives, are likely to be low-titer EV-D68 samples, approaching the limit of detection of both assays. The other false positive was a co-infection with a clear mixture of virus sequences on the sequence analysis chromatogram. The virus identified by GenBank NT BLAST of the readable portion of the sequence was RV-A10. The Ct value for the EV-D68-specific assay was 31.2. One advantage of specific assays is the ability to clearly detect the specific target in a co-infection.

1. Nix, W. A., M. S. Oberste, and M. A. Pallansch. Sensitive, semi-nested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. Journal of Clinical Virology 2006; 44 (8):2698-2704.