Enterovirus D68 2014
Real-Time RT-PCR Assay

Centers for Disease Control and Prevention

For Use Under an Emergency Use Authorization Only

Instructions for Use

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Introduction

Purpose

This document describes the use of a real-time (TaqMan®) RT-PCR (rRT-PCR) assay for detection of the viral protein 1 (VP1) gene of Enterovirus D68 (EV-D68).

Intended Use

The Enterovirus D68 2014 Real-time RT-PCR Assay (EV-D68 2014 rRT-PCR) is intended for the *in vitro* qualitative detection of RNA from the enterovirus D68 (EV-D68) strains detected in North America in 2014. It is intended for use with upper respiratory specimens (such as nasopharyngeal swabs (NP), oropharyngeal swabs (OP), dual NP/OP swabs, and/or nasal washes) and sera in conjunction with patient-matched upper respiratory specimen(s) from individuals with signs and symptoms of EV-D68 infection and/or epidemiologic risk factors, tested in qualified laboratories designated by the Centers for Disease Control and Prevention (CDC).

Positive results generated from direct specimen testing are presumptive for the detection of RNA from the EV-D68 strains detected in North America in 2014. When the Enterovirus D68 2014 rRT-PCR is run in conjunction with a cleared commercial assay (as outlined in the EV-D68 Respiratory Disease Algorithm located at http://www.cdc.gov/non-polio-enterovirus/downloads/ev-d68-2014-respiratory-algorithm.pdf), positive results are considered confirmatory for EV-D68 strains detected in North America in 2014.

Testing with the EV-D68 2014 rRT-PCR should not be performed unless the patient meets clinical and/or epidemiological criteria for testing suspect specimens. Current information on EV-D68, including case definitions, is available at http://www.cdc.gov/non-polio-enterovirus/about/EV-D68.html.

The EV-D68 2014 rRT-PCR is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. Assay results are for the presumptive identification of EV-D68 strains detected in North America in 2014. The definitive identification of EV-D68 requires additional testing. Laboratories are required to report results to CDC.

Negative EV-D68 2014 rRT-PCR results do not preclude EV-D68 infection and should not be used as the sole basis for patient management decisions. Expected diagnostic yield in sera is low; however, EV-D68 has been detected in sera from a few patients aged 2 years to 20 years. Only a limited evaluation of serum specimens has been performed due to the scarcity of serum specimens from individuals with EV-D68 infection. Thus, sera may only be tested if a patient-matched upper respiratory specimen is also tested with this assay.

The EV-D68 2014 rRT-PCR is only for use under the Food and Drug Administration's Emergency Use Authorization. Use is limited to qualified laboratories designated by CDC, which includes qualified laboratories with training, facilities and equipment appropriate for specimen handling, testing and interpretation of the results of this real-time RT-PCR assay.

Specimens

Biosafety information: Infection control precautions should include Standard, Contact, and Droplet Precautions for the current outbreak of EV-D68. See Enterovirus D68 for Health Care Professionals.
http://www.cdc.gov/non-polio-enterovirus/hcp/EV-D68-hcp.html

General Information about Enterovirus D68 2014

See: Non-Polio Enterovirus (Enterovirus D68 Disease) at http://www.cdc.gov/non-polio-enterovirus/index.html

Acceptable Specimens

- Upper Respiratory Specimens (preferred)
  - Nasal washes
  - Swabs (dry or in VTM)
    - Nasopharyngeal (NP) swabs
    - Oropharyngeal (OP) swabs
    - Dual NP/OP swabs

  Note: If testing a dry swab, elute the swab into 500 µL VTM prior to extraction.
- Serum: expected diagnostic yield is low; however, EV-D68 2014 has been detected in sera from a few patients aged 2 years to 20 years.
  NOTE: Sera may only be tested in conjunction with patient-matched upper respiratory specimen(s). See Nucleic Acid Extraction section for use restrictions.

Specimen Collection

Refer to www.CDC.gov for guidance on specimen collection, storage and shipment. Information can be found at http://www.cdc.gov/non-polio-enterovirus/lab-testing/specimen-collection.html.

Specimen Handling and Storage

- Specimens should be kept frozen at ≤ -20°C.
- If PCR cannot be performed the same day as specimen extraction, RNA should be stored at -70°C or lower.
- Extracted total nucleic acid should be stored at -70°C or lower.

Equipment and Consumables

Disclaimer: Names of vendors 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.

Materials provided by CDC

- EV-D68 2014 rRT-PCR Primer and Probe Set (CDC; Catalog #KT0158).
  Refer to product insert for storage and expiration information. Set includes 1 set of primers and 1FAM-labeled probe:
  - VP1.2014 (forward primer [VP1.2014 -F], reverse primer [VP1.2014 -R] and probe [VP1.2014 -P]
- RNase P Real-time PCR Primer and Probe Set (CDC; Catalog #KT0068)
  Refer to product insert for storage and expiration information. Set includes 1 set of primers and 1 FAM-labeled probe:
  - Forward primer (RP-F), reverse primer (RP-R), and probe (RP-P)

Materials provided by CDC, but not included in kit

- EV-D68 2014 rRT-PCR Assay Positive Control (CDC; catalog #KT0159) (1.5 mL/vial)

Materials required but not provided

- SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen; catalog #11732-088)
- Molecular grade water, nuclease-free
- Extraction reagents:
  - Manual
    - QIAamp Viral RNA Mini Kit (QIAGEN; catalog #52904 or 52906) or
    - QIAamp DSP Viral RNA Mini Kit (QIAGEN; catalog #61904)
  - bioMérieux easyMAG
    - Refer to list of reagents and consumables under the easyMAG instrument in Equipment and Consumables section (below).
- Human Genomic DNA: for use as a positive control for the RNase P Primer and Probe Set. Either of the following products may be used:
  - Human Genomic DNA from human blood (buffy coat) (Roche Applied Science; Catalog #11691112001)
  - Human Genomic DNA (Promega Corporation; Catalog #G3041)

Equipment and Consumables

- Acceptable surface decontaminants
    - DNA Away™ (Fisher Scientific; catalog # 21-236-28)
    - RNase Away™ (Fisher Scientific; catalog #21-236-21). This product eliminates RNase and DNA.
    - 10% bleach (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
- 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
- PCR Instrument System and Consumables
    - 7500 Fast Dx Real-Time PCR Systems (Applied Biosystems; catalog #4406984)
        - 0.1 mL PCR reaction strip tubes (Applied Biosystems; catalog #4358293)
        - 0.1 mL PCR reaction plates (Applied Biosystems; catalog #4346907)
        - MicroAmp® Optical 8-cap Strips (Applied Biosystems; catalog # 4323032)
    - 7500 Fast (Applied Biosystems; catalog #4351106)
        - 0.1 mL PCR reaction strip tubes (Applied Biosystems; catalog #4358293)
        - 0.1 mL PCR reaction plates (Applied Biosystems; catalog #4346907
        - MicroAmp® Optical 8-cap Strips (Applied Biosystems; catalog # 4323032)
    - 7500 (Applied Biosystems; catalog #4351104)
        - 0.2 mL PCR reaction strip tubes (Applied Biosystems; catalog #4316567)
        - MicroAmp® Optical 8-cap Strips (Applied Biosystems; catalog #4323032)
        - MicroAmp® Optical 96-Well Reaction Plate with Barcode & Optical Caps (Applied Biosystems catalog #403012)
- Automated Extraction Instrument
    - NucliSENS easyMAG (bioMérieux; catalog #280140)
        - easyMAG Magnetic Silica (48 x 0.6 mL) (bioMérieux; catalog #280133)
        - easyMAG Disposables (48 sets) (bioMérieux: catalog #280135)
        - easyMAG Lysis Buffer (bioMérieux; catalog #280134)
        - easyMAG Buffer 1 (bioMérieux: catalog #280130)
        - easyMAG Buffer 2 (bioMérieux; catalog #280131)
        - easyMAG Buffer 3 (bioMérieux: catalog #280132)
        - BioHit Pipette Tips (bioMérieux; catalog #280146)
        - Micro tubes w/caps (bioMérieux; catalog #200294)

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 the chance of false-positive and false-negative results.

General Considerations

At a minimum, good laboratory practices at the Biosafety Level 2 (BSL-2) should be followed, as described in the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition (http://www.cdc.gov/biosafety/publications/bmbl5/index.htm).

- 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 gowns 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 primer/probes and enzyme mastermix 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).
- To minimize cross-contamination, do not bring extracted nucleic acid or PCR amplicons into the assay setup area. Do not wear any protective clothing that was worn in other parts of the laboratory. Only clean PPE should be worn in this 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.

- EV-D68 2014 rRT-PCR Positive Control –Used as a control for PCR reagent function.EV-D68 2014 target RNA transcript; concentration adjusted to give a consistent Ct value of 30-38. Two (2) positive controls must be run concurrently with all test samples.
- NTC – A known negative template control (sterile, nuclease-free water) added during rRT-PCR reaction set-up. Used as a control for PCR reagent function and cross contamination. Two (2) NTC must be run concurrently with all test samples.
- RNase P – All clinical specimens should be tested for human RNase P gene to control for specimen quality and extraction.

- RP Positive Control –Used as a control for RNase P primer and probe set function. The material should be diluted to 50 ng/5 µL or 10 µg/mL with 10 mM Tris (pH 7.4-8.2) prior to use. An acceptable Ct range is ≤30. Either of the following two products may be used:
  (a) Human Genomic DNA from human blood (buffy coat); Roche Applied Science, Catalog #11691112001
  (b) Human Genomic DNA; Promega Corporation, Catalog #G3041

Table 1: Overview of positive and negative controls and expected results

Control Type	Control Name	Used to Monitor	VP1.2014 result	RP result	Expected Ct Values
Positive	EV-D68 2014 rRT-PCR Pos Ctrl	Performance of the VP1.2014 primer and probe set.	+	-	30-38 Ct for VP1.2014
Positive	RNase P positive control	Performance of the RP primer and probe set.	-	+	Ct ≤ 30 for RP
Negative	NTC	Reagent and/or environmental contamination during PCR set-up	-	-	None detected

Nucleic Acid Extraction

- Upper respiratory and serum specimens should be extracted with either the QIAGEN Viral RNA Mini Kit, following the manufacturer's spin column protocol, or the NucliSENS easyMAG (bioMérieux), following the manufacturer's instructions.
- Sample extractions **must** yield RNA or total nucleic acid of sufficient volume to cover all rRT-PCR assays (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 (preferably -70^{o}C). Thaw only the number of extracts that will be tested in a single day. Do not freeze or thaw extracts more than once before testing.

Testing Algorithm

Use VP1.2014 and RP for specimen testing.

- If the VP1.2014 is positive (Ct <43), the specimen is considered positive for 2014 EV-D68 North America RNA.
- If the VP1.2014 assay is positive (Ct ≥43 and <45), the specimen is considered equivocal for 2014 EV-D68 North America RNA.
- If the VP1.2014 is negative and RP is positive (Ct <43), the specimen is considered negative for 2014 EV-D68 North America RNA.
- If the VP1.2014 and the RP are negative, the specimen is considered inconclusive for 2014 EV-D68 North America RNA.

rRT-PCR Assay

The EV-D68 rRT-PCR primer and probe set targets the coding region for the viral protein 1 (VP1) gene of the Enterovirus D68 and shows ≥91% nucleotide identity with strains circulating within the United States in 2014.

Stock Reagent Preparation

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.

1. **Real-time Primers/Probes**
   - EV-D68 2014 rRT-PCR Primer and Probe Set
     - Precautions:
       These reagents should only be handled 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 must be diluted to the working concentrations in nuclease free H_2O.
       - Primers VP1.2014-F and VP1.2014-R should be diluted to 10 µM (10 pmol/µL). Probe VP1.2014-P should be diluted to 5 µM (5 pmol/µL).
     - Sterilely suspend lyophilized reagents in 0.40 mL PCR grade nuclease-free water (50X working concentration) and allow to rehydrate for 15 min at room temperature in the dark.
     - 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.
     - Each EV-D68 2014 rRT-PCR primer and probe kit will contain material to perform 500 reactions. Store rehydrated aliquots of primers and probes at ≤ -20°C. Do not store in frost-free freezers. Rehydrated primers and probes may be stored frozen for up to 12 months. For complete information on storage conditions, see package insert.
   - RNase P Primer and Probe
     - Sterilely suspend lyophilized reagents in 0.25 mL nuclease-free water and allow to rehydrate for 15 minutes at room temperature.
     - Store rehydrated aliquots of primers and probes at ≤ -20°C. Do not store in frost free freezers. Rehydrated primers and probes may be stored frozen for up to 12 months. For complete information on storage conditions, see package insert.

2. **Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System**
    - Place Invitrogen 2X PCR Master Mix and Superscript III RT/Platinum Taq enzyme mix in a cold rack at 2-8°C.
    - Completely thaw the 2X PCR Master Mix vial.
    - Mix the 2X PCR Master Mix by inversion 10 times.
    - Pulse centrifuge 2X PCR Master Mix and Superscript III RT/Platinum Taq enzyme mix then place in cold rack.

4. **No Template Control (NTCs)** (not provided)
    - Sterile, nuclease-free water
    - Aliquot in small volumes
    - Use to check for contamination during plate set-up

5. **EV-D68 2014 rRT-PCR Positive Control**
    - Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination. Freeze-thaw cycles should be avoided. Maintain on ice when thawed.
    - Used to assess performance of VP1.2014 primer and probe set.
    - Centrifuge tube in microcentrifuge at maximum speed. Ensure pellet is at the bottom (pellet will be a bright pink color).
    - Add 1500 µL of cold nuclease-free water and mix gently. Centrifuge tube. Pellet is in solution when no pink precipitate is visible.
    - To ensure complete rehydration, hold tube on ice for 20 minutes before handling further.
    - Aliquot in 15 µL volumes and store at ≤ -70ºC. These aliquots are the working concentrations.
    - Thaw a single working dilution aliquot for each experiment. Discard any unused portion of the aliquot. Do not refreeze.
    - Add 5 µL of positive control to each specific EV-D68 positive control reaction.
    - Expected Ct value 30-38.
    - For complete use and storage conditions, see package insert.

6. **RNase P Positive Control (Human Genomic DNA)**
    - Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination. Freeze-thaw cycles should be avoided. Maintain on ice when thawed.
    - Used to assess performance of RP primer and probe set.
    - Dilute material to 50 ng/5 µL or 10 µg/mL with 10 mM Tris, pH 7.4-8.2 prior to use.
    - Add 5 µL of human genomic DNA to each specific RP positive control reaction.
    - Expected Ct value ≤30.

Equipment Preparation

1. Turn on AB 7500 Fast Dx (or AB7500 or AB7500 Fast) and allow block to reach optimal temperature.

2. Perform plate set up and select cycling protocol on the instrument

Cycling Conditions

Table 2: rRT-PCR cycling conditions

AB 7500 Fast Dx			
Step	**Cycles**	**Temp**	**Time**
Reverse transcription	1	50°C	30 min
Taq inhibitor inactivation	1	95°C	2 min
PCR Amplification	45	95°C 55°C 72°C	15 sec 1 min 5 sec

- Fluorescent detection at the 55°C annealing step.

Instrument Settings

Table 3: Instrument Settings

7500 Fast Dx	7500 Fast	7500
Reporter: FAM	Reporter: FAM	Reporter: FAM
Quencher: None	Quencher: None	Quencher: None
Passive Reference Dye: None	Passive Reference Dye: None	Passive Reference Dye: None
Run Mode: Standard	Run Mode: Standard	Run Mode: Standard
Sample Volume: 25 µL	Sample Volume: 25 µL	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 work day organization. NTCs, RP positive control, and EV-D68 2014 rRT-PCR positive control must be included in each run.

1. In the reagent set-up room clean hood, place rRT-PCR buffer, enzyme, and primer/probes 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 primer/probes by inversion 5 times.
4. Pulse centrifuge buffer and primers/probes 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 per assay. It is necessary to make excess reaction mix for the NTC, EV-D68-PC, and RP reactions and 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 greater than 15-40, then N = n + 3
   - If the number of samples (n) including controls is >40, then N = n + 5

rRT-PCR Reaction Mix:

For each primer and probe set, calculate the amount of each reagent to be added for each reaction mixture (N = number of reactions).

NOTE: Reactions are singleplex, thus reaction mixtures for VP1.2014 primer/probe set and RP primer/probe set must be prepared separately.

Table 4: rRT-PCR Reaction Master Mix

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

Component		VP1.2014
2X Reaction Mix	= N x	12.50 µL
SS III RT/Platinum Taq Mix	= N x	0.50 µL
Forward primer VP1.2014-F (10µM stock)	= N x	0.80 µL
Reverse primer VP1.2014-R (10 µM stock)	= N x	0.80 µL
Probe VP1.2014-P (5 µM)	= N x	0.80 µL
$MgSO_4$ (50 mM)	= N x	0.50 µL
Water, nuclease-free	= N x	4.10 µL
Total volume	**= N x**	**20.00 µL**
Sample RNA		**5 µL**

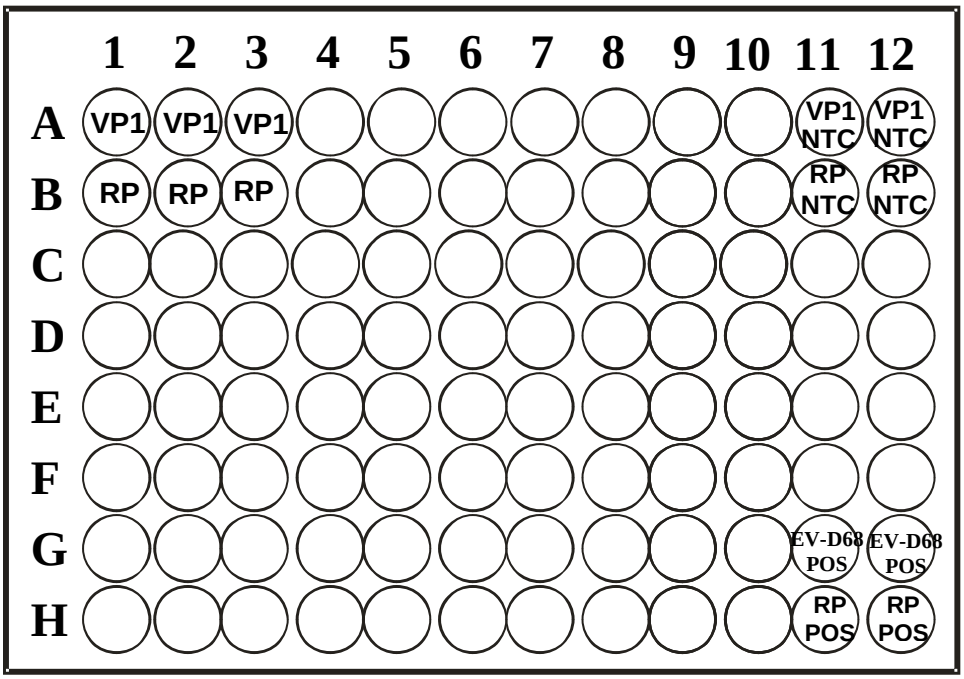
Component		RP
2X Reaction Mix	= N x	12.50 µL
SS III RT/Platinum Taq Mix	= N x	0.50 µL
Forward primer RP-F	= N x	0.25 µL
Reverse primer RP-R	= N x	0.25 µL
Probe RP-P	= N x	0.25 µL
$MgSO_4$ (50 mM)	= N x	0.50 µL
Water, nuclease-free	= N x	5.75 µL
Total volume	**= N x**	**20.00 µL**
Sample RNA		**5 µL**

Note: The reaction mixture is the same for the AB 7500, AB 7500 Fast, and AB 7500 Fast Dx.

7. Mix reaction components by pipetting slowly up and down (avoid bubbles).
8. Add 20 µL of master mix into each well of a chilled optical plate as shown in examples below.

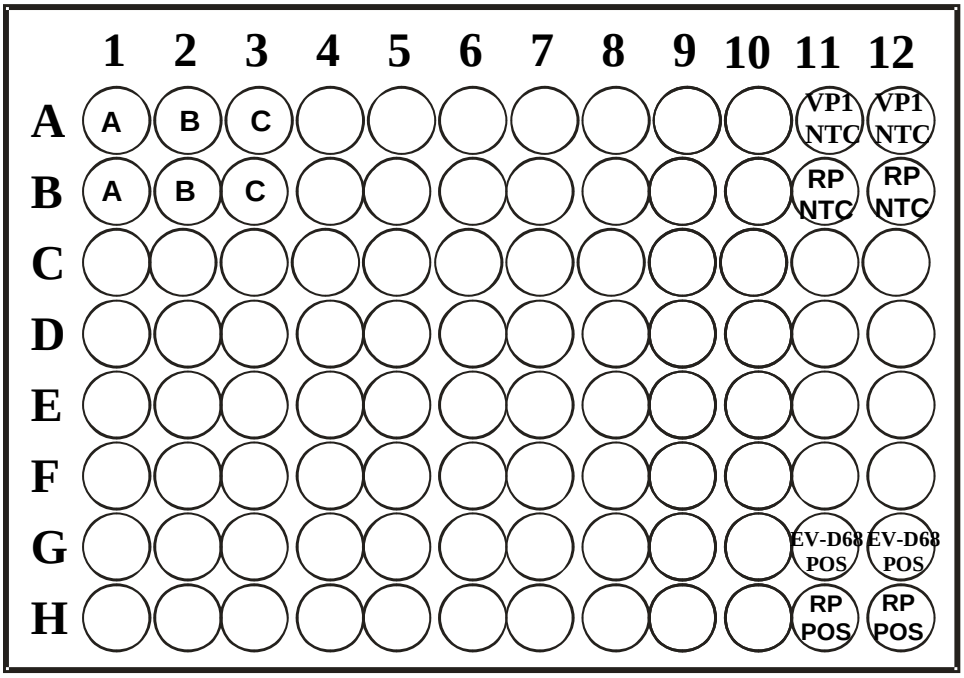
   NOTE: The plate set-ups are provided for example only. Laboratories may configure their plate as best meets the number of samples and standard operations of the laboratory.

Figure 1: Example Plate Set-Up for primers/probes



VP1.2014 (VP1) primer/probe; RNase P (RP) primer/probe; No template reaction mix controls (NTC)

Figure 2: Example Plate Set-Up for testing 3 samples



RNase positive control (RP POS); No template reaction mix controls (NTC);
EV-D68 2014 positive control (EV-D68 POS); A, B and C (samples)

10. Before moving the plate to the nucleic acid handling area, add 5 µL of nuclease-free water to the NTC wells.
11. Loosely apply the 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 positive controls and pulse centrifuge.
13. Set up the extracted RNA specimen reactions.
    (a) Remove the optical strip caps.
    (b) Pipette 5 µL of the first sample into all the wells designated for that sample. For example, dispense 5 µL of sample A into the wells labeled "A" in Fig. 2. Follow in sequential order to your plate/strip tube template.
14. Change tips after each specimen addition.
15. 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.
16. Continue with the remaining samples. Change gloves between samples if you suspect they have become contaminated.
17. Pipette 5 µL of the positive controls (EV-D68 2014 and RNase P positive control) into designated well(s) and cap. Secure all strip caps with capping tool.
18. Transport the plate to the amplification area on cold block.
19. Centrifuge the plate at 500 x *g* for 1 minute at room temperature to remove bubbles or drops that may be present in the wells. Strip tubes may be spun 10-30 seconds in a strip microcentrifuge. Be sure to use a balance plate, if necessary.
20. Place plate/strip tubes on pre-programed AB 7500 Fast Dx (AB 7500 or 7500 Fast) and start run.
22. For detailed instructions on launching and programming the Applied Biosystems 7500 Fast Dx System software, refer to the Programming of the AB 7500 Fast Dx located under Documents/Instrument Programming and Maintenance on the LRN secure website.

Data Analysis

After completion of the run, save and analyze the data following the instrument manufacturer's instructions. Analyses should be performed 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 the setting the threshold should be used consistently.

Interpreting Test Results

Accurate interpretation of rRT-PCR results requires careful consideration of several assay parameters. The following are general guidelines:

Interpret Run Controls:

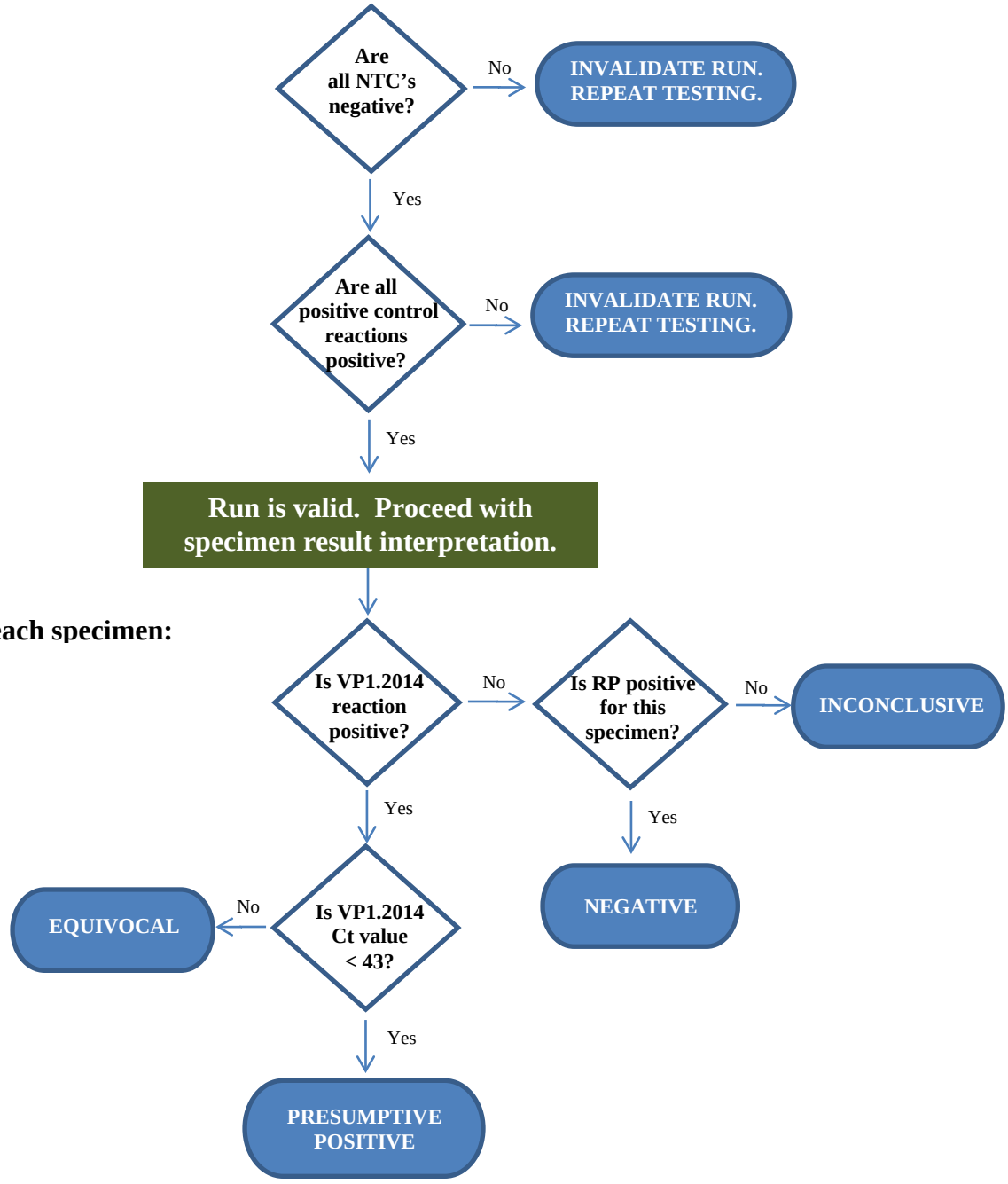


Figure 3: Order of Interpretation of Assay Results

a. Interpretation of Controls
   1. The Positive Controls must be **positive** and with Ct values within the values listed in Table 1 for all primer and probe sets.
      a. If positive controls are *negative*, the testing results for that plate are invalid.
         i. Repeat rRT-PCR test.
         ii. If repeat testing generates negative EV-D68 2014 Positive Control results, send the experiment run file (extension .eds) to LRN@cdc.gov for consultation.
   2. NTCs must be **negative**.
      a. If NTCs are *positive*, the testing results for that plate are invalid.
         i. Clean potential DNA contamination from bench surfaces and pipettes in the reagent setup and template addition work areas.
         ii. Discard working reagent dilutions and remake from fresh stocks.
         iii. Repeat extraction and test multiple NTCs during rRT-PCR run.
         iv. Repeat rRT-PCR test.
   3. RP Assay for each specimen must be **positive (Ct <43)**.
      a. If RP Assay for a specimen sample is *negative* ($Ct \geq 43$ or undetermined) and the EV-D68 2014 Real-time PCR assay is *negative* for specimen samples:
         i. Report result as *Inconclusive*
         ii. Repeat rRT-PCR test of sample using RP and VP1.2014 assay.
         iii. Repeat extraction from new specimen aliquot if RP Assay is *negative* for specimens after repeat testing.
         iv. After repeat extraction and repeat rRT-PCR testing, if VP1.2014 is *positive*, consider the result a true *positive* and continue to follow the testing algorithm.
         v. If you are unable to resolve the results for this specimen, test other specimens from the patient, if available, or request the collection of additional specimens.
      b. If RP Assay for a specimen sample is *negative*, but VP1.2014 is *positive* for specimen samples:
         Do not repeat rRT-PCR test and consider the results of the VP1.2014 assay valid.

If all controls have been performed appropriately, proceed to analyze each target.

- True EV-D68 2014 rRT-PCR positives should produce exponential curves with logarithmic, linear, and plateau phases (Figure 4).
- Note: Weak positives will produce high Ct values that are sometimes devoid of a plateau phase; however the exponential plot will be seen.

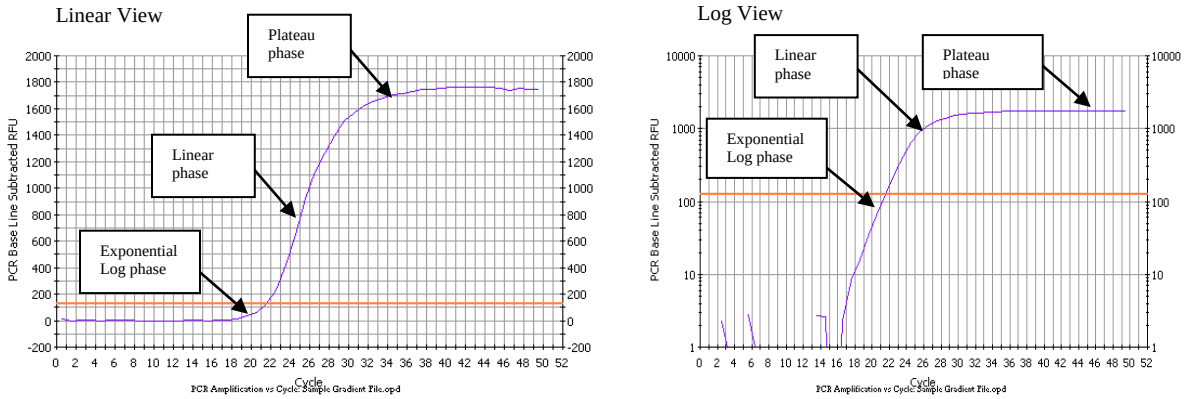


Figure 4: Linear and log views of PCR curves noting each stage of the amplification plots

- For a sample to be a true positive, the curve must cross the threshold in a similar fashion as shown in Figure 4. It must NOT cross the threshold and then dive back below the threshold.
- Figure 5 shows examples of false positives that do not amplify exponentially.

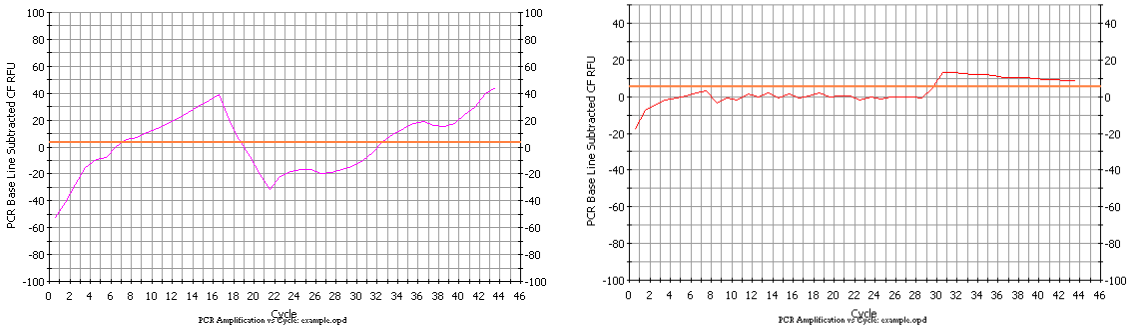


Figure 5: Examples of false positive curves

- To better understand and evaluate challenging curves more effectively, use the background fluorescence view (Rn versus Cycle with AB software) to determine if the curve is actually positive. In this view, a sharp increase in fluorescence indicates a true positive while a flat line (or wandering line) indicates no amplification.

- Figure 6 shows a curve with a Ct value of 29.2 though it is evident that the sample is negative by looking at the background fluorescence view.
- Figure 7 shows an amplification plot with 3 curves: a moderately weak positive (black), a very weak positive (red), and a negative control (blue). The weak positive is verified to be positive by the sharp increase in fluorescence seen in the background fluorescence view.

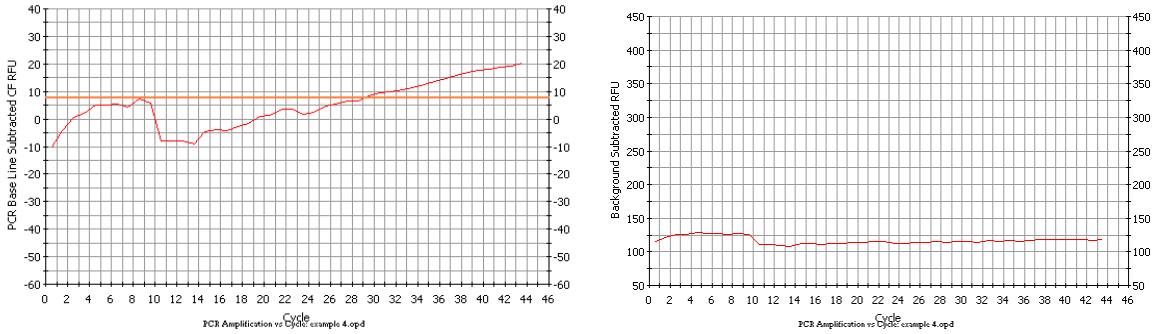


Figure 6: Amplification plot of a sample with a "wandering" curve (left) and the corresponding background fluorescence view (right)

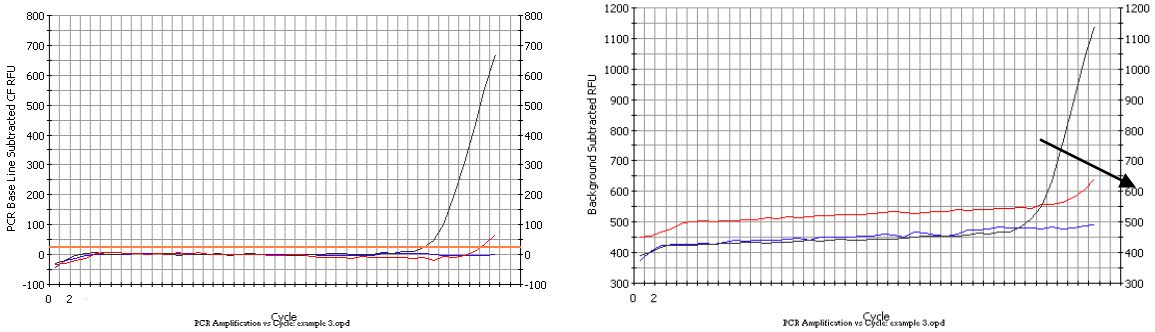


Figure 7: Amplification plot of three samples in the linear view (left) and the corresponding background fluorescence view (right)

- AB software has a spectra component that also can help evaluate challenging curves more efficiently. The spectra component shows the difference in total fluorescence at every cycle. If there is an obvious difference in the fluorescence from cycle 1 to cycle 43, the sample is a true positive. Figure 8 shows the spectra view of a positive sample. Filter A is the FAM filter and indicates if there is an accumulation of fluorescence during the reaction. Filter D is the ROX filter and should remain constant.

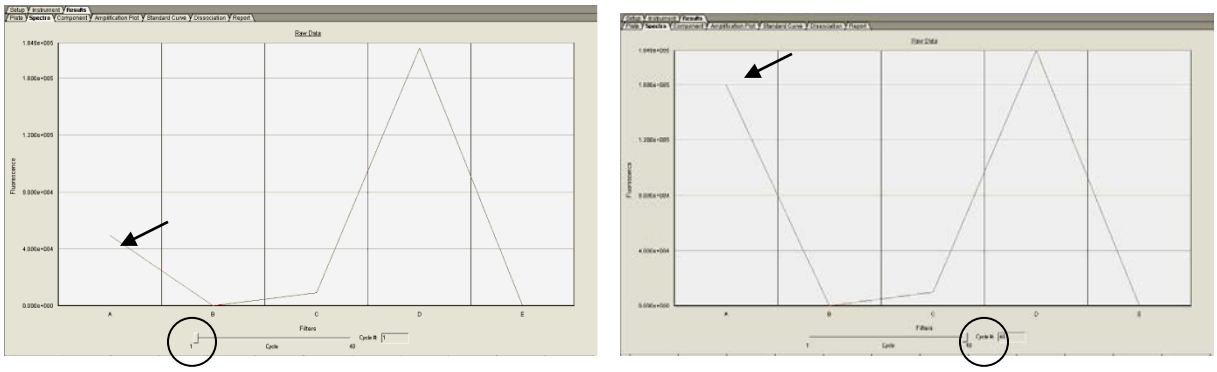


Figure 8: Spectra component of a positive sample. Left screenshot shows fluorescence at cycle 1 and right screenshot shows fluorescence at cycle 40.

- As described above, close examination of the amplification curves can help determine if a sample is truly positive or not and eliminates the need to rely solely on Ct values. However, this does not answer the question of the source of the sample positivity: Is the sample truly positive for the pathogen or did contamination occur during or after sample collection? It is important to be very careful during sample collection, extraction, and rRT-PCR setup to avoid contamination.
- **A note on weak positive samples (Ct ≥38)**
  Weak positives should always be interpreted with caution. Look carefully at the fluorescence curves associated with these results. If curves are true exponential curves, the reaction should be interpreted as positive.
- If repeat testing of a weak specimen is necessary, it is important to repeat the sample in replicates as a single repeat test run has a high likelihood of generating a discrepant result.
- If re-extracting and re-testing the specimen, it may be helpful to elute in a lower volume to concentrate the sample.
- Contact CDC for guidance, to help determine if repeat testing may be warranted and to discuss additional testing strategies as appropriate.

Overall Test Interpretation and Reporting Instructions

Table 5: EV-D68 2014 rRT-PCR Test Interpretation and Reporting Instructions

D68	RP	Interpretation	Reporting	Actions
-	+	EV-D68 Negative	2014 EV-D68 North America RNA not detected by rRT-PCR	Refer to the CDC EV-D68 Respiratory Disease Algorithm. Report results to CDC
-	-	Inconclusive	Inconclusive for 2014 EV-D68 North America RNA by rRT-PCR. An inconclusive result may occur in the case of an inadequate specimen.	If there are no additional specimens available for the patient, request collection of additional specimens. Report results to CDC.
+ Ct <43	+ or -	EV-D68 Presumptive Positive	2014 EV-D68 North America RNA detected by rRT-PCR. Additional analysis required. Refer to CDC EV-D68 Respiratory Disease Algorithm at: http://www.cdc.gov/non-polio-enterovirus/hcp/ev-d68-hcp.html	Refer to the CDC EV-D68 Respiratory Disease Algorithm. Report results to CDC.
+ Ct ≥43 and <45	+/-	Equivocal	Equivocal for 2014 EV-D68 North America RNA. Testing will be repeated and specimen forwarded for additional analysis.	Repeat PCR on extracted nucleic acid from the specimen AND forward specimen to CDC.

NOTE: All test results generated using the EV-D68 2014 rRT-PCR Assay by LRN laboratories must be sent to CDC using LRN Results Messenger. Please refer to the LRN Data Messaging Policy (found under Documents/LRN Specific Information/LRN Policy Statements on the LRN website). For questions regarding this policy, please contact the LRN Helpdesk at LRN@cdc.gov.

NOTE: Please refer to the **Interpreting Test Results** section for detailed guidance on interpreting weak positives or questionable curves.

Results should be used in accordance with the CDC EV-D68 Respiratory Disease Algorithm, available at: http://www.cdc.gov/non-polio-enterovirus/hcp/ev-d68-hcp.html.

Assay Limitations, Warnings and Precautions

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 or
- specimen collection conducted prior to symptom onset or late in illness
- failure to follow the authorized assay procedures
- failure to use authorized extraction kit and platform

Application of appropriate assay controls that identify poor-quality specimens (such as RNase P) and adherence to CDC guidelines for EV-D68 testing (http://www.cdc.gov/non-polio-enterovirus/index.html) can help avoid most false-negative results.

A high Ct observed for a specimen taken during early onset of disease should be followed up with another specimen taken 24-48 hours later. Refer to CDC EV-D68 laboratory guidance for current advice (http://www.cdc.gov/non-polio-enterovirus/index.html) and consultation instructions.

The most common cause of false-positive results is contamination with previously amplified DNA. Liberal use of negative control samples in each assay can help ensure that laboratory contamination is detected and that false positive test results are not reported.

Negative results do not preclude infection with Enterovirus D68 and should not be used as the sole basis of a patient treatment/management decision. All results should be interpreted by a trained professional in conjunction with review of the patient's history and clinical signs and symptoms. Only a limited evaluation of serum specimens has been performed due to the scarcity of serum specimens from individuals with EV-D68 infection. Thus, sera may only be tested in conjunction with patient-matched upper respiratory specimen(s) with the EV-D68 2014 rRT-PCR.

This assay is for *in vitro* diagnostic use under FDA Emergency Use Authorization only and is limited to qualified laboratories designated by CDC.

All specimens should be handled as if infectious. Proper biosafety precautions, including personal protective equipment, must be used when handling specimen materials.

Proper collection, storage and transport of specimens are essential for correct results.

Extraction of nucleic acid from clinical specimens must be performed with either the bioMérieux NucliSENS easyMAG instrument with its associated reagents, the QIAamp Viral RNA Mini Kit, or the QIAamp DSP Viral RNA Mini Kit from QIAGEN. Other extraction kits have not been evaluated for use with this assay.

Performance has only been established with the specimen types listed in the Intended Use. Other specimen types have not been evaluated.

Performance Characteristics

The EV-D68 2014 rRT-PCR has been developed and evaluated by the CDC Picornavirus Laboratory from mid-September to mid-October 2014. The assay and protocol are primarily focused on evaluating respiratory disease due to EV-D68 strains detected in North America in 2014.

Unless otherwise noted, studies presented below were performed following the EV-D68 2014 rRT-PCR test procedure using the Applied Biosystems 7500 Real-time PCR system and the QIAGEN QIAamp Viral RNA Mini Kit for specimen extraction. The comparator method employed in several of the evaluations listed below, the enterovirus VP1 reverse transcription, semi-nested PCR followed by sequencing (EV VP1 Sequencing Assay), is described in the following publication:

> 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 Microbiology 2006, 44(8):2698-2704.

1. VP1.2014 Primers and Probe Set Development

The EV-D68 2014 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).

The Public Health Agency of Canada employs the CDC VP1 Sequencing method and shared five representative EV-D68 Canadian outbreak sequences with CDC in December 2014. These sequences were first analyzed phylogenetically and clustered with US 2014 co-circulating EV-D68 strains. In silico inspection of the VP1.2014 primer sites and probe site showed 100% nucleotide identity with all five Canadian EV-D68 strains.

2. Analytical Sensitivity

a) Preliminary Limit of Detection (LoD) in Minimum Essential Medium (MEM)
Serial ten-fold dilutions of RNA extracted from EV-D68 US/MO/14-18949 strain (10^8 $CCID_{50}$/mL) were prepared in MEM. The serial dilutions from 10^{-1} to 10^{-10} were tested in triplicate using the EV-D68 2014 rRT-PCR. The lowest dilution at which all replicates were positive was at **10^{-7} $CCID_{50}$/mL or $10^{-1.1}$ $CCID_{50}$/5µL** with the EV-D68 2014 rRT-PCR. Results of testing with the EV VP1 Sequencing Assay at each concentration are also included to aid in interpretation of comparative study data presented in the clinical performance sections.

Table 6: EV-D68 2014 rRT-PCR Assay Limits of Detection Estimation with EV-D68 Isolate RNA[a] Diluted in Minimum Essential Medium[b] and Comparison to Limits of Detection Estimation with the EV VP1 Sequencing Assay.

RNA Dilution[c]	Concentration ($CCID_{50}$ / 5 µL)	Replicates (Ct Values)			Result EV-D68 2014 rRT-PCR	Result EV VP1 Sequencing Assay
10^{-1}	$10^{4.9}$	15.8	16.0	16.3	3/3 Positive	3/3 Positive
10^{-2}	$10^{3.9}$	19.3	19.7	19.6	3/3 Positive	3/3 Positive
10^{-3}	$10^{2.9}$	24.5	24.7	25.4	3/3 Positive	3/3 Positive
10^{-4}	$10^{1.9}$	29.1	29.6	30.1	3/3 Positive	3/3 Positive
10^{-5}	$10^{0.9}$	33.7	33.6	34.2	3/3 Positive	3/3 Positive
10^{-6}	$10^{-0.1}$	37.7	36.9	36.8	3/3 Positive	3/3 Positive
10^{-7}	$10^{-1.1}$	40.6	41.9	39.9	3/3 Positive	3/3 Positive
10^{-8}	$10^{-2.1}$	43.5	Negative	Negative	1/3 Equivocal 2/3 Negative	2/3 Positive 1/3 Negative
10^{-9}	$10^{-3.1}$	Negative	Negative	Negative	3/3 Negative	3/3 Negative
10^{-10}	$10^{-4.1}$	Negative	Negative	Negative	3/3 Negative	3/3 Negative

[a] Serial dilutions were prepared from frozen stock.
[b] Minimum essential medium is a cell culture medium that can be substituted for the several commercial formulations of viral transport medium, used for NP swabs
[c] 1:10 dilution series of EV-D68 US/MO/14-18949 control RNA (lot 10/16/14) from titered virus control stock

b) LoD Confirmation in Clinical Matrix – Pooled Dual NP/OP Swabs (PCR Instrument and Extraction Method Bridging Study)

A study was performed to compare the limits of detection for VP1.2014 in pooled leftover dual NP/OP swab specimens in viral transport media across PCR instruments and across extraction methods. Prior to use in the study, the leftover dual NP/OP swab eluents had tested negative for the presence of enterovirus, including D68. The pooled matrix was divided and spiked with EV-D68 US/MO/14-18949 at three concentrations ($10^{0.9}$ $CCID_{50}$/5 µL, $10^{-0.1}$ $CCID_{50}$/5 µL, and $10^{-1.1}$ $CCID_{50}$/5 µL). Each concentration was extracted 20 times by each extraction method. The resulting nucleic acid samples were each tested once on each PCR instrument system. The limits of detection observed for each PCR instrument and extraction method combination are presented in Table 17.

All three PCR instrument systems yielded the same limit of detection, $10^{-0.1}$ $CCID_{50}$/5 µL, with material extracted manually using the QIAGEN QIAamp Viral RNA Mini Kit. With this extraction method, the three instruments achieved equivalent limits of detection for the EV-D68 2014 rRT-PCR and are all considered to be acceptable for use with this assay.

Material extracted on the easyMAG generated the same limit of detection for the EV-D68 2014 rRT-PCR as the QIAamp extracts when tested on the AB 7500. However, the other

two instrument systems (7500 Fast and 7500 Fast Dx) missed the 95% cutoff for this concentration by 1 and 2 reactions, respectively. Thus, the limit of detection for these two instruments with the easyMAG is set at the next highest concentration ($10^{0.9}$ $CCID_{50}$/5 µL). Though the 7500 Fast and Fast Dx instruments, when used with the easyMAG, did not achieve the same LoD as the 7500 Fast, the performance was similar enough to consider these instrument/extraction method combinations acceptable for use with the EV-D68 2014 rRT-PCR.

The RNase P primer and probe set was tested against each nucleic acid sample. Testing was performed exclusively on the AB 7500 instrument and yielded 100% positive results in this evaluation.

Table 7: EV-D68 2014 rRT-PCR Limits of Detection - Spiked Pooled Dual NP/OP Swab Specimen Matrix

Extraction Methods	PCR Platforms		
	7500	7500 Fast	7500 Fast Dx
Qiagen QIAamp	$10^{-0.1}$ $CCID_{50}$/5 µL	$10^{-0.1}$ $CCID_{50}$/5 µL	$10^{-0.1}$ $CCID_{50}$/5 µL
easyMAG	$10^{-0.1}$ $CCID_{50}$/5 µL	$10^{0.9}$ $CCID_{50}$/5 µL	$10^{0.9}$ $CCID_{50}$/5 µL

c) LoD Confirmation in Clinical Matrix – Pooled Serum (Extraction Method Bridging Study)

A study was performed to evaluate the QIAamp, easyMAG and MagNA Pure Compact extraction methods for use in preparing nucleic acid from human sera for subsequent testing with the EV-D68 2014 rRT-PCR. The study was performed using pooled leftover sera. Prior to use in the study, the leftover serum specimens had tested negative for the presence of enterovirus nucleic acid, including EV-D68. The pooled matrix was divided and spiked with EV-D68 US/MO/14-18949 at three concentrations ($10^{0.9}$ $CCID_{50}$/5 µL, $10^{-0.1}$ $CCID_{50}$/5 µL, and $10^{-1.1}$ $CCID_{50}$/5 µL). Each concentration was extracted 20 times by each extraction method. The resulting nucleic acid samples were each tested once by EV-D68 2014 rRT-PCR on the AB 7500 Fast Dx instrument system. The limits of detection observed for each extraction method are presented in Table 19.

Material extracted manually using the QIAGEN QIAamp Viral RNA Mini Kit generated a limit of detection of $10^{-0.1}$ $CCID_{50}$/5 µL. Material extracted on the easyMAG generated a lower limit of detection, $10^{-1.1}$ $CCID_{50}$/5 µL. The MagNA Pure Compact was also included in this evaluation, but data has been excluded as the instrument is not recommended for use with the assay.

As the limits of detection for sera extracted with the QIAamp method and the easyMAG method were the same or lower than the limits of detection achieved in the evaluation of pooled dual NP/OP swabs with the EV-D68 2014 rRT-PCR, both are acceptable for use with serum in preparation of nucleic acid for subsequent testing by this assay.

The RNase P primer and probe set was tested against each nucleic acid sample. RP testing was performed exclusively on the AB 7500 instrument and yielded 100% positive results in this evaluation.

Additional information and line listing data may be found in Attachment E.

Table 7: EV-D68 2014 rRT-PCR Limits of Detection - Spiked Pooled Sera

Extraction Methods	PCR Platform
	7500 Fast Dx
Qiagen QIAamp	$10^{-0.1}$ $CCID_{50}$/5 µL
easyMAG	$10^{-1.1}$ $CCID_{50}$/5 µL

d) Performance of RNase P Primer and Probe Set with Recommended Human DNA Controls

The RNase P Primer and Probe Set (RP) was tested against serial dilutions of two commercial human DNA preparations. A concentration of 50 ng/5 µL (10 ng/µL) should be added to each reaction. The two commercial human DNA preparations have been used as positive controls for RP in other Laboratory Response Network PCR assays, but not with the combination of master mix and cycling conditions used with this assay. The evaluation successfully demonstrated that the human DNA preparations would generate expected results with RP in this assay, and were suitable for use as RP positive control material when used at a concentration of 50 ng/5 µL. Results are presented in Table 20.

Table 9: EV-D68 2014 rRT-PCR Performance of Human DNA Controls with the RNase P Primer and Probe Set

Platform	AB 7500		AB 7500 Fast Dx	
[DNA]	Promega	Roche	Promega	Roche
50 ng / 5 µL	28.4	27.6	26.0	24.8
5.0 ng / 5 µL	31.9	30.9	29.3	28.2
0.5 ng / 5 µL	36.4	34.8	33.2	32.2
0.05 ng / 5 µL	40.7	38.6	37.3	36.4
0.005 ng / 5 µL	Negative	Negative	40.4	39.9

Note: This study was not conducted on the AB 7500 Fast; however, the performance is expected to be similar.

3. Analytical Specificity

a) Cross-Reactivity With Other Enteroviruses

Enterovirus Species D

To evaluate the analytical specificity of the EV-D68 2014 rRT-PCR, we used a panel of enterovirus species D (EV-D) cell culture isolates (n=7) that included the EV-D68, 1962 Fermon prototype strain. Respiratory clinical specimens positive for EV-D68 Fermon, EV-D70, EV-D94, EV-D111 were not available for testing, therefore RNA from these viruses was extracted from frozen undiluted cell culture supernatants and tested. The EV-D68 2014 rRT-PCR did not cross-react with other enterovirus species D viruses including the Fermon prototype strain.

Table 10: EV-D68 2014 rRT-PCR Analytical Specificity Confirmed Using EV-D Cell Culture Isolates

EV-D Cell Culture Isolate[a]	EV-D68 2014 rRT-PCR Result 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

[a] Purified RNA in elution buffer

EV-D120, isolated from gorillas and a chimpanzee, in Cameroon and the Democratic Republic of Congo, respectively, was not available for testing. *In silico* testing was not performed on this strain. To date this virus has not been detected in humans.

Rhinovirus Species A and B

Other genetically related common Rhinoviruses circulating during the EV season were tested using undiluted cell culture supernatants. A total of 95 rhinoviruses (RV), including 70 species A (RV-A) and 25 species B (RV-B) were tested (Table 11). Results demonstrated no cross-reactivity with the Enterovirus species tested.

Table 11: EV-D68 2014 rRT-PCR Analytical Specificity Confirmed Using Undiluted RNA Extracted from Rhinovirus Cell Culture Supernatants

Enterovirus Species[b]	Enterovirus Types	EV-D68 2014 rRT-PCR Result	EV-D68 (neg/total)
RV-A[a]	RV-A1, A2, A7, A8, A9, A10, A11, A12, A13, A15, A16, A18, A19, A20, A21, A22, A23, A24, A25, A28, A29, A30, A31, A32, A33, A34, A36, A38, A39, A40, A41, A43, A45, A46, A47, A49, A50, A51, A53, A54, A55, A56, A57, A58, A59, A60, A61, A62, A63, A64, A65, A66, A67, A68, A71, A73, A74, A75, A76, A77, A78, A80, A82, A85, A88, A89, A90, A94, A96, A100	All Negative	70/70
RV-B	RV-B3, B4, B5, B6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69, B70, B72, B79, B83, B84, B86, B91, B92, B93, B97, B99	All Negative	25/25

[a] RV = rhinovirus; Species A is the most abundant in humans

[b] Undiluted RV RNA extracted from cell culture supernatants was tested.

b) Cross-reactivity with Other Common Respiratory Viruses

RNA extracted from fourteen common respiratory viruses and pooled nasal wash (containing *Streptococcus pneumoniae*, rhinovirus and adenovirus) were tested using the EV-D68 2014 rRT-PCR on both the AB 7500 and the AB 7500 Fast Dx. RNA was extracted from viral cell culture supernatants (except for the pooled nasal wash clinical specimen) and tested undiluted. All results matched expected results.

Table 8: EV-D68 2014 rRT-PCR Cross-Reactivity with Common Respiratory Viruses

Number	Virus	AB 7500	AB 7500 Fast Dx
1	Adenovirus C1 (Ad 71)	Negative	Negative
2	CoV 229E	Negative	Negative
3	CoV OC43	Negative	Negative
4	CoV MERS	Negative	Negative
5	HMPV (CAN99-81)	Negative	Negative
6	Influenza A H1N1	Negative	Negative
7	Influenza A H3N1	Negative	Negative
8	Influenza B	Negative	Negative
9	PIV1 (C35)	Negative	Negative
10	PIV2 (Greer)	Negative	Negative
11	PIV3 (C-43)	Negative	Negative
12	PIV4a (CH 19503)	Negative	Negative
13	RSV Long A	Negative	Negative
14	Rhinovirus 1A	Negative	Negative
15	Pooled nasal wash*	Negative	Negative
16	EV-D68 14-18949	Positive (Ct 32.4)	Positive (Ct 35.7)

* *Streptococcus pneumoniae*; rhinovirus; adenovirus

c) Cross-Reactivity with Other Clinically Relevant Organisms

A panel of nucleic acid samples extracted from clinically relevant organisms was tested using the EV-D68 2014 rRT-PCR. Nucleic acid samples for viral panel members were prepared from undiluted tissue culture grown virus. For bacterial and fungal panel members, nucleic acid was extracted from bacterial and fungal colonies and tested undiluted. Testing was performed by CDC laboratories in Atlanta, Georgia, and Ft. Collins, Colorado. Results are presented in Table 13.

Table 93: EV-D68 2014 rRT-PCR Cross-reactivity with other clinically relevant organisms

Virus/Bacteria/Fungus	VP1.2014 Result
Dengue Virus 1*	Negative
Dengue Virus 2*	Negative
Dengue Virus 3*	Negative
Dengue Virus 4	Negative

Eastern Equine Encephalitis Virus*	Negative
Western Equine Encephalitis Virus*	Negative
Colorado Tick Fever Virus	Negative
Powassan Virus*	Negative
Japanese Encephalitis Virus*	Negative
West Nile Virus*	Negative
Yellow Fever Virus*	Negative
Lacrosse Encephalitis Virus*	Negative
St. Louis Encephalitis Virus*	Negative
Chikungunya Virus*	Negative
Herpes Simplex Virus-1	Negative
Herpes Simplex Virus-2	Negative
Varicella Zoster Virus	Negative
Measles Virus	Negative
Mumps Virus	Negative
Neisseria meningitides	Negative
Listeria monocytogenes	Negative
Escherichia coli	Negative
Bacillus cereus	Negative
Lactobacillus acidophilus	Negative
Crytococcus neoformans	Negative

*Results for these agents were generated using a PCR instrument not validated for use with the EV-D68 2014 rRT-PCR.

d) *In silico Analysis of Common Respiratory Illness-Causing Flora and a Selection of Other Flora Found in Respiratory Specimens and Sera*

Additional evaluation of the analytical specificity of the EV-D68 rRT-PCR was performed through *in silico* analysis of the VP1.2014 primer and probe sequences against common causes of respiratory illness and other clinically relevant organisms. BLASTn analysis queries of the VP1.2014 primers and probe were performed against the GenBank public domain nucleotide sequences and showed no significant combined homologies (primer target and probe target) with other conditions that would predict potential false positive rRT-PCR results. Conditions and associated causative agents covered in the *in silico* specificity analysis are presented in Table 14.

Table 104: VP1.2014 Primer and Probe Set *In silico* Specificity Analysis

Disease/condition	Tax ID	Agent
Human parainfluenza	11226	Parainfluenza virus 4b
Human parechovirus	12063	Parechovirus 1b
Common cold, bronchiolitis, pneumonia	208895	Respiratory syncytial virus B
Sinusitis	1280	*Staphylococcus aureus*
Pertussis (whooping cough)	520	*Bordetella pertussis*
Mycoplasma pneumonia	2104	*Mycoplasma pneumoniae*
Pharyngitis, bronchitis, atypical pneumonia	83558	*Chlamydia pneumoniae* (or *Chlamydophila pneumonia*)

Bacteremia, pneumonia, epiglottitis and acute bacterial meningitis	727	*Haemophilus influenza*
Tuberculosis	1773	*Mycobacterium tuberculosis*
Lyme disease	139	*Borrelia burgdorferi*
Respiratory infection	550	*Enterobacter cloacae*
Septicaemia	1354	*Enterococcus hirae*
Foodborne illness	1502	*Clostridium perfringens*
Abdominal infection (rare)	46503	*Parabacteroides merdae*
Human gastrointestinal bacterium	1680	*Bifidobacterium adolescentis*
Human gastrointestinal bacterium	216816	*Bifidobacterium longum*
Human gastrointestinal bacterium	40518	*Ruminococcus bromii*

4. Precision

Time Period: October 16, 2014- October 19, 2014

Number of Days Tested: 1 assay run (5 replicates/sample) per day for 3 non-consecutive days with two separate PCR instruments (one on Day 1, another on Days 2 and 3).

Number of Operators: 1 operator

Number of Samples Types in Each Run:

- Two concentrations of EV-D68 US/MO/14-18949 control RNA from frozen stock (lot: 10/8/14).
  - A low cell culture 50% infectious dose ($CCID_{50}$) dilution of EV-D68 US/MO/14-18949 control RNA contained 0.79 $CCID_{50}$/ 5µL input RNA
  - A high $CCID_{50}$ dilution contained $10^{2.9}$ $CCID_{50}$ / 5µL input RNA.
- Two clinical NP swab specimens in VTM (one high Ct Positive and one low Ct positive). Swabs were collected from two different patients.
- NTCs

Cumulative results are presented in Table 15 (below).

Table 11: EV-D68 2014 rRT-PCR Precision Testing

Sample	Cumulative Replicates (N)	Results (Positive)	Positive Detection Rate (%)
1: Low Concentration EV-D68 control RNA[a]	15	15/15	100
2: High Concentration EV-D68 control RNA[a]	15	15/15	100
3: NTC control	15	0/15	100
4: EV-D68 Positive Clinical Specimen (low Ct)[b]	15	15/15	100
5: EV-D68 Positive Clinical Specimen (high Ct)[b]	15	15/15	100

[a] purified RNA from virus cell culture isolate

[b] RNA extracted from clinical specimens

5. Clinical Evaluation

The clinical study was conducted from September to October 2014. A total of 128 specimens (116 respiratory and 12 sera) were tested using the EV-D68 2014 rRT-PCR and the enterovirus VP1 reverse transcription semi-nested PCR assay followed by sequencing (EV VP1 Sequencing Assay). All controls were run in duplicate. The same lot of non-infectious control RNA was run concurrently with all test samples. The following standard controls were used during the clinical study.

- Positive control: 2014 outbreak strain EV-D68 US/MO/14-18949 viral RNA, concentration adjusted to yield Ct value of 30-32 (low but consistent positive);
- Negative control: no-template controls (NTC) of nuclease free water
- RNase P (RP) Primer and Probe Set: Included as a control for specimen quality and for the presence of nucleic acid in the sample following extraction. RP was performed on all but 16 specimens (11 NP swab specimens, 5 nasal washes). For specimens without RP results, specimen results were interpreted on the basis of VP1.2014 alone.

Positive percent agreement (PPA) and negative percent agreement (NPA) against the comparator (EV VP1 Sequencing Assay) is presented for upper respiratory specimens in Table 16 and serum in Table 17. A breakdown of the negative respiratory specimens is presented in Table 18.

Table 12: EV-D68 2014 rRT-PCR Clinical Performance Summary for Upper Respiratory Specimens

Specimen Type	EV-D68 Positive by EV VP1 Sequencing Assay				EV-D68 Negative by EV VP1 Sequencing Assay		
	PCR +	PCR E[a]	Total	PPA (95% CI)	PCR -	Total	NPA (95% CI)
NP and OP Swabs[d]	58	1	59	98.3% (91.0% - 99.7%)	40[b]	42	95.2% (84.2% - 98.7%)
Nasal Wash	3	0	3	100% (43.9%- 100%)	11[c]	12	91.6% (64.6% - 98.5%)

[a] PCR E refers to an equivocal result (Ct value ≥ 43 and <45).

[b] One NP swab came up as RV-A10 positive by VP1 sequencing and generated a positive result (Ct 31.2) for VP1.2014. Further testing revealed this to be a specimen from a co-infection, thus the EV-D68 2014 rRT-PCR result was correct. See Table 18 for additional details.
A second NP swab generated a negative result for enterovirus species with the VP1 sequencing assay and a high positive result (Ct 40.9) with the VP1.2014 primer and probe set. This fluorescent signal demonstrated a clear sigmoid curve.

[c] One nasal wash specimen generated a negative result for enterovirus species with the VP1 sequencing assay and a high positive result (Ct 40.1) for VP1.2014. This fluorescent signal demonstrated a clear sigmoid curve.

[d] Line listing represents 100 NP swabs and 1 OP swab specimen

Table 137: EV-D68 2014 rRT-PCR Clinical Performance Summary for Sera Specimens

Specimen Type	EV-D68 Positive by EV VP1 Sequencing Assay				EV-D68 Negative by EV VP1 Sequencing Assay		
	PCR +	PCR E[a]	Total	PPA (95% CI)	PCR -	Total	NPA (95% CI)
Sera	7	0	7	100% (64.6% - 100%)	5	5	100% (56.6% - 100%)

Table 14: EV-D68 2014 rRT-PCR Breakdown of Clinical Respiratory Specimens Negative for EV-D68 by EV VP1 Sequencing Assay

Species[a]	Type(s)	EV-D68 2014 rRT-PCR Results	
		# Tested	# Negative
RV-A[b]	RV-A2, A10, A16, A24, A31, A34, A40, A49, A58, A59, A67, A63, A73, A85	22	21[c]
RV-B	RV-B4, B6, B27, B83, B48	5	5
RV-C	Types not determined	2	2
EV-B	E9, E11, CVB4	3	3
EV-A	CV-A10	1	1
Negative for Enterovirus species		21	19[d]
Total		**54**	**51**

[a] RNA extracted from clinical respiratory specimens
[b] RV = rhinovirus; A species is the most abundant in humans
[c] One specimen gave a positive result with the EV-D68 2014 rRT PCR. Further analyses indicated that this specimen was a co-infection with a clear mixture of virus sequences on the sequence 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 2014 rRT-PCR was 31.2. Data suggests that the EV-D68 2014 rRT-PCR can detect a specific target in a co-infection.
[d] Two "false positive" specimens had high EV-D68 2014 rRT-PCR Ct values (40.1 and 40.9). The fluorescent signal from these specimens demonstrated a clear sigmoid curve.

Contact Information

When questions arise in the real-time testing process, consultation is available via email. Send the AB 7500 experiment file as an attachment to LRN@cdc.gov with an explanation of what the issue is and we will get back to you with comments and suggestions.

For questions or additional information, please contact:

Laboratory Response Network Helpdesk
LRN@cdc.gov