Notice

Novel Coronavirus 2012 Real-time RT-PCR Assay (which detects Middle East Respiratory Syndrome Coronavirus, or MERS-CoV) was developed by the Centers for Disease Control and Prevention's Coronavirus and Other Respiratory Viruses Division. Although this is not a Laboratory Response Network (LRN) procedure, the LRN is coordinating the distribution of this assay within the United States in support of CDC's national preparedness mission.

The LRN Help Desk will serve as the point of contact for questions/comments regarding the procedure; therefore, please address any questions you may have to the LRN Help Desk at (LRN@cdc.gov) or use the Contact Us feature on the LRN Secure Information Hub.

Public information about MERS-CoV, including case definition and information for health care providers, health departments and laboratories, can be found at https://www.cdc.gov/coronavirus/mers/index.html.

Novel Coronavirus 2012 Real-Time RT-PCR Assay

Centers for Disease Control and Prevention

For Use Under an Emergency Use Authorization Only

Instructions for Use

For In-vitro Diagnostic (IVD) Use

Rx Only

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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 Middle East Respiratory Syndrome Coronavirus (MERS-CoV), previously known as Novel Coronavirus 2012 or NCV-2012. Current information on MERS-CoV, including case definitions, is available at https://www.cdc.gov/coronavirus/mers/index.html.

Intended Use

The Centers for Disease Control and Prevention (CDC) Novel Coronavirus 2012 Real-time RT-PCR Assay (NCV-2012 rRT-PCR) is intended for the *in vitro* qualitative detection of MERS-CoV RNA in nasopharyngeal or oropharyngeal swabs, sputa, and lower respiratory aspirates/washes from individuals meeting MERS clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with MERS-CoV infection, contact with a presumptive or confirmed MERS case, or history of travel to geographic locations where MERS cases were detected). Testing is limited to Centers for Disease Control and Prevention designated laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet the requirements to perform high complexity testing.

Testing with the NCV-2012 rRT-PCR Assay should not be performed unless the patient meets clinical and/or epidemiologic criteria for testing suspect specimens.

Results are for the presumptive identification of MERS-CoV RNA. MERS-CoV RNA is generally detectable in upper and lower respiratory specimens during the acute phase of infection. Positive results are indicative of active infection with MERS-CoV but do not rule out bacterial infection or co-infection with other viruses; clinical correlation with patient history, signs, symptoms, exposure likelihood, and other laboratory evidence and diagnostic information is necessary to determine patient infection status. Laboratories are required to report results to the CDC. The definitive identification of MERS-CoV requires additional testing and confirmation to be performed by CDC.

Negative NCV-2012 rRT-PCR Assay results do not preclude MERS-CoV infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and/or epidemiological information. In asymptomatic individuals, a negative result does not exclude the possibility of future illness and does not demonstrate that an individual is not infectious.

The NCV-2012 rRT-PCR Assay is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of PCR and in vitro diagnostic procedures.

The NCV-2012 rRT-PCR Assay is only for use under the Food and Drug Administration’s Emergency Use Authorization.

Specimens

Acceptable Specimens

- Respiratory specimens:
  - Nasopharyngeal and/or Oropharyngeal swabs
  - Sputum specimens
  - Lower respiratory tract aspirates/washes

Specimen Collection

Refer to the *CDC Laboratory Testing for MERS* webpage (https://www.cdc.gov/mers/php/laboratories/index.html).

Specimen Handling and Storage

- Specimens can be stored at 2-8°C for up to 72 hours after collection.
- 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.
- Specimens should be shipped on dry ice.

Equipment and Consumables

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.

Materials Provided by CDC

- NCV-2012 rRT-PCR Assay Primer and Probe Set (CDC; Catalog #KT0136). Refer to product insert for storage and expiration information. Set includes 2 sets of primers and FAM-labeled probes:
  - NCV.upE (forward primer [NCV.upE-F], reverse primer [NCV.upE-R] and probe [NCV.upE-P])
  - NCV.N3 (forward primer [NCV.N3-F], reverse primer [NCV.N3-R] and probe [NCV.N3-P])
- NCV-2012 rRT-PCR Assay Positive Control (CDC; Catalog #KT0137)

Materials Provided by CDC, but Not Included in Kit

- RNase P Real-time PCR Primer and Probe Set (CDC; Catalog #KT0068). Refer to product insert for storage, rehydration and expiration information. Set includes 2 primers and 1 FAM-labeled probe: RNase P forward primer (RP-F), RNase P reverse primer (RP-R) and RNase P probe (RP-P).
- Human Specimen Control (HSC) (CDC; Catalog #KT0189). Refer to product insert for storage and expiration information. Kit includes inactivated human cell material suitable for the detection of RNase P.

Materials Required but Not Provided

- SuperScript™ III Platinum® One-Step qRT-PCR Kit (Invitrogen, Catalog #11732-088)**
- TaqPath 1-Step Multiplex Master Mix (no ROX) (Applied Biosystems, Catalog # A28521, A28522 or A28523)**
- Molecular grade water, nuclease-free
- Pierce DTT (Dithiothreitol), no-weigh formula (Thermo Fisher, Catalog #A39255) or equivalent
- Extraction reagents:
  - NucliSENS® easyMAG® reagents and accessories (bioMérieux)
    - easyMAG® Magnetic Silica (Catalog #280133)
    - easyMAG® Disposables (Catalog #280135)
    - easyMAG® Lysis Buffer (Catalog #280134)
    - easyMAG® Buffer 1 (Catalog #280130)
    - easyMAG® Buffer 2 (Catalog #280131)
    - easyMAG® Buffer 3 (Catalog #280132)
    - BioHit Pipette Tips (Catalog #280146)
    - Micro tubes w/caps (Catalog #200294)
  - EZ1 reagents and accessories (QIAGEN)
    - Qiagen EZ1 DSP Virus Kit (Catalog #62724)
    - AVL buffer (Catalog #19089)
  - QIAamp MinElute Virus Spin Kit and accessories (QIAGEN)
    - QIAamp MinElute Virus Spin Kit (Catalog #57704)**
    - Ethanol (96-100%)

**Only qualified lots may be used. Email CDC at LRN@cdc.gov for approved lots.

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)
  - Ethanol (70%)
  - DNA*Zap*™ (Ambion, Catalog #AM9890) or equivalent.
  - Bleach-Rite (Current Technologies, Catalog #BRSPRAY16)
- Disposable, powder-free gloves and surgical gowns
- Laboratory marking pen
- P2/P10, P200, and P1000 aerosol barrier pipette tips
- 1.5 mL microcentrifuge tubes
- 0.1 mL PCR reaction plates (Applied Biosystems; Catalog #4346906 or #4366932)
- MicroAmp Optical 8-cap Strips (Applied Biosystems; Catalog #4323032)
- Vortex mixer
- Microcentrifuge
- Plate spinner
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- Electronic single-channel pipette (2-20 µL; 20-200 µL)
- Racks for 1.5 mL microcentrifuge tubes
- 2 x 96-well -20°C cold blocks

- 7500 Fast Dx Real-Time PCR Systems (Applied Biosystems; Catalog #4406985 or #4406984)
- Extraction systems (instruments):
    - NucliSENS® easyMAG® (bioMérieux, Catalog #280140)
    - QIAGEN EZ1 Advanced XL Instrument (QIAGEN; Catalog #9001875)

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 chance 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
  i) assay reagent setup,
  ii) handling of extracted nucleic acids and
  iii) rRT-PCR amplification.
  Workflow 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 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 assay 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.

- VTC – NCV-2012 rRT-PCR Assay Positive Control
- NTC_1 – A known negative template control (sterile, nuclease-free water) added during rRT-PCR reaction set-up.
- NTC_2 – A known negative template control (sterile, nuclease-free water) that is **extracted concurrently** with the test samples and included as a sample during rRT-PCR set-up.
- HSC – A known extraction control that is extracted concurrently with the test samples and included as a sample during rRT-PCR set-up.
- RP – All clinical samples should be tested for human RNase P gene (using the LRN RNase P primer and probe set) to control for specimen quality and extraction.

Table 1: Overview of positive, negative and extraction controls

Control Type	Control Name	Used to Monitor	NCV.upE	NCV.N3	RP	Expected Ct Values
Positive	VTC	Substantial reagent failure, including primer and probe integrity	+	+	+	NCV.upE, NCV.N3 and RP Ct<35
Negative	NTC_1	Reagent and/or environmental contamination during PCR set-up	-	-	-	None detected
Extraction	NTC_2	Reagent and/or environmental contamination during extraction or PCR set-up	-	-	-	None detected
Extraction	HSC	Extraction reagent integrity and contamination during extraction or PCR set-up	-	-	+	RP Ct<35

Nucleic Acid Extraction

Respiratory specimens may be extracted using either the NucliSENS® easyMAG®, Qiagen EZ1 Advanced XL Instrument with the QIAGEN EZ1 DSP Virus Kit or the QIAamp MinElute Virus Spin Kit.

- 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).
- Follow the manufacturer's instructions for sample extraction.
- Liquification of sputum specimens using DTT is recommended prior to nucleic acid extraction.
- Nuclease-free water should be included in each extraction run as a sample extraction control (NTC_2) (see below).
- HSC should be included in each extraction run as a sample extraction control (see below).
- Retain specimen extracts in cold block or on ice until testing. If testing will be delayed, freeze immediately at ≤ -70°C. Only thaw 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

NCV.upE and NCV.N3 rRT-PCR assays are used for specimen testing. If both NCV.upE and NCV.N3 are positive, and VTC, NTC and HSC control results are acceptable, the specimens are presumptive positive. Specimens with presumptive positive results should be sent to CDC for confirmation.

Test results should be interpreted in this order:

1. Controls VTC, NTC and HSC results
2. Sample NCV.upE Assay and NCV.N3 Assay results
3. Sample RP Assay result, if NCV.upE and NCV.N3 assays are negative

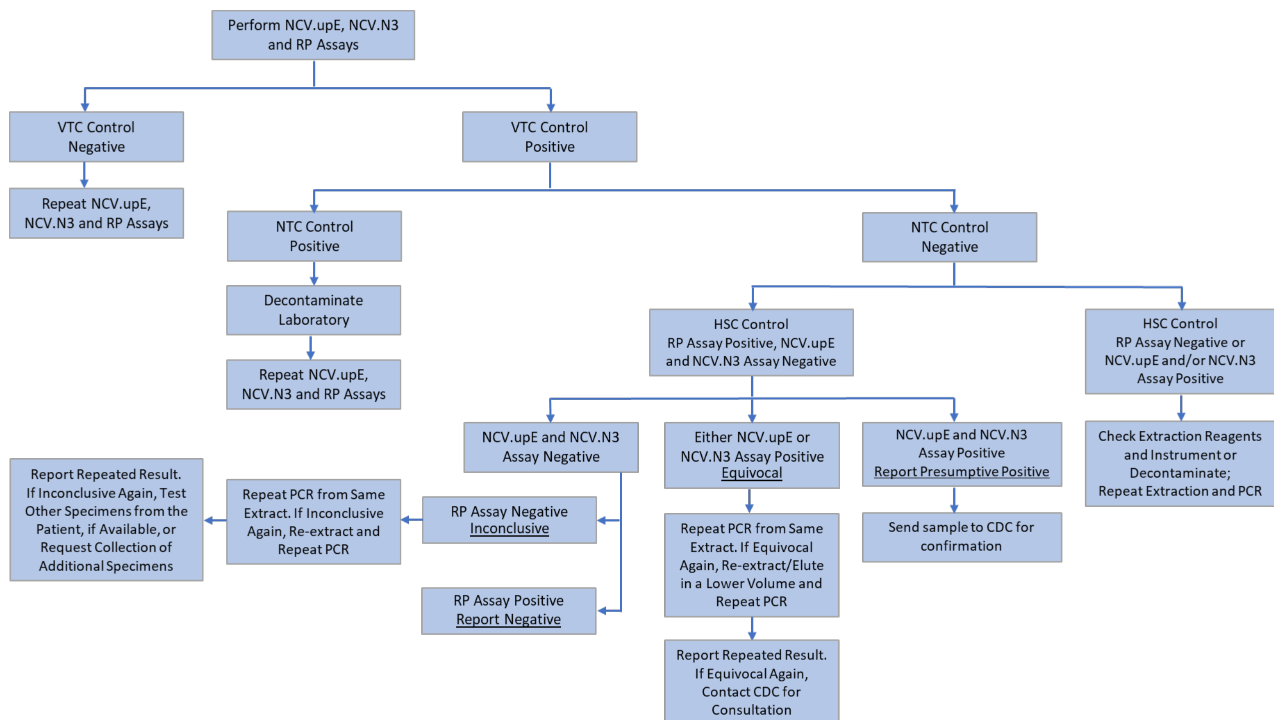


Figure 1: Testing Algorithm

rRT-PCR Assay

The NCV-2012 rRT-PCR primer and probe sets described below target upstream of the MERS- CoV envelope protein gene (NCV.upE) and the nucleocapsid protein gene (NCV.N3).

Stock Reagent Preparation

1. **Real-time Primers/Probes**
    - NCV-2012 rRT-PCR Assay 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.
        - Sterilely suspend lyophilized reagents in 500 µL nuclease-free water (50X working concentration) and allow to rehydrate for 15 min at room temperature in the dark.
        - For each tube in the kit, mix gently and aliquot primers/probe in 100 µL volumes into 5 pre-labeled tubes. Store a single aliquot of primers/probe at 2-8ºC in the dark. Do not refreeze (stable for up to 4 months). Store remaining aliquots at ≤-20ºC in a non-frost-free freezer.
    - RNase P Real-time PCR Primer and Probe Set – refer to package insert for reconstitution instructions.
2. **Viral Template Control (VTC)** - NCV-2012 rRT-PCR Assay 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.
    - Use to assess performance of rRT-PCR assays. Unless already hydrated, sterilely suspend lyophilized reagents in each tube in 1 mL of nuclease-free water to achieve the proper working dilution. Aliquot 100 µL into 10 separate tubes and store at ≤ -70°C.
    - To make working dilutions of VTC, thaw a 100 µL aliquot of concentrated VTC from above and dilute 1:10 by adding 900 µL nuclease-free water (final volume 1.0 mL). Dispense diluted VTC into single use aliquots suitable for your testing needs. Store unused material at ≤ -70°C.
    - Thaw a single aliquot of diluted positive control for each experiment and hold on ice until adding to plate. Discard any unused portion of the aliquot.
    - The VTC also contains RP RNA transcripts that serve as the positive control for the RP assay.

3. **No Template Control (NTCs)** (not provided)
   - Sterile, nuclease-free water
   - Aliquot in small volumes
   - Used to check for contamination during specimen extraction and/or plate set-up

Equipment Preparation

1. Turn on AB 7500 Fast Dx 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

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

Step	Cycles	Temp	Time
Reverse transcription	1	50°C	30 min
Polymerase activation	1	95°C	2 min
Amplification	45	95°C 55°C	15 sec 1 min

B: TaqPath 1-Step Multiplex Master Mix (no ROX)

Step	Cycles	Temp	Time
UNG incubation*	1	25°C	2 min
Reverse transcription	1	53°C	10 min
Polymerase activation	1	95°C	2 min
Amplification	45	95°C 55°C	3 sec 30 sec

*Uracil-N-glycosylase

Instrument Settings

Detector: FAM
Quencher: None
Passive Reference: None
Run Mode: Standard
Sample Volume: 25 µL

1. Remove dedicated 96-well PCR cold-block from reagent set-up room freezer.
2. Remove dedicated 96-well PCR cold-block from the nucleic acid handling area freezer.

Master Mix and Plate Set-Up

Note: Plate set-up configuration can vary with the number of specimens and workday organization. NTC_1 and VTC must be included in each rRT-PCR run. NTC_2 and HSC must be included in at least one rRT-PCR run that covers one extraction 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 the SuperScript® III Platinum® 2X Reaction Mix or 4x TaqPath Multiplex Master Mix prior to use.
3. Mix buffer, enzyme, and primer/probes by inversion 5 times.
4. Briefly centrifuge buffer and primers/probes and return to ice.
5. Label one 1.5 mL microcentrifuge tube for each primer/probe set.
6. Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTCs, VTC and HSC, and for pipetting error. Use the following guide to determine N:
   - If the number of samples (n) including controls equals 14 or less, then N = n + 1
   - If the number of samples (n) including controls is 15 or more, then N = n + 2
7. rRT-PCR Reaction Mix:
   For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture (N = # of reactions).

Table 3: rRT-PCR Reaction Mix

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

Component	Volume
2X Reaction Mix	= N x 12.50 µL
SS III RT/Platinum Taq Mix	= N x 0.50 µL
50X forward primer	= N x 0.50 µL
50X reverse primer	= N x 0.50 µL
50X probe	= N x 0.50 µL
Water, nuclease-free	= N x 5.50 µL
Total volume	= N x 20.00 µL

B: TaqPath 1-Step Multiplex Master Mix (no ROX)

Component	Volume
4x TaqPath Multiplex Master Mix	= N x 6.25 µL
50X forward primer	= N x 0.50 µL
50X reverse primer	= N x 0.50 µL
50X probe	= N x 0.50 µL
Water, nuclease-free	= N x 12.25 µL
Total volume	= N x 20.00 µL

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	NCV.upE	NCV.upE	NCV.upE	NCV.upE	NCV.upE	NCV.upE	NCV.upE	NCV.upE	NCV.upE	NCV.upE	NCV.upE	NCV.upE
B	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3
C	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC_1	S1	S2	S3	S4	S5	S6	S7	S8	HSC	NTC_2	VTC
B	NTC_1	S1	S2	S3	S4	S5	S6	S7	S8	HSC	NTC_2	VTC
C	NTC_1	S1	S2	S3	S4	S5	S6	S7	S8	HSC	NTC_2	VTC
D												
E												
F												
G												
H												

No template reaction mix control (NTC_1) used to check for PCR reagent contamination (column 1); sample extracts (S); HSC extraction control used to check for extraction reagent integrity and contamination occurring during extraction or PCR set-up (column 10); no template extraction control (NTC_2) used to check for contamination occurring during extraction or PCR set-up (column 11); viral template control (VTC) used to assess assay performance (column 12).

Figure 2: Plate Set Up NCV.upE and NCV.N3 Testing (plate lay out example)

10. Before moving the plate to the nucleic acid handling area, add 5 µL of nuclease-free water to the NTC_1 wells in column 1.
11. Loosely apply optical strip caps to the tops of the reaction wells or cover the plate with adhesive film and move plate to the nucleic acid handling area on cold block.
12. Gently vortex sample extracts, VTC, NTC_2 and HSC and centrifuge briefly.
13. Set up the sample extract reactions. Pipette 5 µL of the first sample into all the wells labeled for that sample. Keep the other sample wells covered. Change tips after each sample addition.
14. Cap the column to which the sample has been added. This will enable you to keep track of where you are on the plate.
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 all VTC wells and cap. Secure all strip caps with capping tool.
17. Transport the plate to the amplification area on cold block.
18. Centrifuge the plate briefly to remove bubbles or drops that may be present in the wells.
19. Place plate on pre-programed AB 7500 Fast Dx and start run.

Data Analysis

After completion of the run, save and analyze the data following the instrument manufacturer's instructions. Analyses should be performed separately for each target using a manual threshold setting. Thresholds should be adjusted to fall within exponential phase of the fluorescence curves and above any background signal. The procedure chosen for 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:

1. <u>VTCs</u> should be **positive** and with C_t values within 35 cycles for all primer and probe sets.
    a. If VTCs are *negative*, the testing results for that plate are invalid.
        - Repeat rRT-PCR test.
        - If repeat testing generates negative VTC results, contact the LRN helpdesk for consultation.
2. <u>NTCs</u> should be **negative**.
    a. If NTC_1 is *positive,* the testing results for that plate are invalid.
        - Suggests contamination occurred during the PCR set-up.
        - Clean potential DNA contamination from bench surfaces and pipettes in the reagent set-up and template addition work areas.
        - Discard working reagent dilutions and remake from fresh stocks.
        - Repeat rRT-PCR testing.

    b. If NTC_2 is *positive,* the testing results for that plate are invalid.
        - Suggests contamination occurred during the extraction or PCR set-up
        - Clean potential DNA contamination from bench surfaces and pipettes in the extraction and reagent set-up areas.
        - Repeat extraction and rRT-PCR testing for all specimens that had been extracted alongside the failed NTC_2.
3. <u>HSC</u> should be **positive** for RP assay and **negative** for both NCV.upE and NCV.N3 assays.
    a. If HSC is *negative* for RP assay, the testing results for that plate are invalid.
        - Suggests a failure occurred during the extraction procedure.
        - Check extraction reagents and instrument to make sure they are working properly.
        - Repeat extraction and rRT-PCR testing for all specimens that had been extracted alongside the failed HSC.

    b. If HSC is *positive* for NCV.upE or/and NCV.N3, the testing results for that plate are invalid.
        - Suggests contamination occurred during the extraction or PCR set-up
        - Clean potential DNA contamination from bench surfaces and pipettes in the extraction and reaction set-up areas.
        - Repeat extraction and rRT-PCR testing for all specimens that had been extracted alongside the failed HSC.
4. <u>RP Assay for each specimen</u> should be **positive**.
    a. If RP Assay for a specimen is *negative* and both NCV.upE and NCV.N3 Assays are *negative* for this specimen, consider the result is *inconclusive*. Follow the instructions below:
        - Repeat rRT-PCR testing from the same extract using RP, NCV.upE and NCV.N3 assays.
        - After repeat rRT-PCR testing, if RP, NCV.upE and NCV.N3 assays are

still negative, repeat extraction from new specimen aliquot and repeat rRT-PCR testing.
- After repeat extraction and rRT-PCR testing, if RP assay is *positive* and both NCV.upE and NCV.N3 are *negative,* consider the result a true *negative* and continue to report.
- After repeat extraction and rRT-PCR testing, if both NCV.upE and NCV.N3 are *positive,* consider the result a true *positive* and continue to report.
- After repeat extraction and rRT-PCR testing, if either NCV.upE or NCV.N3 is *positive,* consider the result *equivocal* and continue to report.
- 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 is *negative*, but either NCV.upE Assay or NCV.N3 Assay is *positive* for specimens:
   - Do not repeat rRT-PCR test and consider the results of the NCV-2012 Assays valid.

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

- True positives should produce exponential curves with logarithmic, linear, and plateau phases during the 45 cycles (Figure 3).
- Note: Weak positives will produce high C_t values that are sometimes devoid of a plateau phase; however, the exponential plot will be seen.

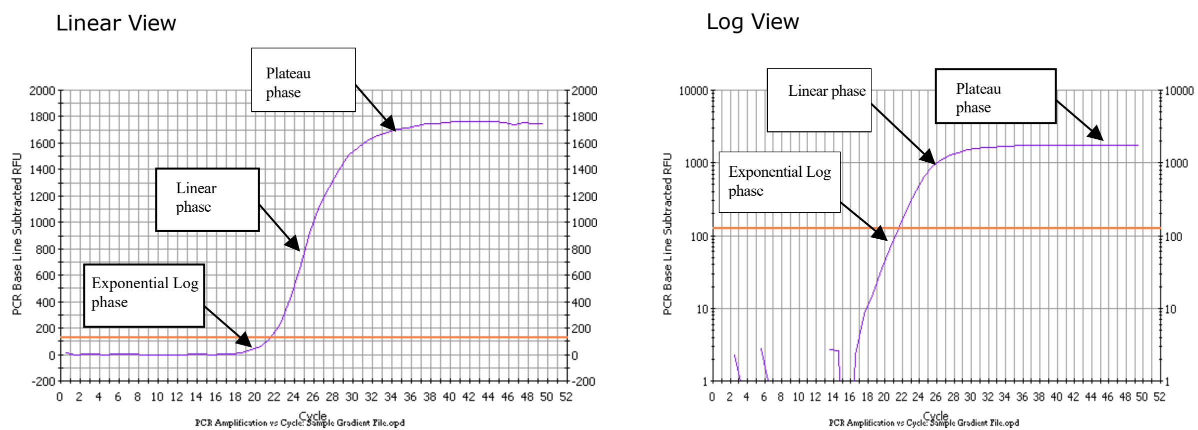
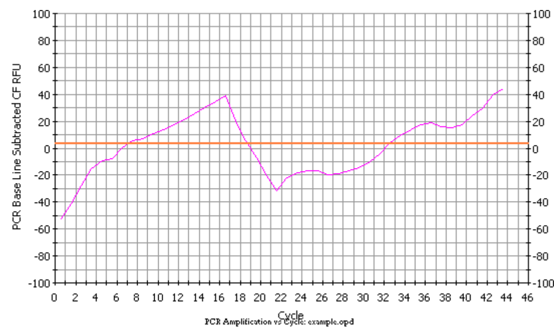


Figure 3: Linear and log views of PCR curves noting each stage of the amplification plots.

- 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 4 shows examples of false positives that do not amplify exponentially.



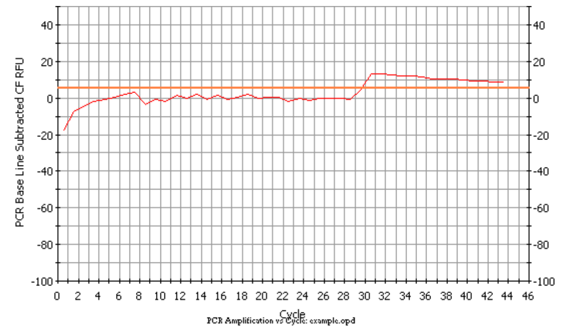
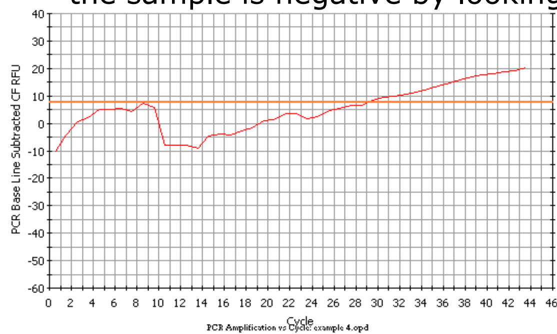


Figure 4: Examples of false positive curves.

- To better understand and evaluate challenging curves more effectively, use the background fluorescence view (Rn versus Cycle with AB 7500Fast system 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 5 shows a curve with a C_t value of 29.2 though it is evident that the sample is negative by looking at the background fluorescence view.



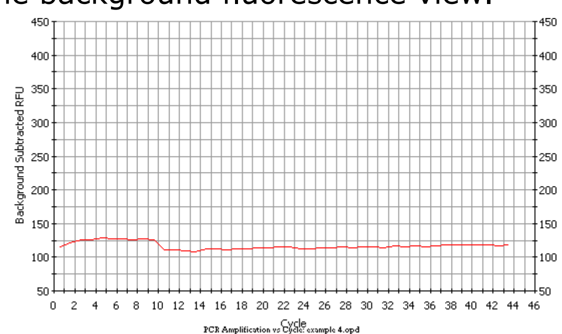
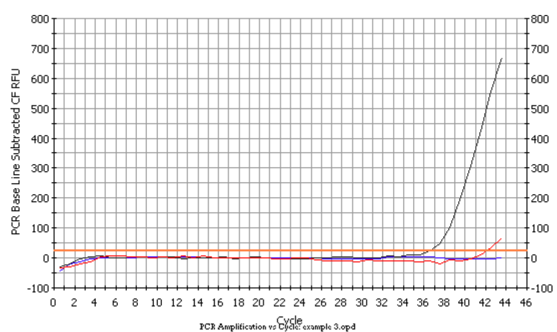


Figure 5: Amplification plot of a sample with a "wandering" curve (left) and the corresponding background fluorescence view (right).

- Figure 6 shows an amplification plot with 3 curves: a moderately weak positive with a Ct of 36.6 (black), a very weak positive with a Ct of 42.1 (red), and a negative control (blue). The weak positive (Ct= 42.1) is verified to be positive by the sharp increase in fluorescence seen in the background fluorescence view.



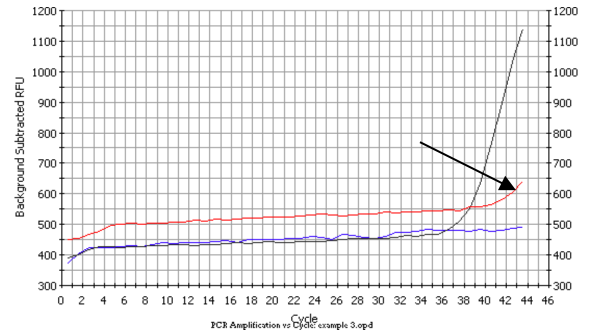


Figure 6: Amplification plot of three samples in the linear view (left) and the corresponding background fluorescence view (right).

- 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 45, the sample is a true positive. Figure 7 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.

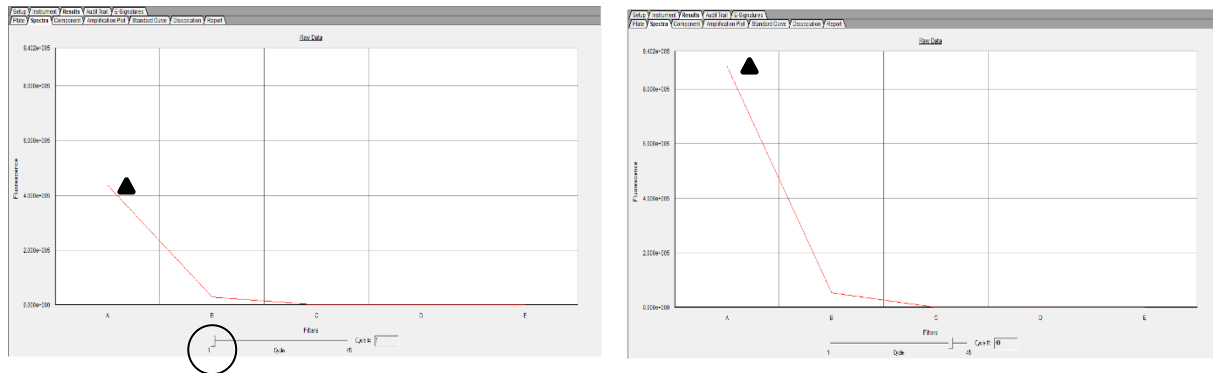


Figure 7: Spectra component of a positive sample. Left screenshot shows fluorescence at cycle 1 and right screenshot shows fluorescence at cycle 40.

- 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 ≥ 37). 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.
  - The LRN helpdesk is available 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 4: NCV-2012 rRT-PCR Assay Test Interpretation and Reporting Instructions

NCV.upE	NCV.N3	RP	Interpretation	Reporting	Actions
-	-	+	MERS-CoV Negative	MERS-CoV RNA not detected by rRT-PCR	Report results to CDC
-	-	-	Inconclusive	Inconclusive for MERS-CoV RNA by rRT-PCR. An inconclusive result may occur in the case of an inadequate specimen.	Repeat rRT-PCR testing from the same extract. If inconclusive again, re-extract and repeat rRT-PCR testing. Report repeated results to CDC. If inconclusive again, test other specimens from the patient, if available, or request collection of additional specimens.
+	+	+/-	MERS-CoV Presumptive Positive	MERS-CoV RNA detected by rRT-PCR. Specimen will be referred to CDC for confirmation.	Send specimen to CDC for confirmation. Report results to CDC
+	-	+/-	Equivocal	MERS-CoV rRT-PCR testing was equivocal.	Repeat rRT-PCR testing from the same extract. If equivocal again, re-extract/elute in a lower volume and repeat rRT-PCR testing. Report repeated results to CDC. If equivocal again, contact CDC for consultation.
-	+	+/-	Equivocal	MERS-CoV rRT-PCR testing was equivocal.	Repeat rRT-PCR testing from the same extract. If equivocal again, re-extract/elute in a lower volume and repeat rRT-PCR testing. Report repeated results to CDC. If equivocal again, contact CDC for consultation.

NOTE: All test results generated using the NCV-2012 rRT-PCR Assay must be sent to CDC. Contact CDC to request a consultation or confirmatory testing as outlined in the LRN notification policy (LRN-1087). Submit all results (positive, negative, equivocal, and inconclusive) to CDC as outlined in the LRN data messaging policy (LRN-1085). Questions may be submitted to the LRN Help Desk using the "Contact Us" feature on the LRN Secure Information Hub.

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

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. Application of appropriate assay controls that identify poor-quality samples can help avoid most false-negative results. A more difficult problem is the apparently low titer of virus shed in specimens collected early in illness, which may 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 can help ensure that laboratory contamination is detected and that false positive test results are not reported. This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use by authorized laboratories;
- This product has been authorized only for the detection of MERS-CoV and not for any other viruses or pathogens; and
- The EUA for this product will be effective until the declaration that circumstances exist justifying the authorization of the emergency use of in vitro diagnostics for detection of MERS-CoV is terminated under Section 564(b)(2) of the Act or the EUA is revoked under Section 564(g) of the Act.

Conditions of Authorization for Laboratories

The Novel Coronavirus 2012 Real-time RT-PCR Assay Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients and Contacts, and authorized labeling are available on the FDA website: https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations-medical-devices#coronavirus2013

However, to assist in using the Novel Coronavirus 2012 Real-time RT-PCR Assay, the relevant Conditions of Authorization are listed below:

- Authorized laboratories* using your product must include, with test result reports, all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating this labeling may be used, which may include mass media.
- Authorized laboratories using your product must use your product as outlined in the authorized labeling. Deviations from the authorized procedures, including authorized instruments, authorized clinical specimen types, authorized control materials, authorized ancillary reagents and authorized materials required to use your product are not permitted.
- Authorized laboratories that receive your product must notify the relevant public health authorities of their intent to run your product prior to initiating testing.
- Authorized laboratories using your product must have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.

- Authorized laboratories must have a process in place to track adverse events and report to you (via email: LRN@cdc.gov) and to FDA pursuant to 21 CFR Part 803.
- All operators using your product must be appropriately trained in performing and interpreting the results of your product, use appropriate personal protective equipment when handling this kit, and use your product in accordance with the authorized labeling.
- You, authorized distributor(s), and authorized laboratories must collect information on the performance of your product and must report any significant deviations from the established performance characteristics of your product of which you or they become aware to DMD/OHT7/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov). In addition, authorized distributor(s) and authorized laboratories must report to you those deviations (via email: LRN@cdc.gov).
- You, authorized distributors, and authorized laboratories using your product must ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to FDA for inspection upon request.

*The letter of authorization refers to, "Centers for Disease Control and Prevention designated laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet the requirements to perform high complexity testing" as "authorized laboratories."

Performance Characteristics

Clinical Performance

Performance characteristics of the NCV-2012 rRT-PCR Assay with human clinical specimens tested during retrospective and prospective MERS-CoV surveillance.

As of April 5, 2013, 318 geographically diverse fresh and frozen human clinical specimens collected between 2011 and 2013 from 308 demographically diverse patients with acute respiratory illness were tested at CDC by the NCV-2012 rRT-PCR Assay (NCV.upE and NCV.N3 assays). Testing was performed using the AB 7500 Fast Dx and Invitrogen Superscript™ III master mix.

Respiratory specimens tested include 281 combined nasopharyngeal/oropharyngeal swabs, 8 individual nasopharyngeal swabs, 1 nasal swab, (all swabs collected in universal transport media), 20 bronchoalveolar lavage (BAL) specimens, 3 tracheal aspirates and 4 sputa. A total of 280 of the combined nasopharyngeal/ oropharyngeal specimens were collected from pediatric cases (infants) with acute respiratory infection in Jordan between April 2011 and March 2012. All other specimens were collected from patients with acute respiratory infection in Kenya, Panama and the United States.

The NCV.upE and NCV.N3 rRT-PCR assays showed no evidence of non-specific amplification with any sample that could be interpreted as a “false” positive test result.

Also included in the data set is one BAL specimen associated with a cluster of acute respiratory infection (ARI) cases at a hospital in Jordan in April 2012. It was positive for MERS-CoV by the NCV.upE and NCV.N3 assays. This specimen was independently confirmed positive for MERS-CoV RNA by virus isolation and/or genome sequencing.

Table 5: Summary of NCV-2012 rRT-PCR Assay Data generated by testing human specimens during retrospective and prospective MERS-CoV surveillance

A: Specimens from Confirmed Cases (Jordan cluster, positive results expected)

Specimen type	#	NCV.upE # pos	NCV.N3 # pos	Overall # pos
NP/OP Swabs	0	-	-	-
Sputum	0	-	-	-
Bronchial or transtracheal aspirates or washes	1	1/1	1/1	1/1

B: Other specimens (Negative results expected)

Specimen type	#	NCV.upE # pos	NCV.N3 # pos	Overall # pos
NP/OP Swabs	290	0/290	0/290	0/290
Sputum	4	0/4	0/4	0/4
Bronchial or transtracheal aspirates or washes	23	0/23	0/23	0/23

Table 6: Percent agreement with expected results

rRT-PCR Result	Expected Result Positive	Expected Result Negative
Positive	1	0
Negative	0	317

Positive percent agreement = 1/1 = 100% (95% CI: 20.7% - 100%)
Negative percent agreement = 317/317 = 100% (95% CI: 98.8% - 100%)
Overall percent agreement = 318/318 = 100% (95% CI: 98.8%-100%)

Performance characteristics of the NCV-2012 rRT-PCR Assay with human clinical specimens tested at CDC and LRN laboratories during prospective MERS-CoV surveillance.

Since April 5, 2013, 2,303 domestic and 7 international (Qatar, Bangladesh and Korea) clinical specimens were tested at CDC and LRN laboratories by the NCV-2012 rRT-PCR Assay. Testing was performed using the AB 7500 Fast Dx and Invitrogen Superscript™ III master mix. Majority of specimens received were swabs (68.7%), 25.3% were sputum and 6.0% were aspirates/washes.

Of the 2,310 respiratory specimens tested, 335 were reanalyzed with the updated algorithm using the NCV.upE and NCV.N3 targets. Of these 335 respiratory specimens, eight were positive for MERS-CoV and associated with two US cases. Of the eight positive clinical specimens, five (62.5%) sputum samples were collected from a Florida case in May 2014. The remaining three (37.5%) were associated with the Indiana case (2 sputum and 1 swab) in May 2014. These 2 cases were independently confirmed positive for MERS-CoV RNA by genome sequencing. Of the remaining 327 respiratory specimens, 325 were negative and 2 inconclusive.

Performance characteristics of the NCV-2012 rRT-PCR Assay using a proficiency panel of contrived respiratory specimens

A panel was developed using contrived specimen constructed from pooled nasopharyngeal and oropharyngeal swabs from 10 individuals. Four contrived specimens (S1 to S4) were spiked with known concentrations of the Jordan-N3/NCV strain of the MERS-CoV, extracted using the Automated NucliSENS® Magnetic Extraction (easyMAG®) and tested with each primer/probe set in triplicate. The specimens were tested by three technicians on three separate days. The data shows comparable performance among operators with low variance in the Ct values for each primer/probe set (**Table 7).**

Table 7: NCV-2012 rRT-PCR Assay results using a proficiency panel of contrived respiratory specimens

A. EQA panel run 1 by XL on 4/10/13

Samples	Virus quantity $TCID_{50}$/mL	NCV.upE Assay Ct	NCV.upE Assay Ct	NCV.upE Assay Ct	NCV.N3 Assay Ct	NCV.N3 Assay Ct	NCV.N3 Assay Ct	RP Assay Ct	RP Assay Ct	RP Assay Ct
S1	$3.4X10^{1}$	20.64	20.81	20.61	18.75	18.82	18.86	33.33	33.04	32.85
S2	0	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
S3	$3.4X10^{-1}$	27.14	26.79	27.14	24.89	25.19	24.89	32.49	32.93	33.06
S4	$3.4X10^{-3}$	35.69	35.79	35.86	33.42	33.73	33.58	32.53	32.77	32.16

B. EQA panel run 2 by BW on 4/11/13

Samples	Virus quantity $TCID_{50}$/mL	NCV.upE Assay Ct	NCV.upE Assay Ct	NCV.upE Assay Ct	NCV.N3 Assay Ct	NCV.N3 Assay Ct	NCV.N3 Assay Ct	RP Assay Ct	RP Assay Ct	RP Assay Ct
S1	$3.4X10^{1}$	22.11	22.1	21.6	20.25	20.49	20.5	32.39	31.88	32.67
S2	0	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
S3	$3.4X10^{-1}$	28.22	28	28.6	27.42	27.22	27.21	32.28	33.47	33.87
S4	$3.4X10^{-3}$	37.59	35.3	35.3	34.38	35.23	34.52	32.12	32.32	32.71

C. EQA panel run 3 by SS on 4/12/13

Samples	Virus quantity $TCID_{50}$/mL	NCV.upE Assay Ct	NCV.upE Assay Ct	NCV.upE Assay Ct	NCV.N3 Assay Ct	NCV.N3 Assay Ct	NCV.N3 Assay Ct	RP Assay Ct	RP Assay Ct	RP Assay Ct
S1	$3.4X10^{1}$	20.68	20.6	20.5	19.18	19.21	19.24	32.34	32.26	32.68
S2	0	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
S3	$3.4X10^{-1}$	25.57	25.4	25.3	24.13	24.08	24.18	31.84	31.76	32.06
S4	$3.4X10^{-3}$	35.75	35.2	36.1	34.62	33.62	35.02	32.12	31.65	32.33

D. EQA panel Average and STD

Samples	NCV.upE Avg.	NCV.upE STD	NCV.N3 Avg.	NCV.N3 STD	RP Avg.	RP STD
S1	21.1	0.676	19.5	0.727	32.6	0.439
S2	Neg	N/A	Neg	N/A	Neg	N/A
S3	26.9	1.244	25.5	1.415	32.6	0.738
S4	35.8	0.719	34.2	0.670	32.3	0.345

MERS-CoV strain tested: NCV-Jordan (2013212797) pass 1 VeroE6; stock virus titer 1.3×10^{4} $TCID_{50}$/mL.
Specimen matrix constructed from combined nasopharyngeal & oropharyngeal swabs obtained from 10 persons.
EQA panel = external quality assessment panel

Analytical Performance

Limit of Detection

The limit of detection for the Novel Coronavirus Real-time RT-PCR Assay (NCV-2012 rRT- PCR) with respiratory specimens is 1.3 x 10^{-2} $TCID_{50}$/mL.

Preliminary Limit of Detection Study

The limit of detection was evaluated in buffer using known concentrations of MERS-CoV strain Jordan-N3/NCV.

RNA was extracted from 100µL of the Jordan-N3/NCV strain (1.3 x 10^4 $TCID_{50}$/mL) using NucliSENS® easyMAG® and eluted in 100µL of elution buffer. Serial ten-fold dilutions of RNA extract were prepared in 10mM TE buffer containing 50 ng/µL yeast tRNA. The serial dilutions from 10^{-4} to 10^{-10} were tested in triplicate by both primer and probe sets in the NCV-2012 rRT- PCR assay kit. NCV-2012 rRT-PCR Assay data were generated using the Applied Biosystems® 7500 Fast Dx and Invitrogen™ SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase master mix.

The lowest dilution at which all replicates were positive for both CDC assays was 1.3 x 10^{-3} $TCID_{50}$/mL.

Table 8: NCV-2012 rRT-PCR Assay Limits of Detection with Jordan-N3/NCV isolate

MERS-CoV RNA dilution[a]	$TCID_{50}$/mL dilution	NCV.upE Assay Ct	NCV.upE Assay Ct	NCV.upE Assay Ct	Call Rate	NCV.N3 Assay Ct	NCV.N3 Assay Ct	NCV.N3 Assay Ct	Call Rate
10^{-4}	$1.3x10^{0}$	27.35	27.46	27.39	3/3	25.98	25.95	25.81	3/3
10^{-5}	$1.3x10^{-1}$	31.06	31.13	31.29	3/3	29.67	29.38	29.40	3/3
10^{-6}	$1.3x10^{-2}$	33.81	34.55	34.67	3/3	32.81	33.06	33.23	3/3
10^{-7}	$1.3x10^{-3}$	37.03	39.01	38.94	3/3	36.60	37.28	35.82	3/3
10^{-8}	$1.3x10^{-4}$	39.07	Neg	Neg	1/3	Neg	Neg	Neg	0/3
10^{-9}	$1.3x10^{-5}$	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3
10^{-10}	$1.3x10^{-6}$	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3

[a]MERS-CoV strain tested: Jordan-N3/NCV pass 1 VeroE6; stock virus titer 1.3x10^4 $TCID_{50}$/mL

Limit of Detection Confirmation Study

The limit of detection for the NCV-2012 rRT-PCR was confirmed in pooled clinical matrix spiked with MERS-CoV strain Jordan-N3/NCV at the estimated limit of detection concentration (1.3×10^{-3} $TCID_{50}$/mL) (from the Preliminary Limit of Detection Study) and to a concentration 10-fold above (1.3×10^{-2} $TCID_{50}$/mL). Each concentration of spiked matrix was extracted 20 times using the easyMAG extraction method. Each extract was tested on the AB 7500 Fast Dx with the Invitrogen SuperScript III master mix. LoD was confirmed at 1.3×10^{-2} $TCID_{50}$/mL.

Table 9: NCV-2012 rRT-PCR Assay Limits of Detection confirmation with Jordan-N3/NCV isolate diluted in clinical matrix.

	Dilution 10^{-6} Virus quantity ($TCID_{50}$/mL) 1.3×10^{-2}	Dilution 10^{-6} Virus quantity ($TCID_{50}$/mL) 1.3×10^{-2}	Dilution 10^{-6} Virus quantity ($TCID_{50}$/mL) 1.3×10^{-2}	Dilution 10^{-7} Virus quantity ($TCID_{50}$/mL) 1.3×10^{-3}	Dilution 10^{-7} Virus quantity ($TCID_{50}$/mL) 1.3×10^{-3}	Dilution 10^{-7} Virus quantity ($TCID_{50}$/mL) 1.3×10^{-3}
Replicates	**NCV.upE Ct**	**NCV.N3 Ct**	**RP Ct**	**NCV.upE Ct**	**NCV.N3 Ct**	**RP Ct**
1	36.15	33.78	37.48	39.90	37.12	38.11
2	37.69	33.53	38.12	Neg	36.24	37.56
3	37.28	33.78	36.75	40.72	38.35	36.98
4	36.49	34.92	37.43	Neg	39.34	38.31
5	36.83	34.05	37.89	Neg	38.25	37.54
6	36.64	34.18	38.16	Neg	37.36	38.11
7	36.14	34.48	37.88	39.77	37.82	37.58
8	37.21	34.38	37.69	Neg	36.62	37.61
9	36.00	34.81	36.77	37.77	36.14	37.29
10	37.86	35.04	37.59	38.76	36.50	37.44
11	36.80	34.07	37.22	Neg	37.41	36.79
12	36.64	34.44	37.58	40.06	Neg	37.50
13	36.90	34.00	39.01	40.15	36.42	38.67
14	36.46	34.76	37.55	44.02	36.75	37.54
15	36.24	34.95	36.88	39.78	36.63	36.91
16	37.76	34.49	37.84	39.33	37.92	37.24
17	36.90	33.94	37.55	38.52	37.66	37.75
18	36.23	34.33	37.58	Neg	36.55	37.74
19	36.26	34.38	36.89	Neg	38.32	37.31
20	37.66	34.17	37.52	40.15	38.45	37.24
Avg.	36.81	34.32	37.57	39.91	37.36	37.56
SD	0.59	0.42	0.52	1.53	0.91	0.46
Call rate	20/20	20/20	20/20	12/20	19/20	20/20

Matrix Comparison Study

To assess the impact of the presence of clinical matrices on the detection of MERS-CoV, pooled and spiked sputum and combined nasopharyngeal/oropharyngeal swab matrices were prepared, extracted and tested by rRT-PCR.

Pooled matrices were constructed from human clinical specimens: NP/OP, combined nasopharyngeal and oropharyngeal swabs in universal transport media (10), and sputum specimens (10). The two pooled matrices (NP/OP and sputum) were each divided and spiked with MERS-CoV virus isolate Jordan-N3/NCV (stock virus titer 1.3 x 10^4 $TCID_{50}$/mL) to concentrations from 1.3 x 10^0 $TCID_{50}$/mL to 1.3 x 10^{-4} $TCID_{50}$/mL. Each concentration of each spiked pooled matrix was extracted three times by easyMAG®. Each extract was then tested against both NCV-2012 primer/probe sets and the RP primer/probe set using the AB 7500 Fast Dx and the Invitrogen Superscript™ III master mix. Data are presented in Table 10 below. These data demonstrate that the detection levels in matrix are comparable to those demonstrated in buffer (1.3x10^{-3} $TCID_{50}$/mL, See Analytical Limit of Detection Studies above).

Table 10: Summary of Matrix Comparison Data

Dil.	Concentration ($TCID_{50}$/mL)	Ct	NCV.upE NP/OP	NCV.upE Sputum	NCV.N3 NP/OP	NCV.N3 Sputum	RP NP/OP	RP Sputum
10^{-4}	1.3 x 10^0	1	26.08	26.62	23.38	23.69	27.62	23.43
10^{-4}	1.3 x 10^0	2	26.35	26.72	23.5	23.74	27.66	23.56
10^{-4}	1.3 x 10^0	3	26.32	26.75	23.51	23.76	27.72	23.61
10^{-4}	1.3 x 10^0	Avg.	26.25	26.7	23.46	23.73	27.67	23.53
10^{-4}	1.3 x 10^0	SD	0.15	0.07	0.07	0.04	0.05	0.09
10^{-4}	1.3 x 10^0	Call rate	3/3	3/3	3/3	3/3	3/3	3/3
10^{-5}	1.3 x 10^{-1}	1	27.9	29.13	25	25.86	27.58	23.02
10^{-5}	1.3 x 10^{-1}	2	27.86	29.05	25.16	25.83	27.62	23.22
10^{-5}	1.3 x 10^{-1}	3	27.85	29.11	24.93	25.9	27.69	23.22
10^{-5}	1.3 x 10^{-1}	Avg.	27.87	29.1	25.03	25.86	27.63	23.15
10^{-5}	1.3 x 10^{-1}	SD	0.03	0.04	0.12	0.03	0.06	0.12
10^{-5}	1.3 x 10^{-1}	Call rate	3/3	3/3	3/3	3/3	3/3	3/3
10^{-6}	1.3 x 10^{-2}	1	34.06	35.29	31.95	32.57	27.84	23.28
10^{-6}	1.3 x 10^{-2}	2	34.51	35.59	31.92	32.69	27.88	23.28
10^{-6}	1.3 x 10^{-2}	3	34.25	34.88	31.9	32.27	27.75	23.28
10^{-6}	1.3 x 10^{-2}	Avg.	34.27	35.25	31.93	32.51	27.82	23.3
10^{-6}	1.3 x 10^{-2}	SD	0.22	0.36	0.03	0.21	0.07	0.03
10^{-6}	1.3 x 10^{-2}	Call rate	3/3	3/3	3/3	3/3	3/3	3/3
10^{-7}	1.3 x 10^{-3}	1	38.7	38.52	36.57	35.49	27.87	23.21
10^{-7}	1.3 x 10^{-3}	2	37.87	38.3	35.43	35.44	28.01	23.25
10^{-7}	1.3 x 10^{-3}	3	39.36	Neg	35.41	34.9	28.03	23.27
10^{-7}	1.3 x 10^{-3}	Avg.	38.64	38.41	35.8	35.27	27.97	23.24
10^{-7}	1.3 x 10^{-3}	SD	0.74	0.16	0.67	0.33	0.09	0.03
10^{-7}	1.3 x 10^{-3}	Call rate	3/3	2/3	3/3	3/3	3/3	3/3
10^{-8}	1.3 x 10^{-4}	1	Neg	Neg	Neg	Neg	28.01	24.74
10^{-8}	1.3 x 10^{-4}	2	Neg	Neg	Neg	Neg	28	24.72
10^{-8}	1.3 x 10^{-4}	3	Neg	Neg	Neg	Neg	27.96	24.69
10^{-8}	1.3 x 10^{-4}	Avg.	N/A	N/A	N/A	N/A	27.99	24.72
10^{-8}	1.3 x 10^{-4}	SD	N/A	N/A	N/A	N/A	0.03	0.02
10^{-8}	1.3 x 10^{-4}	Call rate	0/3	0/3	0/3	0/3	3/3	3/3

Reactivity

Reactivity *(in silico* prediction)

Sequences for the Jordan-N3/NCV strain and three other strains (EMC/2012, England 1, and England-Qatar/2012) were analyzed to verify 100% homology in the NCV.upE and NCV.N3 target regions.

Cross-Reactivity

In silico Analysis

BLASTn analysis queries of the NCV-2012 rRT-PCR Assay primers and probes were performed against public domain nucleotide sequences and showed no significant combined homologies with human genome and other coronaviruses that would predict potential false positive rRT-PCR results.

Database search parameters: GenBank, EMBL, DDBJ, PDB and RefSeq sequences, excluding EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences. The database is partially non-redundant. In some cases identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry. Merged sequences include GenBank and RefSeq entries with identical sequences. Sequences added to the database since April 2011 have also been merged with identical existing entries.

NCV-2012 rRT-PCR Assay Viral and Respiratory Matrix Cross-reactivity Evaluation

Cross-reactivity of the NCV-2012 rRT-PCR Assay was evaluated by testing purified nucleic acid from other respiratory virus isolates or rRT-PCR positive clinical specimens. These samples included other human coronaviruses, including 229E, OC43, NL63, HKU1, SARS-CoV, and SARS-CoV-2 (10 lineages: A, B.1.1.7, P.1, P.2, B.1.351, B.1.617.2, B.1.427, B.1.429, B.1.621 and BA.1), and pooled nasal wash specimen from 20 healthy adults representing diverse microbial respiratory flora. Coronavirus NL63 and coronavirus HKU1 were tested in clinical samples. All data were generated using the AB 7500 Fast Dx and the Invitrogen SuperScript™ III master mix.

Table 11: NCV-2012 rRT-PCR Assay Viral Cross-reactivity

Virus (strain)[a]	NCV.upE	NCV.N3
Adenovirus C1 (Ad.71)	Neg	Neg
Coronavirus 229E	Neg	Neg
Coronavirus OC43	Neg	Neg
Coronavirus SARS (Urbani)	Neg	Neg
Coronavirus HKU1	Neg	Neg
Coronavirus NL63	Neg	Neg
SARS-CoV-2[b]	Neg	Neg
Enterovirus 68	Neg	Neg
HMPV (CAN 99-81)	Neg	Neg
Influenza A H1N1 (A/India/12)	Neg	Neg
Influenza A H3N1 (A/Texas/12)	Neg	Neg
Influenza B (B/Massachusetts/99)	Neg	Neg
Parainfluenza 1 (C35)	Neg	Neg
Parainfluenza 2 (Greer)	Neg	Neg
Parainfluenza 3 (C-43)	Neg	Neg
Parainfluenza 4a (M-25)	Neg	Neg
Parainfluenza 4b (CH 19503)	Neg	Neg
Parechovirus 1b	Neg	Neg
Respiratory syncytial virus A (Long)	Neg	Neg
Rhinovirus 1A	Neg	Neg
Pooled human nasal wash [c]	Neg	Neg

[a] All strains were identified using CDC-developed rRT-PCR assays (for other human respiratory pathogens) and genome sequencing.
[b] Ten SARS-CoV-2 variants (A, B.1.1.7, P.1, P.2, B.1.351, B.1.617.2, B.1.427, B.1.429, B.1.621 and BA.1) were tested. All variants were negative for MERS-CoV mRNA detection.
[c]Pooled nasal wash specimens from 20 consenting healthy new military recruits to represent diverse microbial flora in the human respiratory tract.

Extraction Method and Enzyme Bridging

An extraction/enzyme bridging study was conducted to evaluate the performance of the NucliSENS® easyMAG® and SuperScript™ III Platinum® One-Step qRT-PCR Kit (SuperScript™) which are currently authorized for use with the CDC NCV-2012 rRT-PCR Assay with the following additional extraction methods: QIAGEN EZ1 Advanced XL with QIAGEN EZ1 DSP Virus Kit and QIAGEN QIAamp MinElute Virus Spin kit, and additional enzyme TaqPath™ 1-Step Multiplex Master Mix (No ROX) (TaqPath).

Limit of Detection (LoD)

For range finding, four 3-fold serial dilutions, with 5 individual replicates for each dilution, were prepared in each pooled clinical matrix (swabs in transport medium, BAL and sputum) using a quantified MERS-CoV isolate (Jordan-1 2797; 8.4×10^7 $TCID_{50}$/mL) and tested using NucliSENS® easyMAG® and SuperScript™ to determine an estimated LoD. All assay controls performed as expected. The lowest concentration at which all 5 replicates were positive for all 3 specimen types was 6.22 $TCID_{50}$/mL. The results are presented in Table 12 below.

Table 12. Range Finding LoD – MERS-CoV Jordan-1 2797 Isolate in Clinical Matrices

Concentration ($TCID_{50}$/mL)	Swabs in transport medium NCV.upE	Swabs in transport medium NCV.N3	BAL NCV.upE	BAL NCV.N3	Sputum NCV.upE	Sputum NCV.N3
18.67	33.28	33.13	34.44	32.22	35.30	34.13
18.67	34.39	33.04	34.15	32.40	35.32	33.71
18.67	34.79	33.62	34.33	33.11	35.04	34.21
18.67	35.19	33.44	33.73	32.85	35.21	34.07
18.67	34.50	32.98	34.32	33.88	37.77	33.53
6.22	38.93	36.33	36.20	35.03	37.23	35.13
6.22	38.79	36.28	36.64	34.74	38.80	35.20
6.22	38.66	36.07	34.17	33.71	36.24	36.14
6.22	37.15	36.56	36.24	35.21	37.20	35.58
6.22	38.71	35.09	34.85	35.02	36.88	36.38
2.07	39.83	36.50	38.28	35.45	Neg	Neg
2.07	38.78	36.59	34.89	35.79	Neg	38.06
2.07	Neg	37.41	Neg	35.34	38.19	36.24
2.07	39.39	Neg	36.76	37.70	38.36	Neg
2.07	Neg	37.81	38.37	36.10	42.47	37.15
0.69	Neg	Neg	Neg	37.31	Neg	Neg
0.69	38.87	36.23	Neg	Neg	Neg	Neg
0.69	39.62	36.51	Neg	Neg	Neg	Neg
0.69	Neg	36.35	38.55	37.17	Neg	Neg
0.69	Neg	36.26	39.35	37.61	Neg	Neg

Limit of Detection Confirmation:

The limit of detection for the NCV-2012 rRT-PCR was confirmed in the most complex specimen matrix sputum. Pooled sputum matrix was spiked with MERS-CoV isolate (Jordan-1 2797) at the estimated LoD concentration (6.22 $TCID_{50}$/mL) (from the Preliminary Limit of Detection Study) and to a concentration 3-fold below (2.07 $TCID_{50}$/mL). Twenty (20) individually contrived specimens at each concentration were extracted by three extraction methods: NucliSENS® easyMAG®, QIAGEN EZ1 Advanced XL with QIAGEN EZ1 DSP Virus Kit, and QIAGEN QIAamp MinElute Virus Spin kit. All extracted samples were tested using the CDC NCV-2012 rRT-PCR Assay on the Applied Biosystems 7500 Fast Dx using SuperScript™ and TaqPath.

All new extraction methods and new enzyme performed comparably when compared to the original combination of the easyMAG® extraction platform with the SuperScript™ III enzyme. Summary data are presented in Table 13.

Table 13. Limit of Detection Comparison Between NucliSENS® easyMAG®, QIAGEN EZ1 Advanced XL and QIAGEN QIAamp MinElute Virus Spin kit, Between SuperScript™ and TaqPath – Summary Results: MERS-CoV Positivity

Concentration ($TCID_{50}$/mL)	NucliSENS® easyMAG® / SuperScript™	NucliSENS® easyMAG®/ TaqPath	QIAGEN EZ1 Advanced XL with EZ1 DSP Virus Kit / SuperScript™	QIAGEN EZ1 Advanced XL with EZ1 DSP Virus Kit / TaqPath	QIAGEN QIAamp MinElute Virus Spin kit / SuperScript™	QIAGEN QIAamp MinElute Virus Spin kit / TaqPath
6.22	20/20	20/20	20/20	20/20	20/20	20/20
2.07	11/20	10/20	14/20	13/20	13/20	15/20

Note: The LoD values for different extraction platform and enzyme combination were determined to be 6.22 $TCID_{50}$/mL in this bridging study. However, the LoD was determined to be 1.3×10^{-2} $TCID_{50}$/mL in the original IFU. This variation may be caused by different MERS-CoV isolates. Jordan-1 2797 (8.4×10^{7} $TCID_{50}$/mL) was used in this bridging study. Jordan-N3/NCV (1.3×10^{4} $TCID_{50}$/mL) was used in the original IFU, which was not available for this bridging study. Genome copy numbers of isolate Jordan-1 2797 were determined by droplet digital PCR. The LoD value in this bridging study was 3.6 copies per reaction using NCV.upE assay or 6.9 copies per reaction using NCV.N3 assay, which were consistent with LoDs of 5-10 copies per reaction obtained by using RNA transcripts in CDC MERS publication (Lu et al., JCM 2014).

Spiked Clinical Specimen Testing

Individual clinical matrices (swabs in transport medium, BAL and sputum) were spiked with MERS-CoV isolate (Jordan-1 2797) at high (100x LoD), moderate (10x LoD) and low (3x LoD) concentrations. Twenty negative clinical specimens and 20 positive contrived specimens with different viral loads (6 high, 7 moderate and 7 low concentrations of MERS-CoV) per specimen type were extracted side by side by three extraction methods: NucliSENS® easyMAG®, QIAGEN EZ1 Advanced XL with QIAGEN EZ1 DSP Virus Kit, and QIAGEN QIAamp MinElute Virus Spin kit. All extracted samples were tested using the CDC NCV-2012 rRT-PCR Assay on the Applied Biosystems 7500 Fast Dx using SuperScript® and TaqPath.

All candidate extraction methods and candidate enzyme performed comparably, and controls performed as expected. Summary data are presented by specimen type, in Tables 14 (Swabs in transport medium), 15 (BAL), and 16 (Sputum).

Table 14. Spiked Clinical Specimen Testing – Summary Results – Swabs in Transport Medium

Extraction platform/ Enzyme	Testing Result	Specimen Identity Positive	Specimen Identity Negative	Positive / Negative % Agreement (CI)
NucliSENS® easyMAG®/ SuperScript™	Positive	20	0	PPA: 100 (83.9-100)
NucliSENS® easyMAG®/ SuperScript™	Negative	0	20	NPA: 100 (83.9-100)
NucliSENS® easyMAG®/ TaqPath	Positive	20	0	PPA: 100 (83.9-100)
NucliSENS® easyMAG®/ TaqPath	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ SuperScript™	Positive	20	0*	PPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ SuperScript™	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ TaqPath	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ TaqPath	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / SuperScript™	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / SuperScript™	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / TaqPath	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / TaqPath	Negative	0	20	NPA: 100 (83.9-100)

*Confirmatory testing at CDC resulted in negative result.

Table 15. Spiked Clinical Specimen Testing – Summary Results – BAL

Extraction platform/ Enzyme	Testing Result	Specimen Identity Positive	Specimen Identity Negative	Positive / Negative % Agreement (CI)
NucliSENS® easyMAG®/ SuperScript™	Positive	19*	0	PPA: 95 (76.4-99.1)
NucliSENS® easyMAG®/ SuperScript™	Negative	1*	20	NPA: 100 (83.9-100)
NucliSENS® easyMAG®/ TaqPath	Positive	20	0	PPA: 100 (83.9-100)
NucliSENS® easyMAG®/ TaqPath	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ SuperScript™	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ SuperScript™	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ TaqPath	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ TaqPath	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / SuperScript™	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / SuperScript™	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / TaqPath	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / TaqPath	Negative	0	20	NPA: 100 (83.9-100)

*One positive contrived specimen with low viral load was determined to be equivocal. The specimen was tested negative upon repeat PCR testing.

Table 16. Spiked Clinical Specimen Testing – Summary Results – Sputum

Extraction platform/ Enzyme	Testing Result	Specimen Identity Positive	Specimen Identity Negative	Positive / Negative % Agreement (CI)
NucliSENS® easyMAG® / SuperScript™	Positive	20	0	PPA: 100 (83.9-100)
NucliSENS® easyMAG® / SuperScript™	Negative	0	20	NPA: 100 (83.9-100)
NucliSENS® easyMAG® / TaqPath	Positive	19*	0	PPA: 95 (76.4-99.1)
NucliSENS® easyMAG® / TaqPath	Negative	1*	20	NPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ SuperScript™	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ SuperScript™	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ TaqPath	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN EZ1 Advanced XL/ TaqPath	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / SuperScript™	Positive	20	0	PPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / SuperScript™	Negative	0	20	NPA: 100 (83.9-100)
QIAGEN QIAamp MinElute Virus Spin kit / TaqPath	Positive	19€	0	PPA: 95 (76.4-99.1)
QIAGEN QIAamp MinElute Virus Spin kit / TaqPath	Negative	1€	20	NPA: 100 (83.9-100)

*One positive contrived specimen with moderate viral load was determined to be equivocal. The specimen was tested equivocal again upon repeat PCR testing. The detection failure of this sample might be caused by total nucleic acid loss during extraction. For these studies, we are scoring it as negative to provide a worst-case performance characteristic dataset. If equivocal result is obtained again, re-extract/elute in a lower volume and repeat rRT-PCR testing would occur.
€One positive contrived specimen with low viral load was determined to be equivocal. The specimen was tested equivocal again upon repeat PCR testing. For these studies, we are scoring it as negative to provide a worst-case performance characteristic dataset. If equivocal result is obtained again, re-extract/elute in a lower volume and repeat rRT-PCR testing would occur.

Contact Information

For questions or additional information, please contact the LRN Help Desk using the “Contact Us” feature on the LRN Secure Information Hub.