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

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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 http://www.cdc.gov/coronavirus/mers/index.html.

Intended Use

The 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 respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputa, lower respiratory aspirates/washes), sera and stool from individuals meeting MERS-CoV clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with MERS-CoV infection, contact with a probable or confirmed MERS-CoV case, history of travel to geographic locations where MERS-CoV cases were detected, or other epidemiologic links for which MERS-CoV testing may be indicated as part of a public health investigation).

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. Laboratories are required to report results to the Centers for Disease Control and Prevention (CDC). The definitive identification of MERS-CoV requires additional testing and confirmation to be performed by CDC. The diagnosis of MERS-CoV infection must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence, in addition to the detection of MERS-CoV RNA.

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. 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 trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The level of MERS-CoV that would be present in respiratory specimens, sera or stool from individuals with early systemic infection is unknown. Due to the difficulty in obtaining positive clinical specimens, only limited evaluation of the NCV-2012 rRT-PCR Assay has been made with specimens from individuals with MERS-CoV infection.

The NCV-2012 rRT-PCR Assay is only for use under the Food and Drug Administration's Emergency Use Authorization. Use within the United States is limited to 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: Refer to *Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Middle Ease Respiratory Syndrome Coronavirus (MERS-CoV)* (http://www.cdc.gov/coronavirus/mers/guidelines-lab-biosafety.html)

Acceptable Specimens

- Respiratory specimens, such as:
  - Nasopharyngeal and/or Oropharyngeal swabs
  - Sputum
  - Lower respiratory tract aspirates/washes
- Serum
- Stool (see Nucleic Acid Extraction section for use restrictions)

Specimen Collection

Refer to *Interim Guidelines for Collection, Handling and Testing of Clinical Specimens from Patients Under Investigation (PUIs) for Middle East Respiratory Syndrome Coronavirus (MERS-CoV)* (http://www.cdc.gov/coronavirus/mers/guidelines-clinical-specimens.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.

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 3sets of primers and FAM-labeled probes:
  - NCV.upE (forward primer [NCV.upE-F], reverse primer [NCV.upE-R] and probe [NCV.upE-P])
  - NCV.N2 (forward primer [NCV.N2-F], reverse primer [NCV.N2-R] and probe [NCV.N2-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).

Materials Required but Not Provided

- SuperScript™ III Platinum® One-Step qRT-PCR Kit (Invitrogen, catalog #11732-088)
- Molecular grade water, nuclease-free
- 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)
  - MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Applied Science, catalog #03730964001)

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)
  - DNA*Zap*™ (Ambion, cat. #AM9890) or equivalent.
- Disposable, powder-free gloves and surgical gowns
- Laboratory marking pen
- P2/P10, P200, and P1000 aerosol barrier pipette tips
- 1.5 mL microcentrifuge tubes
- 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)
- MicroAmp Optical 8-cap Strips (Applied Biosystems; catalog #4323032)
- Vortex mixer
- Microcentrifuge
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- Multichannel micropipettes (5-50 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 #446985 or #4406984);
- Extraction systems (instruments):
    - NucliSENS® easyMAG® (bioMérieux, catalog #280140)
    - MagNA Pure Compact (Roche Applied Science, catalog #03731146001)

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.
  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).
- 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.
- 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 and negative controls

Control Type	Control Name	Used to Monitor	NCV.upE	NCV.N2	NCV.N3	RP	Expected C_T Values
Positive	VTC	Substantial reagent failure, including primer and probe integrity	+	+	+	+	<35 C_T
Negative	NTC_1	Reagent and/or environmental contamination during PCR set-up	-	-	-	-	None detected
Negative	NTC_2	Reagent and/or environmental contamination during extraction	-	-	-	-	None detected

Nucleic Acid Extraction

Respiratory specimens and sera may be extracted using either the NucliSENS® easyMAG® or the MagNA Pure Compact (Nucleic Acid Isolation Kit I).

Stool may only be extracted using the NucliSENS® easyMAG® instrument.

NOTE: The MagNA Pure Compact should **not** be used for stool specimens.

- Sample extractions **must** yield RNA or total nucleic acid of sufficient volume to cover all rRT-PCR assays (a minimum of 60µL is recommended).
- Follow the manufacturer's instructions for sample extraction.
- Nuclease-free water should be included in each extraction run as a sample extraction control (NTC_2) (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.N2 rRT-PCR assays are used for specimen screening. If one or both assays are positive, and VTC and NTC control results are acceptable, use the NCV.N3 assay to verify the test results. Specimens with presumptive positive results should be sent to CDC for confirmatory testing.

Test results should be interpreted in this order:

1. NCV Assay VTC and NCV Assay NTC results
2. NCV.upE Assay and NCV.N2 Assay results
3. RP Assay NTC and RP Assay VTC, if NCV.upE and NCV.N2 assays are negative
4. NCV.N3 Assay results

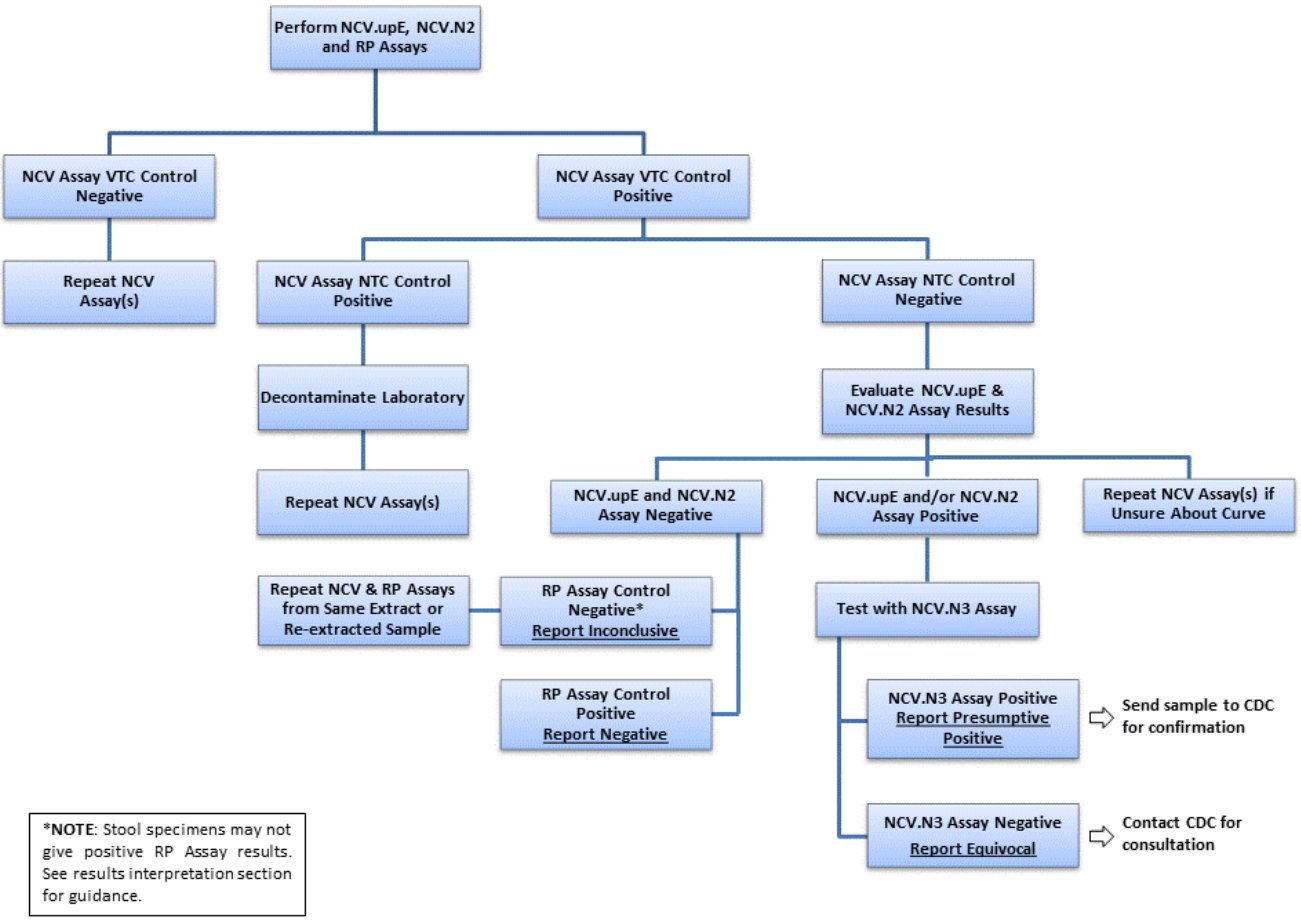

Figure 1: Testing Algorithm

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.N2 and 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.
     - Mix gently and aliquot primers/probe in 100 µL volumes into 5 pre-labeled tubes. Store a single aliquot of primers/probe at 2-8°C in the dark. Do not refreeze (stable for up to 4 months). Store remaining aliquots at ≤-20°C 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.
   - Used 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 (final volume 1.0 mL). Dispense diluted VTC into single use aliquots suitable for your testing needs. Store unused material at ≤ -70ºC.
   - 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 human DNA that serves as the positive control for the RNP 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

AB 7500 Fast Dx			
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

Instrument Settings

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

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 work day organization. NTCs and VTCs 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.
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 NTC, VTC, 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, 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

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

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

9. Add 20 µL of master mix into each well of a chilled optical plate 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.N2	NCV.N2	NCV.N2	NCV.N2	NCV.N2	NCV.N2	NCV.N2	NCV.N2	NCV.N2	NCV.N2	NCV.N2	NCV.N2
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	S9	NTC_2	VTC
B	NTC_1	S1	S2	S3	S4	S5	S6	S7	S8	S9	NTC_2	VTC
C	NTC_1	S1	S2	S3	S4	S5	S6	S7	S8	S9	NTC_2	VTC
D												
E												
F												
G												
H												

No template reaction mix controls (NTC_1) used to check for reagent contamination (column 1); sample extracts (S); no template extraction control (NTC_2) used to check for contamination occurring during extraction or specimen extract handling during plate set-up (column 11); viral template control (VTC) used to assess assay performance (column 12).

Figure 2: Plate Set-Up for Initial NCV.upE and NCV.N2 testing (plate lay out example)

	1	2	3	4	5	6	7	8	9	10	11	12
A	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3	NCV.N3
B	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
C												
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	S9	S10	VTC
B	NTC_1	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	VTC
C												
D												
E												
F												
G												
H												

No template reaction mix controls (NTC_1) used to check for reagent contamination (column 1); sample extracts (S); viral template control (VTC) used to assess assay performance (column 12).

Figure 3: Plate Set-Up for NCV.N3 Testing (Second round; only specimens positive by initial testing; refer to Testing Algorithm above)

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 and move plate to the nucleic acid handling area on cold block.
12. Vortex sample extracts, VTC and NTC_2 briefly and centrifuge for 5 seconds.
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 at 500 x *g* for 1 min at 4°C 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. VTCs 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.
        i. Repeat rRT-PCR test.
        ii. If repeat testing generates negative VTC results, contact the LRN helpdesk for consultation.
2. NTCs should 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 should be **positive**.
    a. If RP Assay for a specimen sample is *negative* and both NCV.upE and NCV.N2 Assays are *negative* for specimen samples :
        i. Report result as *Inconclusive* through LRN Results Messenger
        ii. Follow the specimen-specific instructions below:

Respiratory and Serum samples	Stool samples
1. Repeat rRT-PCR test of sample using RP, NCV.upE and NCV.N2 assays.	1. Repeat testing of the inconclusive specimen is not recommended.
2. Repeat extraction from new specimen aliquot if RP Assay is *negative* for specimens after repeat testing.	2. Test other specimens, respiratory or sera obtained from the patient, if available or request the collection of additional specimens.
3. After repeat extraction and repeat rRT-PCR testing, if either NCV.upE or NCV.N2 assay is *positive*, consider the result a true *positive* and continue to follow the testing algorithm.	
4. 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 either NCV.upE Assay or NCV.N2 Assay is *positive* for specimen samples:
        i. Do not repeat rRT-PCR test and consider the results of the NCV-2012 Assays valid.

Note: Stool specimens may not always generate positive results for the RP Assay.

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

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

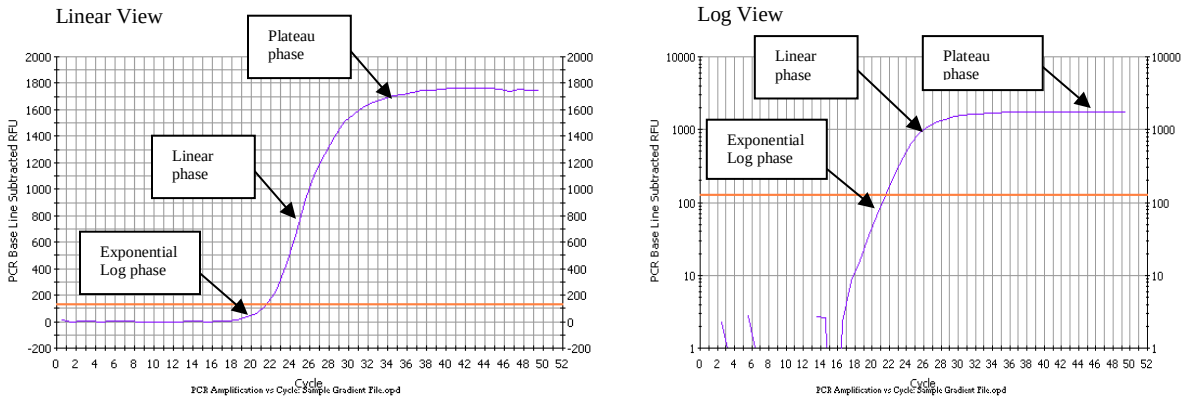

Figure 4: Linear and log views of PCR curves noting each stage of the amplification plots.

- 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.

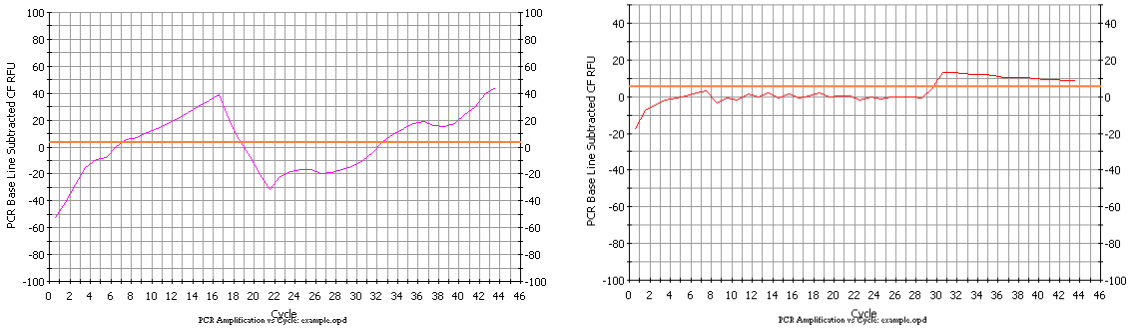

Figure 5: Examples of false positive curves.

- 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 C_T 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 with a C_T of 36.6 (black), a very weak positive with a C_T of 42.1 (red), and a negative control (blue). The weak positive (C_T= 42.1) is verified to be positive by the sharp increase in fluorescence seen in the background fluorescence view.

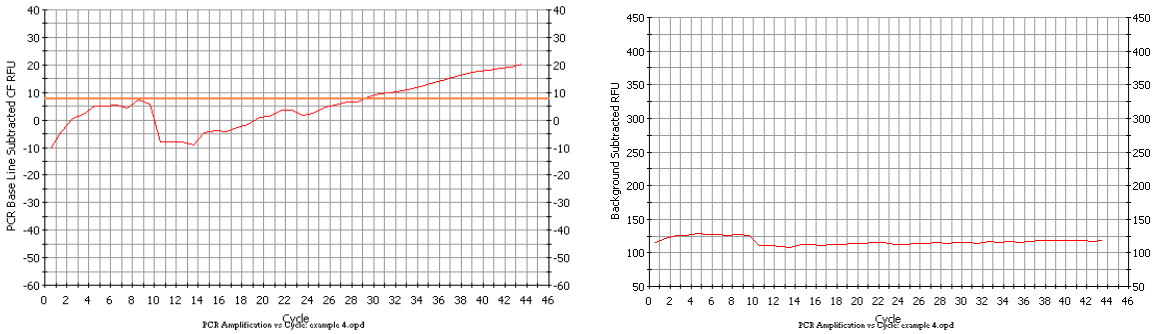

Figure 6: Amplification plot of a sample with a "wandering" curve (left) and the corresponding background fluorescence view (right).

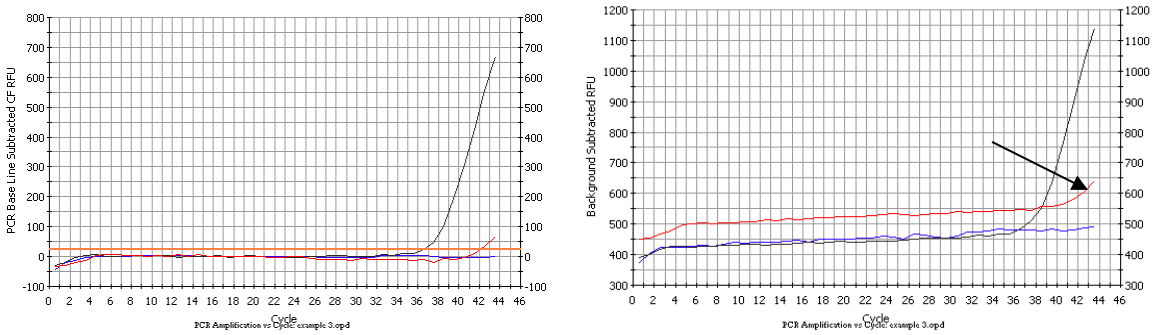

Figure 7: Amplification plot of three samples in the linear view (left) and the corresponding background fluorescence view (right).

- 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 45, 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.

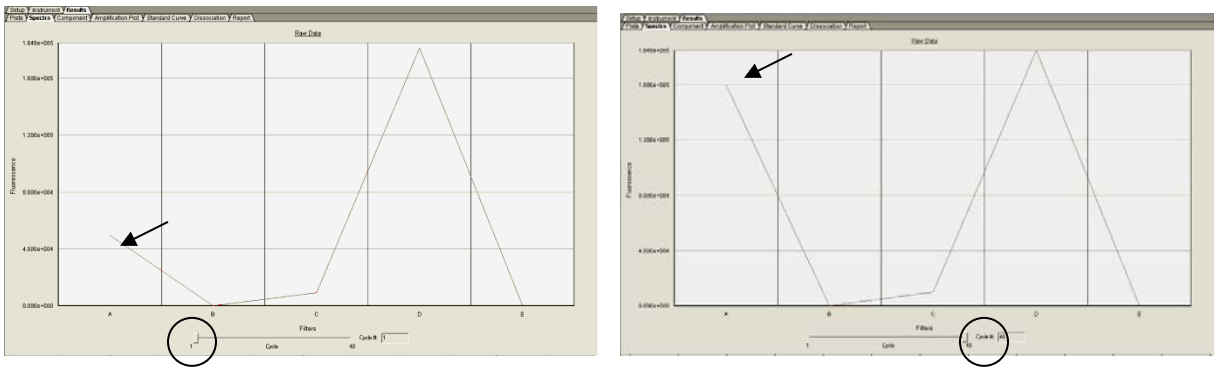

Figure 8: Spectra component of a positive sample. Left screenshot shows fluorescence at cycle 1 and right screenshot shows fluorescence at cycle 40.

- 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 C_T 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 ($C_T \geq 37$). 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.
    - Stool specimens are particularly complex and are known to negatively impact rRT-PCR efficiency, resulting in higher Ct values or false negative rRT-PCR results.

- 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

Interpretation of NCV-2012 rRT-PCR Assay Results

Testing Algorithm Part 1			Algorithm Part 2				
NCV.upE	**NCV.N2**	**RP**	**NCV.N3**	**RP**	**Interpretation**	**Reporting**	**Actions**
-	-	+	Not Done		MERS-CoV Negative	MERS-CoV RNA not detected by rRT-PCR	Report results through LRN Results Messenger
-	-	-	Not Done		Inconclusive	Inconclusive for MERS-CoV 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 through LRN Results Messenger
-	+	+/-	+	+/-	MERS-CoV Presumptive Positive	MERS-CoV RNA detected by rRT-PCR. Confirmatory testing required. Specimen will be referred to CDC for further analysis.	Send specimen to CDC for confirmatory testing. Report results thorugh LRN Results Messenger
+	-						
+	+						
-	+	+/-	-	+/-	Equivocal	NCV-2012 rRT-PCR testing was equivocal. Additional analysis may be conducted by CDC.	Contact CDC for consultation. Report results thorugh LRN Results Messenger
+	-						
+	+						

NOTE: All test results generated using the NCV-2012 rRT-PCR Assay 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.

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 and a well-designed plan for confirmatory testing can help ensure that laboratory contamination is detected and that false positive test results are not reported.

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, 329 geographically diverse fresh and frozen human clinical specimens collected between 2011 and 2013 from 312 demographically diverse patients with acute respiratory illness were tested at CDC by the NCV-2012 rRT-PCR Assay (upE, N2 and N3 assays). Testing was performed using the AB 7500 Fast Dx and Invitrogen Superscript™ III mastermix.

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, 4 sputa, 7 stool/rectal swabs and 3 sera. 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 in Kenya, Panama and the United States.

Though the testing protocol would only indicate the use of NCV.upE and NCV.N2 for initial testing, with no additional testing following negative results, these specimens were tested by all three NCV-2012 rRT-PCR Assay primer and probe sets to give additional confidence in the overall result and performance of the assay.

The upE, N2 and 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 are two (2) specimens from two patients (BAL and serum) associated with a cluster of acute respiratory infection (ARI) cases at a hospital in Jordan in April, 2012. Both were positive for MERS-CoV by the upE, N2 and N3 assays. Both specimens were 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

	Specimens from Confirmed Cases (Jordan cluster, positive results expected)					Other Specimens (Negative results expected)				
Specimen type	#	NCV.upE # pos.	NCV.N2 # pos.	NCV.N3 # pos.	Overall # pos.	#	NCV.upE # pos.	NCV.N2 # pos.	NCV.N3 # pos.	Overall # pos.
NP/OP Swabs	0	-	-	-	-	290	0/290	0/290	0/290	0/290
Sputum	0	-	-	-	-	4	0/4	0/4	0/4	0/4
Bronchial or transtracheal aspirates or washes	1	1/1	1/1	1/1	1/1	23	0/23	0/23	0/23	0/23
Serum	1	1/1	1/1	1/1	1/1	3	0/3	0/3	0/3	0/3
Stool	0	-	-	-	-	7	0/7	0/7	0/7	0/7*

*One stool specimen generated a negative PCR result for RP as well as the NCV primers and probes, thus the result is inconclusive.

Table 6: Percent agreement with expected results

rRT-PCR Result	Expected Result	
	Positive	Negative
Positive	2	0
Inconclusive	0	1*
Negative	0	326

*One stool specimen generated a negative PCR result for RP as well as the NCV primers and probes, thus the result is inconclusive.

Positive percent agreement = 2/2 = 100% (95% CI: 34.2% - 100%)
Negative percent agreement = 327/327 = 100% (95% CI: 98.9% - 100%)
Overall percent agreement = 329/329 = 100% (95% CI: 98.9%-100%)

Performance characteristics of the NCV-2012 rRT-PCR Assay using contrived stool specimens

Due to the lack of stool specimens from patients with MERS-CoV infection, NCV-2012 rRT-PCR Assay performance with stool specimens was determined through an alternative approach. Fifty one (51) stool specimens were selected from a CDC bank of stool specimens collected from patients with an enteric viral infection exhibiting gastrointestinal symptoms. The stool selected for this study was collected from patients presenting with fever, a symptom also associated with MERS-CoV infection. Twenty of these specimens were split into two containers, one left as is, the other spiked with a known concentration of MERS-CoV strain Jordan-N3/NCV. Spiking concentrations included moderate and low concentrations of the virus, with the low concentration near the limit of detection for the assay. The group of 71 samples (51 negatives, 20 spiked positives) were then blinded and tested by the NCV-2012 rRT-PCR Assay. Extractions were performed using the NucliSENS® easyMAG® method and were tested using the AB 7500 Fast Dx and the Invitrogen Superscript™ III master mix.

Although the primers and probes designed to detect the MERS-CoV correctly identified negative retrospective stool specimens, 47 of 51 negative stool samples failed to generate positive results for RP, causing the overall result interpretation to be inconclusive and generating only a 7.8% negative agreement. The failure of the RP control may be due to the storage conditions of the retrospective stool specimens tested in the study resulting in degradation of the human nucleic acids. The performance characteristics of this device with fresh clinical stool specimens that are positive or negative for the MERS-CoV have not been established. Please see page 14 for follow up testing recommendations for stool specimens with inconclusive results using respiratory and serum specimens.

Table 7: Contrived Stool Performance Summary

Assay	Spiked Positive Specimens			Negative Specimens			
	Tested	Positive	Positive Agreement (95% CI)	Tested	Inconclusive	Negative	Negative Agreement (95% CI)
NCV-2012 rRT-PCR	20	20	100% (83.9%-100%)	51	47	4	7.8% (3.1%-18.5%)

Performance characteristics of the NCV-2012 rRT-PCR Assay using contrived serum specimens

Due to the lack of serum specimens from patients with MERS-CoV infection, NCV-2012 rRT-PCR assay performance with serum specimens was determined through an alternative approach. Fifty (50) serum specimens were selected from a CDC bank of serum specimens collected from patients with acute respiratory illness. The serum selected for this study was collected from patients presenting with signs and symptoms associated with MERS-CoV infection. Twenty of these specimens were split into two containers, one left as is, the other spiked with a known concentration of MERS-CoV strain Jordan- N3/NCV. Spiking concentrations included moderate and low concentrations of the virus, with the low concentration near the limit of detection for the assay. The group of 70 samples (50 negatives, 20 spiked positives) were then blinded and tested by the NCV-2012 rRT-PCR. Extractions were performed using the NucliSENS® easyMAG® method and were tested using the AB 7500 Fast Dx and the Invitrogen Superscript™ III master mix.

Table 8: Contrived Sera Performance Summary

Assay	Spiked Positive Specimens			Negative Specimens		
	Tested	Positive	Agreement (95% CI)	Tested	Negative	Agreement (95% CI)
NCV-2012 rRT-PCR	20	20	100% (83.9%-100%)	50	50	100% (92.9%-100%)

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 specimen were spiked with known concentrations of the Jordan-N3/NCV strain of the MERS-CoV, 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 C_T values for each primer/probe set.

Two extraction methods, the Automated NucliSENS® Magnetic Extraction (EasyMAG®) and the MagNA Pure Compact Nucleic Acid Isolation Kit, were also compared in this evaluation. Each NCV-2012 assay yielded similar C_T values with each extraction method, therefore, both are recommended for use with this assay (**Table 9**).

Table 9: NCV-2012 rRT-PCR Assay results using a proficiency panel of contrived respiratory specimens

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

Samples	Virus quantity $TCID_{50}$/mL	EasyMAG												MagNA Pure Compact											
		NCV.upE			NCV.N2			NCV.N3			RNP			NCV.upE			NCV.N2			NCV.N3			RNP		
S1	$3.4X10^{1}$	20.64	20.81	20.61	19.78	19.89	19.64	18.75	18.82	18.86	33.33	33.04	32.85	21.50	21.61	21.29	20.19	20.07	19.98	18.99	19.64	19.65	33.16	33.30	33.45
S2	0		Neg			Neg			Neg			Neg			Neg			Neg			Neg			Neg	
S3	$3.4X10^{-1}$	27.14	26.79	27.14	25.96	25.94	25.87	24.89	25.19	24.89	32.49	32.93	33.06	28.28	28.34	28.20	27.07	27.14	26.67	26.58	26.62	26.58	33.86	34.73	34.12
S4	$3.4X10^{-3}$	35.69	35.79	35.86	35.07	35.21	35.10	33.42	33.73	33.58	32.53	32.77	32.16	36.25	37.28	37.53	35.30	35.29	36.05	34.82	34.57	36.51	33.66	33.75	33.56
	Extraction control		Neg			Neg			Neg			Neg			Neg			Neg			Neg			Neg	
	Negative control		Neg			Neg			Neg			Neg													
	Positive control		25.81			27.12			28.37			28.05													

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

Samples	Virus quantity $TCID_{50}$/mL	EasyMAG												MagNA Pure Compact											
		NCV.upE			NCV.N2			NCV.N3			RNP			NCV.upE			NCV.N2			NCV.N3			RNP		
S1	$3.4X10^{1}$	22.11	22.1	21.6	22.88	22.89	22.74	20.25	20.49	20.5	32.39	31.88	32.67	22.35	22.32	22.17	23.24	23.56	23.14	21.31	21.22	21.24	33.34	33.29	34.21
S2	0		Neg			Neg			Neg			Neg			Neg			Neg			Neg			Neg	
S3	$3.4X10^{-1}$	28.22	28	28.6	29.48	29.19	29.42	27.42	27.22	27.21	32.28	33.47	33.87	26.18	26.07	26.29	27.41	26.17	27.41	25.31	25.26	25.28	32.8	34.37	33.06
S4	$3.4X10^{-3}$	37.59	35.3	35.3	37.63	36.74	37.02	34.38	35.23	34.52	32.12	32.32	32.71	36.69	37.68	36.21	37.49	37.94	36.58	35.76	36.42	36.01	34.28	34.02	32.96
	Extraction control		Neg			Neg			Neg			Neg			Neg			Neg			Neg			Neg	
	Negative control		Neg			Neg			Neg			Neg													
	Positive control		26.39			29.17			29.47			27.59													

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

Samples	Virus quantity $TCID_{50}$/mL	EasyMAG												MagNA Pure Compact											
		NCV.upE			NCV.N2			NCV.N3			RNP			NCV.upE			NCV.N2			NCV.N3			RNP		
S1	$3.4X10^{1}$	20.68	20.6	20.5	19.92	19.28	19.68	19.18	19.21	19.24	32.34	32.26	32.68	21.26	21.25	21.08	19.93	20.15	20.15	20.22	20.24	20.28	34.16	33.78	34.02
S2	0		Neg			Neg			Neg			Neg			Neg			Neg			Neg			Neg	
S3	$3.4X10^{-1}$	25.57	25.4	25.3	24.55	24.6	24.49	24.13	24.08	24.18	31.84	31.76	32.06	27.76	27.56	27.67	26.66	26.67	26.54	26.75	26.65	26.7	33.41	34.36	32.55
S4	$3.4X10^{-3}$	35.75	35.2	36.1	35.62	35.07	35.14	34.62	33.62	35.02	32.12	31.65	32.33	37.59	35.25	36.01	35.04	35.11	34.84	34.59	35.4	35.03	33.14	34.03	33.84
	Extraction control		Neg			Neg			Neg			Neg			Neg			Neg			Neg			Neg	
	Negative control		Neg			Neg			Neg			Neg													
	Positive control		24.75			25.25			27.89			27.54													

Samples	NCV.upE		NCV.N2		NCV.N3		RNP		NCV.upE		NCV.N2		NCV.N3		RNP	
	Avg.	STD	Avg.	STD	Avg.	STD	Avg.	STD	Avg.	STD	Avg.	STD	Avg.	STD	Avg.	STD
S1	21.1	0.676	20.7	1.583	19.5	0.727	32.6	0.439	21.6	0.500	21.2	1.623	20.3	0.817	33.6	0.412
S2																
S3	26.9	1.244	26.6	2.152	25.5	1.415	32.6	0.738	27.4	0.936	26.9	0.421	26.2	0.686	33.7	0.773
S4	35.8	0.719	35.8	1.006	34.2	0.670	32.3	0.345	36.7	0.849	36.0	1.139	35.5	0.754	33.7	0.426

MERS-CoV strain tested: NCV-Jordan (2013212797) pass 1 VeroE6; stock virus titer 1.3 x 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, sera and stool 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 all primer and probe sets in the NCV-2012 rRT-PCR assay kit. NCV-2012 rRT-PCR Assay data were generated using the AB® 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 was for all CDC assays was 1.3 x 10^{-3} $TCID_{50}$/mL.

Table 10: 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 C_T			Call Rate	NCV.N2 Assay C_T			Call Rate	NCV.N3 Assay C_T			Call Rate
10^{-4}	1.3x10^{0}	27.35	27.46	27.39	3/3	27.88	27.98	28.07	3/3	25.98	25.95	25.81	3/3
10^{-5}	1.3x10^{-1}	31.06	31.13	31.29	3/3	31.68	31.64	31.77	3/3	29.67	29.38	29.40	3/3
10^{-6}	1.3x10^{-2}	33.81	34.55	34.67	3/3	35.87	34.62	35.70	3/3	32.81	33.06	33.23	3/3
10^{-7}	1.3x10^{-3}	37.03	39.01	38.94	3/3	38.74	40.01	38.11	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	Neg	Neg	Neg	0/3
10^{-9}	1.3x10^{-5}	Neg	Neg	Neg	0/3	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	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 the most complex specimen matrix included in the intended use, stool. Pooled stool matrix was 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 mastermix using all three primer and probe sets for MERS-CoV. Results confirmed that MERS-CoV could consistently be detected at 1.3×10^{-2} $TCID_{50}$/mL. At this concentration, 100% of replicates were positive across all three primer and probe sets and, thus, overall by the test algorithm. At 1.3×10^{-3} $TCID_{50}$/mL, 18 replicates were positive, one equivocal and one negative when interpreted according to the test algorithm. Results are presented in Table 11: NCV-2012 rRT-PCR Assay limits of detection confirmation with Jordan-N3/NCV isolate diluted in stool matrix.

Table 11: NCV-2012 rRT-PCR Assay limits of detection confirmation with Jordan-N3/NCV isolate diluted in stool matrix.

	Dilution	Virus quantity TCID50/mL			Dilution	Virus quantity TCID50/mL		
	10^{-6}	$1.3x10^{-2}$			10^{-7}	$1.3x10^{-3}$		
Replicates	**NCV.upE C_T**	**NCV.N2 C_T**	**NCV.N3 C_T**	**RP C_T**	**NCV.upE C_T**	**NCV.N2 C_T**	**NCV.N3 C_T**	**RP C_T**
1	36.15	37.47	33.78	37.48	39.90	Neg	37.12	38.11
2	37.69	37.58	33.53	38.12	Neg	Neg	36.24	37.56
3	37.28	36.80	33.78	36.75	40.72	37.89	38.35	36.98
4	36.49	37.99	34.92	37.43	Neg	39.76	39.34	38.31
5	36.83	36.61	34.05	37.89	Neg	38.57	38.25	37.54
6	36.64	37.96	34.18	38.16	Neg	38.75	37.36	38.11
7	36.14	38.34	34.48	37.88	39.77	Neg	37.82	37.58
8	37.21	37.49	34.38	37.69	Neg	39.81	36.62	37.61
9	36.00	37.10	34.81	36.77	37.77	39.64	36.14	37.29
10	37.86	37.15	35.04	37.59	38.76	39.01	36.50	37.44
11	36.80	37.21	34.07	37.22	Neg	37.50	37.41	36.79
12	36.64	38.20	34.44	37.58	40.06	Neg	Neg	37.50
13	36.90	37.54	34.00	39.01	40.15	43.78	36.42	38.67
14	36.46	37.23	34.76	37.55	44.02	37.16	36.75	37.54
15	36.24	38.52	34.95	36.88	39.78	Neg	36.63	36.91
16	37.76	37.93	34.49	37.84	39.33	37.77	37.92	37.24
17	36.90	37.65	33.94	37.55	38.52	39.96	37.66	37.75
18	36.23	38.49	34.33	37.58	Neg	38.98	36.55	37.74
19	36.26	36.66	34.38	36.89	Neg	37.91	38.32	37.31
20	37.66	38.25	34.17	37.52	40.15	37.97	38.45	37.24
Avg.	36.81	37.61	34.32	37.57	39.91	38.96	37.36	37.56
SD	0.59	0.59	0.42	0.52	1.53	1.61	0.91	0.46
Call rate	20/20	20/20	20/20	20/20	12/20	15/20	19/20	20/20

Matrix and Extraction Comparison Study

To assess the impact of the presence of clinical matrices on the detection of MERS-CoV and to compare the performance of the MagNA Pure Compact Nucleic Acid Extraction Kit I to the NucliSENS® easyMAG®, pooled and spiked stool, sputum, serum and combined nasopharyngeal/oropharyngeal swab matrices were prepared, extracted side by side and tested by rRT-PCR.

Pooled matrices were constructed from multiple human clinical specimens: serum, including lipemic and hemolytic samples (10); NP/OP, combined nasopharyngeal and oropharyngeal swabs in universal transport media (10); sputum (10); and stool (15). The four pooled matrices (NP/OP, sputum, serum, stool) were each divided and spiked with MERS-CoV virus isolate Jordan-N3/NCV (stock virus titer 1.3×10^4 $TCID_{50}$/mL) to concentrations from 1.3×10^0 $TCID_{50}$ /mL to 1.3×10^{-4} $TCID_{50}$/mL. Each concentration of each spiked pooled matrix was extracted three times by easyMAG and three times by MagNA Pure Compact (Nucleic Acid Isolation Kit I). Each extract was then tested against all 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 12 below. These data demonstrate that the detection levels in matrix are comparable to those demonstrated in buffer (See Analytical Limit of Detection Studies above).

Data also demonstrate comparable performance between the NucliSENS® easyMAG® and MagNA Pure Compact for extraction of MERS-CoV RNA from respiratory specimens and serum. Data demonstrate that the MagNA Pure Compact does not perform optimally when extracting MERS-CoV RNA from stool specimens. Therefore, the instructions for use of the NCV-2012 rRT-PCR Assay limit use of the MagNA Pure Compact extraction instrument to respiratory specimens and sera.

Table 12: Summary of Matrix and Extraction Comparison Data

				NCV.upE				NCV.N2				NCV.N3				RP			
Extraction	Dil.	Concentration (TCID50/mL)	CT	Serum	NP/OP	Sputum	Stool	Serum	NP/OP	Sputum	Stool	Serum	NP/OP	Sputum	Stool	Serum	NP/OP	Sputum	Stool
NucliSENS® easyMAG®	10^{-4}	1.3 x 10^{0}	1	26.81	26.08	26.62	27.24	26.55	25.88	26.07	28.26	25.71	23.38	23.69	25.13	31.53	27.62	23.43	37.36
			2	26.71	26.35	26.72	27.67	26.46	25.92	26.13	28.34	25.66	23.5	23.74	25.4	31.02	27.66	23.56	38.15
			3	27.07	26.32	26.75	28.05	26.48	25.86	26.15	28.45	25.65	23.51	23.76	25.56	31.04	27.72	23.61	36.74
			Ave	26.86	26.25	26.7	27.65	26.5	25.89	26.12	28.35	25.68	23.46	23.73	25.36	31.2	27.67	23.53	37.42
			SD	0.19	0.15	0.07	0.4	0.05	0.03	0.04	0.09	0.03	0.07	0.04	0.22	0.29	0.05	0.09	0.71
			Call rate	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
NucliSENS® easyMAG®	10^{-5}	1.3 x 10^{-1}	1	30.18	27.9	29.13	30.86	30.11	27.35	28.19	32.84	29.3	25	25.86	29.47	31.19	27.58	23.02	38
			2	30.76	27.86	29.05	31.99	30.26	27.24	28.24	32.12	29.26	25.16	25.83	29.93	31.04	27.62	23.22	38.12
			3	30.75	27.85	29.11	31.62	30.23	27.49	28.23	32.12	29.37	24.93	25.9	29.9	31.42	27.69	23.22	37.47
			Ave	30.56	27.87	29.1	31.49	30.2	27.36	28.22	32.36	29.31	25.03	25.86	29.77	31.22	27.63	23.15	37.86
			SD	0.33	0.03	0.04	0.58	0.08	0.12	0.03	0.42	0.05	0.12	0.03	0.26	0.19	0.06	0.12	0.35
			Call rate	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
NucliSENS® easyMAG®	10^{-6}	1.3 x 10^{-2}	1	33.62	34.06	35.29	34.93	33.03	34.31	35.35	36.95	32.7	31.95	32.57	32.74	31.29	27.84	23.28	36.69
			2	33.67	34.51	35.59	35.74	33	33.98	34.67	37.83	32.43	31.92	32.69	33.87	30.98	27.88	23.28	38.23
			3	33.54	34.25	34.88	35.08	33.33	34.14	35.52	36.72	32.18	31.9	32.27	33.68	31.19	27.75	23.28	38.36
			Ave	33.61	34.27	35.25	35.25	33.12	34.14	35.18	37.17	32.44	31.93	32.51	33.43	31.15	27.82	23.3	37.76
			SD	0.06	0.22	0.36	0.43	0.18	0.17	0.45	0.58	0.26	0.03	0.21	0.6	0.16	0.07	0.03	0.93
			Call rate	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
NucliSENS® easyMAG®	10^{-7}	1.3 x 10^{-3}	1	40.76	38.7	38.52	39.49	36.63	37.5	37.58	40.57	36.78	36.57	35.49	36.41	31.32	27.87	23.21	39.47
			2	37.86	37.87	38.3	neg	37.04	37.11	37.94	40.6	36.69	35.43	35.44	37.52	31.09	28.01	23.25	37.2
			3	37.35	39.36	neg	38.45	37.99	38.1	37.5	40.31	36.6	35.41	34.9	36.57	31.36	28.03	23.27	36.88
			Ave	38.66	38.64	38.41	38.97	37.22	37.57	37.68	40.49	36.69	35.8	35.27	36.83	31.25	27.97	23.24	37.85
			SD	1.84	0.74	0.16	0.74	0.7	0.36	0.23	0.16	0.13	0.67	0.33	0.6	0.14	0.09	0.03	1.42
			Call rate	3/3	3/3	2/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
NucliSENS® easyMAG®	10^{-8}	1.3 x 10^{-4}	1	neg	neg	neg	neg	41.47	neg	neg	neg	neg	neg	neg	neg	31.4	28.01	24.74	37.6
			2	neg	neg	neg	neg	neg	neg	neg	neg	40.44	neg	neg	neg	31.28	28	24.72	37.24
			3	neg	neg	neg	neg	39.72	neg	neg	neg	39.58	neg	neg	neg	31.65	27.96	24.69	36.99
			Ave	NA	NA	NA	NA	40.6	NA	NA	NA	40.01	NA	NA	NA	31.44	27.99	24.72	37.27
			SD	NA	NA	NA	NA	1.23	NA	NA	NA	0.61	NA	NA	NA	0.19	0.03	0.02	0.25
			Call rate	0/3	0/3	0/3	0/3	2/3	0/3	0/3	0/3	2/3	0/3	0/3	0/3	3/3	3/3	3/3	3/3

				NCV.upE				NCV.N2				NCV.N3				RP			
Extraction	Dil.	Concentration (TCID50/mL)	CT	Serum	NP/OP	Sputum	Stool	Serum	NP/OP	Sputum	Stool	Serum	NP/OP	Sputum	Stool	Serum	NP/OP	Sputum	Stool
MagNA Pure Compact Nucleic Acid Isolation Kit I	10^{-4}	1.3 x 10^{0}	1	25.99	25.79	27.33	32.99	25.01	25.66	26.85	32.16	24.87	23.16	24.54	29.86	32.39	28.06	24.2	neg
			2	26.5	25.73	27.35	33.83	24.9	25.76	26.85	32.04	25.16	23.34	24.46	29.67	32.49	28.24	24.34	neg
			3	25.93	25.8	27.44	NA	24.98	25.69	26.85	NA	25.47	23.35	24.52	30.4	32.7	28.16	24.4	neg
			Ave	26.14	25.78	27.37	33.41	24.96	25.7	26.85	32.1	25.17	23.28	24.51	29.99	32.53	28.15	24.31	NA
			SD	0.31	0.04	0.06	0.59	0.05	0.05	0	0.08	0.3	0.1	0.04	0.38	0.15	0.09	0.1	NA
			Call rate	3/3	3/3	3/3	2/2	3/3	3/3	3/3	2/2	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3
MagNA Pure Compact Nucleic Acid Isolation Kit I	10^{-5}	1.3 x 10^{-1}	1	31.26	30.76	32.01	neg	30.84	30.88	31.68	neg	30.35	28.37	29.5	34.99	32.24	28.03	24.69	neg
			2	31.8	30.96	32.12	neg	30.67	31.09	31.97	neg	30.56	28.47	29.51	35.13	32.06	28.1	24.7	neg
			3	31.55	30.9	32.24	neg	30.74	31.06	31.95	neg	30.39	28.66	29.72	34.84	32.05	28.13	24.65	neg
			Ave	31.53	30.88	32.12	NA	30.75	31.01	31.87	NA	30.43	28.5	29.58	34.98	32.12	28.09	24.68	NA
			SD	0.27	0.1	0.11	NA	0.09	0.11	0.16	NA	0.11	0.14	0.12	0.15	0.1	0.05	0.03	NA
			Call rate	3/3	3/3	3/3	0/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3
MagNA Pure Compact Nucleic Acid Isolation Kit I	10^{-6}	1.3 x 10^{-2}	1	33.86	34.22	34.81	neg	33.24	34.07	34.82	neg	32.98	31.82	32.43	38.75	31.64	28	24.51	neg
			2	33.86	35.61	36.05	neg	32.94	34.51	35.36	neg	32.9	32.54	33.01	39.25	32.03	28.04	24.49	neg
			3	33.86	34.78	34.37	neg	33.42	34.66	35.16	neg	33	31.99	32.73	neg	32.21	28.05	24.57	neg
			Ave	33.86	34.87	35.07	NA	33.2	34.42	35.11	NA	32.96	32.12	32.72	39	31.96	28.03	24.52	NA
			SD	0	0.7	0.87	NA	0.24	0.31	0.27	NA	0.06	0.38	0.29	0.35	0.29	0.03	0.04	NA
			Call rate	3/3	3/3	3/3	0/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	2/3	3/3	3/3	3/3	0/3
MagNA Pure Compact Nucleic Acid Isolation Kit I	10^{-7}	1.3 x 10^{-3}	1	37.79	38.35	39.92	neg	39	38.25	37.18	neg	37.66	34.83	35.87	neg	32.23	26.39	24.44	neg
			2	39.06	38.49	38.53	neg	36.9	38.08	37.89	neg	37.55	35.8	35.89	neg	31.99	26.5	24.58	neg
			3	37.88	38.31	37.86	neg	38.68	37.56	39.45	neg	36.75	34.95	35.88	neg	32.24	26.49	24.57	neg
			Ave	38.24	38.39	38.77	NA	38.19	37.96	38.17	NA	37.32	35.2	35.88	NA	32.15	26.46	24.53	NA
			SD	0.71	0.1	1.05	NA	1.13	0.36	1.16	NA	0.5	0.53	0.01	NA	0.14	0.06	0.08	NA
			Call rate	3/3	3/3	3/3	0/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	0/3
MagNA Pure Compact Nucleic Acid Isolation Kit I	10^{-8}	1.3 x 10^{-4}	1	neg	39.07	neg	neg	neg	40.85	neg	neg	neg	neg	neg	neg	32.14	28.05	25.05	neg
			2	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	32.38	28.02	25.08	neg
			3	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	32.35	28.14	25.09	neg
			Ave	NA	39.07	NA	NA	NA	40.85	NA	NA	NA	NA	NA	NA	32.29	28.07	25.07	NA
			SD	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.13	0.06	0.02	NA
			Call rate	0/3	1/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3	3/3	3/3	0/3

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, NCV.N2 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, other coronaviruses or human microflora that would predict potential false positive rRT-PCR results.

Common gastrointestinal flora covered in the *in silico* cross-reactivity analysis are presented in Table 13**.** No cross-reactivity is predicted based on this analysis.

Table 13: NCV-2012 rRT-PCR Assay primer/probe sequence identity with published human microflora bacterial strain genome sequences.

Bacteria (taxid)	Includes	Accession No.
Escherichia coli (taxid: 562)	*Escherichia coli* 0157	CP001368.1
Fusobacterium (taxid: 848)	*Fusobacterium varium*	NZ_GL987995
Enterobacter (taxid: 547)	*Enterobacter cloacae*	NC_014121.1
Enterococcus (taxid: 1350)	*Entercoccus hirae*	CP003504.1
Lactobacillus (taxid: 1578)	*Lactobacillus acidophilus*	NZ_GG669566.1
	Lactobacillus reuteri	NC_010609.1
Bacillus/Clostridium group (taxid: 1239)	*Clostridium perfringens*	NC_008261.1
	Bacillus cereus	NC_003909.8
Bacteroidetes (taxid: 976)	*Parabacteroides* (formally *Bacteriodies*) *merdae*	NZ_JH976453.1
Bifidobacterium (taxid: 1678)	*Bifidobacterium adolescentis*	NC_008618.1
	Bifidobacterium longum	NC_017219.1
Ruminococcus	*Ruminococcus bromii*	NC_021013.1

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 and SARS-CoV, 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 14: NCV-2012 rRT-PCR Assay Viral Cross-reactivity

Virus (strain)[a]	NCV.upE	NCV.N2	NCV.N3
Adenovirus C1 (Ad.71)	Neg	Neg	Neg
Coronavirus 229E	Neg	Neg	Neg
Coronavirus OC43	Neg	Neg	Neg
Coronavirus SARS (Urbani)	Neg	Neg	Neg
Coronavirus HKU1	Neg	Neg	Neg
Coronavirus NL63	Neg	Neg	Neg
Enterovirus 68	Neg	Neg	Neg
HMPV (CAN 99-81)	Neg	Neg	Neg
Influenza A H1N1 (A/India/12)	Neg	Neg	Neg
Influenza A H3N1 (A/Texas/12)	Neg	Neg	Neg
Influenza B (B/Massachusetts/99)	Neg	Neg	Neg
Parainfluenza 1 (C35)	Neg	Neg	Neg
Parainfluenza 2 (Greer)	Neg	Neg	Neg
Parainfluenza 3 (C-43)	Neg	Neg	Neg
Parainfluenza 4a (M-25)	Neg	Neg	Neg
Parainfluenza 4b (CH 19503)	Neg	Neg	Neg
Parechovirus 1b	Neg	Neg	Neg
Respiratory syncytial virus A (Long)	Neg	Neg	Neg
Rhinovirus 1A	Neg	Neg	Neg
Pooled human nasal wash [b]	Neg	Neg	Neg

[a] All strains were identified using CDC-developed real-time RT-PCR assays (for other human respiratory pathogens) and genome sequencing.
[b] Pooled nasal wash specimens from 20 consenting healthy new military recruits to represent diverse microbial flora in the human respiratory tract.

Stool Matrix Cross-reactivity

As presented in the Clinical Performance section (above), all three NCV2012 rRT-PCR Assay primer/probe sets were tested against 51 negative stool specimens collected from patients exhibiting a symptom or symptoms associated with MERS-CoV infection. No false positive results were generated during that evaluation.

Contact Information

For questions or additional information, please contact:

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