Ebola Virus NP 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 NP protein of the Ebola virus (species *Zaire ebolavirus*). Current information on Ebola virus, including case definitions, is available at http://www.cdc.gov/vhf/ebola/index.html.

Intended Use

The Ebola Virus NP Real-time RT-PCR Assay (EBOV NP rRT-PCR) is intended for the in vitro qualitative detection of Ebola virus RNA (species *Zaire ebolavirus* and hereafter referred to as Ebola virus) in clinical specimens, including whole blood, serum, plasma, and urine, from individuals meeting Ebola virus clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with Ebola, contact with a probable or confirmed Ebola virus case, history of travel to geographic locations where Ebola virus cases were detected, or other epidemiologic links for which Ebola virus testing may be indicated as part of a public health investigation), in qualified laboratories designated by the Centers for Disease Control and Prevention (CDC).

Testing with the EBOV NP rRT-PCR should not be performed unless the patient meets clinical and/or epidemiological criteria for testing suspect specimens. Current information on Ebola virus, including case definitions, is available at http://www.cdc.gov/vhf/ebola/index.html.

Assay results are for the presumptive identification of Ebola virus and intended for use as part of a multi-test algorithm to detect the presence of Ebola virus RNA. Laboratories are required to report results to the appropriate public health authorities. Within the United States, results must be reported to CDC. The definitive identification of Ebola virus requires additional testing to be performed by CDC or by designated laboratories in consultation with CDC. The diagnosis of Ebola must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence, in addition to the detection of Ebola virus RNA.

Negative EBOV NP rRT-PCR results do not preclude Ebola virus infection and should not be used as the sole basis for patient management decisions.

The EBOV NP rRT-PCR is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The level of Ebola virus that would be present in whole blood, serum, or plasma specimens from individuals with early systemic infection is variable but generally positive at the time of symptom onset. Due to the difficulty in obtaining positive clinical specimens, only limited evaluation of the EBOV NP rRT-PCR has been made with specimens from individuals with Ebola virus infection.

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

Specimens

Biosafety information: Refer to CDC *Interim Guidance for Specimen Collection, Transport, Testing, and Submission for Patients with Suspected Infection with Ebola Virus Disease* (http://www.cdc.gov/vhf/ebola/hcp/interim-guidance-specimen-collection-submission-patients-suspected-infection-ebola.html)

General Information about Ebola virus

See: Ebola (Ebola Virus Disease) (http://www.cdc.gov/vhf/ebola/index.html)

Acceptable Specimens

- Blood specimens:
  - Whole blood
  - Serum
  - Plasma
- Urine
  NOTE: Urine should not be the sole specimen tested from a patient. If a urine specimen from a patient is tested, it should be tested alongside a blood specimen from the patient.

Specimen Collection

See: *Interim Guidance for Specimen Collection, Transport, Testing, and Submission for Patients with Suspected Infection with Ebola Virus Disease* (http://www.cdc.gov/vhf/ebola/hcp/interim-guidance-specimen-collection-submission-patients-suspected-infection-ebola.html)

Specimen Handling and Storage

- Whole blood can be stored for up to 7 days at 2-8°C prior to extraction.
  - Serum, plasma and urine may be frozen if a delay in extraction is anticipated. Specimens should be frozen at ≤ -70°C, if available.
- RNA should be stored at 2-8°C once extracted.
- If PCR cannot be performed the same day as specimen extraction, RNA should be stored at -70°C or lower.

Equipment and Consumables

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

Materials provided by CDC

- Ebola Virus NP Real-time RT-PCR Primer and Probe Set (CDC; Catalog #KT0152). Refer to product insert for storage and expiration information. Set includes 2 sets of primers and FAM-labeled probes:
    - NP2 (forward primer [NP2-F], reverse primer [NP2-R] and probe [NP2-P])
    - RNase P (RP) (forward primer [RP-F], reverse primer [RP-R] and probe [RP-P])

Materials provided by CDC, but not included in kit

- EBOV NP rRT-PCR Assay Positive Control (CDC; catalog #KT0155) (1 mL/vial)
- Human Specimen Control (HSC) negative extraction control (CDC; catalog #KT0156 (500 µL/vial)

Materials required but not provided

- SuperScript™ III Platinum® One-Step qRT-PCR Kit (Invitrogen; catalog #11732-020 or #11732-088)
- Molecular grade water, nuclease-free
- Extraction reagents (for use with the Dynal BeadRetriever™ System):
    - MagMax Pathogen RNA/DNA kit (Life Technologies; catalog #4462359)
    - Isopropanol
    - Ethanol

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)
- 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 Adhesive Film Kit (Applied Biosystems; catalog # 4311971 or #4360954)
- 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 #4406985);
- Extraction instrument:
  Dynal BeadRetriever™ System (Life Technologies; catalog #159-50)

Quality Control

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

General Considerations

- Personnel must be familiar with the protocol and instruments used.
- Maintain separate areas and dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips, gowns and gloves) for
  - assay reagent setup,
  - handling of extracted nucleic acids and
  - rRT-PCR amplification.
- Work flow must always be from the clean area to the dirty area.
- Wear clean, previously unworn, disposable gowns and new, powder-free gloves during assay reagent setup and handling of extracted nucleic acids. Change gloves whenever you suspect they may be contaminated.
- Store primer/probes and enzyme mastermix at appropriate temperatures (see product inserts). Do not use reagents beyond their expiration dates.
- Keep reagent tubes and reactions capped as much as possible.
- Clean surfaces using an acceptable surface decontaminant (see above).
- Do not bring extracted nucleic acid or PCR amplicons into the assay setup area. Do not wear any protective clothing that was worn in other parts of the lab. Only clean PPE should be worn in this area.
- Use aerosol barrier (filter) pipette tips only.
- Empty all trash daily.

Assay Controls

Assay Controls should be run concurrently with all test samples.

- EBOV NP rRT-PCR Positive Control. Used as a control for PCR reagent function.
- NTC – A known negative template control (sterile, nuclease-free water) added during rRT-PCR reaction set-up. Used as a control for PCR reagent function and cross-contamination.
- HSC – A known negative extraction control (human A549 cells) that is **extracted concurrently** with the test samples and included as a sample during rRT-PCR set-up. Should be negative for NP2 but positive for RP. Used as a control to demonstrate successful extraction and as a control for cross-contamination.
- RP – All clinical specimens should be tested for human RNAse P gene (using the RP primer and probe set included in the EBOV NP rRT-PCR kit) to control for specimen quality and extraction.

Table 1: Overview of positive and negative controls

Control Type	Control Name	Used to Monitor	NP2	RP	Expected C_T Values
Positive	EBOV NP rRT-PCR Pos Ctrl	Substantial reagent failure, including primer and probe integrity	+	+	<35 C_T for NP2 and RP
Negative	NTC	Reagent and/or environmental contamination during PCR set-up	-	-	None detected
Negative	HSC	Reagent and/or environmental contamination during extraction	-	+	None detected for NP2, RP C_T <35

Nucleic Acid Extraction

- Whole blood, serum, plasma and urine may be extracted using the Dynal BeadRetriever™ System.
- 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).
- See the Appendix for instructions for sample extraction.
- 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

Use EBOV NP2 and RP for specimen testing.

- If the NP2 is positive within 38 cycles, the specimen is considered positive for Ebola virus.
- If the NP2 assay is positive at or above 38 cycles, the specimen is considered equivocal.
- If the NP2 is negative and RP is positive (C_T <40), the specimen is considered negative for Ebola virus.
- If the NP2 and the RP are negative, the specimen is considered inconclusive for Ebola virus.
- If this test is utilized for a patient that has symptoms indicative of Ebola virus disease, whether the assay result is positive, negative or equivocal, contact CDC EOC (770-488-7000) for a consultation with a Subject Matter Expert.

rRT-PCR Assay

The EBOV NP rRT-PCR primer and probe set targets the coding region for the nucleoprotein of the Ebola virus and shows 100% homology with currently circulating and historical strains.

Stock Reagent Preparation

1. **Real-time Primers/Probes**
   - Ebola Virus NP Real-time RT-PCR Primer and Probe Set
     - Precautions:
       These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
     - Sterilely suspend lyophilized reagents in 0.25 mL PCR grade nuclease-free water (50X working concentration) and allow to rehydrate for 15 min at room temperature in the dark.
     - Each EBOV NP rRT-PCR primer and probe kit will contain material to perform 500 reactions. Store rehydrated aliquots of primers and probes at ≤ -20° C. Do not store in frost-free freezers. Rehydrated primers and probes may be stored frozen for up to 12 months. For complete information on storage conditions, see package insert.
     - RNase P Primer and Probe – Sterilely suspend lyophilized reagents in 0.25 mL nuclease-free water and allow to rehydrate for 15 minutes at room temperature. Store rehydrated aliquots of primers and probes at ≤ -20° C. Do not store in frost free freezers. Rehydrated primers and probes may be stored frozen for up to 12 months. For complete information on storage conditions, see package insert.
2. **HSC negative extraction control**
   - Human Specimen Control must be extracted and processed with each batch of specimens to be tested.
   - The final volume of eluted RNA should equal the volume of extracted control material. For example, 100 µL of control material should result in 100 µL of RNA extract.
   - Do not dilute extracted RNA before testing.
   - Add 5 µL of extracted RNA to each extraction control (HSC in Fig 2).
   - Expected Ct value <35.
   - For complete use and storage conditions, see package insert.
3. **Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System**
   - Place Invitrogen 2X PCR Master Mix and Superscript III RT/Platinum Taq enzyme mix in a cold rack at 2-8°C.
   - Completely thaw the 2X PCR Master Mix vial.
   - Mix the 2X PCR Master Mix by inversion 10 times.
   - Pulse centrifuge 2X PCR Master Mix and Superscript III RT/Platinum Taq enzyme mix then place in cold rack.

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

5. **EBOV NP rRT-PCR Positive Control** (EBOV-NP-PC)
    - 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.
    - Centrifuge tube in microcentrifuge at maximum speed. Ensure pellet (pellet will be a bright pink color) is at the bottom.
    - Add 1500 µL of cold nuclease-free water and mix gently. Centrifuge tube. Pellet is in solution when no pink precipitate is visible.
    - To ensure complete rehydration, hold tube on ice for 20 minutes before handling further.
    - Aliquot in 15 µL volumes and store at ≤ -70°C. These aliquots are the working concentrations.
    - Thaw a single working dilution aliquot for each experiment. Discard any unused portion of the aliquot. Do not refreeze.
    - Add 5 µL of positive control to each specific positive control reaction.
    - Expected Ct value <35.
    - For complete use and storage conditions, see package insert.

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	15 min
Taq inhibitor inactivation	1	95°C	2 min
PCR Amplification	40	95°C 55°C	15 sec 60 sec

Instrument Settings

Detector: FAM
Quencher: None
Passive Reference: ROX
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, HSC and EBOV NP rRT-PCR positive control must be included in each run.

1. In the reagent set-up room clean hood, place rRT-PCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.
2. Thaw 2X Reaction Mix prior to use.
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, EBOV NP-PC, and RP reactions and for pipetting error. Use the following guide to determine N:
   - If the number of samples (n) including controls equals 1 through 14, then N = n + 1
   - If the number of samples (n) including controls is greater than 15, 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 = number of reactions).

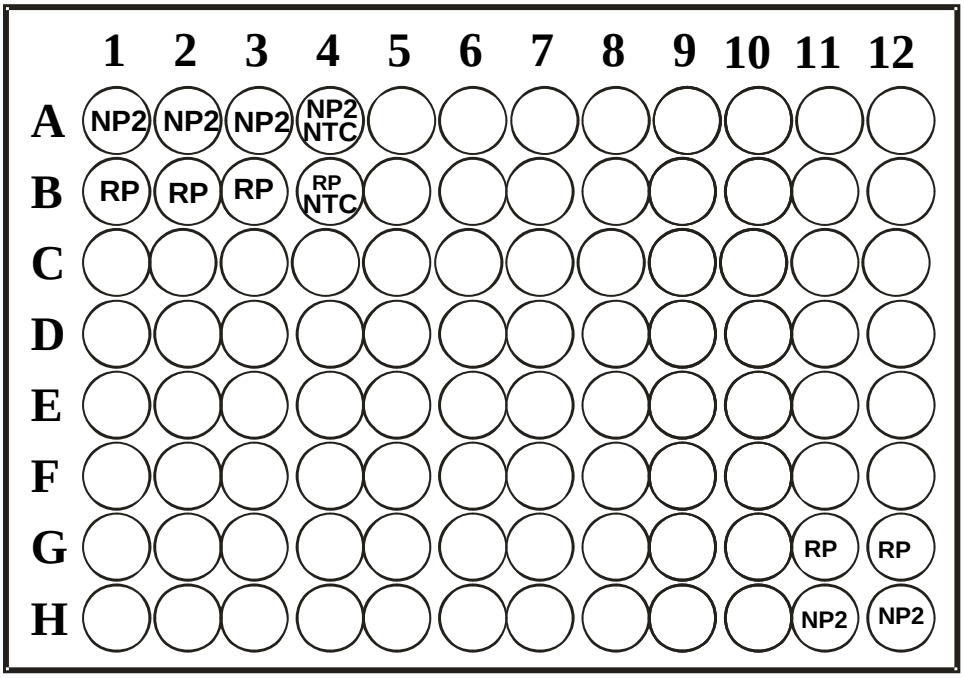
Table 3: rRT-PCR Reaction Mix

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
Forward primer (50µM stock)	= N x 0.50 µL
Reverse primer (50 µM stock)	= N x 0.50 µL
Probe (5 µM)	= N x 0.50 µL
ROX	= N x 0.05 µL
Water, nuclease-free	= N x 5.45 µL
Total volume	**= N x 20.00 µL**
Sample RNA	**5 µL**

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

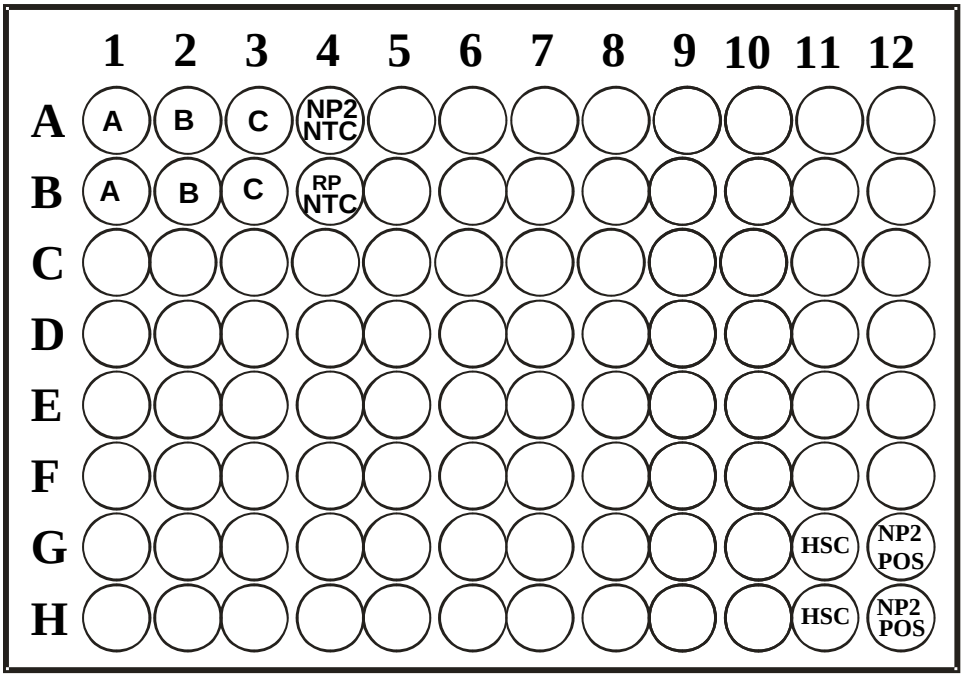
9. Add 20 µL of master mix into each well of a chilled optical plate as shown in examples below:

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



Ebola NP (NP2); RNase P (RP); No template reaction mix controls (NTC);

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



Ebola NP (NP2); RNase P (RP); No template reaction mix controls (NTC); Negative extraction control (HSC); Ebola NP positive control (NP2 POS)

10. Before moving the plate to the nucleic acid handling area, add 5 µL of nuclease-free water to the NTC wells.
11. Gently place the optical adhesive cover atop the plate but **DO NOT** peel off the protective strip and affix yet.
12. Move plate to the nucleic acid handling area on cold block.
13. Vortex sample extracts and HSC briefly and centrifuge for 5 seconds.
14. Set up the extracted DNA sample reactions.
    (a) Remove the optical adhesive cover.
    (b) Pipette 5 µL of the first sample into all the wells labeled for that sample. For example, dispense 5 µL of sample A into the wells labeled "A" in Fig. 2.
    (c) If the sample volume is less than 5 µL, add the necessary volume of PCR-grade water to ensure 5 µL template volume. This will ensure a total reaction volume of 25 µL.
15. Change tips after each sample addition.
16. Continue with the remaining samples. Change gloves between samples if you suspect they have become contaminated.
17. Vortex the EBOV NP positive control and pipette 5 µL into NP2 POS wells.
18. Pipette 5 µL of the extracted HSC RNA into the wells labeled HSC (G11 and H11 in Fig 2).
19. Gently place new optical adhesive cover on plate and seal.
20. Transport the plate to the amplification area on cold block.
21. Look for bubbles at the bottom of the sample wells and liquid drops on the sides of the wells above the reaction mixtures. If either bubbles or drops are seen, gently tap the tray repeatedly until the bubbles are dispersed and drops have fallen back into the main reaction mixture. Alternatively, if your laboratory has a table-top centrifuge with a rotor able to accept plates, centrifuge the plate at 500 x *g* for 1 minute at room temperature. Be sure to use a balance plate, if necessary.
22. Place plate on pre-programed AB 7500 Fast Dx and start run.
22. For detailed instructions on launching and programming the Applied Biosystem 7500 Fast System software, refer to the Programming of the AB® 7500 Fast located under Documents/Instrument Programming and Maintenance on the LRN secure website.

Data Analysis

After completion of the run, save and analyze the data following the instrument manufacturer's instructions. Analyses should be performed using the Auto Ct setting included in the AB 7500 software. This can be accessed by clicking on Analysis Settings- Standard Curve.

Interpreting Test Results

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

1. EBOV NP Positive Controls should be **positive** and with C_T values within 35 cycles for all primer and probe sets.
    a. If EBOV NP positive controls are *negative*, the testing results for that plate are invalid.
        i. Repeat rRT-PCR test.
        ii. If repeat testing generates negative EBOV NP Positive Control results, contact CDC 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. HSC (extraction control) should be
    a. **Positive** with RP primer/probe set due to the human DNA in the HSC
    b. **Negative** with NP2 primer/probe set. A positive result with the HSC and NP2 primer/probes would indicate cross-contamination has occurred. If a positive result is obtained, followed the cleaning procedure described above in 2.a.
4. RP Assay for each specimen should be **positive (C_T <40)**.
    a. If RP Assay for a specimen sample is *negative* and the NP2 assay is *negative* for specimen samples:
        i. Report result as *Inconclusive*
        ii. Repeat rRT-PCR test of sample using RP and NP2 assay.
        iii. Repeat extraction from new specimen aliquot if RP Assay is *negative* for specimens after repeat testing.
        iv. After repeat extraction and repeat rRT-PCR testing, if NP2 is *positive*, consider the result a true *positive* and continue to follow the testing algorithm.
        v. If you are unable to resolve the results for this specimen, test other specimens from the patient, if available, or request the collection of additional specimens.
    b. If RP Assay for a specimen sample is *negative*, but NP2 is *positive* for specimen samples:
    Do not repeat rRT-PCR test and consider the results of the NP2 assay 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 (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.

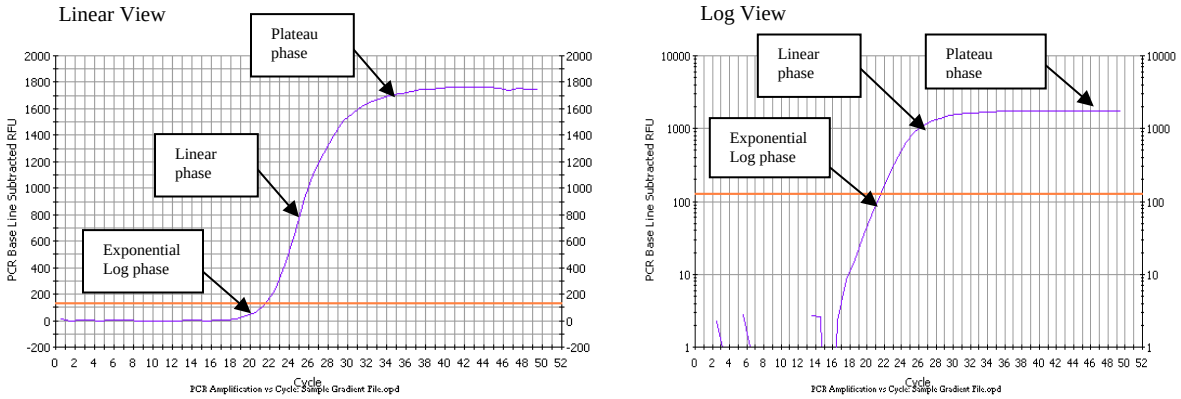


Figure 3: Linear and log views of PCR curves noting each stage of the amplification plots.

- For a sample to be a true positive, the curve must cross the threshold in a similar fashion as shown in Figure 3. 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.

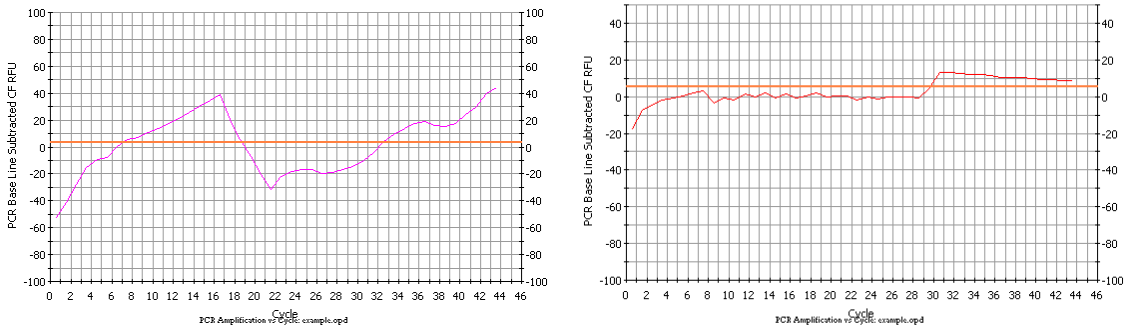


Figure 4: Examples of false positive curves.

- To better understand and evaluate challenging curves more effectively, use the background fluorescence view (Rn versus Cycle with AB 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 6 shows an amplification plot with 3 curves: a moderately weak positive (black), a very weak positive (red), and a negative control (blue). The weak positive is verified to be positive by the sharp increase in fluorescence seen in the background fluorescence view.

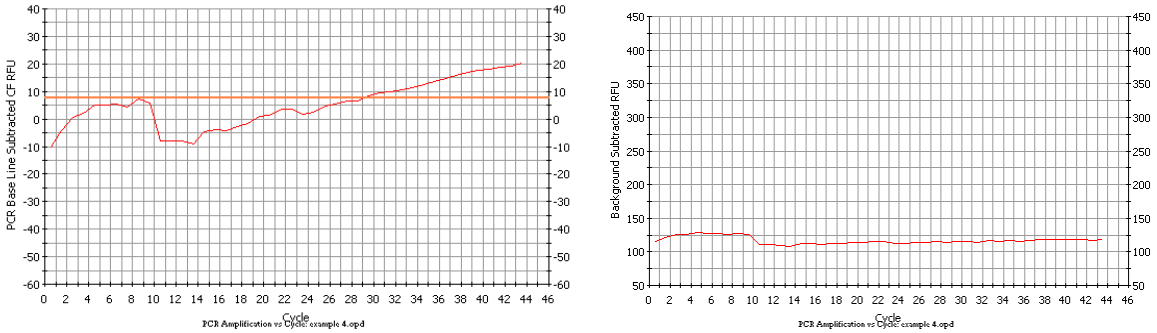


Figure 5: Amplification plot of a sample with a "wandering" curve (left) and the corresponding background fluorescence view (right).

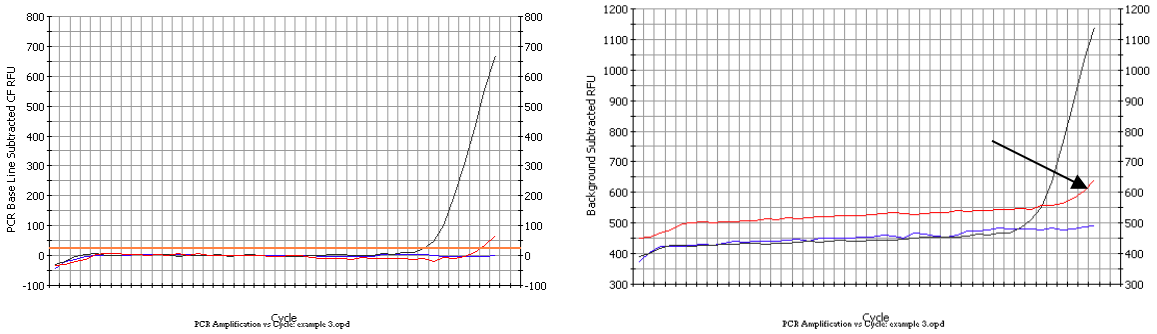


Figure 6: Amplification plot of three samples in the linear view (left) and the corresponding background fluorescence view (right).

- 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 38, 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.

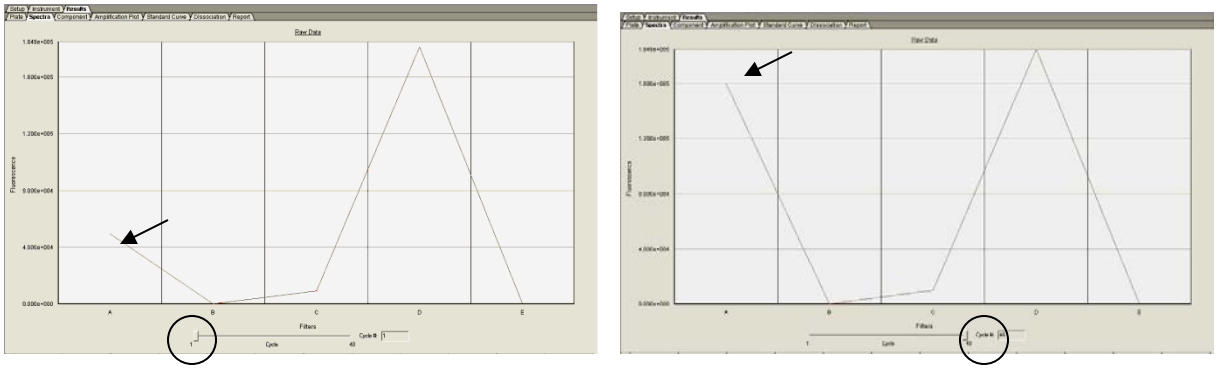


Figure 7: Spectra component of a positive sample. Left screenshot shows fluorescence at cycle 1 and right screenshot shows fluorescence at cycle 40.

- 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 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 ($C_T \geq 35$). 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.
    - During convalescence from Ebola virus infection, the Ct values will be weak and will NOT require repeat testing if all controls react as expected.
    - If repeat testing of a weak specimen is necessary, it is important to repeat the sample in replicates as a single repeat test run has a high likelihood of generating a discrepant result.
    - If re-extracting and re-testing the specimen, it may be helpful to elute in a lower volume to concentrate the sample.
    - Contact CDC for guidance, to help determine if repeat testing may be warranted and to discuss additional testing strategies as appropriate.

Overall Test Interpretation and Reporting Instructions

Table 4: EBOV NP2 rRT-PCR Test Interpretation and Reporting Instructions for testing conducted within the United States.

NP2	RP	Interpretation	Reporting	Actions
-	+	EBOV NP Negative	Ebola virus RNA not detected by rRT-PCR	Report results to CDC
-	-	Inconclusive	Inconclusive for EBOV virus RNA by rRT-PCR. An inconclusive result may occur in the case of an inadequate specimen.	If there are no additional specimens available for the patient, request collection of additional specimens. Report results to CDC.
+ Ct <38	+/-	EBOV NP Presumptive Positive	Ebola virus RNA detected by rRT-PCR. This is a presumptive result. Additional testing required to support definitive identification of Ebola virus.	Contact CDC for consultation and to coordinate additional testing. Report results to CDC.
+ Ct ≥38	+/-	Equivocal	EBOV NP rRT-PCR testing was equivocal. Additional analysis may be required.	Contact CDC for consultation. Report results to CDC.

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

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

Information on additional testing required for the definitive identification of Ebola virus may be found in *Interim Guidance for Specimen Collection, Transport, Testing, and Submission for Patients with Suspected Infection with Ebola Virus Disease* (http://www.cdc.gov/vhf/ebola/hcp/interim-guidance-specimen-collection-submission-patients-suspected-infection-ebola.html)

Assay Limitations, Warnings and Precautions

Interpretation of rRT-PCR test results must account for the possibility of false-negative and false-positive results. False-negative results can arise from:

- poor sample collection or
- degradation of the viral RNA during shipping or storage or
- specimen collection conducted prior to symptom onset.
- failure to follow the authorized assay procedures
- failure to use authorized extraction kit and platform

Application of appropriate assay controls that identify poor-quality specimens (such as RNase P) and adherence to CDC guidelines for Ebola testing (www.cdc.gov/vhf/ebola/index.html) can help avoid most false-negative results.

A high C_T observed for a specimen taken during early onset of disease should be followed up with another specimen taken 24-48 hours later. Refer to CDC Ebola laboratory guidance for current advice (www.cdc.gov/vhf/ebola/index.html) and consultation instructions.

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

The impact of the administration of Ebola virus vaccines and/or therapeutics on the ability to detect Ebola virus RNA in patient specimens has not been evaluated.

Negative results do not preclude infection with Ebola virus and should not be used as the sole basis of a patient treatment/management decision. All results should be interpreted by a trained professional in conjunction with review of the patient's history and clinical signs and symptoms.

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

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

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

Extraction of nucleic acid from clinical specimens must be performed with the specified MagMax Pathogen RNA/DNA kit using the Dynal BeadRetriever™ System. Other extraction kits have not been evaluated for use with this assay.

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

Performance Characteristics

1.Analytical sensitivity evaluation

Limit of Detection – Estimation in PCR Grade Water

Analytical evaluation of the EBOV NP rRT-PCR primer and probe set sensitivity across Ebola virus strains (species *Zaire ebolavirus*) was performed using three strains: EboZ Mayinga 1976, EboZ Kikwit 1995, EboZ Gabon 2002. Each strain was prepared in serial dilution in PCR grade water as live virus, extracted and tested. Aliquots of each stock were then inactivated by gamma irradiation, prepared in serial dilution in PCR grade water, extracted and tested. All materials in this study were extracted using a manual method using Tripure inactivation followed by isolation using the Qiagen RNeasy kit. RT-PCR was performed using the AB 7500 Fast Dx as described in the instructions for use. NP2 performed similarly with live Ebola virus across all three strains, with an estimated limit of detection (LoD) of approximately 3-30 $TCID_{50}$/reaction. Inactivation by gamma irradiation was demonstrated to impact the sensitivity of the assay. Mayinga and Gabon strains saw 10-fold and 100-fold shifts in estimated LoD, respectively. The Kikwit strain maintained the same estimated LoD (10 $TCID_{50}$/rxn), but demonstrated a C_T value shift of approximately 2 cycles at that concentration.

Inactivated stock of the Mayinga 1976 strain was selected for use in blood and urine limit of detection and contrived specimen testing. Data from the LoD evaluation are presented in Tables 5, 6, and 7 below.

Table 5: NP2 Analytical Ebola virus sensitivity evaluation (Mayinga 1976)

$TCID_{50}$/rxn	Live virus (C_T values)			Inactivated virus (C_T values)		
300000	20	21	20	20	20	20
30000	24	24	25	26	26	26
3000	28	28	28	32	32	33
300	32	32	32	34	34	34
30	35	35	35	39	und	38
3	38	40	39	und	und	und
0.3	und	und	und	und	und	und
0.03	und	und	und	und	und	und
Neg. ctrl.	und	und	und	und	und	und

*Und = not detected

Table 6: NP2 Analytical Ebola virus sensitivity evaluation (Kikwit 1995)

$TCID_{50}$/rxn	Live virus (C_T values)			Inactivated virus (C_T values)		
10000	22	22	22	23	23	23
1000	26	26	26	28	29	28
100	30	30	30	32	32	32
10	33	34	33	35	36	35
1	39	37	und	und	und	und
0.1	und	und	und	und	und	und
0.01	und	und	und	und	und	und
0.001	und	und	und	und	und	und
Neg. ctrl.	und	und	und	und	und	und

Table 7: NP2 Analytical Ebola virus sensitivity evaluation (Gabon 2002)

$TCID_{50}$/rxn	Live virus (C_T values)			Inactivated virus (C_T values)		
30000	20	20	21	22	22	22
3000	24	24	25	27	27	27
300	28	28	28	34	34	33
30	31	31	32	38	37	37
3	35	35	35	und	und	und
0.3	und	und	und	und	und	und
0.03	und	und	und	und	und	und
0.003	und	und	und	und	und	und
Neg. ctrl.	und	und	und	und	und	und

Limit of Detection – Confirmation in Whole Blood

The limit of detection of the EBOV NP rRT-PCR primer and probe set in whole blood was confirmed to be 30 $TCID_{50}$/reaction. The limit of detection was determined by testing four pools of spiked whole blood. Each of the four pools was spiked with inactivated Ebola virus (Mayinga 1976) at a different concentration (300 $TCID_{50}$/rxn, 30 $TCID_{50}$/rxn, 3 $TCID_{50}$/rxn, and 0.3 $TCID_{50}$/rxn). Each pool was extracted 20 times using the Dynal BeadRetriever protocol with the MagMAX extraction kit. Each extract was then tested once by the EBOV NP rRT-PCR as described in the instructions for use. Results for the EBOV NP rRT-PCR primer and probe set are presented in Table 8. Those for RP are presented in Table 9.

Table 8: Limit of detection in whole blood – NP2 data

$TCID_{50}$/rxn	NP2 assay C_T values									
300	31	32	34	30	31	30	31	30	32	29
	30	30	30	30	30	29	30	30	30	30
30	33	36	34	35	35	34	34	33	36	34
	34	35	33	35	34	33	35	34	34	35
3	und	und	39	und	und	und	und	und	und	und
	und	39	und	und	und	und	und	40	und	und
0.3	und	und	und	und	und	und	und	und	und	und
	und	und	und	und	und	und	und	und	und	und
Neg. ctrl.	und	und	und	und						

Table 9: Limit of detection in whole blood – RP data

$TCID_{50}$/rxn	RP assay C_T values									
300	25	25	25	25	25	24	24	25	25	25
	25	25	25	25	25	24	24	25	25	25
30	24	25	25	25	25	24	24	24	24	24
	26	26	24	25	25	24	24	25	25	24
3	24	25	24	25	25	25	24	24	24	24
	25	25	24	25	25	25	24	24	24	24
0.3	25	25	26	25	25	25	24	24	23	25
	26	25	25	25	25	25	25	24	25	24
Neg. ctrl.	und	und	und	und						

Limit of Detection – Confirmation in Urine

The limit of detection of the EBOV NP rRT-PCR primer and probe set in urine was confirmed to be 30 $TCID_{50}$/reaction. The limit of detection was determined by testing four pools of spiked urine. The urine used for this evaluation was a leftover clinical specimen from a febrile pediatric patient. Each of the four pools was spiked with inactivated Ebola virus (Mayinga 1976) at a different concentration (300 $TCID_{50}$/rxn, 30 $TCID_{50}$/rxn, 3 $TCID_{50}$/rxn, and 0.3 $TCID_{50}$/rxn). Each pool was extracted 20 times using the Dynal BeadRetriever protocol with the MagMAX extraction kit. Each extract was then tested once by the EBOV NP rRT-PCR as described in the instructions for use. Results for the EBOV NP rRT-PCR primer and probe set are presented in Table 10. Those for RP are presented in Table 11.

Table 10: Limit of detection in urine – NP2 data

$TCID_{50}$/rxn	NP2 assay C_T values									
300	29	31	29	29	31	30	30	29	31	30
	28	28	28	28	28	28	28	28	28	29
30	32	31	31	31	31	31	31	32	32	34
	32	33	33	32	31	34	33	33	33	34
3	38	39	38	38	39	38	38	und	und	38
	39	38	38	37	39	37	38	39	38	38
0.3	und	40	40	und	38	40	37	und	und	38
	38	39	39	und	40	und	und	und	und	und
Neg. ctrl.	und	und	und	und						

Table 11: Limit of detection in urine – RP data

$TCID_{50}$/rxn	RP assay C_T values									
300	36	36	36	36	34	36	36	35	36	36
	38	35	35	36	35	36	38	35	35	35
30	36	36	36	35	35	36	36	35	35	35
	37	35	36	35	35	37	36	35	35	38
3	35	36	35	35	35	35	34	35	35	35
	35	36	35	36	35	36	35	36	35	36
0.3	34	35	36	34	35	35	34	35	35	35
	36	35	35	36	35	35	36	35	35	35
Neg. ctrl.	und	und	und	und						

2. Analytical Reactivity Evaluation – *In silico* Analysis

In silico analysis of the EBOV NP rRT-PCR Assay primer and probe sequences was performed to verify reagent sequence homology with the target region of five current and eight historical Ebola outbreak strains. All primer and probe sequences showed 100%

alignment, predicting no false negative results are likely to occur. Table 12 below contains a summary of the findings.

Table 12: *In silico* Reactivity based on sequence identity

Strain	Genbank #	Primer/Probe Sequence Identity		
		NP2-F	NP2-R	NP2-P
Liberia 2014		100%	100%	100%
SierraLeone 2014	KM233053	100%	100%	100%
SierraLeone 2014	KM233035	100%	100%	100%
Guinea 2014	KJ660347	100%	100%	100%
Guinea 2014	KJ660346	100%	100%	100%
DRC Luebo 2007	KC242788	100%	100%	100%
DRC Luebo 2007	KC242784	100%	100%	100%
Gabon 1996	KC242793	100%	100%	100%
Gabon 1996	KC242794	100%	100%	100%
DRC Kikwit 1995	AY354458	100%	100%	100%
DRC Kikwit 1995	KC242796	100%	100%	100%
DRC Mayinga 1976	KC242791	100%	100%	100%
DRC Mayinga 1976	NC_002549	100%	100%	100%

3. Analytical Specificity

The EBOV NP rRT-PCR primer and probe set has been observed to be 100% specific to Ebola virus (*Zaire ebolavirus*). Specificity was evaluated through testing of a set of near neighbor viruses. Each live virus was prepared at a concentration of 10^5 $TCID_{50}$/mL, extracted and tested as described in the EBOV NP rRT-PCR Instructions for Use in quadruplicate. Included in the PCR testing were a positive control (EboZ Mayinga 1976) and a negative extraction control.

Table 13: NP2 Specificity

Virus	NP2 Results (C_T values)			
	Replicate 1	Replicate 2	Replicate 3	Replicate 4
EboZ Mayinga 1976	26	26	27	27
Neg. ctrl.	und	und	und	und
Sudan virus	und	und	und	und
Bundibugyo	und	und	und	und
Reston Ebolavirus	und	und	und	und
Taï Forest virus	und	und	und	und
Marburg virus	und	und	und	und
RAVN Marburgvirus	und	und	und	und
Rift Valley Fever virus	und	und	und	und
Crimean Congo Hemorrhagic Fever virus	und	und	und	und
Lassa virus	und	und	und	und

Additional evaluation of the analytical specificity of the EBOV NP rRT-PCR was performed through *in silico* analysis of the EBOV NP rRT-PCR primer and probe sequences against other common causes of fever in persons returning from Africa as well as hemorrhagic fever-causing viruses. BLASTn analysis queries of the EBOV NP rRT-PCR Assay primers and probe were performed against the GenBank public domain nucleotide sequences and showed no significant combined homologies (primer target and probe target) with other conditions that would predict potential false positive rRT-PCR results. Conditions and associated causative agents covered in the *in silico* specificity analysis are presented in Table 14.

Table 14: *In silico* Specificity

Disease/condition	Organism (taxid)	Agent
Malaria	5883	Plasmodium falciparum
Typhoid Fever	90370	Salmonella typhi
Meningococcal disease	487	Neisseria meningitidis
Pneumonia	1313	Streptococcus pneumoniae
Pneumonia	727	Hemophilus influenzae
Dengue	12637	Dengue virus
African trypanosomiasis	31285	Trypanosoma brucei gambiense
Tickborne rickettsiae	782	Rickettsia prowazekii
Tickborne rickettsiae	785	Rickettsia typhi
Tickborne rickettsiae	781	Rickettsia conorii
Tickborne rickettsiae	35788	Rickettsia africae
Acute schistosomasis	6183	Schistosoma mansoni
Acute schistosomasis	6187	Schistosoma intercalatum
Acute schistosomasis	6185	Schistosoma heamatobium
Influenza	197911	Influenza virus A
Influenza	197912	Influenza virus B
Leptospirosis	171	Leptospira genus
Lassa Fever	11620	Lassa virus
VHF	11269	Marburg virus
VHF	186539	Reston virus
VHF	186540	Sudan virus
VHF	565995	Bundibugyo virus
VHF	186541	Taï Forest virus
VHF	11593	Crimean Congo Hemorrhagic Fever

4. Contrived Clinical Specimen Studies

Contrived Urine Specimens

Clinical evaluation of the EBOV NP rRT-PCR primer and probe set was performed using the Mayinga 1976 strain of species *Zaire ebolavirus*. Urine specimens from 50 febrile pediatric patients were separated into two 100µL aliquots. One 100µL aliquot was left neat (no virus added) and the other 100µL aliquot was spiked with either a low concentration (100 $TCID_{50}$/rxn; approximately 3x LoD) or high concentration (1,000 $TCID_{50}$/rxn; approximately 30x LoD) of inactivated Ebola virus. These 100 specimens were then blinded and passed off for testing to a technician not involved in specimen preparation. Each specimen was extracted using the Dynal BeadRetriever instrument, and tested using the AB 7500 Fast Dx as described in the instructions for use. A summary of the data generated is presented in Table 15. One inconclusive result and one false-negative result were generated.

Table 15: NP2 Contrived Urine Specimens: Summary of Results

$TCID_{50}$/rxn	Total #	Positive	Equivocal	Inconclusive	Negative
Low Concentration ($TCID_{50}$ = 100)	25	24	0	0	1
High Concentration ($TCID_{50}$ = 1000)	25	25	0	0	0
No Virus Added	50	0	0	1	49

Positive Percent Agreement: 98% (49/50) (95% CI: 90%, 100%)
Negative Percent Agreement: 100% (49/49) (95% CI: 93%, 100%)

Contrived Whole Blood (EDTA)

Clinical evaluation of the EBOV NP rRT-PCR primer and probe set was performed using the Mayinga 1976 strain of species *Zaire ebolavirus*. Whole blood (EDTA) specimens from 50 donors were separated into two 100µL aliquots. One 100µL aliquot was left neat (no virus added) and the other 100µL aliquot was spiked with either a low concentration (100 $TCID_{50}$/rxn; approximately 3x LoD) or high concentration (1,000 $TCID_{50}$/rxn; approximately 30x LoD) of inactivated Ebola virus. These 100 specimens were then blinded and passed off for testing to a technician not involved in specimen preparation. Each specimen was extracted using the Dynal BeadRetriever instrument, and tested using the AB 7500 Fast Dx as described in the instructions for use. A summary of the data generated is presented in Table 16. No erroneous results were obtained.

Table 16: NP2 Contrived Whole Blood (EDTA) Specimens: Summary of Results

$TCID_{50}$/rxn	Total #	Positive	Equivocal	Inconclusive	Negative
Low Concentration ($TCID_{50}$ = 100)	25	25	0	0	0
High Concentration ($TCID_{50}$ = 1000)	25	25	0	0	0
No Virus Added	50	0	0	0	50

Positive Percent Agreement: 100% (50/50) (95% CI: 93%, 100%)

Negative Percent Agreement: 100% (50/50) (95% CI: 93%, 100%)

Contact Information

For questions or additional information, please contact:

Within the United States:

Laboratory Response Network Helpdesk
LRN@cdc.gov

International users:

Centers for Disease Control and Prevention
EBOV@cdc.gov

Appendix: RNA Extraction Protocol Using the Dynal BeadRetriever™ System

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, but Not Included in Kit

- Human Specimen Control (HSC) negative extraction control (CDC; catalog # HS0096 (500 µL/vial)

Materials Required but Not Provided

- 100 % isopropanol
- 100 % ethanol
- MagMax Pathogen RNA/DNA kit (Life Technologies, Cat # 4462359)

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)
- Dynal BeadRetriever™ System (Life Technologies, catalog #159-50)
- Tip combs
- 5-well reagent reservoir
- Disposable, powder-free gloves and surgical gowns
- Laboratory marking pen
- P2/P10, P200, and P1000 aerosol barrier pipette tips
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- 1.7 mL microcentrifuge tubes (Eppendorf or equivalent)
- Racks for 1.7 mL microcentrifuge tubes
- 2 x 96-well -20 °C cold blocks
- Vortex

Nucleic Acid Extraction

Biosafety information: Refer to CDC *Interim Guidance for Specimen Collection, Transport, Testing, and Submission for Patients with Suspected Infection with Ebola Virus Disease* (http://www.cdc.gov/vhf/ebola/hcp/interim-guidance-specimen-collection-submission-patients-suspected-infection-ebola.html)

- Whole blood, serum, plasma and urine may be extracted using the Dynal BeadRetriever™ System.
- Sample extractions **must** yield RNA or total nucleic acid of sufficient volume to cover all rRT-PCR assays (a minimum of 60µL is recommended).
- HSC should be included in each extraction run as a negative extraction control.
- 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.

Extraction Reagent Preparation

All reagent preparation must be performed in a CLEAN area.

1. Wash Solution 1: Add 125 mL of 100% **isopropanol** to the Wash Solution 1 Concentrate. Mix well by shaking and mark the bottle to indicate that this has been done. Store at room temperature for up to 6 weeks.
2. Wash Solution 2: Add 232 mL of 100% **ethanol** to each of the Wash Solution 2 Concentrate bottles. Mix well by shaking and mark on the bottle to indicate that this has been done. Store at room temperature for up to 6 weeks.
3. Lysis/binding solution: Prepare enough of the Lysis/binding solution for all specimens to be extracted in the run. Remember to prepare enough solution for extraction of one HSC alongside the specimens in each extraction run.
    a. Label one appropriately sized tube: Lysis/binding Solution
    b. For each specimen, add 200 µL lysis binding concentrate + 200 µL 100% **isopropanol** + 2 µL carrier RNA.
    c. Store at room temperature for up to 8 hours.
4. Bead mix:
    a. Label one 1.7 mL Eppendorf tube: Bead Solution
    b. Votex beads thoroughly before making dilution.
    c. For each specimen, add 10 µL of beads + 10 µL of lysis/binding enhancer to tube.
    d. Store on ice for up to 4 hours.
5. Human Specimen Control (HSC):
    a. Prepare as instructed in product insert.

Equipment Preparation

1. Turn on the Dynal BeadRetriever™ instrument.
2. Choose Program "custom 1836_bloodv2".
   NOTE: This is a custom program and must be installed by the instrument manufacturer.
3. Add tip combs to the slot below the magnets until you feel a click.
4. Add the 5 well reagents reservoirs to the metal tray according to the number of samples. Label the tab of the reservoir with the sample number. The tab should face to the left side of the machine. Label each of one of the 5-well reservoirs starting from left to right with #1-5.

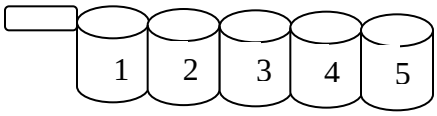
Extraction Program for the Dynal BeadRetriever™ System

Table 1A: Extraction Protocol (programmed into BeadRetriever by manufacturer)

Protocol Name			**custom 1836_bloodv2**
Machine configuration			BeadRetriever
Tip Comb / Cat#			
Sample Volume (µl)			100
Well Position		**Reagents addition Order and Usage**	
		Sample	100 µL
		Beads	20 µL
1	Lysis/Binding	Lysis/Binding Soln	400 µL
2	1st Wash 1	Wash Soln 1	300 µL
3	2nd Wash 1	Wash Soln 1	300 µL
4	Wash 2	Wash Soln 2	900 µL
5	Elution	Elution Buffer	90 µL

Extraction Protocol

Note: HSC extraction controls must be included in each run. Treat HSC as another specimen to be extracted.



1. Add the following reagents to the sample reservoir:
   a. Aliquot **300 µL Wash Solution 1** to **well #2** of each sample reservoir.
   a. Aliquot **300 µL** of **Wash Solution 1** to **well #3** of each sample reservoir
   b. Aliquot **900 µL** of **Wash Solution 2** to **well #4** of each sample reservoir
   c. Aliquot **90 µL** of **Elution Solution** to **well #5** of each sample reservoir

2. Prepare specimens for extraction:
    a. Label a 1.7 mL Eppendorf tube for each specimen and for the HSC.
    b. Pipette **100 µL** of the appropriate **specimen** into each tube
    c. Add to **400 µL** of **Lysis/binding Solution**.to each tube. Pipette up and down to mix.

    NOTE: This mixture ratio of lysis buffer and sample has been shown to inactivate Ebola virus.

3. Once samples are ready, vortex the Bead mix then aliquot **20 µL** of the **Bead Mix** into **well #1** of each sample reservoir.

4. Load specimens:
    NOTE: Do not mix samples with the bead mix in the well; just add the samples directly onto the bead mix.
    a. Add the **HSC** into **well #1** of the appropriate reservoir
    b. Add **specimens** (pre-mixed with lysis/binding solution) to well #1 of the appropriate reservoirs. Volume added should be **500 µL**.

5. Press START once all samples and reagents are loaded.

6. Press START again to start run.

7. Label a sterile 1.7 mL Eppendorf tube for each sample being extracted + 1 for the HSC.

8. When run is complete, carefully remove the RNA from **well #5** and place into appropriately labeled 1.7 mL tube and place on ice.
    NOTE: If unable to perform rRT-PCR immediately store RNA at ≤-70°C.