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 *Guidance for Collection, Transport and Submission of Specimens for Ebola Virus Testing* (http://www.cdc.gov/vhf/ebola/healthcare-us/laboratories/specimens.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: *Guidance for Collection, Transport and Submission of Specimens for Ebola Virus Testing* (http://www.cdc.gov/vhf/ebola/healthcare-us/laboratories/specimens.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 must be stored at 2-8°C once extracted.
- If PCR cannot be performed the same day as specimen extraction, RNA must 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
- Reagents required for automated extraction (for use with the Dynal BeadRetriever™ System and/or the MagMAX™ Express-96 Deep Well Magnetic Particle Processor):
  - MagMax Pathogen RNA/DNA kit (Life Technologies; catalog #4462359)
  - Isopropanol
  - Ethanol
- Reagents required for manual extraction:
  - QIAamp DSP Viral RNA Mini Kit (QIAGEN; Catalog #61904)
  - TRIzol® LS reagent (Life Technologies Catalog #10296-010, 100 mL) or TriPure Isolation Reagent (Roche Life Science Catalog #11667165001, 200 mL)

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
- 1.7 mL microcentrifuge tubes
- Vortex mixer
- Microcentrifuge
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- Multichannel micropipettes (5-50 µL)
- Racks for 1.7 mL microcentrifuge tubes
- 2 x 96-well -20°C cold blocks

- Real-time PCR instrument and consumables:
    - 7500 Fast Dx Real-Time PCR Systems (Applied Biosystems; catalog #4406985)
        - 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)
        - MicroAmp® Optical Adhesive Film Kit (Applied Biosystems; catalog # 4311971 or #4360954)
    - CFX96 Touch Real-time PCR Detection System (Bio-Rad; catalog #185-5196)
        - Hard-shell PCR Plates 96-well WHT/WHT (Bio-Rad; catalog #HSP9655 or equivalent)
        - Microseal 'B' Adhesive Plate Seal (Bio-Rad; catalog #MSB-1001)
- Extraction instruments and consumables:
    - Dynal BeadRetriever™ System (Life Technologies; catalog #159-50)
    - MagMAX Express-96 Deep Well Magnetic Particle Processor (Life Technologies, catalog #4400079)
        - MagMAX Express-96 Microtiter Deep Well Plate (Life Technologies, catalog #4388476)
        - MagMAX Express-96 Tip Combs for DW Magnets (Life Technologies, catalog #4388487)
        - MagMAX Express-96 Plate (200µL) (Life Technologies, catalog #4388475)

Quality Control

rRT-PCR can be 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. HSC must 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

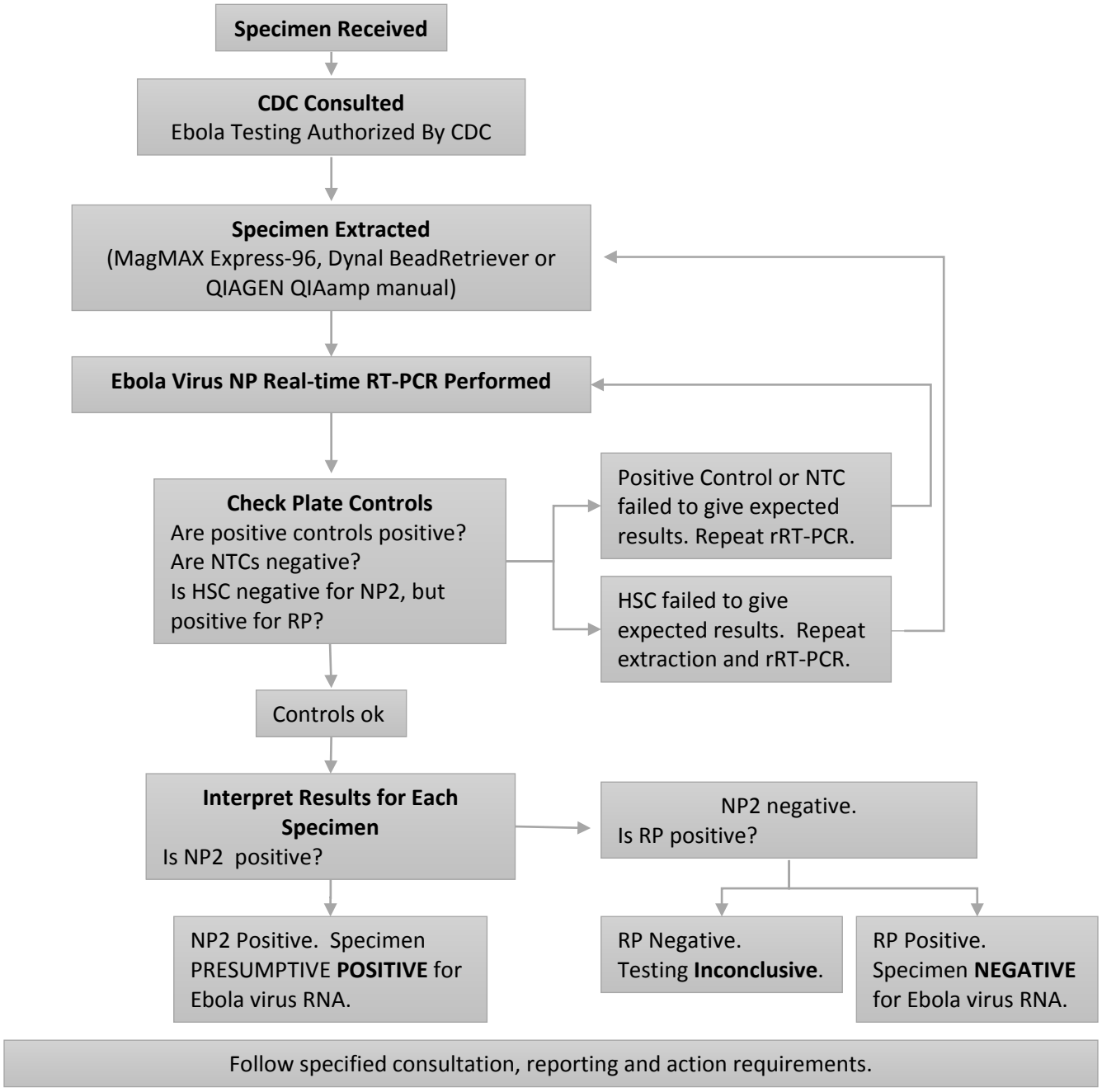
Control Type	Control Name	Used to Monitor	NP2	RP	Expected C_T Values
Positive	EBOV NP rRT-PCR Pos Ctrl	Substantial reagent failure, including primer and probe integrity	+	+	<35 C_T for NP2 and RP
Negative	NTC	Reagent and/or environmental contamination during PCR set-up	-	-	None detected
Negative	HSC	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 either of the following extraction options:
  - Dynal BeadRetriever™ System
    - Up to 15 specimens/run
    - See Appendix A for sample extraction instructions
  - MagMAX™ Express-96 Deep Well Magnetic Particle Processor
    - Up to 96 specimens/run
    - See Appendix B for sample extraction instructions
  - QIAGEN QIAamp DSP Viral RNA Mini Kit
    - Up to 50 RNA preps
    - See Appendix D for specimen inactivation

- 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).
- 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



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 Kit
     - 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 rehydration and 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.
   - 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 is at the bottom. Pellet will be a bright pink color.
    - Add 1500 µL of cold nuclease-free water and mix gently. Centrifuge tube. Pellet is in solution when no pink precipitate is visible.
    - To ensure complete rehydration, hold tube on ice for 20 minutes before handling further.
    - Aliquot in 15 µL volumes and store at ≤ -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 rehydration and storage conditions, see package insert.

Equipment Preparation

1. Turn on AB 7500 Fast Dx or Bio-Rad CFX96 Touch.
2. Perform plate set up and select cycling protocol on the instrument

7500 Fast Dx

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

For detailed instructions on launching and programming the Applied Biosystems 7500 Fast Dx System software, refer to the Programming of the AB® 7500 Fast Dx located under Documents/Instrument Programming and Maintenance on the LRN secure website.

CFX96 Touch

Instrument Settings
Detector: FAM
Sample Volume: 25 µL

For detailed instructions on programming the CFX96 Touch, refer to Appendix E.

Cycling Conditions

Table 2: rRT-PCR cycling conditions

AB 7500 Fast Dx and CFX96 Touch			
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

Collect Cold Blocks

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 1.7 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

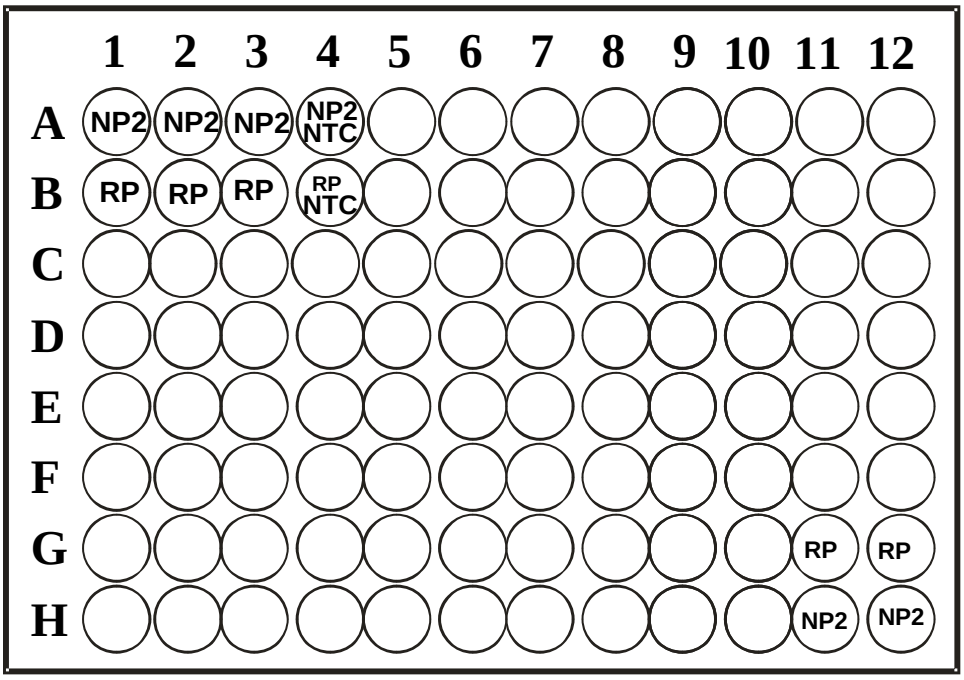
7. rRT-PCR Reaction Mix:
   For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture (N = number of reactions).

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**

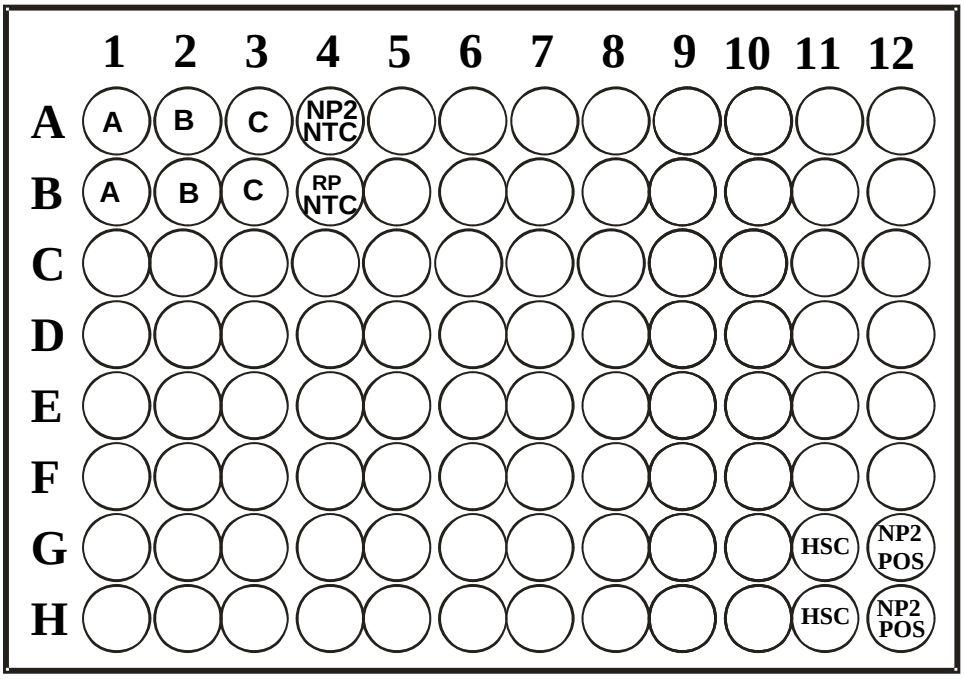
   NOTE: The reaction mixture is the same for both the 7500 Fast Dx and CFX96 Touch.
8. Mix reaction components by pipetting slowly up and down (avoid bubbles).
9. Add 20 µL of master mix into each well of a chilled optical plate as shown in examples below.
   NOTE: Plate lay-outs are examples only. Laboratory should create a plate lay-out that best accommodates the number of samples being tested.

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.
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 or CFX96 Touch 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 using the Auto Ct setting included in the 7500 Fast Dx and CFX96 Touch software.

NOTE: On the 7500 Fast Dx, the Auto Ct setting 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 for the HSC with the NP2 primer/probes would indicate cross-contamination has occurred. If a positive result is obtained, all results in the run are invalid. Follow the cleaning procedure described above in 2.a. and repeat extraction and testing.
4. RP Assay for each specimen should be **positive (C_T <40)**.
    a. If the RP assay for a specimen is *negative* and the NP2 assay is *negative* for specimen samples:
        i. Report result as *Inconclusive*
        ii. Repeat extraction from new specimen aliquot.

iii. 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.

iv. 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.

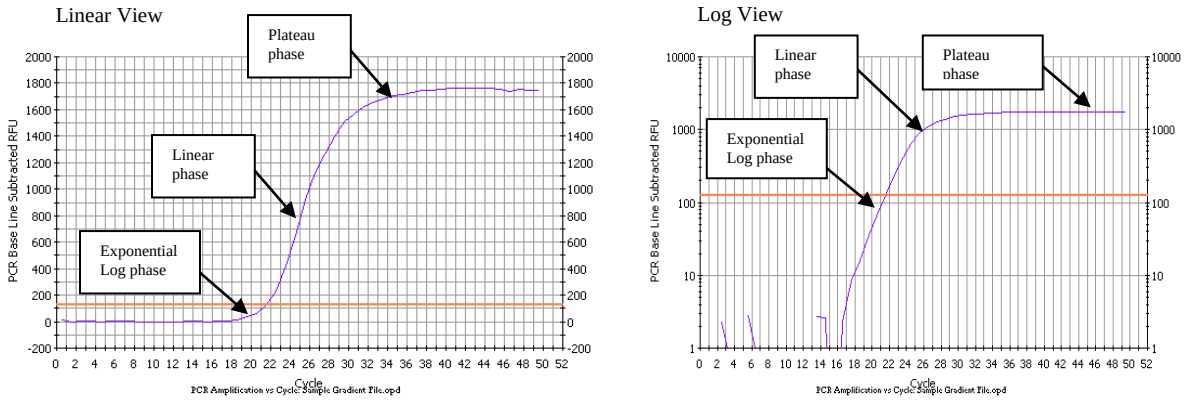


Figure 3: Linear and log views of PCR curves noting each stage of the amplification plots.

- 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.

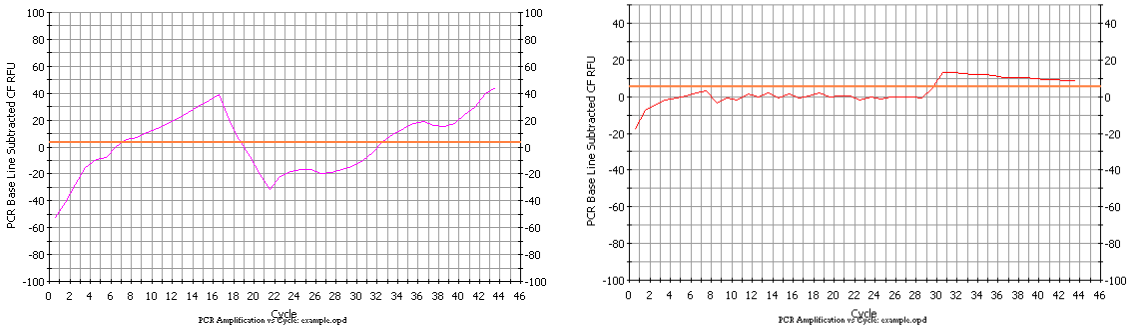


Figure 4: Examples of false positive curves.

- To better understand and evaluate challenging curves more effectively, use the background fluorescence view (Rn versus Cycle with AB software, RFU versus Cycle on CFX96 Touch 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.

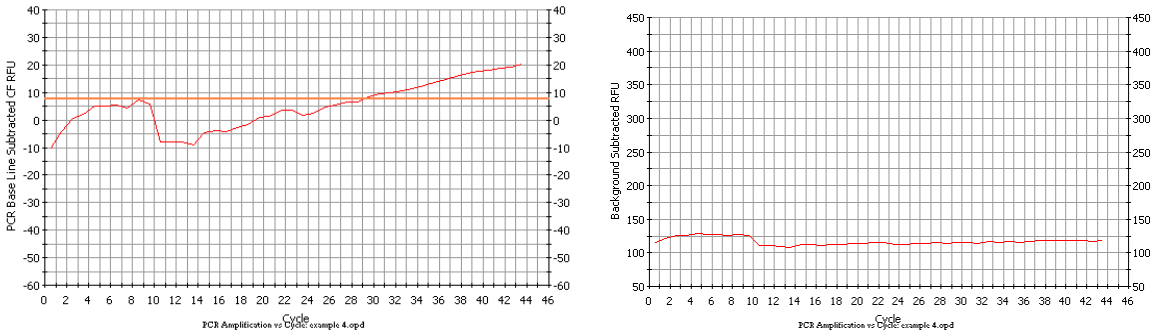


Figure 5: Amplification plot of a sample with a "wandering" curve (left) and the corresponding background fluorescence view (right).

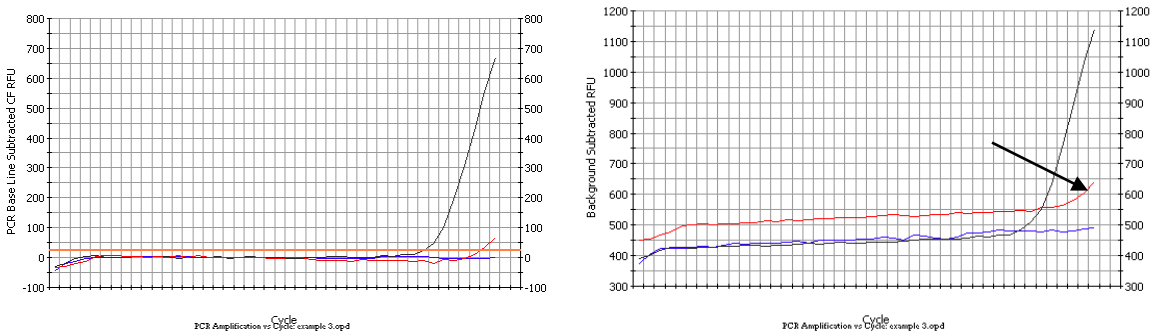


Figure 6: Amplification plot of three samples in the linear view (left) and the corresponding background fluorescence view (right).

- 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 40, 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.
  NOTE: This feature is not available on the CFX96 Touch.

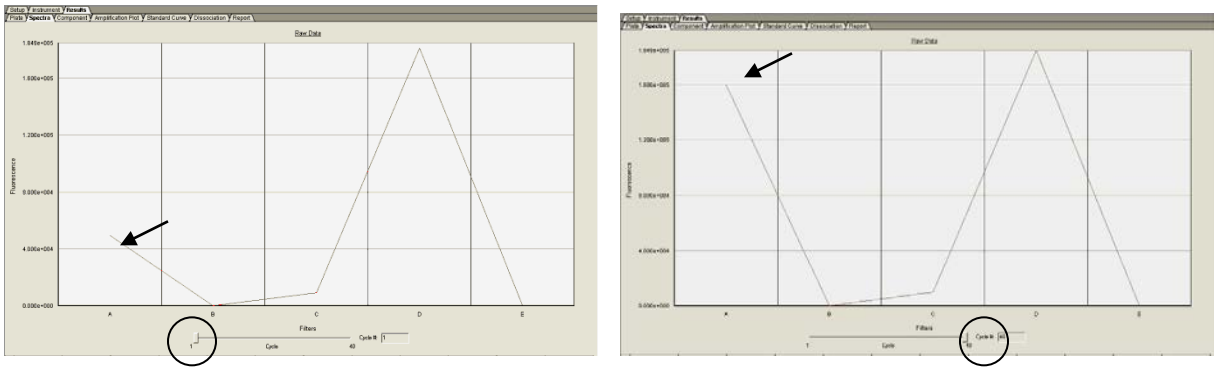


Figure 7: Spectra component of a positive sample. Left screenshot shows fluorescence at cycle 1 and right screenshot shows fluorescence at cycle 40.

- 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 <u>interpreted with caution</u>. 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
-	+	Ebola Virus NP Negative	Ebola virus RNA not detected by rRT-PCR	Report results to CDC Consult CDC to determine if additional patient testing is required.
-	-	Inconclusive	Inconclusive for Ebola 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 <40	+/-	Ebola Virus 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.

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 *Guidance for Collection, Transport and Submission of Specimens for Ebola Virus Testing* (http://www.cdc.gov/vhf/ebola/healthcare-us/laboratories/specimens.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, the MagMAX™ Express-96 Deep Well Magnetic Particle Processor, or the QIAamp DSP Viral RNA Mini Kit for manual extraction. Other extraction methods 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 600-6000 $TCID_{50}$/mL. Inactivation by gamma irradiation was demonstrated to impact the sensitivity of the assay. Mayinga and Gabon strains saw 10-fold shifts in estimated LoD. The Kikwit strain maintained the same estimated LoD (2000 $TCID_{50}$/mL), 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)

$TCID_{50}$/mL	Live virus (C_T values)			Inactivated virus (C_T values)		
6×10^7	20	21	20	20	20	20
6×10^6	24	24	25	26	26	26
6×10^5	28	28	28	32	32	33
6×10^4	32	32	32	34	34	34
6×10^3	35	35	35	39	und	38
600	38	40	39	und	und	und
60	und	und	und	und	und	und
6	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)

$TCID_{50}$/mL	Live virus (C_T values)			Inactivated virus (C_T values)		
2×10^6	22	22	22	23	23	23
2×10^5	26	26	26	28	29	28
2×10^4	30	30	30	32	32	32
2×10^3	33	34	33	35	36	35
200	39	37	und	und	und	und
20	und	und	und	und	und	und
2	und	und	und	und	und	und
0.2	und	und	und	und	und	und
Neg. ctrl.	und	und	und	und	und	und

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

$TCID_{50}$/mL	Live virus (C_T values)			Inactivated virus (C_T values)		
6×10^6	20	20	21	22	22	22
6×10^5	24	24	25	27	27	27
6×10^4	28	28	28	34	34	33
6×10^3	31	31	32	38	37	37
600	35	35	35	und	und	und
60	und	und	und	und	und	und
6	und	und	und	und	und	und
0.6	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 6 x 10^3 $TCID_{50}$/mL. 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 (6 x 10^4 $TCID_{50}$/mL, 6 x 10^3 $TCID_{50}$/mL, 600 $TCID_{50}$/mL, and 60 $TCID_{50}$/mL). 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 on the AB 7500 Fast Dx 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

$TCID_{50}$/mL	NP2 assay C_T values									
6 x 10^4	31	32	34	30	31	30	31	30	32	29
	30	30	30	30	30	29	30	30	30	30
6 x 10^3	33	36	34	35	35	34	34	33	36	34
	34	35	33	35	34	33	35	34	34	35
600	und	und	39	und	und	und	und	und	und	und
	und	39	und	und	und	und	und	40	und	und
60	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

$TCID_{50}$/mL	RP assay C_T values									
6 x 10^4	25	25	25	25	25	24	24	25	25	25
	25	25	25	25	25	24	24	25	25	25
6 x 10^3	24	25	25	25	25	24	24	24	24	24
	26	26	24	25	25	24	24	25	25	24
600	24	25	24	25	25	25	24	24	24	24
	25	25	24	25	25	25	24	24	24	24
60	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 6 x 10^3 $TCID_{50}$/mL. 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 (6 x 10^4 $TCID_{50}$/mL, 6 x 10^3 $TCID_{50}$/mL, 600 $TCID_{50}$/mL, and 60 $TCID_{50}$/mL). 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 on the AB 7500 Fast Dx 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

$TCID_{50}$/mL	NP2 assay C_T values									
6 x 10^4	29	31	29	29	31	30	30	29	31	30
	28	28	28	28	28	28	28	28	28	29
6 x 10^3	32	31	31	31	31	31	31	32	32	34
	32	33	33	32	31	34	33	33	33	34
600	38	39	38	38	39	38	38	und	und	38
	39	38	38	37	39	37	38	39	38	38
60	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

$TCID_{50}$/mL	RP assay C_T values									
6 x 10^4	36	36	36	36	34	36	36	35	36	36
	38	35	35	36	35	36	38	35	35	35
6 x 10^3	36	36	36	35	35	36	36	35	35	35
	37	35	36	35	35	37	36	35	35	38
600	35	36	35	35	35	35	34	35	35	35
	35	36	35	36	35	36	35	36	35	36
60	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 using the AB 7500 Fast Dx 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 below contains a summary of the findings.

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 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 100 µL aliquots. One 100 µL aliquot was left neat (no virus added) and the other 100 µL aliquot was spiked with either a low concentration (2 x 10^4 $TCID_{50}$/mL) or high concentration (2 x 10^5 $TCID_{50}$/mL) 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 15: NP2 Contrived Whole Blood (EDTA) Specimens: Summary of Results

$TCID_{50}$/mL	Total #	Positive	Inconclusive	Negative
Low Concentration (2 x 10^4 $TCID_{50}$/mL)	25	25	0	0
High Concentration (2 x 10^5 $TCID_{50}$/mL)	25	25	0	0
No Virus Added	50	0	0	50

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

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

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 100 µL aliquots. One 100 µL aliquot was left neat (no virus added) and the other 100 µL aliquot was spiked with either a low concentration (2 x 10^4 $TCID_{50}$/mL) or high concentration (2 x 10^5 $TCID_{50}$/rxn) 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 16: NP2 Contrived Urine Specimens: Summary of Results

$TCID_{50}$/rxn	Total #	Positive	Inconclusive	Negative
Low Concentration (2 x 10^4 $TCID_{50}$/mL)	25	24	0	1
High Concentration (2 x 10^5 $TCID_{50}$/mL)	25	25	0	0
No Virus Added	50	0	1	49

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

Negative Percent Agreement: 100% (49/49) (95% CI: 93%, 100%)

5. Bio-Rad CFX96 Bridging Study

CFX96 Bridging Study – LoD Estimation in Whole Blood

A stock of Ebola virus prepared from a strain obtained in the outbreak in West Africa (Liberia 2014) was quantified and then inactivated by gamma irradiation. Serial 10-fold dilutions of this quantified inactivated stock was prepared in whole blood and extracted according to the MagMax/Dynal method defined in the authorized Instructions for Use. Each dilution was then tested side-by-side in duplicate using the NP2 primer and probe set on both the AB 7500 Fast Dx and the Bio-Rad CFX96. Results of this testing are presented in Table 17.

Table 17: 7500 Fast DX and CFX96 Side-by-Side Serial Dilution Data

Ebola virus (Liberia 2014)		NP2 Ct Values (duplicate testing)			
Dilution	$TCID_{50}$/mL	AB 7500 Fast Dx		Bio-Rad CFX96 Touch	
-1	40,000,000	20	20	20	20
-2	4,000,000	24	24	23	23
-3	400,000	28	28	27	27
-4	40,000	30	30	30	29
-5	4,000	34	34	34	33
-6	400	37	38	39	37
-7	40	und	und	und	und
-8	4	und	und	und	und
neg.ctrl.	0	und	und	und	und

CFX96 Bridging Study – LoD Verification in Whole Blood

The lowest concentration generating 100% positive results in the initial range-finding step (400 $TCID_{50}$/mL) was tested a further 20 times on each PCR instrument to verify the limit of detection. These data are presented in Table 18.

Table 18: 7500 Fast Dx and CFX96 Side-by-Side Limit of Detection Verification

Instrument	NP2 Ct Values - Replicate Testing (400 $TCID_{50}$/mL)										Negative Control
AB 7500 Fast Dx	36	35	35	35	35	35	36	35	35	35	und
	35	35	36	35	35	36	35	36	36	35	und
Bio-Rad CFX96 Touch	37	37	37	37	37	37	37	37	37	37	und
	36	37	37	37	37	36	36	37	37	36	und

The limit of detection for both the CFX96 and the AB 7500 Fast Dx in this evaluation is 400 $TCID_{50}$/mL. These data suggest that the performance of the CFX96 is comparable to the performance of the AB7500 Fast Dx with this assay.

6. Extraction Instrument Bridging Study

MagMAX Express-96 Deep Well Magnetic Particle Processor – Side-by-side Serial Dilution Testing

A stock of Ebola virus prepared from a strain obtained in the outbreak in West Africa (Liberia 2014) was quantified and then inactivated by gamma irradiation. Serial 10-fold dilutions of the quantified, inactivated stock was prepared in whole blood. Each concentration was extracted three times by each extraction method: the MagMAX Pathogen RNA/DNA Kit on the Dynal BeadRetriever and the same extraction kit on the MagMAX Express-96 Deep Well Magnetic Particle Processor. Resulting nucleic acid samples were tested once by EBOV NP rRT-PCR. Data demonstrate comparable performance between the two automated extraction instruments. Results of this evaluation are presented in Table 19 (NP2) and Table 20 (RP).

Table 19: Dynal BeadRetriever and MagMAX Express-96 Side-by-side NP2 Serial Dilution Data

Ebola virus (Liberia 2014)		NP2 Ct Values (triplicate extractions)					
Dilution	$TCID_{50}$/mL	MagMAX Express-96 Deep Well Magnetic Particle Processor			Dynal BeadRetriever		
-2	4,000,000	26	25	25	25	25	25
-3	400,000	28	28	28	28	28	28
-4	40,000	31	32	31	31	32	31
-5	4,000	35	35	35	und	35	35
-6	400	und	und	und	und	38	39
HSC	0	und	und	und	und	und	und

Table 20: Dynal BeadRetriever and MagMAX Express-96 Side-by-side RP Serial Dilution Data

Ebola virus (Liberia 2014)		RP Ct Values (triplicate extractions)					
Dilution	$TCID_{50}$/mL	MagMAX Express-96 Deep Well Magnetic Particle Processor			Dynal BeadRetriever		
-2	4,000,000	27	26	27	28	27	27
-3	400,000	27	27	26	27	27	27
-4	40,000	27	27	26	27	27	27
-5	4,000	27	27	27	30	28	27
-6	400	27	27	27	27	27	27
HSC	0	27	27	27	26	27	26

7. Manual Extraction Method Bridging Study

QIAGEN QIAamp DSP Viral RNA Mini Kit – Limit of Detection Study

A stock of Ebola virus prepared from a strain used in the original limit of detection study (Mayinga 1976) was quantified and inactivated by gamma irradiation. Four pools of serial 10-fold dilutions of this stock were prepared, then inactivated with TRIzol® LS (Life Technologies, catalog #10296-010) using a 5:1 dilution of TRIzol to specimen. Each concentration was extracted five times using the QIAamp DSP Viral RNA Mini Kit (QIAGEN, catalog #61904) to obtain an estimated LoD result. Resulting nucleic acid samples were tested by EBOV NP rRT-PCR Assay. An additional 15 extractions at the estimated LoD concentration were performed to confirm the limit of detection. Confirmation of LoD was achieved at 6 x 10^3 $TCID_{50}$/mL since 100% (20/20) results were positive at this concentration. Results of this LoD evaluation are presented in Table 21.

Table 21: LoD Study Data

$TCID_{50}$/mL	NP Positive Results	
	Range-finding	Verification
6 x 10^5	5/5	
6 x 10^4	5/5	
6 x 10^3	5/5	15/15
600	2/5	
HSC	0/1	0/1
NTC	0/1	0/1

***RP generated similar results across pools as all pools were prepared with human whole blood.**
HSC – Human Specimen Control
NTC – No-Template Control

This data demonstrate comparable performance between using the QIAGEN QIAamp DSP Viral RNA Mini Kit and using the MagMAX Pathogen RNA/DNA Kit on the Dynal BeadRetriever™ System. A comparison of the limit of detection for both methods is presented in Table 22.

Table 22: Summary of Observed Limit of Detection by Method in Whole Blood

Extraction Method	Limit of Detection
QIAGEN QIAamp DSP Viral Mini Kit	6,000 $TCID_{50}$/mL
Dynal BeadRetriever™ System using the MagMax Pathogen RNA/DNA kit	6,000 $TCID_{50}$/mL*

*LoD for Dynal BeadRetriever taken from the analytical sensitivity study data presented in Table 8 above.

8. Assay Performance Verification - Bo, Sierra Leone

In April 2015, CDC conducted a study in Bo, Sierra Leone, to demonstrate the similarity of performance characteristics of the Ebola Virus NP Real-time RT-PCR Assay (EBOV NP rRT-PCR) in the context of a field laboratory. The study evaluated the limit of detection, precision, and repeatability of the NP2 primer and probe set. The data generated in these performance verifications are presented below.

Limit of Detection – Field Laboratory Verification

Limit of detection for the EBOV NP rRT-PCR Assay primer and probe set was verified in the CDC Bo field laboratory using the high concentration lyophilized spiked serum specimens from the Proficiency Testing (PT) Panel. This human serum specimen, spiked with inactivated (gamma irradiated) Ebola virus (Mayinga 1976) at 250,000 $TCID_{50}$/mL prior to lyophilization, was rehydrated per PT Panel package directions and used to prepare 10-fold serial dilutions in healthy human sera. Four dilutions were prepared, from 25,000 $TCID_{50}$/mL to 25 $TCID_{50}$/mL. Each dilution was extracted in triplicate using the MagMax Pathogen RNA/DNA Kit on the Dynal BeadRetriever System as described in the EBOV NP rRT-PCR Assay Instructions for Use. Each resulting nucleic acid sample was then tested one time by the EBOV NP rRT-PCR assay on the CFX96 Touch to determine the LoD range to be evaluated. Two concentrations, 250 $TCID_{50}$/mL and 2500 $TCID_{50}$/mL, were selected for further testing. These two concentrations were extracted an additional 20 times by the same extraction method. Each nucleic acid sample was tested once by the EBOV NP rRT-PCR assay on the CFX96 Touch. All controls (HSC, NTC and Positive Control) generated expected results. The lowest concentration generating at least 95% positive results for NP2 is considered the confirmed limit of detection for the EBOV NP rRT-PCR assay.

Table 23: Limit of Detection Field Laboratory Verification – Confirmed Limit of Detection Summary

Primer and Probe Set	Extraction Method	PCR Instrument	Limit of Detection
NP2	Dynal BeadRetriever	CFX96 Touch	2500 $TCID_{50}$/mL

Data generated in support of the addition of the CFX96 Touch to the FDA Emergency Use Authorizations for the EBOV NP rRT-PCR demonstrated a LoD of 400 $TCID_{50}$/mL in whole blood for the NP2 primer and probe set testing samples prepared using a stock of gamma-irradiated Ebola virus from a strain obtained in the outbreak in West Africa (Liberia 2014). Although the confirmed LoD from this field evaluation based on the at least 95% positivity criterion is 2500 $TCID_{50}$/mL, positivity of 91.3% (21/23) (95% CI: 73.2% - 97.6%) was observed at 250 $TCID_{50}$/mL. Thus, the true LoD from this field evaluation most likely falls between 250 $TCID_{50}$/mL and 2500 $TCID_{50}$/mL. Therefore, the LoD observed in this field evaluation is consistent with the LoD data generated with the same PCR instrument in Atlanta for the EUA amendment authorized on March 2, 2015.

Precision and Repeatability - Field Laboratory Verification

Precision of the EBOV NP rRT-PCR Assay was evaluated in the CDC field laboratory in Bo, Sierra Leone using a Proficiency Testing (PT) Panel containing 6 lyphilized contrived serum specimens. Of the 6 specimens, two had been spiked with inactivated (gamma irradiated) Ebola virus (Mayinga 1976) at 2.5 x 10^5 $TCID_{50}$/mL prior to lyophilization, two had been spiked with this inactivated Ebola virus at 2.5 x 10^3 $TCID_{50}$/mL, and two contained no Ebola virus. Each day of testing, a fresh PT panel was rehydrated per PT panel package directions. Two operators each tested the PT panel members once a day for five days. Each panel member was extracted once using the MagMax Pathogen RNA/DNA Kit on the Dynal BeadRetriever™ System as described in the Instructions for Use. Each

extracted RNA sample was then tested in triplicates on the CFX96 Touch Real-Time PCR instrument. A total of 30 data points for each concentration level tested per operator were generated for the precision study.

Summaries of the qualitative results of this precision study are presented in Table 24 below.

Table 24: Qualitative summary (Percent agreement with expected result, each PCR replicate considered separately)

Concentration (samples)	Operator 1		Operator 2	
	NP2	RP	NP2	RP
Neat	100% (30/30)	100% (30/30)	100% (30/30)	100% (30/30)
2500 $TCID_{50}$/mL	96.67% (29/30)*	100% (30/30)	100% (30/30)	100% (30/30)
250,000 $TCID_{50}$/mL	100% (30/30)	100% (30/30)	100% (30/30)	100% (30/30)

*One replicate on Day 1 failed to generate a positive result due to the presence of a deceased insect in the lid of the CFX96 Touch directly over well B1, obstructing the view of the optical unit.

Assessment of the qualitative results of the study demonstrates that the assay is repeatable in hands of multiple operators and at a site separate from the facility where the assay was developed. All but one result matched expected results.

Additional analysis of the Ct values generated for the NP2 primer and probe set was conducted for each run, for each operator and overall. To evaluate precision of the assay, standard deviation (SD) was calculated for NP2 Ct values at each concentration generated by Operator 1. This data was evaluated for each run, as well as for the entire data set generated by Operator 1. No standard deviation calculated for either concentration for any run or combination of runs ever exceeded 1, demonstrating the assay to be sufficiently precise in the hands of users in the CDC field laboratory in Bo, Sierra Leone.

To evaluate the repeatability of the assay, data from both operators was evaluated based on coefficients of variance (CV). For both Ebola virus concentrations, the CV was calculated for each run, for each operator and for the overall data set. The CV never exceeded 3% for any run or combination of runs, demonstrating the assay to be repeatable. The overall coefficient of variance at the high concentration was 1.79%. The overall coefficient of variance at the low concentration was 1.76%. Summaries by run and by operator are presented in Table 25 below.

Table 25: Precision and repeatability data for NP2 Primer and Probe Set

				Run 1	Run 2	Run 3	Run 4	Run 5	All Days
NP2	250,000 TCID50/mL	Operator 1	Replicates	6	6	6	6	6	30
			# Positive	6	6	6	6	6	30
			Average Ct	27.36	28.28	28.37	28.59	28.48	28.22
			SD	0.131	0.165	0.183	0.172	0.147	0.473
			%CV	0.48%	0.59%	0.64%	0.60%	0.52%	1.68%
		Operator 2	Replicates	6	6	6	6	6	30
			# Positive	6	6	6	6	6	30
			Average Ct	28.37	28.03	28.72	27.36	27.69	28.04
			SD	0.156	0.253	0.193	0.249	0.096	0.524
			%CV	0.55%	0.90%	0.67%	0.91%	0.34%	1.87%
		All Operators	Replicates						60
			# Positive						60
			Average Ct						28.13
			SD						0.503
			%CV						1.79%
NP2	2500 TCID50/mL	Operator 1	Replicates	6	6	6	6	6	30
			# Positive	5	6	6	6	6	29
			Average Ct	33.83	34.73	35.01	35.32	34.96	34.80
			SD	0.068	0.161	0.305	0.184	0.136	0.521
			%CV	0.20%	0.46%	0.87%	0.52%	0.39%	1.50%
		Operator 2	Replicates	6	6	6	6	6	30
			# Positive	6	6	6	6	6	30
			Average Ct	35.34	35.23	35.78	34.79	34.42	35.11
			SD	0.219	0.338	0.447	0.938	0.060	0.666
			%CV	0.62%	0.96%	1.25%	2.70%	0.18%	1.90%
		All Operators	Replicates						60
			# Positive						59
			Average Ct						34.96
			SD						0.614
			%CV						1.76%

One run performed by Operator 2 was excluded from the analysis. A power interruption was encountered and curves generated during the run were abnormal. The results generated did not meet the criteria for acceptable PCR curves outlined in the instructions for use. The run was repeated and the repeat data are presented in Table 25.

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 A: 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 *Guidance for Collection, Transport and Submission of Specimens for Ebola Virus Testing* (http://www.cdc.gov/vhf/ebola/healthcare-us/laboratories/specimens.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.

Upon receipt of extraction kit: Prepare the reagents included in the kit according to manufacturer's recommendation which is the following:

NOTE: This is done once upon receiving the kit.

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.
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.
3. Prepare the Lysis/binding solution by adding **240 mL of 100% isopropanol** which is a 1:1 dilution of buffer to isopropanol (carrier added later as needed)

Store the wash solutions, the elution buffer and the lysis solution at room temperature. Store the RNA binding beads at 4°C and the lysis enhancer and carrier RNA at -20°C.

On day of extraction:

1. 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 clean tube: Lysis/binding Solution
    b. For each specimen, add 400 µL lysis binding solution + 2 µL carrier RNA.
    c. Store at room temperature for up to 8 hours.
    d. Label one 1.7 mL Eppendorf tube for each specimen to be extracted and one for the HSC.
    e. Aliquot 400 uL of Lysis binding/carrier RNA solution into each tube.
    f. Close and transfer to HOT LAB.

2. Bead mix:
    a. Label one 1.7 mL Eppendorf tube: Bead Solution
    b. Vortex 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.
3. Human Specimen Control (HSC):
    a. Prepare as instructed in product insert.

Specimen Preparation (HOT LAB)

1. Add 100 µL of sample to the appropriately labeled tube containing Lysis Binding/Carrier RNA solution.
2. Pipette up and down to mix (**DO NOT Vortex**). Let the mixture sit for 10 minutes to ensure inactivation.
3. Repeat process for remaining specimens and for the HSC.
4. Decontaminate exterior of tubes and transfer to extraction set-up area. (Ex. By dunking tubes out in 5% Microchem dunk tank)

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.

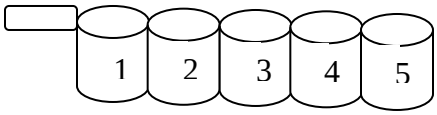
Extraction Program for the Dynal BeadRetriever™ System

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 of Wash Solution 1** to **well #2** of each sample reservoir
   b. Aliquot **300 µL** of **Wash Solution 1** to **well #3** of each sample reservoir
   c. Aliquot **900 µL** of **Wash Solution 2** to **well #4** of each sample reservoir
   d. Aliquot **90 µL** of **Elution Solution** to **well #5** of each sample reservoir

2. Once samples are ready (from "Specimen Preparation (HOT LAB)"), vortex the Bead mix then aliquot **20 µL** of the **Bead Mix** into **well #1** of each sample reservoir.

3. 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**.

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

5. Press START again to start run.

6. 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.

Appendix B: RNA Extraction Protocol Using the MagMAX™ Express-96 Deep Well Magnetic Particle Processor

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)
- MagMAX Express-96 Deep Well Magnetic Particle Processor (Life Technologies, catalog #4400079)
- MagMAX Express-96 Microtiter Deep Well Plate (Life Technologies, catalog #4388476)
- MagMAX Express-96 Plate (200µL) (Life Technologies, catalog #4388475)
- MagMAX Express-96 Tip Combs for DW Magnets (Life Technologies, catalog #4388487)
- 96-well plate optical adhesive cover (either of the following or equivalent):
  - MicroAmp Optical Adhesive Film Kit (Applied Biosystems, catalog #4311971 or #4360954)
  - Microseal 'B' Adhesive Plate Seal (Bio-Rad, catalog #MSB-1001)
- 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)
- 12-channel 20µL, 200µL, and 1000µL pipettes
- 2 mL Starstedt tubes
- 1.7 mL microcentrifuge tubes (Eppendorf or equivalent)
- Racks for 1.7 mL microcentrifuge tubes
- 2 x 96-well -20 °C cold blocks
- RNase-DNase-free reagent trays
- Vortex

Nucleic Acid Extraction

Biosafety information: Refer to CDC *Guidance for Collection, Transport and Submission of Specimens for Ebola Virus Testing* (http://www.cdc.gov/vhf/ebola/healthcare-us/laboratories/specimens.html)

- Whole blood, serum, plasma and urine may be extracted using the MagMAX Express-96 Deep Well Magnetic Particle Processor.
- 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.

Upon receipt of extraction kit: Prepare the reagents included in the kit according to manufacturer's recommendation which is the following:

NOTE: This is done once upon receiving the kit.

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.
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.
3. Prepare the Lysis/binding solution by adding **240 mL of 100% isopropanol** which is a 1:1 dilution of buffer to isopropanol (carrier added later as needed)

Store the wash solutions, the elution buffer and the lysis solution at room temperature. Store the RNA binding beads at 4°C and the lysis enhancer and carrier RNA at -20°C.

On day of extraction:

1. 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 clean tube: Lysis/binding Solution
    b. For each specimen, add 400 µL lysis binding solution + 2 µL carrier RNA.
    c. Store at room temperature for up to 8 hours.
    d. Label one 2 mL Starstedt tube for each specimen to be extracted and one for the HSC.
    e. Aliquot 400 µL of Lysis binding/carrier RNA solution into each tube.
    f. Close and transfer to HOT LAB.

2. Bead mix:
    a. Label one 1.7 mL Eppendorf tube: Bead Solution
    b. Vortex beads thoroughly before making dilution.
    c. For each specimen, add 10 µL of beads + 10 µL of lysis/binding enhancer to tube.
    NOTE: Prepare enough for overage, enough for approximately 10 extra samples if using a multichannel pipette.
    d. Store on ice for up to 4 hours.
    e. When ready to prepare instrument for extraction run, aliquot 20 µL of bead mix per well (one well per sample) into a 96-well deep-well plate.
    f. Lightly cover plate with an ABI optical adhesive cover and place in refrigerator until ready to add samples.
4. Human Specimen Control (HSC):
    a. Prepare as instructed in product insert.

Specimen Preparation (HOT LAB)

1. Add 100 µL of sample to the appropriately labeled Sarstedt tube containing Lysis Binding/Carrier RNA solution.
2. Pipette up and down to mix (**DO NOT Vortex**). Let the mixture sit for 10 minutes to ensure inactivation.
3. Repeat process for remaining specimens and for the HSC.
4. Decontaminate exterior of Starstedt tubes and move tubes to extraction set-up area. (Ex. By dunking tubes out in 5% Microchem dunk tank)

Equipment Preparation and Extraction

1. Prepare the machine with the reagents in the appropriate 96-well plates:
NOTE: This can be done with a multi-channel pipet and reservoir trays.
    a. Add 90 µL of elution buffer to a standard plate, label and cover lightly with an optical adhesive cover
    b. Add 450 µL of Wash 2 to a deep-well plate and label
    c. Add 450 µL of Wash 2 to another deep-well plate, label and stack the plate on top of the other Wash 2 plate and cover top plate lightly with the optical adhesive cover
    d. Add 300 µL of Wash 1 to a deep-well plate and label
    e. Add 300 µL of Wash 1 to another deep-well plate, label plate and stack the plate on top of the other Wash 1 plate and cover top plate lightly with the optical adhesive cover
    f. Set plates next to the machine until you are ready to load

2. Once the samples have been prepared as described in Specimen Preparation (above), add the entire amount (500 µL) of the sample/lysis buffer mix to the appropriate wells of the deep-well plate with beads (prepare a plate template with sample numbers ahead of time).
NOTE: Add the sample directly to the beads- DO NOT mix or pipet up and down

HSC extraction control must be included in each run. Treat HSC as another specimen to be extracted.

3. Turn on the MagMAX™ Express-96 Deep Well Magnetic Particle Processor instrument.
   a. Choose Program "4462359_DW__HV".
      NOTE: This is a custom program and must be installed by the instrument manufacturer.
   b. Press start and follow the prompts to load the machine with the pre-prepared buffer plates.
   c. Load tip comb in deep-well plate in position 7, press START
   d. Remove cover and load the plate containing elution buffer in position 6 and press START
   e. Remove cover from wash 2 plates and load one of the plates in position 5 and press START
   f. Load the other wash 2 plate in position 4 and press START
   g. Remove cover from wash 1 plates and load one of the plates in position 3 and press START
   h. Load the other wash 1 plate in position 2 and press START
   i. Load the sample plate in position 1 and press START
   j. The run will take ~25 min

4. Once the run is complete you will need to CAREFULLY remove the RNA plate (elution plate) from the machine and proceed to adding the RNA to the qRT-PCR plate. Label the RNA plate and lightly cover with the optical adhesive covers and place in freezer until the PCR run is complete. If you do not have samples that need to be repeated you can place the adhesive plate covers on the plate for long-term storage.
   NOTE: If unable to perform rRT-PCR immediately, store RNA at ≤-70°C.

5. Follow machine prompts to remove and discard the other plates.

Extraction Program for the MagMAX™ Express-96 Deep Well Magnetic Particle Processor

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

Protocol Name			custom 4462359_DW__HV
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	1st Wash 2	Wash Soln 2	450 µL
5	2nd Wash 2	Wash Soln 2	450 µL
6	Elution	Elution Buffer	90 µL
7	Tip Combs	N/A	N/A

Appendix C: Whole Blood Inactivation Procedure for Subsequent Manual Extraction

Ebola virus (*species Zaire ebolavirus*) Trizol/TriPure Inactivation Procedure

This specimen inactivation procedure is for use with whole blood, serum and plasma specimens *only*.

Whole blood (EDTA anticoagulant, lavender top collection tube), serum or plasma is to be treated with Trizol® LS reagent (Life Technologies) or TriPure Isolation Reagent (Roche Life Science).

This procedure is required prior to manual extraction with the QIAGEN QIAamp method. It is not required for specimens prior to extraction with either automated extraction method authorized for use with this assay.

Biosafety Information: Specimens should be collected using appropriate infection control precautions for Ebola or other hemorrhagic fever viruses and according to the manufacturer's instructions for the specimen collection device. Shipping should be performed according to the policies of the shipping performer, customs regulations, and the requirements of the receiving laboratory.

Safety References:

http://www.who.int/csr/resources/publications/ebola/filovirus_infection_control/en/

http://www.cdc.gov/vhf/ebola/healthcare-us/laboratories/specimens.html

http://www.cdc.gov/vhf/abroad/pdf/african-healthcare-setting-vhf.pdf

Protocol should be performed in a Class II or higher biosafety cabinet (BSC) or glove box.

Specimens potentially infected with Ebola virus (all species and strains) can be inactivated (i.e., rendered non-infectious) by the addition of 10 parts Trizol with 1 part specimen following the method below:

Procedure

a. Add 1250 µL of Trizol LS or TriPure to a 1.7 mL microcentrifuge tube.

b. Within a BSC, and using appropriate personal protective equipment, add 125 µL of specimen to the microcentrifuge tube containing Trizol LS.

c. Vortex the tube for at least 5 seconds and incubate at ambient temperature for 5 minutes +/-30 seconds.

d. Once the procedure is complete, samples can be handled following appropriate safety precautions defined by the testing laboratory.

IMPORTANT: While treatment with Trizol has been shown to be an effective method to disrupt viruses and to stabilize the target nucleic acid, specimens should still be handled as if they were infectious and present a potential safety hazard.

Appendix D: RNA Extraction Protocol Using the Qiagen QIAamp DSP Viral RNA Mini Kit

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
- Qiagen QIAamp DSP Viral RNA Mini Kit (Qiagen Catalog #61904)
- TRIzol® LS reagent (Life Technologies Catalog #10296-010, 100 mL) or TriPure Isolation Reagent (Roche Life Science Catalog #11667165001, 200 mL)

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
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- 12-channel 20µL, 200µL, and 1000µL pipettes
- 1.7 mL microcentrifuge tubes (Eppendorf or equivalent)
- Racks for 1.7 mL microcentrifuge tubes
- 2 x 96-well -20 °C cold blocks
- RNase-DNase-free reagent trays
- Vortex
- Molecular grade water (nuclease-free water – Ambion; Catalog # AM9937 or equivalent)

Nucleic Acid Extraction

Biosafety information: All blood samples must be processed according to the Trizol/TriPure Inactivation Procedure (Appendix C) prior to extraction with the QIAamp DSP Viral RNA Mini Kit.

Refer to CDC *Guidance for Collection, Transport and Submission of Specimens for Ebola Virus Testing* (http://www.cdc.gov/vhf/ebola/healthcare-us/laboratories/specimens.html)

- This extraction method **must** be preceded by a specimen inactivation step using a 10:1 ratio of TRIzol to specimen (see Appendix C for inactivation protocol).
- 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).
- HSC should be included in each extraction run as a negative extraction control.
- HSC should not be treated with TRIzol prior to extraction as it is already inactivated.
- Retain specimen extracts in a cold block or on ice until testing.

Extraction Reagent Preparation

All reagent preparation must be performed in a CLEAN area.

Appropriate specimens are Trizol-inactivated whole blood, serum or plasma specimens (see Appendix C).

Specimens should be of sufficient volume to support testing for all targets in singlet (one well per test), with some reserve for re-testing or re-extraction if necessary.

RNA Extraction using the QIAamp DSP Viral RNA Mini Kit **Buffer Preparation:**
Note: Follow manufacturer's instructions for preparation of reagents and buffers.

- Prepare QIAGEN Buffers AW1, AW2, and Carrier RNA as directed in QIAamp DSP Viral RNA kit package insert.
- Equilibrate QIAGEN buffer AVE to room temperature, if necessary.
- If precipitate has formed in QIAGEN Buffer AVL, dissolve by warming at 80 ± 3°C until precipitate is dissolved.
    - Prepare AVL-carrier RNA mix as described in manufacturer instructions.

IMPORTANT NOTE: In order to avoid cross-contamination, change gloves between samples if you suspect they have become contaminated.

1. Pipette **280 µL of prepared Buffer AVL containing carrier RNA** into a 1.7 mL labeled microcentrifuge tube.
2. Add **70 µL of inactivated specimen or 70 µL of HSC** to the 1.7 mL labeled RNase-free microcentrifuge tube and mix by pulse-vortexing for 15 seconds.
3. Incubate specimen(s) and control at room temperature (15–25°C) for 10 minutes.

4. Briefly centrifuge the tubes to remove drops from the inside of the lid.
5. Add **280 µL of 96-100% ethanol** to each specimen and control tube, and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tubes to remove drops from inside the lid.
6. For each specimen and control, place a QIAamp spin column into a 2 mL collection tube (from the QIAamp Viral RNA Mini Kit). Be sure to label the top of the columns clearly.
7. Carefully transfer the mixture from step 5, including any precipitate, to the QIAamp spin column WITHOUT moistening the rim of the column.
8. Centrifuge 1-2 minutes at 6,000 x g. If the specimen has not cleared the filter after the first run, repeat centrifugation until the specimen has cleared the filter.
9. For each specimen and control, place the QIAamp spin column into a second, clean 2 mL collection tube (from the QIAamp Mini Kit) and add **500 µL of Buffer AW1**. Discard the tube containing the filtrate from the previous step.
10. Centrifuge 1-2 minutes at 6,000 x g. If the buffer has not cleared the filter after 1-2 minutes, repeat centrifugation until buffer has cleared the filter.
11. Place each QIAamp spin column into a third clean 2 mL collection tube (from the QIAamp Mini Kit). Carefully open the QIAamp spin column and add **500 µL of Buffer AW2**.
12. Centrifuge at full speed (approx. 14,000 x g) for 3 minutes. Discard the tube containing the filtrate from the previous step.
13. To eliminate any possible Buffer AW2 carryover, place the QIAamp spin column into a new collection tube, discard the old collection tube, and centrifuge at full speed (approx. 14,000 x g) for 1 minute.
14. Place the QIAamp Mini column in a clean, clearly labeled 1.7 mL RNase-free microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate.
15. Carefully open the QIAamp Mini column and **add 70 µL of Buffer AVE** that has been equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 minute.
16. Centrifuge at 6,000 x g for 1 minute. RNA is now present in the eluate and ready to test. Store specimens and controls at 2-8°C until PCR master mixes are prepared.
17. Extracted specimens should be tested by PCR within 6 hours of completing the extraction process. Residual unextracted specimens should be stored at 2-8°C while testing is in progress. Long-term storage of extracted specimens (>6 hours) should be at -20°C (preferably -80°C). Minimize (not to exceed 3) repeated freeze-thaw cycles.

Appendix E: Programming the CFX96 Touch

Equipment Preparation

1. Turn on computer and CFX96 Touch instrument.
2. Once computer has booted, open the Bio-Rad CFX Manager
3. Initiate start-up wizard.
4. Select **Create a New Experiment**: run type: user-defined

Set Thermal Cycling Conditions

5. In Experiment Setup, under Options, select the **Protocol** tab.
6. Set thermal cycling parameters for the run:
   a. If thermal cycling parameters have not previously been established on the instrument:
      i. select **Create New**
      ii. adjust thermal cycling parameters to match those specified in this document
      iii. be sure that at 55°C **Plate Read** is selected

      NOTE: It may be helpful to save the cycling parameters as a protocol on the CFX96 Touch so they can be accessed quickly from the Express Load drop down menu.
   b. If the thermal cycling parameters for this assay have previously been programmed into the instrument, select your EBOV NP rRT-PCR parameters from the Express Load drop down menu.
   c. Press **OK**

Edit Plate Setup

7. In Experiment Setup, under Options, select the **Plate** tab.
8. Click **Edit Selected**
9. If the wells in the plate menu contain default settings, highlight all wells and click **Clear Wells**
10. Choose **Select Fluorophores**.
11. Make sure **FAM** is the only fluorophore selected. Click **OK**.

Well Settings – may be entered before or after the run.

12. Enter target information
    a. Select all wells that will contain NP2 reactions.
    b. Click on the box next to FAM under Target Name
    c. Select **NP2** from the drop down menu or, if it is not there, type it into the box.
    d. Select all wells that will contain RP reactions.
    e. Click on the box next to FAM under Target Name
    f. Select **RP** from the drop down menu or, if it is not there, type it into the box.

13. Enter specimen type information
    a. Select all wells that will contain the no template control reactions (NTCs)
    b. Select **NTC** from the Sample Type drop down menu.
    c. Follow steps 13a.-b. to label wells that will contain positive control reactions, negative control (HSC) reactions, and unknowns.
14. Enter specimen name or identifier information
    a. One-by-one, highlight each well that will contain an unknown
    b. Add the appropriate specimen name or identifier in the box under Sample Name
15. Once all plate setup information has been entered, click **OK** and save the plate setup.