Ebola Zaire (EZ1) rRT-PCR (TaqMan®) Assay
On
ABI® 7500 Fast Dx, LightCycler®, and JBAIDS

INSTRUCTION BOOKLET

Version 2.0
14 August 2014

For Use Under an Emergency Use Authorization (EUA) Only

Manufactured by the Naval Medical Research Center for
The U.S. Department of Defense

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COMMON INFORMATION

1. INTENDED USE STATEMENT

The Ebola Zaire Target 1 (EZ1) real-time reverse transcription (rRT) polymerase chain reaction (PCR) (TaqMan®) assay (EZ1 assay) is for the presumptive detection of Ebola Zaire virus (detected in the West Africa outbreak in 2014) on specified instruments in individuals in affected areas with signs and symptoms of Ebola virus infection or who are at risk for exposure or may have been exposed to the Ebola Zaire virus (detected in the West Africa outbreak in 2014) in conjunction with epidemiological risk factors. The EZ1 assay is intended for use only on authorized platforms by laboratories designated by DoD.

Testing with the EZ1 assay should not be performed unless the individual has been exposed to or is at risk for exposure to Ebola Zaire virus or has signs and symptoms of infection with Ebola Zaire virus (detected in the West Africa outbreak in 2014) that meet clinical and epidemiologic criteria for testing suspect specimens.

The level of Ebola Zaire virus (detected in the West Africa outbreak in 2014) present in blood from individuals with early systemic infection is unknown. Negative results do not preclude Ebola Zaire virus (detected in the West Africa outbreak in 2014) infection and should not be used as the sole basis for patient management decisions. Results are for the presumptive identification of the Ebola Zaire virus (detected in the West Africa outbreak in 2014). The definitive identification of the Ebola Zaire virus (detected in the West Africa outbreak in 2014) requires additional testing and confirmation procedures in consultation with public health or other authorities for whom reporting is required. The diagnosis of Ebola Zaire virus (detected in the West Africa outbreak in 2014) infection must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence in addition to the identification of the Ebola Zaire virus (detected in the West Africa outbreak in 2014) by this test.

The EZ1 assay is for use only under Emergency Use Authorization (EUA) by specified laboratories and clinical laboratory personnel who have been trained on authorized instruments.

2. INTRODUCTION

The EZ1 assay has been authorized for use on the Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument (ABI 7500 Fast Dx), Roche LightCycler® (LightCycler), and Biofire Defense Joint Biological Agent Identification and Diagnostic System (JBAIDS) to provide Ebola Zaire virus testing capability to sites that currently perform PCR testing on these instruments.

All users, analysts, and any person reporting diagnostic results from the use of these devices should be trained in rRT-PCR on the specified instrument. Use of this assay is limited to laboratories designated by DoD.

3. KIT LABELS & STORAGE OF CONTENTS

3.1 Examples of Labels Found on the Naval Medical Research Center (NMRC) Reagent Vials

Figure 1. PEUA140014

EUA Assay Ebola Zaire (EZ1)
Master Mix
Lot: XXXXXX—XXM Exp XX/XX
40 Rxns 584 µL Store -20°C

RNAseP Master Mix
Lot: XXXXXX—XXM Exp XX/XX
40 Rxns 584 µL Store -20°C

RT-Taq Polymerase
Lot: XXXXXXXXX Store -20°C
80 Rxns 35.5 µL Exp XX/XX

EUA Assay Ebola Zaire (EZ1)
100X Positive Control
Lot: XXXXXX—XXS XXXXXX
50 µL Exp XX/XX Store -20°C

100X RNAseP
Positive Control
Lot: XXXXXX—XXS XXXXXX
50 µL Exp XX/XX Store -20°C

Individual Tube Labels, 5 reagents make up one kit

EUA Assay Ebola Zaire (EZ1)
Reagents
Total Tubes enclosed: 3

Label for the outside of the bag containing Master Mix, RNAseP Master Mix, and RT-Taq Polymerase

EUA Assay Ebola Zaire (EZ1)
Positive Controls
Total Tubes enclosed: 2

Label for the outside of the bag containing Master Mix Positive Control and RNAseP Positive Control

EUA Assay Ebola Zaire (EZ1)
For *in vitro* Diagnostic Use Only
Under EUA On Authorized Platforms

Manufactured by:
Naval Medical Research Center — Frederick

Catalog Number: EZ1-PEUA140014

Storage: -20°C No. of tests: 40

Label for the outside of the bag that contains the smaller bags of reagents and positive controls

3.2 Storage Instructions

3.2.1 Store controls and all kit components at -20°C upon receipt.

3.2.2 Do not use any product past the labeled expiration date.

3.2.3 Do not store in frost-free freezers. Store in a non-defrosting freezer.

3.3 Instructions for Diluting and Aliquoting Controls

3.3.1 Upon initial thaw, prepare 1X controls by adding 10 µL of 100X control material to 990 µL of molecular grade water and vortexing for 5 seconds. Discard remaining 100X control.

3.3.2 Dispense into 100 µL aliquots (10 individual vials)

- Place proper labels on each of the tubes clearly indicating a name and date.
- Store aliquots of controls at -20°C or below.
- Aliquots of 1X controls are single-use vials.

3.4 Blood Specimens will be extracted using the Qiagen QIAamp Viral RNA Mini Kit

Each extracted Trizol-inactivated blood or Trizol-inactivated plasma specimen should be tested with both EZ1 Master Mix and RNase P (RP) Master Mix.

Note: It is very important that all blood samples are inactivated according to the Trizol Inactivation Procedure **(Appendix A).**

3.5 Reagents

EZ1 Master Mix contains primer and probe sequences that specifically detect Ebola Zaire in Trizol-inactivated whole blood or Trizol-inactivated plasma specimens.

The EZ1 detection assay also includes the following control materials:

3.5.1 **RNase P (RP)** detects human RP and is used as a positive control with human clinical specimens to indicate that adequate isolation of nucleic acid resulted from the extraction of the clinical specimen.

3.5.2 **EZ1 Positive Template Control (EZ1-PTC)** is a PTC designed to react with the EZ1-PCR reagents to indicate whether the Ebola Zaire PCR reaction worked. This PTC material consists of synthetic template RNA.

3.5.3 **RP Positive Template Control (RP-PTC)** is designed to react with the RP rRT-PCR reagents to indicate whether the RP PCR reaction worked. This PTC material consists of synthetic template RNA. The RP-PTC should only be positive for the RP assay.

Other controls that should be included in each run include:

3.5.4 **Negative Processing Control (NPC)** is a water sample and will serve as an external negative sample processing control.

3.5.5 **Reagent Negative Template Control (RNTC)** is a water sample that will be prepared in the reagent area and will serve as a check for contamination in the reagents.

3.5.6 **Sample No Template Control (SNTC)** is a water sample that will be prepared in the reagent area and will not be capped until after all samples have been loaded and will serve as a check for cross contamination while loading samples.

4. RNA EXTRACTION PROCEDURE

4.1 Equipment

Table 1. REQUIRED EXTRACTION Equipment

Refrigerator, 2-8°C
Pipettes (0.5 µL-1000 µL)
Vortexer (VWR 58810-163 or equivalent)
Microcentrifuge (Eppendorf 5415C or equivalent)
Heat block or water bath, ambient-85 °C
Class II biosafety cabinet (or glove box)

4.2 Materials

Table 2. Extraction Materials Required but not Provided

Personal protective equipment: Powder-free gloves, lab coat, eye protection, etc.
Tube racks, microcentrifuge tube racks
1.5 mL nuclease-free microcentrifuge tubes
Assorted aerosol barrier filter, nuclease-free pipette tips
Molecular grade water (nuclease-free water—Ambion Cat. No. AM9937 or equivalent)
Absolute ethanol (96-100%)
Qiagen QIAamp Viral RNA Mini Kit (Qiagen Cat. No. 52906 or 52904)
TRIzol® LS reagent (Life Technologies Cat. No. 10296-010, 100 mL) or TRI Reagent® LS (Sigma Cat. No. T3934-100mL, 100mL)

Note: Prior to extraction, all specimens should be handled in a Class II biological safety cabinet, if available.

4.3 Obtaining Specimens

4.3.1 Appropriate specimens are Trizol-inactivated whole blood or Trizol-inactivated plasma specimens, collected in lavender-top EDTA tubes (see Appendix A).

4.3.2 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. To support the extraction protocol as described **below** (70 µL input volume), an initial minimum specimen volume of 150 µL of Trizol-inactivated whole blood or Trizol-inactivated plasma is recommended.

4.4 RNA Extraction using the QIAamp® Viral RNA Mini Kit

Prepare QIAamp Viral RNA Mini kit reagents in accordance with manufacturer guidelines (Qiagen manual).

Note: Specimens can be extracted in sets of 12, plus a NPC (see 3.5.4).
Qiagen Buffer AVL with carrier RNA is stable at room temperature for 3-4 hours; however, store reconstituted Buffer AVL/Carrier RNA at 2-8°C for longer periods. A precipitate will form and must be redissolved by warming at 80°C before use. DO NOT warm solution more than 6 times and DO NOT incubate for more than 5 minutes (see manufacturer guidelines for more information). It is recommended to aliquot out the Qiagen Buffer AVL containing carrier RNA so as to prevent warming the reagent vial more than 6 times. If aliquoting in larger than single use volumes, then mark on the vial how many times the vial has undergone warming.

4.4.1 Pipette **280 µL of prepared Buffer AVL containing carrier RNA** into a 1.5 mL labeled microcentrifuge tube.

4.4.2 Add **70 µL of specimen or 70 µL of water for NPC** to be extracted to the 1.5 mL labeled RNase-free microcentrifuge tube and mix by by pulse-vortexing for 15 seconds.

4.4.3 Incubate specimen(s) and control at room temperature (15–25°C) for 10 minutes.

4.4.4 Briefly centrifuge the tubes to remove drops from the inside of the lid.

4.4.5 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.

4.4.6 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.

4.4.7 Carefully transfer the mixture from step 4.4.5, including any precipitate, to the QIAamp spin column WITHOUT moistening the rim of the column.

4.4.8 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.

4.4.9 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.

4.4.10 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.

4.4.11 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**.

4.4.12 Centrifuge at full speed (approx. 14,000 x g) for 3 minutes. Discard the tube containing the filtrate from the previous step.

4.4.13 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.

4.4.14 Place the QIAamp Mini column in a clean, clearly labeled 1.5 mL RNase-free microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate.

4.4.15 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.

4.4.16 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.

4.4.17 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.

INSTRUMENT SPECIFIC INSTRUCTIONS

5. TESTING SPECIMENS ON ABI 7500 FAST DX

5.1 Equipment

Table 3. EZ1 Equipment

Freezer, -20°C
Refrigerator, 4°C
Pipettes (ranges from 0.5 µL-1,000 µL)
ABI 7500 Fast Dx
ABI 7500 Fast Dx software (Version 1.4 or later)
Class II biosafety cabinet (or glove box)
ABI 7500 Plate Sealer

5.2 Materials

5.2.1 The EZ1 Assay

NMRC Catalog Number: EZ1-PEUA140014. The kit reagents consist of the following:

Table 4. EZ1 Kit Reagents

Components	Quantity per vial
EZ1 Master Mix containing primers, probe, and reaction buffer	584 µL
RP Master Mix containing primers, probe, and reaction buffer	584 µL
RT-Taq Polymerase	35.5 µL
EZ1 Positive Template Control (EZ1-PTC)	50 µL
RP Positive Template Control (RP-PTC)	50 µL

- Probes in master mix contain 6-FAM reporter and TAMRA quencher.
- Approximate number of tests (specimens and control tests) per kit: 40.
- Master mixes and RT-Taq Polymerase should be stored at -20°C upon arrival until use.
- Store EZ1-PTC and RP-PTC at -20°C upon receipt. After first thaw, aliquot into single-use volumes and store at -20°C. Minimize (not to exceed 3) repeated freeze-thaw cycles.

5.2.2 Other Materials Required but Not Provided in Kit

Table 5. EZ1 Materials Required but not Provided

Personal protective equipment: Powder-free gloves, lab coat, etc.
Tube racks, microcentrifuge tube racks
1.5 mL nuclease-free microcentrifuge tubes
Assorted aerosol barrier filter, nuclease-free pipette tips
Molecular grade water (nuclease-free water—Ambion Cat. No. AM9937 or equivalent)
96 Well ABI 7500 Plates (Cat. No. 4346906)
ABI 7500 96 Well Plate Optical Adhesive Film (Cat. No. 4311971) or ABI MicroAmp® Optical 8-Cap Strip (Cat. No. 4323032)

5.3 Procedure

5.3.1 Reaction Setup

5.3.1.1 Determine the number of reactions (N) to set up per assay. Calculate required volumes of master mixes and RT-Taq Polymerasebased on what is shown below. It is necessary to make excess reaction cocktail to allow for the NTC, PTC, and NPC reactions and pipetting error.

- If number of specimens (n) including controls = 1 to 14, then N=n+1
- If number of specimens (n) including controls >15, then N=n+2

5.3.1.2 Master mixes come premade with primers, probes, and reaction buffer included. Dispense calculated volumes of EZ1 and RP Master Mixes into individually labeled 1.5 mL microcentrifuge tubes. Each reaction requires 14.6 µL of master mix.

5.3.1.3 Based on the number of reactions, add corresponding amounts of RT-Taq Polymerase (includes both reverse transcriptase and Taq polymerase) to both master mix tubes. Each reaction requires 0.4 µL of RT-Taq Polymerase.

5.3.1.4 Master Mix (+): Indicating master mix with RT-Taq Polymerase added: Calculate each master mix volume and amount of RT-Taq Polymerase to be added to each microcentrifuge tube. The calculations are as follows:

Table 6. EZ1 Master Mix

Reagent	Volume of Reagent Added per Reaction
Ebola Zaire Master Mix	N x 14.6 µL
RT-Taq Polymerase	N x 0.4 µL
Total Volume of Ebola Zaire Master Mix (+)	N x 15 µL

OR

Table 7. RP Master Mix

Reagent	Volume of Reagent Added per Reaction
RP Master Mix	N x 14.6 µL
RT-Taq Polymerase	N x 0.4 µL
Total Volume of RP Master Mix (+)	N x 15 µL

- Due to the viscosity of the RT-Taq Polymerase, pipette slowly and mix by gently pipetting up and down.

5.3.1.5 After addition of the components, mix reaction mixtures by gently pipetting up and down. Do not vortex.

5.3.1.6 Pulse centrifuge at full speed for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack (2-8°C).

5.3.1.7 Set up 96 well plates.

5.3.1.8 Dispense 15 µL of each master mix (+) into each well on the plate, as indicated in the diagrams below. Dispense one master mix (+) into all of the required positions before moving on to the next master mix (+). For example, dispense 15 µL EZ1 Master Mix (+) into the appropriate wells before moving onto the RP Master Mix (+).

5.3.2 Plate Setup

5.3.2.1 Tables 8 and 9 show the test setup for a 96 well plate system. This test assumes testing a clinical specimen in one single well (i.e., a "singlet testing"). Once all master mixes (+) are prepared, it is pertinent that the user place the appropriate master mix (+) in the correct positions. Add all of the master mix (+) combinations from the first table into the appropriate positions. Once this is complete, specimens and controls will then be added according to the illustrations below.

Table 8. The Positions for the Two Master Mixes (+): The EZ1 Master Mix (+) should be dispensed into rows A, C, E, and G while the RP Master Mix (+) should be dispensed into rows B, D, F, and H as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1
B	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
C	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1
D	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
E	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1
F	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
G	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1	EZ1
H	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP

Assuming singlet testing, up to 44 specimens can be extracted and batched on 1 plate.

Table 9. Plate 1. (contains 44 specimens plus RNTC, SNTC, NPC, EZ1-PTC, & RP-PTC)

	1	2	3	4	5	6	7	8	9	10	11	12
A	RNTC EZ1	UNK 2	UNK 6	UNK 10	UNK 14	UNK 18	UNK 22	UNK 26	UNK 30	UNK 34	UNK 38	UNK 42
B	RNTC RP	UNK 2	UNK 6	UNK 10	UNK 14	UNK 18	UNK 22	UNK 26	UNK 30	UNK 34	UNK 38	UNK 42
C	SNTC EZ1	UNK 3	UNK 7	UNK 11	UNK 15	UNK 19	UNK 23	UNK 27	UNK 31	UNK 35	UNK 39	UNK 43
D	SNTC RP	UNK 3	UNK 7	UNK 11	UNK 15	UNK 19	UNK 23	UNK 27	UNK 31	UNK 35	UNK 39	UNK 43
E	NPC EZ1	UNK 4	UNK 8	UNK 12	UNK 16	UNK 20	UNK 24	UNK 28	UNK 32	UNK 36	UNK 40	UNK 44
F	NPC RP	UNK 4	UNK 8	UNK 12	UNK 16	UNK 20	UNK 24	UNK 28	UNK 32	UNK 36	UNK 40	UNK 44
G	UNK 1	UNK 5	UNK 9	UNK 13	UNK 17	UNK 21	UNK 25	UNK 29	UNK 33	UNK 37	UNK 41	EZ1 PTC
H	UNK 1	UNK 5	UNK 9	UNK 13	UNK 17	UNK 21	UNK 25	UNK 29	UNK 33	UNK 37	UNK 41	RP PTC

Note: The NTCs (RNTC, SNTC) should be added first before any of the clinical specimens are added. The negative external processing control should be added next. Positive template controls (PTC) should be added last.

5.3.2.2 Before moving the plate to the nucleic acid handling area, set up the NTC reactions in the reagent prep area.

5.3.2.3 Pipette 5 µL of nuclease-free water into the NTC wells.

Note: If using the MicroAmp® Optical 8-Cap Strip instead of the ABI 7500 96 Well Plate Optical Adhesive Film, cut two caps from the strip and cap RNTC wells.

5.3.2.4 Change personal protective equipment (PPE) such as gloves and lab coat when moving from the reagent area to the nucleic acid handling area.

5.4 Specimen Setup

5.4.1 Set up the extracted nucleic acid specimens in the cold rack (2-8°C).

5.4.2 **Pipette 5 µL of the first specimen** into both the EZ1 well and RP well labeled for that specimen, beginning with the NPC followed by specimen 1 (e.g., Unknown Specimen 1 would go into boxes labeled "UNK 1" shown in Table 9).

5.4.3 Change tips after each addition (since there is no way of capping the specimens in the plate, take extra precaution not to cross contaminate the specimens when loading). Change gloves between specimens and before starting extraction, processing, and the specimen run.

5.4.4 Repeat previous steps for the remaining specimens.

Note: If using the 8-Cap Strip, cap samples, NPC, and SNTC prior to addition of PTCs.

5.4.5 Add 5 µL of RP-PTC (RP Positive control) to the RP-PTC well.

5.4.6 Finally, pipette 5 µL of EZ1 positive template control RNA into EZ1 PTC well.

Note: If using the 8-Cap Strip, cap PTCs. Proceed to 5.4.9.

5.4.7 Once all of the specimens are added, remove the backing of the adhesive plate seal and place it over the entire plate.

5.4.8 Use the plate sealer to seal the adhesive to the plate.

5.4.9 Push on the dimple on the right side of the tray door on the ABI 7500 Fast Dx to open.

5.4.10 Place the reaction plate into the plate holder. Verify that the row headings (A through H) are on the left. The bar code on the plate should be facing out. Note that the Fast plates have a notch at the top left corner.

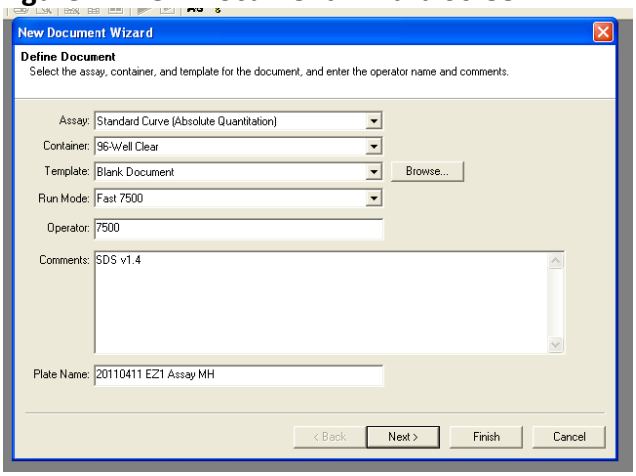
5.4.11 Push on the dimple on the right side of the tray door to close.

5.4.12 Launch the ABI 7500 Fast Dx Real-Time PCR System by double clicking on the ABI 7500 Fast Dx System SDS icon on the desktop.

5.4.13 Select “Create New Document” from the Quick start up menu.

5.4.14 The “New Document Wizard” screen will appear. Confirm that all the information is correct as shown below:

Figure 2. New Document Wizard Screen



5.4.15 Change the Plate name to the following format: Year Month Day EZ1 assay Operator's initials. For example: (11APR11 EZ1Assay MH).

5.4.16 Select "Next" and a new screen should appear to select detectors. Click on "New Detector."

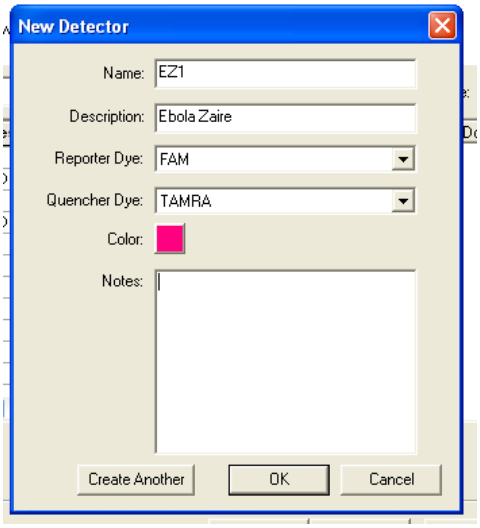
5.4.17 Name the "New Detector." Type in the "Name" space.

5.4.18 Select "FAM" for the Reporter Dye and "TAMRA" for the Quencher Dye.

5.4.19 Click on the "Colors" icon to display the color pallet, and select a color to associate with your detector.

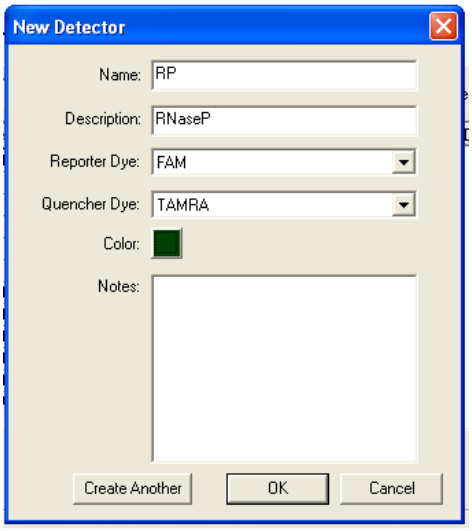
5.4.20 Confirm the detector information is correct as shown below:

Figure 3. EZ1 Detector Screen



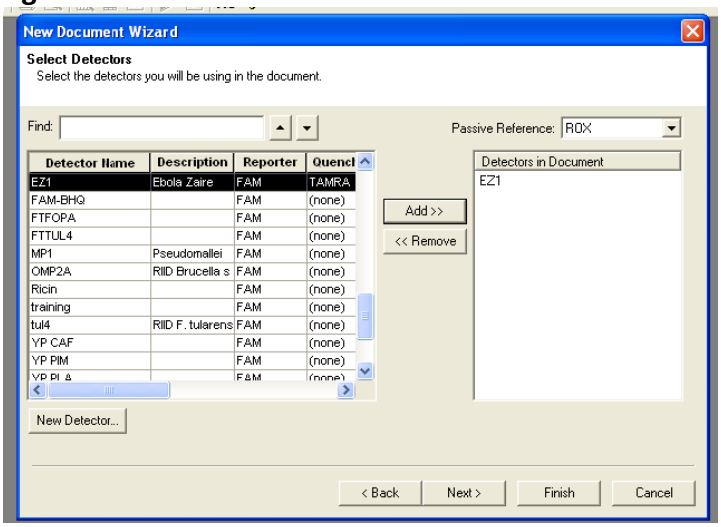
5.4.21 Repeat steps 5.4.16 through 5.4.20 to add the "RP" detector.

Figure 4. RP Detector Screen



5.4.22 When you are done, click "OK," then locate the EZ1 detector and add it to the right column as shown below:

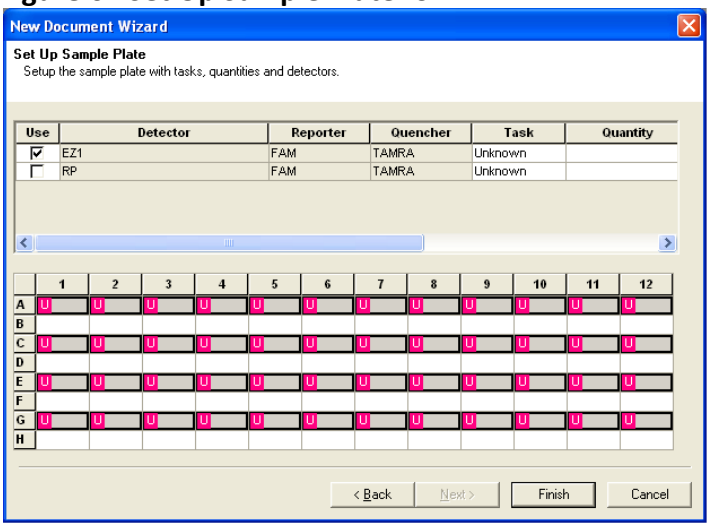
Figure 5. New Document Wizard Screen



5.4.23 Click on the "Passive Reference" box which should be displaying "ROX." Select "none" from the drop-down menu.

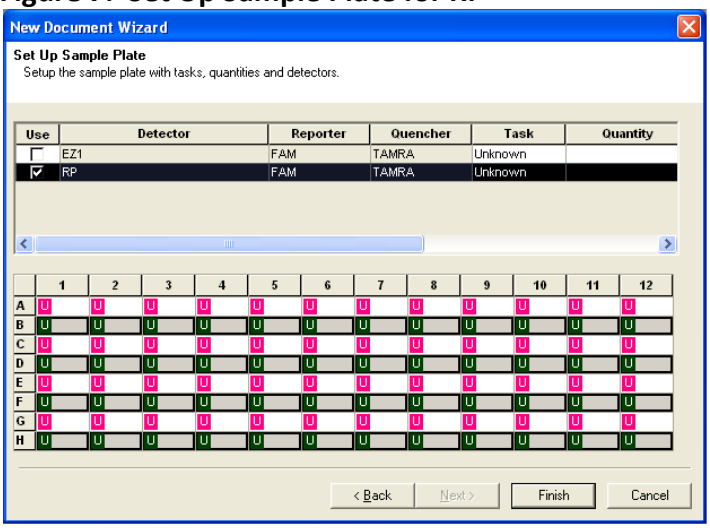
5.4.24 Using the mouse, press and hold the Ctrl button and click on rows A, C, E, and G to highlight boxes corresponding to all of the wells containing specimens and controls for the EZ1 detector. While the boxes are highlighted, click on the box beside the EZ1 detector so that a check mark appears in the box.

Figure 6. Set Up Sample Plate for EZ1



5.4.24.1 Using the mouse, press and hold the Ctrl button and click on rows B, D, F, and H to highlight boxes corresponding to all of the wells containing specimens and controls for the RP detector. While the boxes are highlighted, click on the box beside the RP detector so that a check mark appears in the box.

Figure 7. Set Up Sample Plate for RP



5.4.25 When this is completed, all the selected wells will show a “U” for “Unknown.”

5.4.26 Select “Finish,” there will be a pause while the ABI 7500 Fast initializes, followed by a “clicking” noise. This will confirm to you that the instrument is connected and ready to run. If the instrument is not found or has trouble connecting, refer back to your ABI 7500 Instruction Manual.

5.4.27 Using the mouse, click boxes that correspond to wells containing NTCs. Open the "Well Inspector" by right clicking the selected wells; click the "Task" icon where it says "unknown" to generate a drop down window, select "NTC." Repeat for all negative controls. The selected specimen wells should then show an "N" for the NTC's instead of a "U" as shown in the other wells. Close the window by clicking "x" in the upper right-hand corner.

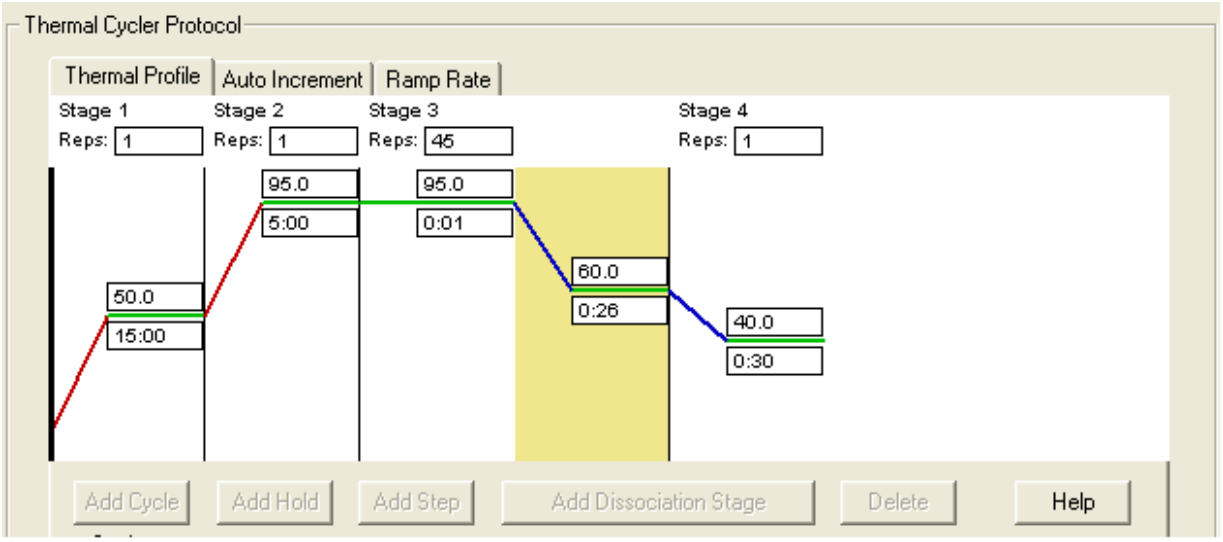
5.4.28 Enter control and sample IDs by single clicking on the corresponding box and typing the identification label (i.e., "R-NTC," "S-NTC," "EZ1-PTC," "RP-PTC," and sample ID numbers).

5.4.29 Select the "Instrument" tab.

5.4.30 Modify the thermal cycler conditions as follows:

- In Stage 1 — set to 50°C for 15 minutes
- In Stage 2 — set to 95°C for 5 minutes
- In Stage 3 — set to 95°C for 1 second
- In Stage 3 — click on "Add Step" set to 60°C for 26 seconds
- Change "Reps" to 45 cycles of Stage 3
- Click on "Add Cycle" and in Stage 4, set to 40°C for 30 seconds (cool down step)
- Make sure specimen volume in the bottom left-hand box shows 20µL
- In data collection, make sure it reads "Stage 3, Step 2 (60.0 @ 0:26)"
- Ensure that the "Run Mode" window selection is "Fast 7500"
- Confirm cycles are as shown below:

Figure 8. Thermal Cycler Protocol Screen



5.4.31 Before starting the run, the file must first be saved. Select "File" and then "Save As." Give the experiment a file name: Year Month Day EZ1Assay Operator's initials. For example: (11APR11 EZ1Assay MH) and click the "Save" icon.

5.4.32 Click the "Start" icon on the left hand side of the screen. The run should take approximately 50 minutes.

5.5 Amplification Conditions

5.5.1 The reaction volume is 20 µL. Program the thermocycler as follows:

Table 10. Thermocycler Programming:

Reverse Transcription (1 cycle)	50°C for 15 minutes
Taq Inhibitor Inactivation (1 cycle)	95°C for 5 minutes
PCR Amplification (45 cycles)	95°C for 1 second 60°C for 26 seconds
Cool Down (1 cycle)	40°C for 30 seconds

5.5.2 Fluorescence data (FAM) is collected during the 60°C amplification step.

5.6 PCR Analysis Using ABI 7500 Fast Dx Software

5.6.1 After the run is completed, click on the "Results" icon.

5.6.2 Click on the "Amplification Plot" tab and view and adjust the raw data.

- Highlight all the specimens by clicking on the upper left hand box of the specimen wells (all the growth curves should appear on the graph).
- In the "Data" window, "Delta Rn vs Cycle" should be selected.
- In the "Detector" window, "All" should be selected.
- The "Start (cycle)" window should read "3."
- The "End (cycle)" needs to be adjusted such that the value corresponds to the cycle just before exponential phase of the PCR is observed.
- Lastly, be sure to click the "Analyze" icon to update the analysis.

5.6.3 Using the mouse, click and drag the red threshold line until it lies within the exponential phase of the fluorescence curves and above any background noise.

5.6.4 Click the "Analyze" icon on the right. The red threshold line will turn to green, indicating the data has been analyzed.

5.6.5 Click the "Report" icon above the graph to display the cycle threshold (C_t) values.

5.6.6 Save file prior to printing.

5.6.7 The report may be printed by selecting "File" then "Print."

5.6.8 The report can also be printed by first exporting the data, opening the exported file using Microsoft Excel, and then printing from Excel.

5.6.9 A positive amplification result on the ABI 7500 Fast Dx generates a C_t value. A negative amplification result does not generate a C_t value (shown as *Undet*).

5.7 Points of Contact

5.7.1 For questions regarding operations of the ABI 7500 Fast Dx, instrument, please contact ABI directly (Dial 1-800-831-6844, select option 5, and then option 3).

Web address: http://www6.appliedbiosystems.com/support/contact/index.cfm

5.7.2 For questions regarding components of the EZ1 assay for use on authorized instruments, please contact:

U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID)
Diagnostics Systems Division
Field Operations and Training
1425 Porter Street
Fort Detrick, MD 21702-5011
Commercial: 301-619-6357
DSN: 343-6357

Director, Field Operations and Training: 301-619-4738
DSN: 343-6357/4738

Deputy Director, Field Operations and Training: 301-619-6357
DSN: 343-6357

Chief, Diagnostic Systems Division: 301-619-4721
DSN: 343-6357/4721

6. TESTING SPECIMENS ON LIGHTCYCLER

6.1 Equipment

Table 11. EZ1 Equipment

Freezer, -20°C
Refrigerator, 4°C
Pipettes (ranges from 0.5 µL-1000 µL)
Microcentrifuge with capillary tube inserts (Eppendorf 5415C or equivalent)
Chilled Capillary Tube Racks (Roche)
LightCycler 1.5
LightCycler Software: LightCycler 3 Front v. 3.5.17 LightCycler 3 Run v. 5.32 LightCycler 3 Data Analysis v. 3.5.28
Class II biosafety cabinet (or glove box)
Capillary capping tool or forceps

6.2 Materials

6.2.1 The EZ1 Assay

NMRC Catalog Number: EZ1-PEUA140014. The kit reagents consist of the following:

Table 12. EZ1 Kit Reagents

Components	Quantity per vial
EZ1 Master Mix containing primers, probe, and reaction buffer	584 µL
RP Master Mix containing primers, probe, and reaction buffer	584 µL
RT-Taq Polymerase	35.5 µL
EZ1 Positive Template Control (EZ1-PTC)	50 µL
RP Positive Template Control (RP-PTC)	50 µL

- Probes in master mix contain 6-FAM reporter and TAMRA quencher.
- Approximate number of tests (specimens and control tests) per kit: 40.
- Master mixes and RT-Taq Polymerase should be stored at -20°C upon arrival until use.
- Store EZ1-PTC and RP-PTC at -20°C upon receipt. After first thaw, aliquot into single-use volumes and store at -20°C. Minimize (not to exceed 3) repeated freeze-thaw cycles.

6.2.2 Other Materials Required but Not Provided in kit

Table 13. EZ1 Materials Required but not Provided

Personal protective equipment: Powder-free gloves, lab coat, etc.
Tube racks, microcentrifuge tube racks
1.5 mL nuclease-free microcentrifuge tubes
Assorted aerosol barrier filter, nuclease-free pipette tips
Molecular grade water (nuclease-free water—Ambion Cat. No. AM9937 or equivalent)
LightCycler glass capillaries (Roche Cat. No. 04929292001)

6.3 Procedure

6.3.1 Reaction Setup

6.3.1.1 Determine the number of reactions (N) to set up per assay. Calculate required volumes of master mixes and RT-Taq Polymerase based shown below. It is necessary to make excess reaction cocktail to allow for the NTC, PTC, and NPC reactions and pipetting error.

- If number of specimens (n) including controls = 1 to 14, then N=n+1
- If number of specimens (n) including controls >15, then N=n+2

6.3.1.2 Master mixes come premade with primers, probes, and reaction buffer included. Dispense calculated volumes of EZ1 and RP master mixes into individually labeled 1.5 mL microcentrifuge tubes. Each reaction requires 14.6 µL of master mix

6.3.1.3 Based on the number of reactions, add corresponding amounts of Taq Enyme Mix (includes both reverse transcriptase and Taq polymerase) to both master mix tubes. Each reaction requires 0.4 µL of RT-Taq Polymerase.

6.3.1.4 Master Mix (+): Indicating master mix with RT-Taq Polymerase added: Calculate each master mix volume and amount of RT-Taq Polymerase to be added to each microcentrifuge tube. The calculations are as follows:

Table 14. EZ1 Master Mix

Reagent	Volume of Reagent Added per Reaction
Ebola Zaire Master Mix	N x 14.6 µL
RT-Taq Polymerase	N x 0.4 µL
Total Volume of Ebola Zaire Master Mix (+)	N x 15 µL

OR

Table 15. RP Master Mix

Reagent	Volume of Reagent Added per Reaction
RP Master Mix	N x 14.6 µL
RT-Taq Polymerase	N x 0.4 µL
Total Volume of RP Master Mix (+)	N x 15.0 µL

- Due to the viscosity of the RT-Taq Polymerase, pipette slowly and mix by gently pipetting up and down.

6.3.1.5 After addition of the components, mix reaction mixtures by gently pipetting up and down. Do not vortex.

6.3.1.6 Pulse centrifuge at full speed for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack (2-8°C).

6.3.1.7 Set up capillaries or PCR reaction tubes, as appropriate, in a cold rack.

6.3.1.8 Dispense 15 µL of each master mix (+) into each capillary, as indicated in the diagrams below. Dispense one master mix (+) into all of the required positions before moving on to the next master mix (+). For example, dispense 15 µL EZ1 Master Mix (+) into the appropriate capillaries before moving onto the RP Master Mix (+).

6.3.2 Run Setup

6.3.2.1 Tables 16 and 17 show the test setup for a 32-position Carousel-based system. This test assumes testing a clinical specimen in one single capillary (i.e., a “singlet testing”). Once all master mixes (+) are prepared, it is pertinent that the user place the appropriate master mix (+) in the correct positions. Add all of the master mix (+) combinations from the first table into the appropriate positions. Once this is complete, specimens and controls will then be added according to the illustrations below.

Table 16. The Positions for the Two Master Mixes (+): The EZ1 Master Mix (+) should be dispensed into all odd positions while the RP Master Mix (+) should be dispensed into all even positions shown below:

Position		Position		Position		Position	
1	EZ1	9	EZ1	17	EZ1	25	EZ1
2	RP	10	RP	18	RP	26	RP
3	EZ1	11	EZ1	19	EZ1	27	EZ1
4	RP	12	RP	20	RP	28	RP
5	EZ1	13	EZ1	21	EZ1	29	EZ1
6	RP	14	RP	22	RP	30	RP
7	EZ1	15	EZ1	23	EZ1	31	EZ1
8	RP	16	RP	24	RP	32	RP

Assuming singlet testing, up to 12 specimens can be extracted and batched on 1 rotor.

Table 17. Rotor 1. (contains 12 specimens plus RNTC, NPC, SNTC, EZ1-PTC, & RP-PTC)

Position		Position		Position		Position	
1	RNTC	**9**	S4	**17**	S8	**25**	S12
2	RNTC	**10**	S4	**18**	S8	**26**	S12
3	S1	**11**	S5	**19**	S9	**27**	NPC
4	S1	**12**	S5	**20**	S9	**28**	NPC
5	S2	**13**	S6	**21**	S10	**29**	SNTC
6	S2	**14**	S6	**22**	S10	**30**	SNTC
7	S3	**15**	S7	**23**	S11	**31**	EZ1-PTC
8	S3	**16**	S7	**24**	S11	**32**	RP-PTC

Note: The NTCs should be added first before any of the clinical specimens are added. The RNTC should be capped in the reagent prep area before proceeding. The SNTC controls should be added next and not capped until all clinical specimens are loaded to check for possible cross contamination during the loading of specimens. The negative external processing control should be added next. Positive template controls (PTC) should be added last, after all clinical specimens and NTCs are sealed.

6.3.2.2 Before moving the capillaries to the nucleic acid handling area, set up the NTC reactions in the reagent prep area. As shown above, specimens can be added by column.

6.3.2.3 Pipette 5 µL of nuclease-free water into the NTC capillaries. Cap theRNTC capillaries.

6.3.2.4 Close the lid on the capillary box and move the capillaries to the nucleic acid handling area.

6.3.2.5 Change personal protective equipment (PPE) such as gloves and lab coat when moving from the reagent area to the nucleic acid handling area.

6.4 Specimen Setup

6.4.1 Set up the extracted nucleic acid specimens in the cold rack (2-8°C).

6.4.2 Pipette 5 µL of nuclease-free water into the SNTC capillaries. Do not cap the SNTC capillaries until all specimens have been added.

6.4.3 Pipette 5 µL of the first specimen into all the capillaries labeled for that specimen (e.g., Specimen "S1" as shown in Table 17). Change tips after each addition.

6.4.4 Cap the tubes to which the specimen has been added. This will help to prevent specimen cross contamination and enable you to keep track of where you are in the series.

6.4.5 Change gloves when necessary to avoid contamination, especially between specimens. Repeat previous steps for the remaining specimens.

6.4.6 Cap the SNTC capillaries before loading the positive controls.

6.4.7 Add 5 µL of RP-PTC to the RP-PTC capillary. Cap the RP-PTC tube.

6.4.8 Finally, pipette 5 µL of EZ1-PTC RNA into EZ1-PTC capillary. Cap the EZ1-PTC tube. All capillaries should now be capped.

6.4.9 Briefly centrifuge capillaries in microcentrifuge capillary inserts on soft mode for 10-15 seconds to ensure complete reaction mixture is in the bottom of the capillary. Return capillaries to cold rack (2-8°C).

6.4.10 Remove the LightCycler 32-position carousel from the system.

6.4.11 Load capillaries into carousel, beginning at position one and continue to load in sequential order.

6.4.12 Confirm that you have loaded the carousel with the reaction capillaries, according to the pattern you have entered on the screen. Once all capillaries are loaded, check to verify that all are equal in volume (20 µL) by lining up the liquid volume line in the capillaries loaded in the carousel. If a capillary is significantly low or high in volume (indicating non-addition or over-addition), appropriately discard the capillary and re-load its contents.

6.4.13 Replace the carousel into the LightCycler system, ensuring that the white dot inside the machine lines up with the notch on the carousel.

6.4.14 Push down to lock the carousel in place.

6.4.15 Start the run and wait for the instrument to detect all of the capillaries.

6.4.16 Ensure that the “samples count” at the top of the page correctly corresponds to the number of specimens in the run.

6.4.17 Start the run and wait for the instrument to detect all of the capillaries.

6.5 Amplification Conditions

6.5.1 The reaction volume is 20 µL. Program the thermocycler as follows:

Table 18. Thermocycler Programming

Reverse Transcription (1 cycle)	50°C for 15 minutes
Taq Inhibitor Inactivation (1 cycle)	95°C for 5 minutes
PCR Amplification (45 cycles)	95°C for 1 second 60°C for 20 seconds

6.5.2 Fluorescence data (FAM) is collected during the 60°C amplification step.

6.6 PCR Analysis Using LightCycler Software

6.6.1 When the LightCycler run is complete, perform an analysis by selecting ''Quantification' button. Select 'Second Derivitive Maximum' under Analysis, and 'Arithmetic' for baseline adjustment. Print a copy of the LightCycler Report.

6.6.2 A positive amplification result on the LightCycler 1.5 generates a C_p value. A negative amplification result does not generate a C_p value.

6.7 Points of Contact

6.7.1 For questions regarding operations of the LightCycler instrument, please contact:

Roche Diagnostics Corporation
9115 Hague Road
P.O. Box 50414
Indianapolis, IN 46250-0414
USA
1-800-262-4911
www.lifescience.roche.com

6.7.2 For questions regarding components of the EZ1 assay for use on authorized instruments, please contact:

USAMRIID, Diagnostics Systems Division
Field Operations and Training
1425 Porter Street
Fort Detrick, MD 21702-5011
Commercial: 301-619-6357
DSN: 343-6357

Director, Field Operations and Training: 301-619-4738
DSN: 343-6357/4738

Deputy Director, Field Operations and Training: 301-619-6357
DSN: 343-6357

Chief, Diagnostic Systems Division: 301-619-4721
DSN: 343-6357/4721

7. TESTING SPECIMENS ON JBAIDS

7.1 Equipment

Table 19. EZ1 Equipment

Freezer, -20°C
Refrigerator, 4°C
Pipettes (ranges from 0.5 µL-1000 µL)
Microcentrifuge with capillary tube inserts (Eppendorf 5415C or equivalent)
Chilled Capillary Tube Racks (Roche)
JBAIDS (Model JB01)
JBAIDS Software (Version 2.5 or later)
Class II biosafety cabinet (or glove box)
Capillary capping tool or forceps

7.2 Materials

7.2.1 The EZ1 Assay

NMRC Catalog Number: EZ1-PEUA140014. The kit reagents consist of the following:

Table 20. EZ1 Kit Reagents

Components	Quantity per vial
EZ1 Master Mix containing primers, probe, and reaction buffer	584 µL
RP Master Mix containing primers, probe, and reaction buffer	584 µL
RT-Taq Polymerase	35.5 µL
EZ1 Positive Template Control (EZ1-PTC)	50 µL
RP Positive Template Control (RP-PTC)	50 µL

- Probes in master mix contain 6-FAM reporter and TAMRA quencher.
- Approximate number of tests (specimens and control tests) per kit: 40.
- Master mixes and RT-Taq Polymerase should be stored at -20°C upon arrival until use.
- Store EZ-PTC and RP-PTC at -20°C upon receipt. After first thaw, aliquot into single-use volumes and store at -20°C. Minimize (not to exceed 3) repeated freeze-thaw cycles.

7.2.2 Other Materials Required but Not Provided in kit

Table 21. EZ1 Materials Required but not Provided

Personal protective equipment: Powder-free gloves, lab coat, etc.
Tube racks, microcentrifuge tube racks
1.5 mL nuclease-free microcentrifuge tubes
Assorted aerosol barrier filter, nuclease-free pipette tips
Molecular grade water (nuclease-free water—Ambion Cat. No. AM9937 or equivalent)
LightCycler glass capillaries (Roche Cat. No. 04929292001)

7.3 Procedure

7.3.1 Reaction Setup

7.3.1.1 Determine the number of reactions (N) to set up per assay. Calculate required volumes of master mixes and RT-Taq Polymerase based shown below. It is necessary to make excess reaction cocktail to allow for the NTC, PTC, and NPC reactions and pipetting error.

- If number of specimens (n) including controls = 1 to 14, then N=n+1
- If number of specimens (n) including controls >15, then N=n+2

7.3.1.2 Master mixes come premade with primers, probes, and reaction buffer included. Dispense calculated volumes of EZ1 and RP master mixes into individually labeled 1.5 mL microcentrifuge tubes. Each reaction requires 14.6 µL of master mix

7.3.1.3 Based on the number of reactions, add corresponding amounts of Enyme Mix (includes both reverse transcriptase and Taq polymerase) to both master mix tubes. Each reaction requires 0.4 µL of RT-Taq Polymerase.

7.3.1.4 Master Mix (+): Indicating master mix with RT-Taq Polymerase added: Calculate each master mix volume and amount of RT-Taq Polymerase to be added to each microcentrifuge tube. The calculations are as follows:

Table 22. EZ1 Master Mix

Reagent	Volume of Reagent Added per Reaction
Ebola Zaire Master Mix	N x 14.6 µL
RT-Taq Polymerase	N x 0.4 µL
Total Volume of Ebola Zaire Master Mix (+)	N x 15 µL

OR

Table 23. RP Master Mix

Reagent	Volume of Reagent Added per Reaction
RP Master Mix	N x 14.6 µL
RT-Taq Polymerase	N x 0.4 µL
Total Volume of RP Master Mix (+)	N x 15.0 µL

- Due to the viscosity of the RT-Taq Polymerase, pipette slowly and mix by gently pipetting up and down.

7.3.1.5 After addition of the components, mix reaction mixtures by gently pipetting up and down. Do not vortex.

7.3.1.6 Pulse centrifuge at full speed for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack (2-8°C).

7.3.1.7 Set up capillaries or PCR reaction tubes, as appropriate, in a cold rack.

7.3.1.8 Dispense 15 µL of each master mix (+) into each capillary, as indicated in the diagrams below. Dispense one master mix (+) into all of the required positions before moving on to the next master mix (+). For example, dispense 15 µL EZ1 Master Mix (+) into the appropriate capillaries before moving onto the RP Master Mix (+).

7.3.2 Run Setup

7.3.2.1 Tables 24 and 25 show the test setup for a 32-position Carousel-based system. This test assumes testing a clinical specimen in one single capillary (i.e., a "singlet testing"). Once all master mixes (+) are prepared, it is pertinent that the user place the appropriate master mix (+) in the correct positions. Add all of the master mix (+) combinations from the first table into the appropriate positions. Once this is complete, specimens and controls will then be added according to the illustrations below.

Table 24. The Positions for the Two Master Mixes (+): The EZ1 Master Mix (+) should be dispensed into all odd positions while the RP Master Mix (+) should be dispensed into all even positions shown below:

Position		Position		Position		Position	
1	EZ1	9	EZ1	17	EZ1	25	EZ1
2	RP	10	RP	18	RP	26	RP
3	EZ1	11	EZ1	19	EZ1	27	EZ1
4	RP	12	RP	20	RP	28	RP
5	EZ1	13	EZ1	21	EZ1	29	EZ1
6	RP	14	RP	22	RP	30	RP
7	EZ1	15	EZ1	23	EZ1	31	EZ1
8	RP	16	RP	24	RP	32	RP

Assuming singlet testing, up to 12 specimens can be extracted and batched on 1 rotor.

Table 25. Rotor 1. (contains 12 specimens plus RNTC, NPC, SNTC, EZ1-PTC, & RP-PTC)

Position		Position		Position		Position	
1	RNTC	**9**	S4	**17**	S8	**25**	S12
2	RNTC	**10**	S4	**18**	S8	**26**	S12
3	S1	**11**	S5	**19**	S9	**27**	NPC
4	S1	**12**	S5	**20**	S9	**28**	NPC
5	S2	**13**	S6	**21**	S10	**29**	SNTC
6	S2	**14**	S6	**22**	S10	**30**	SNTC
7	S3	**15**	S7	**23**	S11	**31**	EZ1-PTC
8	S3	**16**	S7	**24**	S11	**32**	RP-PTC

Note: The NTCs should be added first before any of the clinical specimens are added. The RNTC should be capped in the reagent prep area before proceeding. The SNTC controls should be added next and not capped until all clinical specimens are loaded to check for possible cross contamination during the loading of specimens. The negative external processing control should be added next. Positive template controls (PTC) should be added last, after all clinical specimens and NTCs are sealed.

7.3.2.2 Before moving the capillaries to the nucleic acid handling area, set up the NTC reactions in the reagent prep area. As shown above, specimens can be added by column.

7.3.2.3 Pipette 5 µL of nuclease-free water into the NTC capillaries. Cap the RNTC capillaries.

7.3.2.4 Close the lid on the capillary box and move the capillaries to the nucleic acid handling area.

7.3.2.5 Change personal protective equipment (PPE) such as gloves and lab coat when moving from the reagent area to the nucleic acid handling area.

7.4 Specimen Setup

7.4.1 Set up the extracted nucleic acid specimens in the cold rack (2-8°C).

7.4.2 Pipette 5 µL of nuclease-free water into the SNTC capillaries. Do not cap the SNTC capillaries until all specimens have been added.

7.4.3 Pipette 5 µL of the first specimen into all the capillaries labeled for that specimen (e.g., Specimen "S1" as shown in Table 25). Change tips after each addition.

7.4.4 Cap the tubes to which the specimen has been added. This will help to prevent specimen cross contamination and enable you to keep track of where you are in the series.

7.4.5 Change gloves when necessary to avoid contamination, especially between specimens. Repeat previous steps for the remaining specimens.

7.4.6 Cap the SNTC capillaries before loading the positive controls.

7.4.7 Add 5 µL of RP-PTC to the RP-PTC capillary. Cap the RP-PTC tube.

7.4.8 Finally, pipette 5 µL of EZ1-PTC RNA into EZ1-PTC capillary. Cap the EZ1-PTC tube. All capillaries should now be capped.

7.4.9 Briefly centrifuge capillaries in microcentrifuge capillary inserts on soft mode for 10-15 seconds to ensure complete reaction mixture is in the bottom of the capillary. Return capillaries to cold rack (2-8°C).

7.4.10 Remove the JBAIDS 32-position carousel from the system.

7.4.11 Load capillaries into carousel, beginning at position one and continue to load in sequential order.

7.4.12 Confirm that you have loaded the carousel with the reaction capillaries, according to the pattern you have entered on the screen. Once all capillaries are loaded, check to verify that all are equal in volume (20 µL) by lining up the liquid volume line in the capillaries loaded in the carousel. If a capillary is significantly low or high in volume (indicating non-addition or over-addition), appropriately discard the capillary and re-load its contents.

7.4.13 Replace the carousel into the JBAIDS system, ensuring that the arrows line up.

7.4.14 Lock the carousel in place.

7.4.15 Ensure that the “samples count” at the top of the page correctly corresponds to the number of specimens in the run.

7.4.16 Start the run and wait for the instrument to detect all of the capillaries.

7.5 Amplification Conditions

7.5.1 The reaction volume is 20 µL. Program the thermocycler as follows:

Table 26. Thermocycler Programming

Reverse Transcription (1 cycle)	50°C for 15 minutes
Taq Inhibitor Inactivation (1 cycle)	95°C for 5 minutes
PCR Amplification (45 cycles)	95°C for 1 second 60°C for 20 seconds

7.5.2 Fluorescence data (FAM) is collected during the 60°C amplification step.

7.6 PCR Analysis Using JBAIDS Software

7.6.1 When the JBAIDS run is complete, select "Finish." Perform an analysis by selecting "Analysis" then "Qualitative Detection." Print a copy of the JBAIDS report; choose "No" when asked to set up specimen settings prior to analysis.

7.6.2 The JBAIDS software analyzes the data and gives an automated determination. A *positive* target call by the software is considered a positive amplification result. A *negative* or *uncertain* target call by the software is a negative amplification result.

7.7 Points of Contact

7.7.1 For questions on the JBAIDS instrument and operations of the JBAIDS instrument, please contact the director of JBAIDS/NGDS Training Program:

Telephone: 210-221-7702 or 210-808-3215
Email: usarmy.jbsa.medcom-ameddcs.list.jbaids-training@mail.mil

7.7.2 For questions regarding components of the EZ1 assay for use on authorized instruments, please contact:

USAMRIID, Diagnostics Systems Division
Field Operations and Training
1425 Porter Street
Fort Detrick, MD 21702-5011
Commercial: 301-619-6357
DSN: 343-6357

Director, Field Operations and Training: 301-619-4738
DSN: 343-6357/4738

Deputy Director, Field Operations and Training: 301-619-6357
DSN: 343-6357

Chief, Diagnostic Systems Division: 301-619-4721
DSN: 343-6357/4721

INTERPRETATION OF RESULTS

8. INTERPRETATION OF CONTROLS AND CLINICAL SPECIMENS

8.1 Test Run is Valid when all Controls Meet the Following Stated Standards

8.1.1 In order for a test run to be valid, all NTC reactions must be negative without an amplification curve. If one or more NTC fails, the entire run is invalid and potential sources of contamination should be identified and corrected. Re-test the purified specimen and controls, and re-analyze.

8.1.2 Failure to achieve a positive PTC reaction for any of the assays invalidates the entire run. Obtain new PTC reagents and repeat the run.

8.1.3 The RP-PTC should be positive for the RP assay.

All clinical specimens should exhibit a positive RP reaction, indicating sufficient recovery of acceptable quality RNA from the specimen. A negative RP reaction could indicate improper RNA extraction, low recovery of RNA, improper reaction setup, reagent or equipment malfunction, PCR inhibition, or absence of sufficient human cellular material in the specimen to enable detection.

Positive results for the EZ1 assay should override a failed (negative or uncertain) RP assay result for that clinical specimen. A failed RP control assay reaction that accompanies a negative or uncertain target result requires the purified specimen to be re-tested, 1) undiluted and 2) at a 1:10 dilution (using nuclease-free water), with all assays (EZ1 and RP). Valid positive or negative re-test results for the undiluted specimen will be taken as the final result. Valid positive target assay results for the 1:10 dilution will also be considered the final result. Negative or uncertain results for the target assays or another failure of the control assay suggest possible PCR inhibition and require the original clinical specimens to be re-purified and re-tested.

8.1.4 The external negative processing control associated with each extraction batch should be negative with no amplification curves. If the external sample processing negative control for a set of extracted specimens is positive, all results for those specimens are invalid. Re-test the purified specimen and controls and re-analyze. If the re-test for the processing control results is positive, then re-extract the specimens and controls.

8.2 Unknown Specimen Interpretation

8.2.1 When all controls meet stated requirements (see Table 27), the run is valid.

8.2.2 A specimen is considered positive for Ebola Zaire virus if the specimen has a positive amplification result for the EZ1 assay. The RP assay may or may not have a positive amplification result as described above. A positive test specimen has priority over a negative RP assay result (see Table 27).

8.2.3 A specimen is considered negative for Ebola Zaire if the EZ1 assay does not have a positive amplification result and the RP assay has a positive amplification result (see Table 27).

Table 27. Interpretation of Control & Specimen Calls

Controls/Specimens	EZ1	RP	Action
RNTC/SNTC	-	-	Any (+) calls invalidate the run. Re-test from purified specimen.
NPC	-	-	Any (+) calls invalidate the run. Re-purify specimen and re-test.
EZ1-PTC	+	**N/A**	Any failures invalidate the run. Re-test from purified specimen. If EZ1-PTC fails again, obtain a NEW EZ1 kit.
RP-PTC	**N/A**	+	Should be (+) for RP. If (-), run is invalid. Re-run. If still (-), obtain new EZ1 kit.
Unknown specimens	+	+	If the EZ1assay and RP assay are (+), then specimen is (+) for Ebola Zaire.
Unknown specimens	+	-	If the EZ1 assay is (+) and the RP assay is (-), then the specimen is (+) for Ebola Zaire.
Unknown specimens	-	+	If is the EZ1 assay is (-) and the RP assay is (+), then the specimen is then considered (-) for Ebola Zaire.
Unknown specimens	-	-	Results for that specimen are invalid. Re-test extracted specimen undiluted and diluted 1:10. If both still come up (-), repeat extraction of specimen.

N/A = Not applicable

Assay Limitations

1. All results should be interpreted by a trained professional in conjunction with the patient's history and clinical signs and symptoms.
2. Interpretation of results from the EZ1 assay must account for the possibility of false-negative and false-positive results.
3. Negative results do not preclude infection with Ebola virus and should not be the sole basis of a patient treatment/management decision.
4. False positive results may occur from cross-contamination by target organisms, their nucleic acids or amplified product.
5. Failure to follow the assay procedures may lead to false negative results.
6. Improper collection, storage, or transport of specimens may lead to false negative results.
7. Inhibitors present in the samples may lead to false negative results.

Warnings and Precautions

1. For *In Vitro* Diagnostic Use under Emergency Use Authorization only.
2. Use of this assay should be limited to designated personnel.
3. Treat all specimens as potentially infectious. Follow all necessary precautions when handling samples and assay reagents.
4. Ensure that all samples have been inactivated with Trizol reagent prior to any testing (see Appendix A for further information).
5. Performance of the EZ1 assay has only been established with the specimen types listed in the Intended Use. Performance with other specimens types has not been evaluated.
6. Proper sample collection, storage, and transport are essential for correct results.
7. Store assay reagents as indicated on their individual labels.
8. Do not use reagents from other manufacturers with this assay.
9. Use appropriate laboratory and personal protective equipment when using this kit.

PERFORMANCE CHARACTERISTICS

9. ABI 7500 FAST DX

9.1 Analytical Sensitivity/Limit of Detection (LoD)

Limit of Detection (LoD): Serial dilutions in buffer of nucleic acid purified from Ebola Zaire virus obtained from the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) RNA reference panel were tested in triplicate. The lowest concentration at which all replicates were positive was treated as the tentative LoD for each test.

Table 28. Tentative LoD: EZ1 Assay

Strain tested	PFU Equivalents/Rxn	Call rate	Run 1 C_p	Run 2 C_p	Run 3 C_p
Ebola Zaire	1.0	3/3	23.10	22.86	22.79
	0.1	3/3	26.84	26.71	26.86
	0.01	3/3	30.52	30.48	30.46
	0.001	3/3	33.57	33.98	33.79
	0.0001	3/3	35.90	35.95	35.50
	0.00001	1/3	36.64	N/D	N/D
	0.000001	0/3	N/D	N/D	N/D
	0.0000001	0/3	N/D	N/D	N/D
	0.00000001	0/3	N/D	N/D	N/D

N/D = Not detected

Analytical sensitivity LoD studies determine the lowest detectable concentration of Ebola Zaire viral RNA at which approximately 95% of all (true positive) replicates test positive. The LoD was determined by limiting dilution studies using characterized specimens.

Table 29. Titers and Strains of Viral Stocks for LoD Study

Strain of viral stock	Ebola Zaire (Mayinga) Inactivated (UCC#: Ebola001, R3828s)
Titer (PFU/mL)	1.70e07
How were stocks prepared and inactivated	Viral Stocks were cultured, treated with β-Propiolactone, and gamma irradiated
How were titers determined	Plaque assay prior to inactivation of the viral stock

Strain of viral stock	Ebola Zaire (Mayinga) LIVE (UCC#: Ebola001, R3816)
Titer (PFU/mL)	1.70e07
How were stocks prepared	Viral stocks were cultured, frozen, thawed, spiked into whole blood under BSL-4 containment, and placed into Qiagen's AVL buffer containing guanidine thiocyanate
How were titers determined	Plaque assay prior to freezing of stock

Strain of viral stock	Ebola Zaire (95) LIVE (UCC#: Ebola007, R4308)
Titer (PFU/mL)	5.50e06
How were stocks prepared	Viral stocks were cultured, frozen, thawed, spiked into whole blood under BSL-4 containment, and placed into Qiagen's AVL buffer containing guanidine thiocyanate
How were titers determined	Plaque assay prior to freezing of stock

The nucleic acid was extracted using QIAamp Viral RNA Mini Kit from each of the serial dilutions.

LoD Estimation

The LoD was determined by limiting dilution studies using characterized specimens. Four serial dilutions of characterized Ebola Zaire (Mayinga) were tested with 3 replicates of each dilution. The lowest concentration at which all 3 replicates were positive was considered the initial estimate of LoD for each test. The LoD estimate was refined with 3 dilutions (4 replicates each) near the initial estimated LoD. The refined LoD of each test was then confirmed by testing 20 replicates with concentrations at the tentative LoD. The final LoD of each test was determined to be the lowest concentration resulting in positive detection of 19 out of 20 replicates (95%).

Initial LoD Estimation — Inactivated Ebola Zaire Mayinga

For an initial estimate of LoD, inactivated virus was spiked at eleven concentrations in triplicate ranging from (10,000 PFU/mL down to 1.0 PFU/mL Individual extracted specimens were analyzed according to conditions described in the product insert. Estimated LoD results can be found in Table 30.

Table 30. Inactivated Ebola Zaire Mayinga Virus Initial LoD Estimation

Assay	PFU/mL	Results	C_t			*Mean C_t (n=3)*	*SD*
EZ1	10,000	3/3	29.11	29.23	29.07	29.14	0.08
	1,000	3/3	34.04	33.15	31.25	32.81	1.43
	100	1/3	35.40	N/D	N/D	35.40	N/D
	10	2/3	35.98	43.01	N/D	39.50	4.97
	1.0	0/3	N/D	N/D	N/D	N/D	N/D

N/D = Not detected

Refined LoD Estimation – Inactivated Ebola Zaire Mayinga

For a refinement ofLoD, inactivated Ebola virus was spiked at three concentrations in quadruplicate based on LoD estimation at 1,000, 750, and 250 PFU/mL. The highest concentration of 1,000 PFU/mL resulted in variable and weak positives; therefore, LoD refinement was repeated at higher concentrations. The virus was spiked at three concentrations in quadruplicate based on LoD estimation above 1,000 PFU/mL at 10,000, 7,500 and 5,000 PFU/mL. Results from the refined LoD estimation can be found in Table 31. The EZ1 assay detected 4/4 specimens at 5,000 PFU/mL. The LoD will be confirmed at 5,000 PFU/mL.

Table 31. Inactivated Ebola Zaire Mayinga Virus Refined LoD Estimations

Assay	PFU/mL	Results	C_t				*Mean C_t (n=4)*	*SD*
EZ1	10,000	4/4	31.37	31.35	31.15	31.14	31.25	0.12
	7,500	4/4	31.89	31.85	31.73	31.73	31.80	0.08
	5,000	4/4	32.66	32.25	32.45	32.74	32.53	0.22

Confirmation of LoD Estimate – Inactivated Ebola Zaire Mayinga

Based on the initial and refined estimates of LoD for this virus previously reported, twenty independent whole blood specimens were spiked with Trizol-inactivated virus at 5,000 PFU/mL, extracted and analyzed. At this concentration, 20/20 test results were positive. The results of the analysis can be found in Table 32.

Table 32. Inactivated Ebola Zaire Mayinga Virus LoD Confirmation

Virus Concentration = 5,000 PFU/mL		
Specimen	**Pos/Neg**	**C_t**
1	+	32.19
2	+	32.72
3	+	32.10
4	+	32.31
5	+	32.12
6	+	32.52
7	+	32.52
8	+	32.37
9	+	33.22
10	+	32.36
11	+	32.92
12	+	32.44
13	+	32.44
14	+	33.08
15	+	34.13
16	+	32.69
17	+	32.57
18	+	32.40
19	+	32.55
20	+	32.19
Mean C_t (n=20)		32.59
SD		0.47
CV (%)		1.44
Results	20/20	

Conclusion:

Successful detection of 20/20 spiked specimens (100% success) confirms the LoD of the Ebola Zaire Virus rRT-PCR detection assay for Ebola Zaire Mayinga virus on ABI 7500 Fast Dx in Trizol-inactivated whole blood or Trizol-inactivated plasma specimens to be 5,000 PFU/mL.

LoD Estimation Live Ebola Zaire Mayinga

Inactivated Ebola virus was used for most studies for reasons of safety. However, virus inactivation, such as gamma irradiation, can impact viral genome integrity and present as an artifact in estimations of PCR assay performance. To provide an indication of assay performance against live virus, an estimation of the LoD of live Ebola Zaire Mayinga was conducted. The virus was spiked under BSL-4 containment at five concentrations in

triplicate ranging from 100,000 PFU/mL down to 10 PFU/mL. Results closest to LoD can be found in Table 33.

Table 33. Live Ebola Zaire Mayinga Virus Initial LoD Estimation

Assay	PFU/mL	Results	C_t			*Mean C_t (n=3)*	*SD*
EZ1	100,000	3/3	22.31	22.38	22.38	22.36	0.04
	10,000	3/3	26.28	25.50	26.00	25.93	0.40
	1,000	3/3	29.36	28.52	28.38	28.75	0.53
	100	3/3	33.1	33.78	33.55	33.48	0.35
	10	0/3	N/D	N/D	N/D	N/D	N/D

N/D = Not detected

Conclusion

Successful detection of 3/3 spiked specimens (100% success) at 100 PFU/mL indicates the LoD of the Ebola Zaire virus rRT-PCR detection assay for live Ebola Zaire Mayinga virus on the ABI 7500 Fast Dx in Trizol-inactivated whole blood or Trizol-inactivated plasma specimens will be more sensitive than was indicated for those specimens spiked using inactivated material, where the LoD was confirmed at 5,000 PFU/mL.

LoD Estimation — Live Ebola Zaire 95

The Ebola Zaire virus rRT-PCR detection assay is intended to be specific for the Zaire clade of Ebola virus. However, the particular viruses responsible for natural outbreaks of Ebola hemorrhagic fever have exhibited some variability, insufficient to define a new clade. To evaluate assay performance against a different isolate of Ebola Zaire, LoD for live Ebola Zaire 95 virus had been estimated and refined previously at 1,000 PFU/mL on the JBAIDS PCR platform. Confirmation specimens previously analyzed on JBAIDS were run on the ABI 7500 Fast Dx to verify detection of Ebola Zaire 95 at 1,000 PFU/mL on this instrument. Results can be found in Table 34.

Table 34. Live Ebola Zaire 95 LoD Confirmation on ABI 7500 Fast Dx

Virus Concentration = 1,000 PFU/mL		
Specimen	**Pos/Neg**	**C_t**
1	+	30.13
2	+	30.33
3	+	30.13
4	+	29.95
5	+	30.23
6	+	30.14
7	+	30.16
8	+	29.88
9	+	30.02
10	+	30.50
11	+	30.02
12	+	30.36
13	+	30.14
14	+	30.44
15	+	30.12
16	+	30.20
17	+	30.48
18	+	30.03
19	+	29.98
20	+	30.88
Mean C_t (n=20)		30.21
SD		0.24
CV (%)		0.78
Results	20/20	

Conclusion

Successful detection of 20/20 spiked specimens (100% success) verifies the LoD of the Ebola Zaire virus rRT-PCR detection assay for live Ebola Zaire 95 virus on ABI 7500 Fast Dx in Trizol-inactivated whole blood or Trizol-inactivated plasma specimens to be at least 1,000 PFU/mL.

Performance Comparison Between the ABI 7500 Fast Dx and LightCycler Using Whole Blood and Trizol-inactivated Whole Blood

Analytical sensitivity of the EZ1 assay was demonstrated and compared when performed on the ABI 7500 Fast Dx and LightCycler. The comparison of the two PCR systems was performed using virus-spiked whole blood with and without Trizol treatment (TRI Reagent LS).

Inactivated Ebola Zaire Mayinga virus stock was used to spike human whole blood, collected on EDTA, at a concentration of 5,000 PFU/mL, which was the previously determined LoD for the assay. One set of 20 extractions was performed with virus-spiked whole blood and a second set of extractions was performed with virus-spiked whole blood treated with Trizol at a ratio of 1:3 v/v; whole blood: Trizol. The resultant purified RNA was analyzed using the EZ1 assay and RP control assay on the ABI 7500 Fast Dx and the LightCycler 1.5. In order to confirm the LoD at least 19 of 20 samples (≥95%) at the selected virus concentration must generate a positive result.

All controls and RP assays achieved expected results (data not shown; Tables 35-38).

Table 35. Whole human blood spiked with Ebola virus analyzed with ABI 7500 Fast Dx.

Virus Concentration = 5,000 PFU/mL		
Sample	**Pos/Neg**	**C_t**
2	+	34.60
3	+	32.44
4	+	34.73
5	+	40.17
6	+	34.10
7	-	N/D
8	+	33.60
9	+	43.87
10	+	32.80
11	+	34.24
12	+	32.09
13	+	32.72
14	+	32.97
15	+	33.67
16	+	33.82
17	+	33.07
18	+	37.02
19	+	36.28
20	+	33.88
21	+	34.45
Mean C_t (n=19)		34.77
SD		2.98
CV (%)		8.58
Correct Results	19/20 (95%)	

Note: Sample 1 was a negative control (not shown) and had no C_t value.
N/D = Not detected

Table 36. Trizol treated whole blood spiked with Ebola virus analyzed with ABI 7500 Fast Dx.

Virus Concentration = 5000 PFU/mL		
Sample	**Pos/Neg**	**C_t**
23	+	35.29
24	+	34.77
25	+	35.13
26	+	34.42
27	+	35.17
28	+	34.47
29	+	36.40
30	+	35.43
31	+	35.80
32	+	37.14
33	+	34.92
34	+	35.07
35	+	35.15
36	+	35.11
37	+	34.80
38	+	34.92
39	+	34.17
40	+	34.30
41	+	34.97
42	+	35.24
Mean C_t (n=20)		35.12
SD		0.71
CV (%)		2.03
Correct Results	20/20 (100%)	

Note: Sample 22 was a negative control (not shown) and had no C_t value

Table 37. Whole human blood spiked with Ebola analyzed with LightCycler 1.5.

Virus Concentration = 5000 pfu/ml		
Sample Name	**Pos/Neg**	**C_p**
2	+	33.76
3	+	32.32
4	+	33.21
5	+	32.78
6	+	32.59
7	+	33.34
8	+	32.24
9	+	33.28
10	+	34.14
11	+	33.86
12	+	33.19
13	+	32.28
14	+	32.73
15	+	32.58
16	+	33.34
17	+	32.80
18	+	32.46
19	+	33.04
20	+	32.80
21	+	32.85
Mean C_p (n=20)		32.99
SD		0.55
CV (%)		1.66
Correct Results	20/20 (100%)	

Note: Sample 1 was a negative control (not shown) and had no C_p value.

Table 38. Trizol treated whole blood spiked with Ebola virus analyzed with LightCycler 1.5

Virus Concentration = 5,000 pfu/ml		
Sample Name	**Pos/Neg**	**C_p**
23	+	34.16
24	+	33.12
25	+	34.08
26	+	33.56
27	+	33.42
28	+	33.61
29	+	33.89
30	+	34.26
31	+	35.88
32	+	34.85
33	+	34.60
34	+	34.04
35	+	34.18
36	+	33.77
37	+	33.18
38	+	33.90
39	+	34.68
40	+	34.60
41	+	30.27
42	+	34.72
Mean C_p (n=20)		33.90
SD		1.09
CV (%)		3.22
Correct Results	20/20 (100%)	

Note: Sample 22 was a negative control (not shown) and had no C_p value

Conclusions

This study successfully confirmed the previously determined LoD of 5,000 PFU/mL of Ebola Zaire virus in whole blood using both the ABI 7500 Fast DX and LightCycler 1.5 instruments, which demonstrates equivalence between these two systems. Additionally, the treatment of blood samples with Trizol to inactivate the virus prior to extraction had no observable

effect on the LoD of the assay. The C_t and C_p values are slightly increased in Trizol-inactivated samples due to the 1:4 dilution factor that results from the Trizol-inactivation procedure. This slight increase is not clinically or statistically significant and will not adversely affect the diagnostic capability of the assay in Trizol treated samples.

9.2 Analytical Specificity

Analytical reactivity and cross-reactivity studies were conducted using the JBAIDS, using the identical reagents described in this report. The cross-reactivity results from JBAIDS are applicable to ABI 7500 Fast Dx as the same kits, including the primers and probe and other reagents, are used for both analyzers.

9.2.1 Reactivity:

Reactivity of the EZ1 assay was evaluated for one additional isolate of the Ebola Zaire virus as described above (Table 38). The level of performance, including the C_t value, is very similar to that observed for live Ebola Zaire Mayinga virus (Table 33).

9.2.2 Cross-Reactivity:

9.2.2.1 *Virus Cross-reactivity:* Cross-reactivity of the EZ1 assay was evaluated on the JBAIDS thermocycler using the same reagents by testing additional strains of Ebola virus (10,000 PFU/reaction), other strains in Ebola's taxonomic family known as Marburg viruses, and various other RNA viruses (10,000 PFU equivalents /reaction). **No cross-reactivity was observed for any of the viruses tested (Table 50).**

9.2.2.2 *Bacterial Cross-Reactivity:* Bacterial cross-reactivity of the EZ1 assay was evaluated by testing purified nucleic acid of bacteria that potentially could be infecting the majority of the population. **No cross-reactivity was observed in the human DNA or any of the bacteria tested (Table 51).**

9.2.2.3 *Human DNA Cross-Reactivity:* Human DNA cross-reactivity of the EZ1 assay was evaluated by testing purified human DNA at a concentration of 100 pg (31 human genome equivalents) per reaction. **No cross-reactivity with human DNA was observed (Table 52).**

9.3 ABI 7500 Fast Dx Mock Clinical Study for Inactivated Ebola Zaire Mayinga Virus Spiked into Whole Blood

Mock Clinical Study Results

Data from the LoD study confirmed that the LoD of the EZ1 assay for inactivated Ebola Zaire Mayinga virus was 5,000 PFU/mL. To predict clinical performance at the 95% confidence interval (CI), 45 independent whole blood specimens were blindly spiked with Trizol-inactivated virus at the concentrations shown on Table 39. Fifteen specimens each were spiked at 2.25x LoD (11,300 PFU/mL), at 3.0x LoD (15,000 PFU/mL), and at a high concentration (MAX; 1,700,000 PFU/mL). Another 100 whole Trizol-inactivated blood

specimens were spiked with viral transport media (VTM) alone. All blinded spiked specimens were extracted using the Qiagen QIAamp Viral RNA Mini kit, and analyzed on the ABI 7500 Fast Dx instrument using the EZ1 assay according to instructions in the product inserts. The blinded spiking key was unmasked after the results were complete. The results of the analysis can be found in Table 39 and the 95% CI is calculated in Table 40.

Table 39. Blinded Mock Clinical Study: EZ1 Assay using the ABI 7500 Fast Dx Analyzer

Spiking KEY		Virus Concentration (PFU/mL)	C_t Value	Call	RP Control C_t	Agreement with Expected Result
1	VTM	N/A	N/A	-	25.2419	YES
2	VTM	N/A	N/A	-	25.1521	YES
3	VTM	N/A	N/A	-	24.5481	YES
4	2.25x LoD	1.13e04	39.645	+	25.2269	YES
5	3x	1.50e04	38.0162	+	25.2231	YES
6	MAX	1.70e06	41.7358	+	25.4025	YES
7	VTM	N/A	N/A	-	25.0663	YES
8	MAX	1.70e06	33.7636	+	24.9611	YES
9	VTM	N/A	N/A	-	24.8638	YES
10	MAX	1.70e06	33.3511	+	25.4925	YES
11	VTM	N/A	N/A	-	25.0865	YES
12	VTM	N/A	N/A	-	25.0473	YES
13	VTM	N/A	N/A	-	25.0626	YES
14	VTM	N/A	N/A	-	25.6011	YES
15	VTM	N/A	N/A	-	24.7087	YES
16	VTM	N/A	N/A	-	25.4359	YES
17	VTM	N/A	N/A	-	25.2273	YES
18	MAX	1.70e06	33.4872	+	25.5844	YES
19	MAX	1.70e06	30.5533	+	25.9218	YES
20	2.25x LoD	1.13e04	36.5131	+	24.8867	YES
21	VTM	N/A	N/A	-	25.3297	YES
22	VTM	N/A	N/A	-	26.0798	YES
23	VTM	N/A	N/A	-	25.2508	YES
24	VTM	N/A	N/A	-	25.2094	YES
25	VTM	N/A	N/A	-	25.2745	YES
26	VTM	N/A	N/A	-	25.5649	YES
27	VTM	N/A	N/A	-	25.2244	YES
28	VTM	N/A	N/A	-	25.2085	YES
29	VTM	N/A	N/A	-	25.2094	YES
30	VTM	N/A	N/A	-	25.4865	YES
31	VTM	N/A	N/A	-	25.3621	YES
32	3x LoD	1.50e04	34.6494	+	25.1512	YES
33	VTM	N/A	N/A	-	25.2922	YES

Table 39. Blinded Mock Clinical Study: EZ1 Assay using the ABI 7500 Fast Dx Analyzer

Spiking KEY		Virus Concentration (PFU/mL)	C_t Value	Call	RP Control C_t	Agreement with Expected Result
34	VTM	N/A	N/A	-	26.2594	YES
35	VTM	N/A	N/A	-	25.1714	YES
36	VTM	N/A	N/A	-	25.4585	YES
37	VTM	N/A	N/A	-	25.3089	YES
38	VTM	N/A	N/A	-	25.0271	YES
39	VTM	N/A	N/A	-	25.5189	YES
40	3x LoD	1.50e04	35.3559	+	25.5489	YES
41	VTM	N/A	N/A	-	25.4509	YES
42	VTM	N/A	N/A	-	25.2702	YES
43	3x LoD	1.50e04	34.3196	+	26.2123	YES
44	VTM	N/A	N/A	-	25.5534	YES
45	VTM	N/A	N/A	-	25.6448	YES
46	VTM	N/A	N/A	-	25.4339	YES
47	3x LoD	1.50e04	35.5714	+	25.4957	YES
48	VTM	N/A	N/A	-	26.2898	YES
49	VTM	N/A	N/A	-	25.3379	YES
50	VTM	N/A	N/A	-	25.1504	YES
51	VTM	N/A	N/A	-	25.4089	YES
52	MAX	1.70e06	30.6377	+	25.5127	YES
53	VTM	N/A	N/A	-	25.1921	YES
54	VTM	N/A	N/A	-	25.2955	YES
55	VTM	N/A	N/A	-	24.9416	YES
56	VTM	N/A	N/A	-	25.2163	YES
57	MAX	1.70e06	30.1226	+	25.1719	YES
58	VTM	N/A	N/A	-	25.0288	YES
59	VTM	N/A	N/A	-	25.0723	YES
60	VTM	N/A	N/A	-	25.0332	YES
61	VTM	N/A	N/A	-	25.2542	YES
62	MAX	1.70e06	28.5661	+	25.3825	YES
63	3x LoD	1.50e04	33.0379	+	25.1482	YES
64	VTM	N/A	N/A	-	25.5086	YES
65	VTM	N/A	N/A	-	26.038	YES
66	3x LoD	1.50e04	33.646	+	25.9504	YES
67	VTM	N/A	N/A	-	25.7259	YES
68	VTM	N/A	N/A	-	25.5467	YES
69	3x LoD	1.50e04	33.208	+	25.5986	YES
70	3x LoD	1.50e04	33.4403	+	25.3427	YES
71	VTM	N/A	N/A	-	25.8031	YES
72	VTM	N/A	N/A	-	25.546	YES

Table 39. Blinded Mock Clinical Study: EZ1 Assay using the ABI 7500 Fast Dx Analyzer

Spiking KEY		Virus Concentration (PFU/mL)	C_t Value	Call	RP Control C_t	Agreement with Expected Result
73	VTM	N/A	N/A	-	25.771	YES
74	VTM	N/A	N/A	-	26.1368	YES
75	2.25x LoD	1.13e04	34.3655	+	25.6828	YES
76	3x LoD	1.50e04	33.834	+	25.2315	YES
77	VTM	N/A	N/A	-	25.4494	YES
78	2.25x LoD	1.13e04	34.6365	+	25.792	YES
79	VTM	N/A	N/A	-	24.9817	YES
80	VTM	N/A	N/A	-	24.674	YES
81	VTM	N/A	N/A	-	25.1389	YES
82	VTM	N/A	N/A	-	24.3683	YES
83	VTM	N/A	N/A	-	24.6693	YES
84	2.25x LoD	1.13e04	33.4827	+	24.6001	YES
85	VTM	N/A	N/A	-	24.3906	YES
86	VTM	N/A	N/A	-	24.3717	YES
87	MAX	1.70e06	27.8982	+	24.7202	YES
88	VTM	N/A	N/A	-	25.8732	YES
89	VTM	N/A	N/A	-	24.7589	YES
90	VTM	N/A	N/A	-	24.4913	YES
91	VTM	N/A	N/A	-	24.9409	YES
92	3x LoD	1.50e04	32.4086	+	25.1555	YES
93	VTM	N/A	N/A	-	24.6983	YES
94	VTM	N/A	N/A	-	24.7585	YES
95	2.25x LoD	1.13e04	34.2155	+	24.7689	YES
96	VTM	N/A	N/A	-	25.2819	YES
97	VTM	N/A	N/A	-	25.0312	YES
98	VTM	N/A	N/A	-	24.6786	YES
99	VTM	N/A	N/A	-	24.9321	YES
100	3x LoD	1.50e04	32.1961	+	25.1794	YES
101	MAX	1.70e06	28.175	+	24.8647	YES
102	2.25x LoD	1.13e04	32.3227	+	24.7821	YES
103	3x LoD	1.50e04	32.945	+	24.7197	YES
104	VTM	N/A	N/A	-	25.1498	YES
105	2.25x LoD	1.13e04	33.9424	+	25.978	YES
106	VTM	N/A	N/A	-	25.5806	YES
107	MAX	1.70e06	29.2473	+	26.433	YES
108	VTM	N/A	N/A	-	25.6961	YES
109	MAX	1.70e06	30.6115	+	26.3428	YES
110	VTM	N/A	N/A	-	25.5373	YES
111	VTM	N/A	N/A	-	25.7146	YES

Table 39. Blinded Mock Clinical Study: EZ1 Assay using the ABI 7500 Fast Dx Analyzer

Spiking KEY		Virus Concentration (PFU/mL)	C_t Value	Call	RP Control C_t	Agreement with Expected Result
112	VTM	N/A	N/A	-	25.509	YES
113	MAX	1.70e06	29.6618	+	25.393	YES
114	VTM	N/A	N/A	-	25.5363	YES
115	VTM	N/A	N/A	-	25.9197	YES
116	VTM	N/A	N/A	-	25.5517	YES
117	2.25x LoD	1.13e04	34.5261	+	25.6411	YES
118	VTM	N/A	N/A	-	25.8912	YES
119	VTM	N/A	N/A	-	25.8357	YES
120	2.25x LoD	1.13e04	34.8585	+	25.6586	YES
121	VTM	N/A	N/A	-	25.9715	YES
122	VTM	N/A	N/A	-	26.1255	YES
123	2.25x LoD	1.13e04	35.4119	+	26.2353	YES
124	VTM	N/A	N/A	-	25.6372	YES
125	VTM	N/A	N/A	-	26.2748	YES
126	VTM	N/A	N/A	-	25.6701	YES
127	3x LoD	1.50e04	32.6004	+	26.4094	YES
128	MAX	1.70e06	28.4556	+	25.2442	YES
129	MAX	1.70e06	28.8294	+	25.4014	YES
130	VTM	N/A	N/A	-	25.633	YES
131	VTM	N/A	N/A	-	27.0139	YES
132	3x LoD	1.50e04	33.2311	+	25.2804	YES
133	VTM	N/A	N/A	-	25.4974	YES
134	VTM	N/A	N/A	-	25.4641	YES
135	2.25x LoD	1.13e04	34.461	+	27.299	YES
136	VTM	N/A	N/A	-	25.4481	YES
137	2.25x LoD	1.13e04	34.5633	+	25.5888	YES
138	VTM	N/A	N/A	-	25.8074	YES
139	2.25x LoD	1.13e04	33.6471	+	25.4474	YES
140	VTM	N/A	N/A	-	25.6956	YES
141	VTM	N/A	N/A	-	25.6831	YES
142	VTM	N/A	N/A	-	25.6724	YES
143	2.25x LoD	1.13e04	33.3755	+	25.3774	YES
144	VTM	N/A	N/A	-	25.4757	YES
145	VTM	N/A	N/A	-	26.5641	YES

N/A = Not applicable

Table 40. Mock Clinical Study Summary Statistics

EZ1 Detection Assay on the ABI 7500 Fast Dx Platform	Positive Results		Negative Results
Positive Specimens (2.25 x LoD)	15		0
Positive Specimens (3 x LoD)	15		0
Positive Specimens (MAX)	15		0
Negative Specimens (100)	0		100
Total (145)	45		100
			95% CI
Positive Percent Agreement	45/45	100%	92.13%-100%
Negative Percent Agreement	100/100	100%	96.38%-100%

Conclusion:

The EZ1 assay correctly identified 45 of 45 specimens spiked with inactivated Ebola Zaire virus at the concentrations shown, including concentrations near the limit of detection of the assay. No negatively spiked specimen rendered a positive reading.

10. LIGHTCYCLER

The LoD of the EZ1 assay using the LightCycler was demonstrated to be equivalent to the ABI 7500 Fast Dx (Section 9.1). The JBAIDS is a ruggedized version of the LightCycler. The EZ1 assay performance (inclusivity and exclusivity) are expected to be interchangeable between the two instruments (see Section 11).

11. JBAIDS

11.1 Analytical Sensitivity/Limit of Detection (LoD)

Limit of Detection (LoD): Serial dilutions in buffer of nucleic acid purified from Ebola Zaire virus obtained from the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) RNA reference panel were tested in triplicate. The lowest concentration at which all replicates were positive was treated as the tentative LoD for each test.

Table 41. Tentative LoD: EZ1 Assay

Strain tested	PFU Equivalents/Rxn	Call rate	Run 1 C_p	Run 2 C_p	Run 3 C_p
Ebola Zaire	1.0	3/3	23.10	22.86	22.79
	0.1	3/3	26.84	26.71	26.86
	0.01	3/3	30.52	30.48	30.46
	0.001	3/3	33.57	33.98	33.79
	0.0001	3/3	35.90	35.95	35.5
	0.00001	1/3	36.64	N/D	N/D
	0.000001	0/3	N/D	N/D	N/D
	0.0000001	0/3	N/D	N/D	N/D
	0.00000001	0/3	N/D	N/D	N/D

N/D = Not detected

Analytical sensitivity LoD studies determine the lowest detectable concentration of Ebola Zaire virus at which approximately 95% of all (true positive) replicates test positive. The LoD was determined by limiting dilution studies using characterized specimens.

Table 42. Titers and Strains of Viral Stocks for LoD Study

Strain of viral stock	Ebola Zaire (Mayinga) Inactivated (UCC#: Ebola001, R3828s)
Titer (PFU/mL)	1.70e07
How were stocks prepared and inactivated	Viral Stocks were cultured, treated with β-Propiolactone, and gamma irradiated
How were titers determined	Plaque Assay prior to inactivation of the viral stock

Strain of viral stock	Ebola Zaire (Mayinga) LIVE (UCC#: Ebola001, R3816)
Titer (PFU/mL)	1.70e07
How were stocks prepared	Viral stocks were cultured, frozen, thawed, spiked into whole blood under BSL-4 containment, and placed into Qiagen's AVL buffer containing guanidine thiocyanate
How were titers determined	Plaque assay prior to freezing of stock

Strain of viral stock	Ebola Zaire (95) LIVE (UCC#: Ebola007, R4308)
Titer (PFU/mL)	5.50e06
How were stocks prepared	Viral stocks were cultured, frozen, thawed, spiked into whole blood under BSL-4 containment, and placed into Qiagen's AVL buffer containing guanidine thiocyanate
How were titers determined	Plaque assay prior to freezing of stock

The nucleic acid was extracted using **QIAamp Viral RNA Mini Kit** from each of the serial dilutions.

LoD Estimation

The LoD was determined by limiting dilution studies using characterized specimens. Four serial dilutions of characterized Ebola Zaire (Mayinga) were tested with 3 replicates of each dilution. The lowest concentration at which all 3 replicates were positive was considered the initial estimate of LoD for each test. The LoD estimate was refined with 3 dilutions (4 replicates each) near the initial estimated LoD. The refined LoD of each test was then confirmed by testing 20 replicates with concentrations at the tentative LoD. The final LoD of each test was determined to be the lowest concentration resulting in positive detection of 19 out of 20 replicates (95%).

Initial LoD Estimation — Inactivated Ebola Zaire Mayinga

For an initial estimate of LoD, inactivated virus was spiked at four concentrations in triplicate ranging from (10,000 PFU/mL down to 10 PFU/mL). Individual extracted specimens were analyzed according to conditions described in the product insert. Estimated LoD results can be found in Table 43.

Table 43. Inactivated Ebola Zaire Mayinga Virus Initial LoD Estimation

Assay	PFU/mL	Results	C_p			*Mean C_p (n=3)*	*SD*
EZ1	10,000	3/3	30.55	30.72	30.26	30.51	0.23
	1,000	2/3	31.45	30.97	N/D	31.21	0.34
	100	0/3	N/D	N/D	N/D	N/D	N/D
	10	0/3	N/D	N/D	N/D	N/D	N/D

N/D = Not detected

Refined LoD Estimation — Inactivated Ebola Zaire Mayinga

For a refinement of LoD, inactivated Ebola virus was spiked at 1,000, 5,000, and 10,000 PFU/mL in quadruplicate based on the initial LoD estimation. Results from the refined LoD estimation can be found in Table 44. The EZ1 assay detected 4/4 specimens at 5,000 PFU/mL and 2/4 at 1,000 PFU/mL. The LoD will be confirmed at 5,000 PFU/mL.

Table 44. Inactivated Ebola Zaire Mayinga Virus Refined LoD Estimation

Assay	PFU/mL	Results	C_p				*Mean C_p (n=4)*	*SD*
EZ1	10,000	4/4	29.65	29.73	29.90	30.19	29.87	0.24
	5,000	4/4	29.16	29.96	30.31	29.71	29.79	0.48
	1,000	2/4	31.41	29.84	N/D	N/D	30.63	1.11

N/D = Not detected

Confirmation of LoD Estimate — Inactivated Ebola Zaire Mayinga

Based on the initial and refined estimates of LoD for this virus, twenty independent whole blood specimens were spiked with inactivated virus at 5,000 PFU/mL, extracted and analyzed. Initial LoD Confirmation at 5,000 PFU/mL resulted in only 16/20 (+); therefore, we increased LoD confirmation to 7,500 PFU/mL. Twenty additional independent whole blood specimens were spiked at 7,500 PFU/mL, extracted and analyzed. The results of the analysis can be found in Table 45.

Table 45. Inactivated Ebola Zaire Mayinga Virus LoD Confirmation

Virus Concentration = 7,500 PFU/mL		
Specimen	**Pos/Neg**	**C_p**
1	+	30.25
2	+	30.76
3	+	30.28
4	+	30.58
5	+	30.72
6	+	30.12
7	+	30.55
8	+	30.57
9	+	30.38
10	+	29.92
11	+	31.10
12	+	30.94
13	+	30.49
14	+	30.67
15	+	31.05
16	+	30.21
17	+	31.00
18	+	30.84
19	+	30.06
20	+	30.18
Mean C_p (n=20)		30.65
SD		0.39
CV (%)		1.28
Results	20/20	

Conclusion:

Successful detection of 20/20 spiked specimens (100% success) confirms the LoD of the Ebola Zaire Virus rRT-PCR detection assay for inactivated Ebola Zaire Mayinga virus on JBAIDS in whole blood specimens to be 7,500 PFU/mL.

LoD Estimation Live Ebola Zaire Mayinga

Inactivated Ebola virus was used for most studies for reasons of safety. However, virus inactivation, such as gamma irradiation, can impact viral genome integrity and result in

increased LoD determinations as an artifact of the inactivation. To provide an indication of assay performance against live virus, an LoD estimation of live Ebola Zaire Mayinga was conducted. The virus was spiked under BSL-4 containment at six concentrations in triplicate ranging from 100,000 PFU/mL down to 1 PFU/mL. Results can be found in Table 46.

Table 46. Live Ebola Zaire Mayinga Virus Initial LoD Estimation

Assay	PFU/mL	Results	C_p			*Mean C_p (n=3)*	*SD*
EZ1	100,000	3/3	22.96	22.51	22.47	22.65	0.27
	10,000	3/3	26.38	26.08	26.53	26.33	0.23
	1,000	3/3	29.34	29.08	28.95	29.12	0.20
	100	3/3	31.16	31.00	30.88	31.01	0.14
	10	0/3	N/D	N/D	N/D	N/D	N/D
	1	0/3	N/D	N/D	N/D	N/D	N/D

N/D = Not Detected

Conclusion:

Successful detection of 3/3 spiked specimens (100% success) at 100 PFU/mL indicates the assay LoD testing live Ebola Zaire Mayinga virus in whole blood specimens on JBAIDS will be more sensitive than was determined using inactivated material.

Initial LoD Estimation — Live Ebola Zaire 95

The EZ1 assay is intended to be specific for the Zaire clade of Ebola virus. However, the particular viruses responsible for natural outbreaks of Ebola hemorrhagic fever have exhibited some variability within the Zaire clade. To evaluate assay performance against a different isolate of Ebola Zaire, the LoD was estimated for Ebola Zaire 95.
The LoD was initially estimated by spiking four concentrations in triplicate ranging from (10,000 PFU/mL to 100 PFU/mL). Results can be found in Table 47.

Table 47. Live Ebola Zaire 95 Initial LoD Estimation

Assay	PFU/mL	Results	C_p			*Mean C_p (n=3)*	*SD*
EZ1	10,000	3/3	27.97	27.87	27.89	27.91	0.05
	5,000	3/3	28.58	28.27	28.37	28.41	0.56
	1,000	3/3	29.87	29.95	29.82	29.88	0.07
	100	0/3	N/D	N/D	N/D	N/D	N/D

N/D = Not Detected

The LoD of the EZ1 assay was initially estimated to be 1,000 PFU/mL when detecting Ebola Zaire 95.

Refined LoD Estimation — Live Ebola Zaire 95

The initial LoD estimation was further refined by spiking three concentrations in quadruplicate (5,000, 2,500, and 1,000 PFU/mL). Results from the refined LoD estimation can be found in Table 48.

Table 48. Live Ebola Zaire 95 Refined LoD Estimation

Assay	PFU/mL	Results	C_p				*Mean C_p (n=4)*	*SD*
EZ1	5,000	4/4	28.98	28.98	29.07	27.57	28.65	0.72
	2,500	4/4	29.32	29.27	29.13	29.22	29.23	0.08
	1,000	4/4	30.10	30.17	30.43	30.11	30.19	0.16

The EZ1 assay detected 4/4 specimens at 1,000 PFU/mL.

Confirmation of LoD Estimate — Live Ebola Zaire 95

Based on the initial and refined estimates of LoD for this virus , twenty independent whole blood specimens were spiked at 1,000 PFU/mL, extracted and analyzed.

Table 49. Live Ebola Zaire 95 LoD Confirmation

Virus Concentration = 1,000 PFU/ml		
Specimen	**Pos/Neg**	**C_p**
1	+	30.32
2	+	29.98
3	+	30.10
4	+	29.91
5	+	29.73
6	+	29.77
7	+	30.09
8	+	30.32
9	+	29.97
10	+	29.78
11	+	29.79
12	+	29.59
13	+	30.34
14	+	29.91
15	+	30.20
16	+	29.50
17	+	29.58
18	+	29.16
19	+	29.48
20	+	29.96
Mean C_p (n=20)		29.87
SD		0.314
CV (%)		1.05
Results	20/20	

Conclusion:

Successful detection of 20/20 spiked specimens (100% success) confirms the LoD of the EZ1 assay for live Ebola Zaire 95 virus in whole blood specimens on JBAIDS was 1,000 PFU/mL.

11.2 Analytical Specificity

11.2.1 Reactivity:

Reactivity of the EZ1 assay was evaluated against Ebola Zaire Mayinga and Ebola Zaire 95. The level of performance against both strains was similar (Table 46-49).

11.2.2 Virus Cross-reactivity:

Cross-reactivity of the EZ1 assay was evaluated by testing additional non-Zaire strains of Ebola virus (10,000 PFU/reaction), other strains in Ebola's taxonomic family known as Marburg viruses (10,000 PFU/reaction), and various other RNA viruses (10,000 PFU/reaction). **No cross-reactivity was observed for any of the viruses tested (Table 50).**

Table 50. Virus Cross-Reactivity: EZ1 Assay

Inactivated Virus	Strain	Concentration (PFU Equivalents/ Reaction)	Concentration (PFU Equivalents/ mL)	C_p Value
Ebola	Ivory Coast	2.05e03*	4.1e04*	N/D
Ebola	Reston	1.0e04	2.0e06	N/D
Ebola	Sudan-Gulu	1.0e04	2.0e06	N/D
Ebola	Sudan-Boniface	4.9e03*	9.8e05*	N/D
Ebola	Uganda-Bunidbugyo	1.0e04	2.0e06	N/D
Marburg	Angola	1.0e04	2.0e06	N/D
Marburg	Ci67	1.0e04	2.0e06	N/D
Marburg	Musoke	1.0e04	2.0e06	N/D
Marburg	Ravn	1.0e04	2.0e06	N/D
Sindbis	UgMP6440	1.0e04	2.0e06	N/D
VEE 1 A/B	TC83	1.0e04	2.0e06	N/D
VEE I A/B	Trinidad Donkey	1.0e04	2.0e06	N/D
VEE VI	AG80-663	1.0e04	2.0e06	N/D
VEE II	Everglades Fe3-7c	1.0e04	2.0e06	N/D
VEE IE	68U201	1.0e04	2.0e06	N/D
VEE IV	Pixuna BeAr40403	1.0e04	2.0e06	N/D
VEE IF	78V3531	1.0e04	2.0e06	N/D
VEE IV	BeAr436087	1.0e04	2.0e06	N/D
VEE IC	CO951006	1.0e04	2.0e06	N/D
VEE ID	V209A-TVP-1163	1.0e04	2.0e06	N/D
VEE IIIA	Mucambo BeAn8	1.0e04	2.0e06	N/D
VEE V	Cabassou aAr508	1.0e04	2.0e06	N/D
EEE	76V25343	1.0e04	2.0e06	N/D
EEE	ARG-LL	1.0e04	2.0e06	N/D
EEE	Georgia97	1.0e04	2.0e06	N/D
EEE	PE6	1.0e04	2.0e06	N/D
WEE	CBA 87/4	1.0e04	2.0e06	N/D
WEE	McMillan	1.0e04	2.0e06	N/D
WEE	B11	1.0e04	2.0e06	N/D
WEE	71V1658	1.0e04	2.0e06	N/D
Semliki Forest	Original	1.0e04	2.0e06	N/D
Ross River Virus	T49	1.0e04	2.0e06	N/D

Table 50. Virus Cross-Reactivity: EZ1 Assay

Inactivated Virus	Strain	Concentration (PFU Equivalents/ Reaction)	Concentration (PFU Equivalents/ mL)	C_p Value
Highlands J	B230	1.0e04	2.0e06	N/D
Mayaro	BeH256	1.0e04	2.0e06	N/D
O'nyong nyong	Gulu	1.0e04	2.0e06	N/D
Chikungunya	INDO23574	1.0e04	2.0e06	N/D
Chikungunya	B8635	1.0e04	2.0e06	N/D
Getah	MM 2021	1.0e04	2.0e06	N/D
Middleburg	SA Ar749	1.0e04	2.0e06	N/D
Barmah Forest	BH2193	1.0e04	2.0e06	N/D
Ndumu	SA Ar2210	1.0e04	2.0e06	N/D
Vaccinia virus	R1352	5.0e05	1.0e08	N/D

**The concentration of two virus stocks was too low to be tested at 1.0e04 PFU/reaction. Actual concentrations tested are indicated.*

N/D = Not Detected

11.2.3 Bacterial Cross-Reactivity: Cross-reactivity of the EZ1 assay with bacterial species was evaluated by testing purified nucleic acid of bacteria that potentially could be present in a whole blood specimen. No cross-reactivity was observed with any of the bacteria tested.

Table 51. Bacteria Cross Reactivity: EZ1 Assay

Purified DNA from Bacteria	Concentration (pg DNA/Rxn)	Genomic Equivalents per Reaction	Concentration (pg/mL)	C_p Value
Acinetobacter baumanni	100	2.5e04	2.0e04	N/D
Actinomyces naeslundii	100	3.5e04	2.0e04	N/D
Actinobacillus pleuropneumoniae	100	4.1e04	2.0e04	N/D
Alcaligenes xylosoxidans	100	1.0e05	2.0e04	N/D
Bacillus anthracis	100	5.4e05	2.0e04	N/D
Bacillus (Geobacillus) stearothermophilus	100	5.2e08	2.0e04	N/D
Bacillus thuringiensis	100	8.9e05	2.0e04	N/D
Bacillus subtilis var niger	100	2.4e04	2.0e04	N/D
Bacillus cereus	100	1.9e04	2.0e04	N/D
Bacteroides fragilis	100	1.8e04	2.0e04	N/D
Bartonella henselae	100	5.1e04	2.0e04	N/D

Table 51. Bacteria Cross Reactivity: EZ1 Assay

Purified DNA from Bacteria	Concentration (pg DNA/Rxn)	Genomic Equivalents per Reaction	Concentration (pg/mL)	C_p Value
Bifidobacterium infantis	100	3.5e04	2.0e04	N/D
Bordetella bronchiseptica	100	1.8e04	2.0e04	N/D
Bordetella pertussis	100	2.4e04	2.0e04	N/D
Borrelia burgdorferi	100	1.1e05	2.0e04	N/D
Brucella melitensis	100	8.2e04	2.0e04	N/D
Budvicia aquatica	100	3.0e04	2.0e04	N/D
Burkholderia mallei	100	2.8e04	2.0e04	N/D
Campylobacter jejuni	100	5.8e04	2.0e04	N/D
Candida albicans	100	1.0e05	2.0e04	N/D
Chryseobacterium meningosepticum	100	1.8e04	2.0e04	N/D
Clostridium botulinum type A	100	2.4e04	2.0e04	N/D
Clostridium perfringens	100	1.4e06	2.0e04	N/D
Clostridium difficile	100	2.3e04	2.0e04	N/D
Comamonas (Delftia) acidovorans	100	1.4e04	2.0e04	N/D
Corynebacterium diphtheriae	100	3.9e04	2.0e04	N/D
Coxiella burnetii	100	4.9e04	2.0e04	N/D
Deinococcus radiodurans	100	2.4e05	2.0e04	N/D
Enterobacter aerogenes	100	1.9e04	2.0e04	N/D
Enterobacter (Paoea) agglomerans	100	2.1e04	2.0e04	N/D
Enterococcus faecalis	100	3.3e04	2.0e04	N/D
Escherichia coli	100	1.8e04	2.0e04	N/D
Francisella tularensis	100	4.8e04	2.0e04	N/D
Haemophilus influenzae	100	5.4e04	2.0e04	N/D
Klebsiella pneumoniae	100	1.7e04	2.0e04	N/D
Legionella pneumophila	100	2.9e04	2.0e04	N/D
Listeria monocytogenes	100	3.3e04	2.0e04	N/D
Moraxella catarrhalis	100	5.2e04	2.0e04	N/D
Mycoplasma pneumoniae	100	1.2e05	2.0e04	N/D
Neisseria meningitidis	100	4.6e04	2.0e04	N/D
Pasteurella multocida	100	4.3e04	2.0e04	N/D
Porphyromonas gingivalis	100	4.2e04	2.0e04	N/D
Proteus mirabilis	100	2.4e04	2.0e04	N/D
Pseudomonas aeruginosa	100	1.6e04	2.0e04	N/D

Table 51. Bacteria Cross Reactivity: EZ1 Assay

Purified DNA from Bacteria	Concentration (pg DNA/Rxn)	Genomic Equivalents per Reaction	Concentration (pg/mL)	C_p Value
Rhizobium radiobacter	100	2.4e04	2.0e04	N/D
Salmonella choleraesuis serotype *Paratyphi*	100	2.0e04	2.0e04	N/D
Serratia marcescens	100	1.8e04	2.0e04	N/D
Shigella flexneri	100	2.1e04	2.0e04	N/D
Staphylococcus epidermidis	100	3.7e04	2.0e04	N/D
Staphylococcus aureus	100	3.4e04	2.0e04	N/D
Stenotrophomonas maltophilia	100	2.1e04	2.0e04	N/D
Streptococcus pyogenes	100	5.1e04	2.0e04	N/D
Streptococcus pneumoniae	100	4.8e04	2.0e04	N/D
Ureaplasma parvum (urealyticum)	100	1.3e05	2.0e04	N/D
Vibrio cholerae	100	3.1e04	2.0e04	N/D
Yersinia pseudotuberculosis	100	2.1e04	2.0e04	N/D
Yersinia enterocolitica	100	2.1e04	2.0e04	N/D
Yersinia pestis (CO92;PW)	100	2.1e04	2.0e04	N/D

N/D = Not detected

11.2.4 Human DNA Cross-Reactivity: Human DNA cross-reactivity of the EZ1 assay was evaluated by testing purified human DNA at a concentration of 100 pg (31 human genome equivalents) per reaction. No cross-reactivity with human DNA was observed (Table 52).

Table 52. Human DNA Cross Reactivity: EZ1 Assay

Purified DNA from Human	Concentration (pg/Rxn)	Genomic Equivalents per Reaction	Concentration (pg DNA/mL)	C_p Value
Human DNA	100	31	2.0e04	N/D

N/D = Not detected

11.3 JBAIDS Mock Clinical Study for Inactivated Ebola Zaire Mayinga Virus Spiked into Whole Blood

Mock Clinical Study Results

Data from the LoD study confirmed that the LoD of the EZ1 assay for inactivated Ebola Zaire Mayinga virus was 7,500 PFU/mL. To predict clinical performance at the 95% confidence interval (CI), 45 independent whole blood specimens were blindly spiked with inactivated virus at the concentrations shown on Table 53. Fifteen specimens each were spiked at 1.5x LoD (11,250 PFU/mL), at 2.0x LoD (15,000 PFU/mL), and at a high concentration (MAX; 1,700,000 PFU/mL). Another 100 whole blood specimens were spiked with viral transport media (VTM) alone. All blinded spiked specimens were extracted using the Qiagen QIAamp Viral RNA Mini kit, and analyzed on the JBAIDS instrument using the EZ1 assay according to instructions in the product inserts. The blinded spiking key was unmasked after the results were complete. The results of the analysis can be found in Table 53 and the 95% CI is calculated in Table 54.

Table 53. Blinded Mock Clinical Study: EZ1 Assay using the JBAIDS

Spiking KEY		Virus concentration (PFU/mL)	C_p Value	Call	Max Fluorescence	RP Control C_p	Agreement with Expected Result
1	VTM	N/A	N/A	-	0.01	25.15	YES
2	1.5x LoD	1.125e04	30.26	+	3.14	24.79	YES
3	VTM	N/A	N/A	-	0.05	25.08	YES
4	2x	1.50e04	30.47	+	2.8	24.95	YES
5	VTM	N/A	N/A	-	0.13	24.97	YES
6	1.5x	1.125e04	30.36	+	2.15	24.93	YES
7	MAX	1.70e06	28.37	+	9.83	24.81	YES
8	1.5x	1.125e04	30.28	+	2.33	24.83	YES
9	VTM	N/A	N/A	-	0.21	24.93	YES
10	VTM	N/A	N/A	-	0.15	24.95	YES
11	VTM	N/A	N/A	-	0.03	24.65	YES
12	VTM	N/A	N/A	-	0.06	24.9	YES
13	VTM	N/A	N/A	-	0.04	24.75	YES
14	VTM	N/A	N/A	-	0.24	26.15	YES
15	VTM	N/A	N/A	-	0.37	26.62	YES
16	1.5x	1.125e04	30.17	+	3.32	25.06	YES
17	VTM	N/A	N/A	-	0.21	24.87	YES
18	VTM	N/A	N/A	-	0.26	24.78	YES
19	VTM	N/A	N/A	-	0.13	24.75	YES
20	MAX	1.70e06	29.09	+	2.81	28.21	YES
21	1.5x	1.125e04	30	+	3.83	24.64	YES
22	VTM	N/A	N/A	-	0.13	24.61	YES
23	2x	1.50e04	29.71	+	3.68	24.72	YES
24	VTM	N/A	N/A	-	0.02	24.37	YES
25	VTM	N/A	N/A	-	0.04	24.29	YES
26	VTM	N/A	N/A	-	0.1	24.35	YES

Table 53. Blinded Mock Clinical Study: EZ1 Assay using the JBAIDS

Spiking KEY		Virus concentration (PFU/mL)	C_p Value	Call	Max Fluorescence	RP Control C_p	Agreement with Expected Result
27	VTM	N/A	N/A	-	0.07	24.18	YES
28	VTM	N/A	N/A	-	0.07	24.23	YES
29	VTM	N/A	N/A	-	0.09	24.13	YES
30	VTM	N/A	N/A	-	0.09	24.24	YES
31	VTM	N/A	N/A	-	0.18	24.06	YES
32	VTM	N/A	N/A	-	0.12	24.68	YES
33	1.5x	1.125e04	29.75	+	2.42	24.08	YES
34	MAX	1.70e06	27.85	+	13.37	24.53	YES
35	2x	1.50e04	29.78	+	2.67	24.25	YES
36	VTM	N/A	N/A	-	0.43	23.94	YES
37	VTM	N/A	N/A	-	0.05	24.03	YES
38	VTM	N/A	N/A	-	0.09	23.74	YES
39	1.5x	1.125e04	30.22	+	7.01	24.09	YES
40	VTM	N/A	N/A	-	0.07	24.29	YES
41	MAX	1.70e06	28.58	+	15.48	24.06	YES
42	VTM	N/A	N/A	-	0.23	24.48	YES
43	VTM	N/A	N/A	-	0.38	24.72	YES
44	1.5x	1.125e04	28.51	+	1.17	24.74	YES
45	MAX	1.70e06	27.99	+	15.78	24.7	YES
46	VTM	N/A	N/A	-	0.34	24.27	YES
47	2x	1.50e04	28.66	+	1.74	25.01	YES
48	VTM	N/A	N/A	-	0.12	24.23	YES
49	VTM	N/A	N/A	-	0.07	24.04	YES
50	1.5x	1.125e04	29.54	+	2.74	23.83	YES
51	1.5x	1.125e04	29.94	+	1.99	24.1	YES
52	2x	1.50e04	29.9	+	4.27	24.11	YES
53	1.5x	1.125e04	N/D	-	0.16	24.99	NO
54	MAX	1.70e06	29.56	+	12.57	25.36	YES
55	VTM	N/A	N/A	-	0.09	25.02	YES
56	1.5x	1.125e04	N/D	-	0.25	24.73	NO
57	1.5x	1.125e04	N/D	-	0.03	24.98	NO
58	VTM	N/A	N/A	-	0.2	24.65	YES
59	2x	1.50e04	32.03	+	1.73	24.84	YES
60	VTM	N/A	N/A	-	0.14	24.58	YES
61	VTM	N/A	N/A	-	0.04	24.75	YES
62	MAX	1.70e06	28.89	+	13.29	25.06	YES
63	VTM	N/A	N/A	-	0.08	24.87	YES
64	1.5x	1.125e04	N/D	-	0.35	24.64	NO
65	VTM	N/A	N/A	-	0.31	24.68	YES
66	VTM	N/A	N/A	-	0.11	25.18	YES
67	MAX	1.70e06	29.02	+	10.94	25.17	YES
68	VTM	N/A	N/A	-	0.14	25.17	YES
69	MAX	1.70e06	28.9	+	14.52	25.17	YES
70	VTM	N/A	N/A	-	0.35	25.17	YES

Table 53. Blinded Mock Clinical Study: EZ1 Assay using the JBAIDS

Spiking KEY		Virus concentration (PFU/mL)	C_p Value	Call	Max Fluorescence	RP Control C_p	Agreement with Expected Result
71	VTM	N/A	N/A	-	0.26	24.66	YES
72	VTM	N/A	N/A	-	0.28	24.76	YES
73	MAX	1.70e06	28.64	+	11.57	24.6	YES
74	MAX	1.70e06	29.08	+	13.15	25.1	YES
75	VTM	N/A	N/A	-	0.24	24.99	YES
76	MAX	1.70e06	28.74	+	15.09	24.68	YES
77	VTM	N/A	N/A	-	0.09	24.86	YES
78	VTM	N/A	N/A	-	0.03	24.56	YES
79	VTM	N/A	N/A	-	0.04	24.52	YES
80	2x	1.50e04	31.22	+	0.63	24.91	YES
81	VTM	N/A	N/A	-	0.06	24.76	YES
82	VTM	N/A	N/A	-	0.04	24.9	YES
83	VTM	N/A	N/A	-	0.17	24.55	YES
84	VTM	N/A	N/A	-	0.15	24.69	YES
85	2x	1.50e04	32.61	+	0.85	24.21	YES
86	VTM	N/A	N/A	-	0.15	24.48	YES
87	VTM	N/A	N/A	-	0.07	24.51	YES
88	2x	1.50e04	N/D	-	0.45	24.66	NO
89	VTM	N/A	N/A	-	0.03	24.4	YES
90	VTM	N/A	N/A	-	0.08	24.1	YES
91	VTM	N/A	N/A	-	0.07	24.43	YES
92	VTM	N/A	N/A	-	0.03	24.86	YES
93	VTM	N/A	N/A	-	0.24	24.49	YES
94	VTM	N/A	N/A	-	0.14	24.63	YES
95	VTM	N/A	N/A	-	0.31	25.09	YES
96	VTM	N/A	N/A	-	0.29	24.66	YES
97	VTM	N/A	N/A	-	0.31	24.72	YES
98	VTM	N/A	N/A	-	0.26	24.68	YES
99	VTM	N/A	N/A	-	0.35	24.65	YES
100	VTM	N/A	N/A	-	0.07	24.85	YES
101	VTM	N/A	N/A	-	0.12	24.63	YES
102	VTM	N/A	N/A	-	0.08	24.64	YES
103	VTM	N/A	N/A	-	0.07	24.52	YES
104	VTM	N/A	N/A	-	0.43	24.75	YES
105	MAX	1.70e06	29.13	+	16.47	25.67	YES
106	VTM	N/A	N/A	-	0.14	25.8	YES
107	VTM	N/A	N/A	-	0.07	25.96	YES
108	VTM	N/A	N/A	-	0.13	25.3	YES
109	VTM	N/A	N/A	-	0.04	25.39	YES
110	VTM	N/A	N/A	-	0.01	25.09	YES
111	VTM	N/A	N/A	-	0.02	24.99	YES
112	VTM	N/A	N/A	-	0.07	25.23	YES
113	VTM	N/A	N/A	-	0.03	25.3	YES
114	VTM	N/A	N/A	-	0.05	25.19	YES

Table 53. Blinded Mock Clinical Study: EZ1 Assay using the JBAIDS

Spiking KEY		Virus concentration (PFU/mL)	C_p Value	Call	Max Fluorescence	RP Control C_p	Agreement with Expected Result
115	VTM	N/A	N/A	-	0.08	25.48	YES
116	MAX	1.70e06	29.14	+	16.29	25.45	YES
117	2x	1.50e04	31.24	+	5.5	25.1	YES
118	2x	1.50e04	31.34	+	8	25.68	YES
119	VTM	N/A	N/A	-	0.04	25.46	YES
120	VTM	N/A	N/A	-	0.23	25.7	YES
121	VTM	N/A	N/A	-	0.09	25.27	YES
122	VTM	N/A	N/A	-	0.27	25.82	YES
123	VTM	N/A	N/A	-	0.31	25.56	YES
124	VTM	N/A	N/A	-	0.26	25.21	YES
125	2x	1.50e04	N/D	-	0.77	27.71	NO
126	2x	1.50e04	31.42	+	6.9	25.26	YES
127	2x	1.50e04	31.31	+	8.51	25.5	YES
128	VTM	N/A	N/A	-	0.16	24.99	YES
129	1.5x	1.125e04	32.29	+	3.6	25.05	YES
130	VTM	N/A	N/A	-	0.04	24.94	YES
131	VTM	N/A	N/A	-	0.03	24.98	YES
132	VTM	N/A	N/A	-	0.09	25.11	YES
133	VTM	N/A	N/A	-	0.05	25.03	YES
134	2x	1.50e04	30.92	+	7.73	25.06	YES
135	VTM	N/A	N/A	-	0.11	25.29	YES
136	VTM	N/A	N/A	-	0.07	24.88	YES
137	VTM	N/A	N/A	-	0.05	24.79	YES
138	VTM	N/A	N/A	-	0.12	25.1	YES
139	VTM	N/A	N/A	-	0.05	25.02	YES
140	VTM	N/A	N/A	-	0.14	25.07	YES
141	VTM	N/A	N/A	-	0.03	24.59	YES
142	VTM	N/A	N/A	-	0.07	24.81	YES
143	VTM	N/A	N/A	-	0.06	24.86	YES
144	MAX	1.70e06	29.5	+	13.24	25.24	YES
145	VTM	N/A	N/A	-	0.03	27.82	YES

N/A = Not applicable
VTM = Viral transport media
Max = High concentration

Table 54. Mock Clinical Study Summary Statistics

EZ1 Detection Assay on the JBAIDS Platform	Positive Results		Negative Results
Positive Specimens (1.5 x LoD)	11		4
Positive Specimens (2 x LoD)	13		2
Positive Specimens (MAX)	15		0
Negative Specimens (100)	0		100
Total (145)	39		106
			95% CI
Positive Percent Agreement	39/45	86.70%	73.21%-94.95%
Negative Percent Agreement	100/100	100%	96.38%-100%

Conclusion:

The EZ1 assay correctly identified 39 of 45 specimens spiked with inactivated Ebola Zaire virus at the concentrations shown, including concentrations near the limit of detection of the assay. No negatively spiked specimens rendered a positive reading.

APPENDIX A

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Joint Project Management Office Medical Countermeasure Systems

Ebola Zaire (EZ1) Sample Collection Procedure

The Ebola Zaire (EZ1) real-time reverse transcription (rRT) polymerase chain reaction (PCR) (TaqMan®) assay is intended for the qualitative detection of Ebla Zaire RNA from whole blood (EDTA anticoagulant) treated with Trizol (Life Technologies TRIzol® LS reagent or Sigma TRI Reagent® LS) and plasma (EDTA anticoagulant) treated with Trizol. The EZ1 assay is for use only under Emergency Use Authorization (EUA) in specified populations by specified laboratories and clinical laboratory personnel who have been trained on authorized instruments.

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/bioriskreduction/interim_recommendations_filovirus.pdfua=1
http://whqlibdoc.who.int/publications/2010/9789241599221_eng.pdf?ua=1
http://www.cdc.gov/vhf/abroad/pdf/african-healthcare-setting-vhf.pdf

Trizol Inactivation Procedure

Note: Protocol should be performed in a Class II or higher BioSafety Cabinet (BSC) or Glove Box

Blood and plasma samples potentially infected with Ebola virus (all species and strains) can be inactivated (i.e., rendered non-infectious) by the addition of 3 parts Trizol with 1 part whole blood or plasma following the method below:

1. **Procedure**
   a. Add 0.75mL of Trizol LS to a microcentrifuge tube.
   b. Within a BSC, and using appropriate personal protective equipment, add 0.25mL of whole blood or plasma sample 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.