ALDATU BIOSCIENCES

REF 4011096

# PANDAA® Ebola

Pan-species detection of ebolavirus RNA

## Instructions for Use

PANDAA® Ebola is for Research Use Only (RUO)

**NOT FOR RESALE**

RUO For research use only

REF 4011096

−15 ˚C to −25 ˚C

Contents sufficient for 96 reactions

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# Overview

## Intended Use (RUO)

This technical user guide is for PANDAA® Ebola, a Research Use Only (RUO) real-time RT-PCR assay for the amplification and qualitative **pan-species detection of orthoebolavirus RNA**. PANDAA Ebola is uniquely designed to provide accurate and rapid information for the identification of ebolaviruses, and PANDAA VHF Interpretation Software may be used to support analysis and interpretation of results. Use the most current version of this Instructions for Use available at www.aldatu.bio/downloads.

**This kit is for Research Use Only and is not intended for use in diagnostic procedures.**

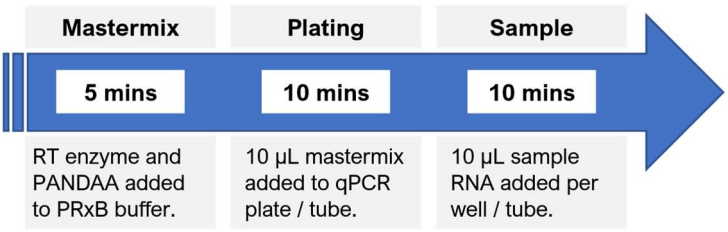
## PANDAA Ebola Test Principle

PANDAA Ebola assay is comprised of single reaction mix containing PANDAA primers to amplify and a FAM-labelled hydrolysis probe to detect all known species of ebolavirus.

An exogenous, non-competitive internal control is included to monitor for successful nucleic acid extraction and for the presence of RT-PCR inhibitors. A sample adequacy control (SAC) detects endogenous human RNase P in samples derived from human biological material to control for sample quality and to ensure adequate addition of sample to the reaction.

**The entire PANDAA Ebola workflow can be completed in two hours.**

cAssay preparation takes ~30 minutes with a PCR run time ~70 minutes. Prepare the PANDAA mastermix by adding Stop-Start RT enzyme and PANDAA to the PRxB buffer. Dispense 10 µL of mastermix into each PCR well/tube, then add sample RNA to each well/tube. **No mixing is necessary.**




## Compatible Real-Time PCR Instruments

Any real-time PCR instrument that can detect fluorophores in the green, yellow, and red channels, such as those listed in Appendix B.

## PANDAA Technology

Aldatu Biosciences' detection and genotyping PANDAA technology uniquely compensates for evolving pathogen diversity, ensuring that PCR diagnostic integrity isn't affected by genomic variation now, or in the future. PANDAA is designed to mitigate genomic variability by normalizing probe-binding regions. During the initial real-time PCR cycles, the target genome is adapted through site-directed mutagenesis to replace any polymorphisms that could cause false negative results. Read the methods publication here: https://www.nature.com/articles/s42003-021-01751-9.

# Contents and Storage

## Reagents

The kit includes all amplification reagents for 96 reactions and includes one (1) each of the Positive Control, Negative Control, and Internal Control RNA. **Refer to Appendix A for a list of materials required but not included.**

| Contents | Cap color | Top Label | Quantity | Volume |
|---|---|---|---|---|
| PRxB III Buffer | Clear | PRxB | 2 | 500 µL |
| Ebolavirus PANDAA | White | PAN EBOV | 2 | 35 µL |
| Stop-Start RT Enzyme | Blue | RT | 1 | 8 µL |
| Internal Control RNA | Yellow | INT CTRL | 1 | 120 µL |
| EBOV Positive Control | Red | POS | 1 | 100 µL |
| Negative Control | Green | NEG | 1 | 100 µL |

## Storage and Stability

PANDAA Ebola is shipped on dry ice, and kit components arrive frozen. Store all components at −15 °C to −25 °C upon receipt, protected from light. Stop-Start RT enzyme contains glycerol and may remain liquid at −15 °C to −25 °C. Keep reagents in the original packaging when not in use and return them promptly to recommended storage conditions. For receiving inspection and handling, see *General Guidelines → Shipping, Storage and Handling* (page 25).

**A unique feature of PANDAA assays is that, *after thawing*, all components except Stop-Start RT enzyme may be stored at 2 °C to 8 °C for up to 7 days,** protected from light. To maintain assay performance, avoid more than two freeze-thaw cycles for any reagent.

| Contents | Stability at 2 °C to 8 °C |
|---|---|
| PRxB III Buffer | **7 days** |
| Ebolavirus PANDAA | |
| Internal Control RNA | |
| EBOV Positive Control | |
| Negative Control | |
| Stop-Start RT Enzyme | ***Should only be stored at −15 °C to −25 °C*** |

**NOTE:** All reagents, *other than the Stop-Start RT enzyme*, may be stored at 2 °C to 8 °C for up to one week after thawing.

## Reagents Description

### PANDAA Reaction Buffer (PRxB III)

A complete custom assay buffer that is compatible with high, low and no ROX real-time PCR machines. Contact Technical Support (support@aldatubio.com) with questions about your real-time PCR instrument requirements and configuration.

### Stop-Start RT Enzyme

A specially formulated hot-start reverse transcriptase optimized for PANDAA.

### Ebolavirus PANDAA

Includes uniquely designed PANDAA primers and probes for the amplification and detection of three distinct targets:

- **Ebolavirus (PAN EBOV):** targeted to the nucleoprotein (NP) region of the ebolavirus genome.
- **Internal Control (IC):** detects the internal control RNA, which serves as a process control for both RNA extraction and PCR amplification.
- **Sample Adequacy Control (SAC):** detects human RNase P in samples derived from human biological material to control for the quantity, quality and adequacy of the specimen.

### Extraction and Amplification Controls

- **Internal Control** (IC) is an exogenous, non-competitive control that detects MS2 phage-specific RNA, which should be either spiked into the lysis buffer prior to extraction or added directly to the real-time PCR reaction mix.
- **EBOV Positive Control** consists of synthetic, non-infectious RNA covering the assay target regions in the genome of Ebola virus, isolate Mayinga, species *Orthoebolavirus zairense*. The control is provided at 150 copies/µL to yield 1,500 copies/reaction when using 10 µL control per reaction. It is prepared in a background of human genomic DNA and Internal Control RNA.
- **Negative Control** comprises ~0.1 ng/µL human genomic DNA, which is ~30 copies/µL to yield ~300 copies/reaction when using 10 µL control per reaction, and includes Internal Control RNA.

# Sample Preparation for PANDAA Ebola

The starting material for the PANDAA Ebola kit is isolated viral RNA. Read the *Safety* section. If samples have already been extracted then proceed to *PANDAA Ebola Kit Step-by-Step Instructions* on page 9.

## Nucleic Acid Extraction

### Compatible Extraction Reagents

Many commercially available nucleic acid extraction systems are compatible with real-time PCR and are suitable for sample preparation prior to PANDAA Ebola testing, including systems that purify total nucleic acid (DNA and RNA). Follow the extraction kit manufacturer's instructions for the nucleic acid extraction kit or workflow used for viral RNA and refer to the safety guidelines in this user guide.

### Sample Volume

The recommended sample input volume for nucleic acid extraction is 140 µL. Sample input volume may vary by platform and should be maximized to optimize test sensitivity.

| | |
|---|---|
| **Recommended specimen type** | EDTA plasma |
| **Recommended sample volume** | 140 µL |
| **Recommended elution volume** | 60 µL |

### Required Elution Volume

Elution volume may vary by nucleic acid extraction protocol and should be optimized to maximize RNA recovery while minimizing dilution. Typical elution volumes range from **50 µL to 100 µL**. Each PANDAA Ebola reaction requires **10 µL eluted RNA**.

### Extracted RNA Handling

Use extracted RNA as soon as possible after extraction. If testing is delayed, extracted RNA may be stored at 2 – 8 °C for up to 6 hours. Extraction eluants and storage conditions can affect RNA stability, which may reduce detection in low-copy-number samples.

### Internal Control for RNA Extraction

The Internal Control (IC) RNA provided can be added to the sample extraction kit lysis buffer to serve as a full process control and verify successful RNA extraction as well as downstream amplification/detection. For each sample being processed, add **1 µL IC** to the sample extraction lysis buffer e.g., if processing 48 samples then add 48 µL IC to the lysis buffer.

# PANDAA Ebola Kit Step-by-Step Instructions

## A. Before Beginning

PANDAA Ebola is a highly sensitive molecular assay. To prevent false-positive results, follow a strict unidirectional workflow. Always use separate, dedicated lab areas and equipment for reagent preparation and for sample/control addition.

**CAUTION**

**In a dedicated lab area for setting up PCR reaction mixes**

Remove reagents from storage and place at room temperature to thaw. Estimated thawing times are 10 minutes for **PRxB buffer** and 5 minutes for **PANDAA tubes**. Remove **Stop-Start RT enzyme (RT)** from the freezer and place it directly on ice. *Tubes should be placed on ice after thawing.*

## B. PANDAA Ebola Mastermix Setup (48 Reactions)

1. **Prepare reagents:** Spin the **reagent tubes** briefly to collect drops on the interior sides of the tubes. Gently vortex the reagent tubes and spin briefly again.
2. **Add reagents to PRxB tube:**
   - Add **30 µL Ebolavirus PANDAA** directly to the PRxB tube.
   - Add **3 µL Stop-Start RT enzyme** directly to the PRxB tube.
     2..1 Submerge only the tip of the pipette to ensure that no excess RT droplets are transferred to the exterior of the tip.
   - **If Internal Control (IC) was not added to the lysis buffer prior to sample extraction**, it should be added to the PRxB tube here.
     - For 48 samples, add **3 µL Internal Control (IC) to the PRxB tube** if the IC was not added to the lysis buffer prior to sample extraction.
3. **Mix reagents:** Gently vortex the **PRxB tube** containing the Stop-Start RT enzyme, PANDAA, and Internal Control (if not added to the sample extraction lysis buffer). Spin to collect any droplets.

**NOTE: 24 reactions modification**

Remove **250 µL PRxB buffer** and transfer to a clean tube. Add **15 µL Ebolavirus PANDAA** and **1.5 µL Stop-Start RT Enzyme**. Add 1.5 µL IC RNA only if the IC was not added to the lysis buffer.

Follow the storage instructions on page 6 for the remaining reagents.

**NOTE: Fewer than 48 reactions**

If you require fewer than 48 reactions, prepare mastermix for 48 samples and store any unused volume. The complete PANDAA Ebola mastermix (PRxB, Stop-Start RT enzyme, and PANDAA (with or without Internal Control) is **stable for up to 48 hours when stored at 2–8 °C**.

## C. Real-time PCR Plate Setup

1. **Dispense mastermix:** Add 10 µL of the complete mastermix from the PRxB tube into each required well of an optical 96-well reaction plate or to each optical reaction tube compatible with your real-time PCR instrument.

**CAUTION**

**Perform the remaining steps in a pre-amplification area designated for RNA handling in your laboratory**

2. **Add sample or controls:** Add 10 µL sample RNA, Positive Control, or Negative Control into the top of the mastermix in each well / tube. Do *not* pipette up and down to mix.
3. **Seal and spin (plate only):** Seal the plate with optical adhesive film or cap the reaction tubes. If using a 96-well plate, spin the plate briefly before loading into the real-time PCR instrument.

## D. Real-time PCR Protocol

1. Proceed immediately to loading the plate / tubes into the real-time PCR instrument.
2. Use the real-time PCR template provided by Aldatu Biosciences, which can be downloaded from www.aldatu.bio/qpcr-templates. Total run time should be 60 to 80 minutes.

## E. Manual Real-time PCR Settings

Use the real-time PCR run template provided by Aldatu Biosciences, available at www.aldatu.bio/qpcr-templates. **Aldatu Biosciences cannot accept any responsibility for inaccurate data that has been generated through incorrect manual programming of the real-time PCR instrument.**

Use the following parameters and proceed to run the PANDAA real-time PCR. **Refer to Appendix B (page 28) for detection channel settings for compatible real-time PCR instruments.** For assistance with instrument configuration, contact Aldatu Biosciences Technical Support (support@aldatubio.com).

### Real-Time PCR Instrument Settings

| Setting | Value |
|---|---|
| Reaction volume | 20 µL |
| Ramp rate | Fast, if compatible with your real-time PCR instrument. Otherwise use standard ramp rates. Refer to page 17 for ramp temperatures. |
| Passive reference (if applicable) | ROX |

| Target Name | Ex / Em (nm) | Channel | Reporter |
|---|---|---|---|
| Ebolavirus (PAN EBOV) | 495 / 520 | Blue/Green* | Refer to Appendix B (page 28) for instrument-specific reporter / dye settings. |
| Internal Control (IC) | 650 / 670 | Red | |
| Sample Adequacy Control (SAC) | 554 / 576 | Yellow | |

*Channel name may differ between instruments.

### Cycling Conditions

During the 60 °C anneal and extension phase of the *Amplification and Detection* step, acquire fluorescence data in the green, yellow, and red channels.

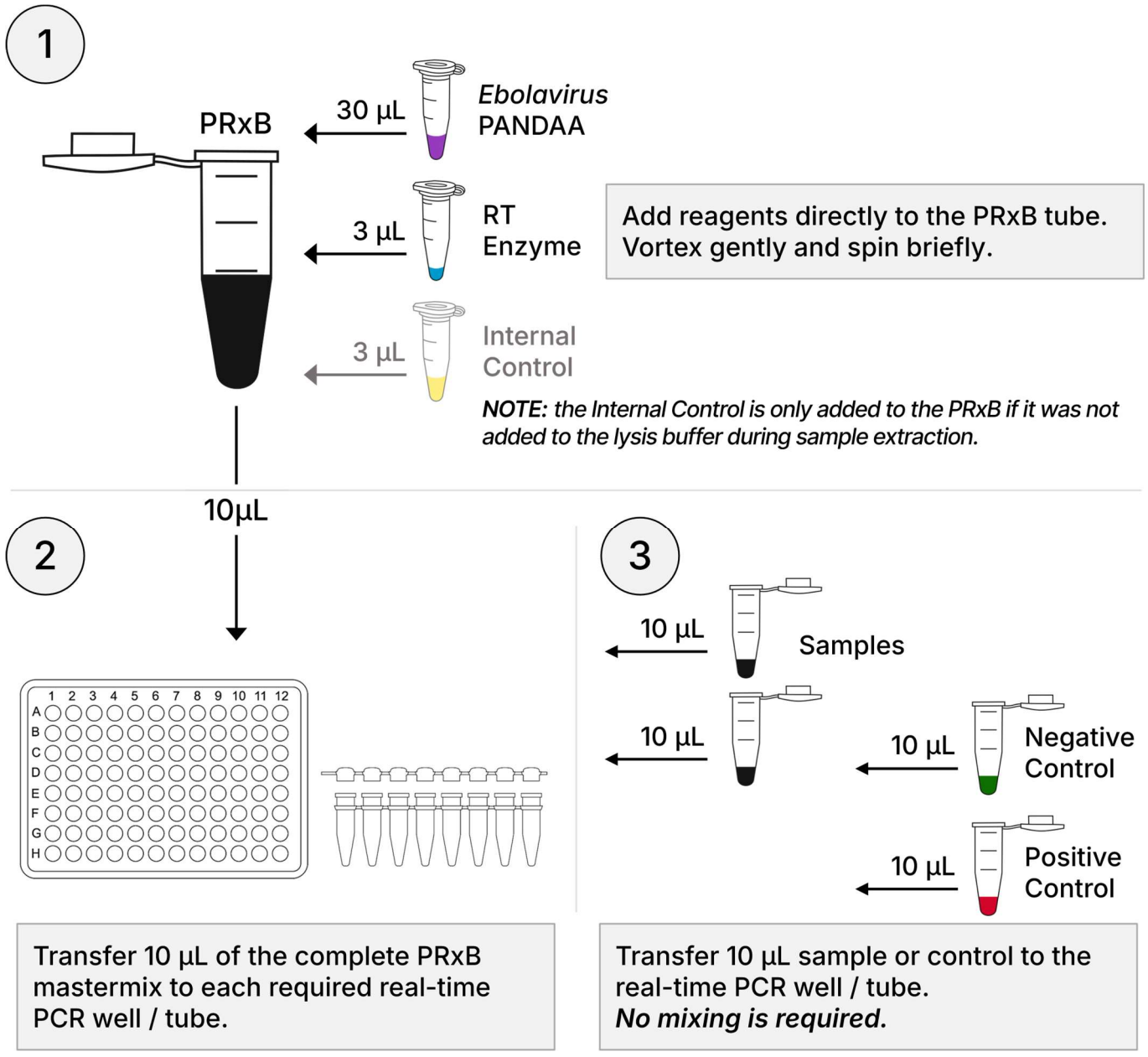
| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Reverse transcription | 50 °C | 15 minutes | 1 |
| Enzyme activation | 95 °C | 2 minutes | 1 |
| PANDAA adaptation | 95 °C<br>55 °C<br>60 °C | 1 second<br>30 seconds<br>30 seconds | 10 |
| Amplification and detection | 95 °C<br>60 °C | 1 second<br>(*acquire*) 60 seconds | 35 |

## PANDAA EBOV Qualification Panel

**NOTE: PANDAA EBOV Qualification Panel**

For first-time instrument use, perform a preliminary run with the PANDAA EBOV Qualification Panel (catalog #4111096), **provided free with any Aldatu assay** order. The panel contains ten synthetic, non-infectious RNA samples: nine positive controls covering all five ebolavirus species and one negative control (Marburg virus). Contact support@aldatubio.com for details.

## PANDAA Ebola Kit Quick Guide



# Data Analysis and Interpretation of Results

## Real-Time PCR Analysis Parameters

Refer to Appendix B (page 28) for instrument-specific analysis settings, including threshold settings for determination of Ct values for the ebolavirus, Internal Control, and Sample Adequacy Control targets. For technical assistance, contact Aldatu Biosciences at support@aldatubio.com.

## Threshold Settings

Adjust the EBOV threshold following the instrument-specific analysis settings in Appendix B (page 28). Use the instrument's automatic threshold for IC and SAC (recommended). If set manually, place the IC and SAC thresholds in the exponential (log-linear) region above baseline and below plateau; apply the same setting to all samples and controls.

## Fluorescence Cutoff

Minor non-specific increases in fluorescence due to probe hydrolysis, dye crosstalk, or signal drift can produce spurious Ct values in the absence of genuine target amplification. The fluorescence cutoff distinguishes these artefactual signals from true positive results.

At the end of the PCR run, **a fluorescence cutoff of 2% should be applied to confirm negative results**. A well is called negative if its baseline-subtracted endpoint fluorescence (ΔRn on Applied Biosystems and Thermo Fisher instruments; baseline-subtracted endpoint RFU on Bio-Rad CFX instruments; fluorescence change on Qiagen Rotor-Gene and Mic instruments) is less than 2% of the highest baseline-subtracted endpoint fluorescence detected on the plate. The highest value on the plate is typically the on-plate Positive Control. Refer to Appendix B (page 28) for instrument-specific instructions. **The fluorescence cutoff is determined automatically when using the *PANDAA VHF Interpretation Software*.**

## PANDAA VHF Interpretation Software

*PANDAA VHF Interpretation Software* is a free, automated tool for reviewing and interpreting Aldatu PANDAA real-time PCR run files for viral hemorrhagic fever assays. The software can import data directly from instrument files generated by supported real-time PCR instruments, apply standardized analysis thresholds, evaluate control validity, and generate summary reports, **typically in a few seconds after importing a run file**. Refer to the *PANDAA VHF Interpretation Software* instructions for use for installation, supported instruments, and detailed operating instructions.

Download the *PANDAA VHF Interpretation Software* from www.aldatu.bio/vhf-software.

### Key Capabilities

- Automatically analyzes results directly from instrument data files from multiple real-time PCR instrument platforms.
- Applies standardized analysis criteria (Ct and fluorescence cutoff thresholds).
- Validates quality controls with a structured flag framework.
- Generates formatted reports (PDF and Excel) for documentation and review.
- Provides "Detected/Not Detected" interpretations with appropriate validity flags.

### Supported Instruments and File Types

The software can currently accept the analyzed instrument run file directly from multiple instruments and accepts results files from the following real-time PCR instruments in the formats below.

| Instrument | Supported File Formats |
|---|---|
| QuantStudio 5 | .eds, .xlsx |
| QuantStudio 7 Pro | .eds, .xlsx |
| 7500 / 7500 Fast | .eds, .xls |
| Rotor-Gene Q | .rex, .csv |
| Mic | .micrun, .xlsx |
| CFX96 | .pcrd, .xlsx |
| LightCycler 96 | .lc96, .txt |
| LightCycler 480 II | .ixo, .txt |

## Positive and Negative Controls Interpretation

These PCR controls must be included in every run for the results to be valid. The Positive Control must have a Ct < 30 cycles for the PAN EBOV target to pass quality control; the PAN EBOV target must not be detected in the Negative Control.

| Control | Target | | Call | Results Interpretation |
|---|---|---|---|---|
| **POS** | PAN EBOV | + | PASS | Positive Control *passed* QC as the EBOV Ct is *below* the cut-off. |
| | IC | + | | |
| | SAC | + | | |
| **POS** | PAN EBOV | - | FAIL | Positive Control *failed* QC as the EBOV Ct is *above* the cut-off. This may indicate an error during setup, inefficient sample extraction or amplification reagent issues. |
| | IC | + | | |
| | SAC | + | | |
| **NEG** | PAN EBOV | - | PASS | Negative Control *passed* QC as the EBOV Ct is *above* the cut-off. |
| | IC | + | | |
| | SAC | + | | |
| **NEG** | PAN EBOV | + | FAIL | Negative Control *failed* QC as the EBOV Ct is *below* the cut-off. This may indicate an error during setup or cross-contamination. |
| | IC | + | | |
| | SAC | + | | |

## Results Interpretation

A Ct value < 30 cycles for the ebolavirus (PAN EBOV) target indicates that ebolavirus RNA is present in the sample. If the PAN EBOV target is ≥ 30 cycles, or not detected, *and* the Internal Control and Sample Adequacy Control Ct values are < 30 cycles then ebolavirus RNA is *not* present.

If both the PAN EBOV *and* the Internal control or Sample Adequacy Control Ct is ≥ 30 cycles, or not detected, then there has been a reagent or extraction failure and the sample should be repeated.

| PAN EBOV | IC | SAC | Call | Results Interpretation / Recommended Action |
|---|---|---|---|---|
| + | ±* | ±* | Positive | Ebolavirus RNA detected. Detection of IC or SAC is not required for a positive result. |
| - | + | + | Negative | Ebolavirus RNA not detected; successful RNA extraction and sample adequacy verified. |
| - | + | - | Invalid Result | SAC not detected; inadequate human biological material, inappropriate specimen collection, or compromised specimen integrity. Repeat extraction and/or obtain a new specimen. |
| - | - | + | Invalid Result | Internal control not detected; indicates a potential reagent or extraction failure. Repeat extraction and/or obtain a new specimen. |
| - | + | N/A† | Negative | Ebolavirus RNA not detected; successful RNA extraction verified. Sample adequacy control is not applicable for non-human biological specimens. |
| - | - | N/A† | Invalid Result | Internal control not detected; indicates potential reagent or extraction failure. Repeat extraction and/or obtain a new specimen. |

±*: Detection of Internal Control (IC) and Sample Adequacy Control (SAC) is not required to interpret a positive result for Ebolavirus, as high viral load can suppress these signals.

N/A†: SAC is not applicable when testing samples that are not derived from human biological material. In these cases, only the Internal Control is required for result interpretation.

## * Internal Control and Sample Adequacy Control Interpretation

PANDAA Ebola contains an exogenous, non-competitive extraction control (Internal Control) that is either spiked into the lysis buffer prior to extraction *or* added directly to the PRxB buffer. Additionally, PANDAA Ebola contains amplification and detection reagents for the Sample Adequacy Control (SAC), which detects human nucleic acid. A high ebolavirus viral load may lead to the biased consumption of reaction amplification components and cause a delayed or absent Internal Control signal and/or Sample Adequacy Control signal. Therefore, detection of the Internal Control or Sample Adequacy Control is not required to call a positive result for ebolavirus.

# Performance Characteristics

## Analytical Sensitivity (inactivated virus, PFU/mL)

The limit of detection (LoD) of PANDAA Ebola was estimated using gamma-irradiated cell lysate and supernatant from Vero E6 cells infected with Bundibugyo ebolavirus, Sudan ebolavirus, or Zaire ebolavirus obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Analytical sensitivity was defined as the concentration (plaque forming units (PFU) per ml) detected with a positivity rate of ≥ 95% for Sudan ebolavirus and Zaire ebolavirus. For Bundibugyo ebolavirus, the 95% limit of detection was determined by probit.

| Virus isolate tested | Common name | Limit of Detection |
|---|---|---|
| Bundibugyo virus, Butalya-811250 | Bundibugyo ebolavirus (BDBV) | 5.7 PFU/mL |
| Sudan virus, Boniface | Sudan ebolavirus (SUDV) | 200 PFU/mL |
| Ebola virus, Makona | Zaire ebolavirus (EBOV/ZEBOV) | 50 PFU/mL |

### Tentative Limit of Detection (LoD) Estimation

For the tentative LoD estimation, inactivated virus was spiked into pooled EDTA plasma from 1,000 PFU/mL to 1 PFU/mL in half-log dilutions, or with PBS as the extraction negative control. Nucleic acid was extracted from 140 µL sample using the QIAamp Viral RNA Mini Kit (Qiagen). For each concentration, 3 independent extractions were performed and a single replicate per extraction was used with the PANDAA Ebola assay on the ABI 7500 Fast (ABI 7500), Mic real-time PCR Cycler (Mic), and QuantStudio 5 (0.1 mL, 96-well; QS5). The tentative LoD estimation was refined at two chosen concentrations.

### Limit of Detection (LoD) Confirmation

Based on the results obtained from the tentative LoD of Bundibugyo ebolavirus, Sudan ebolavirus, or Zaire ebolavirus, 20 individually spiked plasma samples were extracted from 140 µL sample using the QIAamp Viral RNA Mini Kit (Qiagen). A single replicate per extraction was used with the PANDAA Ebola assay on the ABI 7500, Mic, QS5, and the Qiagen Rotor-Gene Q 5-Plex (RGQ). An equal number of negative extraction controls were performed and specificity was 100%.

| Instrument | Run Time (average) | Software Used |
|---|---|---|
| **ABI 7500** | Fast: 78 min | 7500/7500 Fast Real-Time PCR System Software v2.3 |
| **Mic** | Fast: 87 min | micPCR Software v2.12.7 |
| **QS5 *** | Fast: 75 min | QuantStudio Design & Analysis Software version 1.6.1 |
| **RGQ** | Standard: 100 min | Rotor-Gene Q Software version 2.3.5 |

* Fast thermal ramp rates on the QuantStudio 5 were 4.1 °C/s (heating) and 3.1 °C/s (cooling).

## Bundibugyo ebolavirus, Butalya-811250

Serial dilutions were performed with irradiated infected cell lysate and supernatant from Vero E6 cells infected with Bundibugyo ebolavirus, Prototype Isolate #811250 (BEI Resources, NR-49813) with a pre-irradiation titer by plaque assay of $7.9 \times 10^6$ PFU/mL.

| Conc. (PFU/mL) | ABI 7500 | Mic | QS5 |
|---|---|---|---|
| **1,000** | 3/3 | 3/3 | 3/3 |
| **500** | 3/3 | 3/3 | 3/3 |
| **100** | 3/3 | 3/3 | 3/3 |
| **50** | 3/3 | 3/3 | 3/3 |
| **10** | 2/3 | 3/3 | 3/3 |
| **5** | 1/3 | 3/3 | 3/3 |
| **1** | 0/3 | 1/3 | 2/3 |
| **Negative** | 0/3 | 0/3 | 0/3 |

| Conc. (PFU/mL) | ABI 7500 | Mic | QS5 |
|---|---|---|---|
| **4** | 1/3 | 0/3 | 1/3 |
| **2** | 1/3 | 0/3 | 0/3 |

Limit of detection confirmation (5 PFU/mL, Bundibugyo ebolavirus)

| Replicate | ABI 7500 Ct | Mic Ct | QS5 Ct | RGQ Ct |
|---|---|---|---|---|
| **1** | 26.7 | 26.4 | 26.4 | 29.3 |
| **2** | 26.0 | 26.4 | 28.2 | 26.2 |
| **3** | N.D. | 26.9 | N.D. | 28.8 |
| **4** | 28.4 | 27.0 | 29.2 | 28.3 |
| **5** | 28.2 | 26.0 | 28.8 | 27.9 |
| **6** | 27.5 | 26.7 | 27.2 | 28.4 |
| **7** | 28.5 | 26.3 | N.D. | 26.6 |
| **8** | 26.8 | 26.4 | 28.6 | 29.2 |
| **9** | 27.3 | N.D. | 28.9 | N.D. |
| **10** | 27.9 | 26.4 | 27.5 | 27.2 |
| **11** | N.D. | N.D. | 28.7 | N.D. |
| **12** | 28.6 | 28.5 | 26.4 | 29.2 |
| **13** | 27.8 | N.D. | N.D. | 28.0 |
| **14** | 26.7 | 26.7 | 26.4 | N.D. |
| **15** | 28.5 | 27.1 | 26.2 | 26.8 |
| **16** | 26.1 | 28.1 | 27.5 | 28.0 |
| **17** | 28.6 | N.D. | N.D. | 30.0 |
| **18** | 27.8 | 27.6 | 27.4 | 28.0 |
| **19** | N.D. | 30.1 | 28.4 | 29.6 |
| **20** | 28.9 | 29.1 | 29.6 | 29.3 |
| **Hit Rate** | **17/20** | **17/20** | **16/20** | **17/20** |

Limit of detection confirmation (10 PFU/mL, Bundibugyo ebolavirus)

| Replicate | ABI 7500 Ct | Mic Ct | QS5 Ct | RGQ Ct |
|---|---|---|---|---|
| **1** | 26.9 | 27.8 | 25.8 | 27.8 |
| **2** | 27.8 | 26.7 | 26.5 | 27.8 |
| **3** | 26.2 | 26.0 | 26.5 | 25.5 |
| **4** | 26.2 | 26.3 | 25.7 | 26.0 |
| **5** | 25.4 | 26.8 | 25.8 | 26.1 |
| **6** | 26.4 | 28.1 | 26.4 | 26.0 |
| **7** | 26.7 | 26.5 | 26.6 | 26.2 |
| **8** | 27.1 | 28.6 | 27.5 | 27.2 |
| **9** | 26.2 | 26.8 | 26.2 | 26.7 |
| **10** | 27.7 | 28.1 | 27.8 | 27.7 |
| **11** | 28.3 | 27.6 | 28.7 | 26.2 |
| **12** | 25.8 | 27.1 | 26.3 | 27.1 |
| **13** | 27.5 | 26.5 | 26.3 | 26.4 |
| **14** | 26.5 | 27.0 | 26.8 | N.D. |
| **15** | 27.3 | N.D. | 29.4 | 27.8 |
| **16** | 27.6 | 27.0 | 27.4 | 27.8 |
| **17** | 27.7 | 27.7 | 27.3 | 27.6 |
| **18** | 28.6 | 29.5 | 28.7 | 28.4 |
| **19** | 26.4 | 26.9 | 26.7 | 28.1 |
| **20** | 27.9 | 28.7 | 29.1 | 28.1 |
| **Hit Rate** | **20/20** | **19/20** | **20/20** | **19/20** |

The 95% limit of detection of the PANDAA Ebola assay for Bundibugyo ebolavirus, Butalya-811250 is **5.7 PFU/mL** (95% CI: 5.1 – 11.2 PFU/mL), as determined by probit.

## Sudan ebolavirus, Boniface

Serial dilutions were performed with irradiated infected cell lysate and supernatant from Vero E6 cells infected with Sudan ebolavirus, Boniface (BEI Resources, NR-49810) with a pre-irradiation titer by plaque assay of $6.3 \times 10^6$ PFU/mL. **The 95% limit of detection of the PANDAA Ebola assay for Sudan ebolavirus, Boniface is 200 PFU/mL**, based on the lowest concentration at which ≥ 95% were positive.

| Conc. (PFU/mL) | ABI 7500 | Mic | QS5 |
|---|---|---|---|
| **1,000** | 3/3 | 3/3 | 3/3 |
| **500** | 3/3 | 3/3 | 3/3 |
| **100** | 1/3 | 3/3 | 3/3 |
| **50** | 1/3 | 0/3 | 1/3 |
| **10** | 1/3 | 1/3 | 0/3 |
| **5** | 0/3 | 0/3 | 0/3 |
| **1** | 0/3 | 0/3 | 1/3 |
| **Negative** | 0/3 | 0/3 | 0/3 |

| Conc. (PFU/mL) | ABI 7500 | Mic | QS5 |
|---|---|---|---|
| **200** | 3/3 | 3/3 | 3/3 |
| **100** | 1/3 | 1/3 | 0/3 |

Limit of detection confirmation (200 PFU/mL, Sudan ebolavirus)

| Replicate | ABI 7500 Ct | Mic Ct | QS5 Ct | RGQ Ct |
|---|---|---|---|---|
| **1** | 25.9 | 27.2 | 26.3 | 28.0 |
| **2** | 27.7 | 27.6 | 27.4 | N.D. |
| **3** | 26.5 | 27.2 | 26.2 | 25.4 |
| **4** | 27.6 | 28.6 | 26.8 | 24.9 |
| **5** | 25.8 | 27.1 | 26.0 | 26.1 |
| **6** | 26.6 | 29.4 | N.D. | 26.1 |
| **7** | 27.0 | N.D. | 27.5 | 25.4 |
| **8** | 26.5 | 28.0 | 28.2 | 27.6 |
| **9** | 28.9 | 28.1 | 26.9 | 26.0 |
| **10** | 27.7 | 29.0 | 28.6 | 25.9 |
| **11** | 27.7 | 27.1 | 26.7 | 27.5 |
| **12** | 26.6 | 26.5 | 26.1 | 26.4 |
| **13** | 27.6 | 29.6 | 26.5 | 26.1 |
| **14** | 26.0 | 26.6 | 26.6 | 25.6 |
| **15** | 25.9 | 27.2 | 27.6 | 28.8 |
| **16** | 28.7 | 28.0 | 29.7 | N.D. |
| **17** | 28.6 | 26.4 | 26.7 | 26.1 |
| **18** | 26.9 | 28.4 | 27.2 | 26.8 |
| **19** | 27.6 | 29.1 | 27.3 | 29.0 |
| **20** | 27.4 | 28.2 | 28.0 | 25.4 |
| **Hit Rate** | **20/20** | **19/20** | **19/20** | **18/20** |

## Zaire ebolavirus, Mayinga

Serial dilutions were performed with irradiated infected cell lysate and supernatant from Vero E6 cells infected with Zaire ebolavirus, Mayinga (BEI Resources, NR-49809) with a pre-irradiation titer by plaque assay of $1.0 \times 10^8$ PFU/mL. **The 95% limit of detection of the PANDAA Ebola assay for Zaire ebolavirus, Mayinga is 50 PFU/mL**, based on the lowest concentration at which ≥ 95% were positive.

| Conc. (PFU/mL) | ABI 7500 | Mic | QS5 |
|---|---|---|---|
| **1,000** | 3/3 | 3/3 | 3/3 |
| **500** | 3/3 | 3/3 | 3/3 |
| **100** | 3/3 | 3/3 | 3/3 |
| **50** | 3/3 | 3/3 | 3/3 |
| **10** | 2/3 | 2/3 | 2/3 |
| **5** | 0/3 | 0/3 | 1/3 |
| **1** | 0/3 | 0/3 | 0/3 |
| **Negative** | 0/3 | 0/3 | 0/3 |

| Conc. (PFU/mL) | ABI 7500 | Mic | QS5 |
|---|---|---|---|
| **40** | 3/3 | 2/3 | 2/3 |
| **20** | 1/3 | 1/3 | 1/3 |

Limit of detection confirmation (50 PFU/mL, Zaire ebolavirus)

| Replicate | ABI 7500 Ct | Mic Ct | QS5 Ct | RGQ Ct |
|---|---|---|---|---|
| **1** | 24.6 | 25.8 | 25.5 | 26.0 |
| **2** | 26.6 | 25.9 | 25.5 | 26.2 |
| **3** | 24.6 | 26.4 | 24.9 | 26.5 |
| **4** | 25.8 | 25.8 | 25.2 | 27.8 |
| **5** | 25.5 | 26.5 | 26.0 | 25.2 |
| **6** | 26.5 | 25.5 | 25.8 | 25.4 |
| **7** | 25.6 | 25.2 | 25.3 | 25.5 |
| **8** | 26.4 | 26.0 | 27.0 | 25.2 |
| **9** | 25.7 | 26.0 | 26.0 | 26.4 |
| **10** | 27.0 | 26.2 | 27.0 | 25.8 |
| **11** | 26.2 | 26.0 | 25.9 | 25.7 |
| **12** | 26.5 | 25.1 | 26.1 | 28.6 |
| **13** | 26.5 | 26.2 | 25.7 | 25.5 |
| **14** | 24.7 | 25.9 | 24.5 | 25.5 |
| **15** | 25.6 | 25.6 | 25.7 | 25.8 |
| **16** | 25.6 | 25.9 | 25.5 | 25.9 |
| **17** | 25.9 | 25.8 | 25.7 | 27.1 |
| **18** | 26.3 | 27.1 | 26.5 | 25.0 |
| **19** | 25.5 | 26.5 | 25.3 | 25.8 |
| **20** | 25.8 | 25.4 | 25.9 | 25.6 |
| **Hit Rate** | **20/20** | **20/20** | **20/20** | **20/20** |

## Analytical Sensitivity and Inclusivity (synthetic RNA, copies/reaction)

Analytical sensitivity and inclusivity were evaluated using *in vitro* transcribed RNA representing multiple isolates and ebolavirus species. RNA copy numbers were assigned by RT-qPCR using digital PCR (dPCR)-traceable calibration. Among 900 negative control replicates, analytical specificity was 99.3% (95% CI: 98.5% to 99.8%).

### Bundibugyo ebolavirus

| Isolate | 20 copies/rxn | 10 copies/rxn | 5 copies/rxn |
|---|---|---|---|
| Butalya-811250 | 100% (36/36) | 97.2% (35/36) | 75.0% (27/36) |

### Reston ebolavirus

| Isolate | 20 copies/rxn | 10 copies/rxn | 5 copies/rxn |
|---|---|---|---|
| Pennsylvania | 100% (36/36) | 100% (36/36) | 97.2% (35/36) |
| R08 | 100% (36/36) | 100% (36/36) | 77.8% (28/36) |

### Sudan ebolavirus

| Isolate | 20 copies/rxn | 10 copies/rxn | 5 copies/rxn |
|---|---|---|---|
| Boniface | 100% (36/36) | 100% (36/36) | 97.2% (35/36) |
| Gulu | 100% (36/36) | 100% (36/36) | 86.1% (31/36) |
| Maleo | 100% (36/36) | 100% (36/36) | 69.4% (25/36) |
| Nakisamata | 100% (36/36) | 95.7% (34/36) | 83.3% (30/36) |
| Uganda | 100% (36/36) | 97.2% (35/36) | 80.6% (29/36) |
| Yambio | 100% (36/36) | 100% (36/36) | 91.7% (33/36) |

### Taï Forest ebolavirus

| Isolate | 20 copies/rxn | 10 copies/rxn | 5 copies/rxn |
|---|---|---|---|
| Pauléoula-CI | 100% (36/36) | 95.8% (34/36) | 45.8% (17/36) |

### Zaire ebolavirus

| Isolate | 20 copies/rxn | 10 copies/rxn | 5 copies/rxn |
|---|---|---|---|
| Boende | 100% (36/36) | 100% (36/36) | 100% (36/36) |
| Gabon | 100% (36/36) | 100% (36/36) | 91.7% (33/36) |
| Ilembe | 100% (36/36) | 100% (36/36) | 100% (36/36) |
| Makona | 100% (36/36) | 100% (36/36) | 94.4% (34/36) |
| M-M | 100% (36/36) | 100% (36/36) | 100% (36/36) |
| Yambuku | 100% (36/36) | 100% (36/36) | 100% (36/36) |

## Analytical Specificity (Exclusivity)

A panel of 29 pathogens was evaluated, representing genetically related filoviruses, including Marburg virus and Ravn virus, and co-endemic viral, bacterial, or parasitic agents associated with febrile illness. Organisms were tested at high, medically relevant concentrations: viruses were tested at ≥ $10^5$ PFU/mL or ≥ $10^5$ $TCID_{50}$/mL and bacteria and parasites were tested at ≥ $10^6$ CFU/mL. Where purified nucleic acids was used in lieu of whole organisms, equivalent high-titer genomic copy levels were tested: viral nucleic acids at ≥ $10^7$ copies/mL and bacterial/parasitic DNA at ≥ $10^6$ copies/mL.

The results summarized in the table below demonstrate that **all tested pathogens were non-reactive with the PANDAA Ebola assay.**

| Pathogen (Isolate) | Titer | Result |
|---|---|---|
| Adenovirus Type 5 (Adenoid 75) | $3.4 \times 10^9$ $TCID_{50}$/mL | Negative |
| Chikungunya virus (181/25) | $4.7 \times 10^7$ NDU/mL | Negative |
| Crimean-Congo hemorrhagic fever virus (IbAr10200) | $6.3 \times 10^6$ copies/mL | Negative |
| Dengue virus type 1 (Hawaii) | $6.2 \times 10^5$ $TCID_{50}$/mL | Negative |
| Dengue virus type 2 (New Guinea C) | $5.9 \times 10^5$ $TCID_{50}$/mL | Negative |
| Dengue virus type 3 (Philippines/H87/1956) | $9.8 \times 10^5$ $TCID_{50}$/mL | Negative |
| Dengue virus type 4 (H241) | $5.9 \times 10^5$ $TCID_{50}$/mL | Negative |
| Hepatitis A virus (HM175/18f) | $1.5 \times 10^6$ $TCID_{50}$/mL | Negative |
| Hepatitis B virus (N/A) | $2.3 \times 10^6$ IU/mL | Negative |
| Hepatitis C virus (N/A) | $1.8 \times 10^6$ IU/mL | Negative |
| HIV-1 (DE00210CM019) | $3.5 \times 10^7$ copies/mL | Negative |
| HIV-2 (subtype A, NIH-Z) | $5.3 \times 10^8$ copies/mL | Negative |
| Human herpesvirus 4 (Epstein-Barr virus, B95-8) | $1.0 \times 10^5$ copies/mL | Negative |
| Human herpesvirus 5 (CMV, AD-169) | $1.0 \times 10^5$ copies/mL | Negative |
| Human herpesvirus 6 (Z29) | $1.0 \times 10^6$ copies/mL | Negative |
| La Crosse virus (R97841d) | $1.6 \times 10^8$ $TCID_{50}$/mL | Negative |
| Lassa Fever virus (Guinea Faranah 9615289) | $1.9 \times 10^7$ copies/mL | Negative |
| Marburg virus (Musoke) | $7.6 \times 10^5$ copies/mL | Negative |
| Marburg virus (Voege) | $8.5 \times 10^7$ copies/mL | Negative |
| Measles virus (MVs/Ohio.USA/17.14/3) | $8.9 \times 10^6$ $TCID_{50}$/mL | Negative |
| Mumps virus (MuV/Iowa.US/2006) | $7.3 \times 10^6$ PFU/mL | Negative |
| *Plasmodium falciparum* (FCB) | $8.3 \times 10^6$ copies/mL | Negative |
| Ravn virus (Kitum Cave-810040) | $2.3 \times 10^7$ copies/mL | Negative |
| Rift Valley fever virus (ZH501) | $1.6 \times 10^7$ copies/mL | Negative |
| St. Louis Encephalitis virus (V 08449) | $1.6 \times 10^6$ $TCID_{50}$/mL | Negative |
| Usutu virus (SAAR 1776) | $3.5 \times 10^6$ $TCID_{50}$/mL | Negative |
| West Nile virus (TX 9410 (D0325)) | $9.8 \times 10^6$ $TCID_{50}$/mL | Negative |
| Yellow fever virus (17D) | $6.6 \times 10^6$ copies/mL | Negative |
| Zika virus (IB H 30656) | $3.3 \times 10^7$ copies/mL | Negative |

## Procedural Limitations

The PANDAA Ebola kit is released for Research Use Only (RUO) and is not intended for use in diagnostic procedures. The assay is intended for use by lab personnel trained in the PANDAA Ebola assay procedure and in the operation of nucleic acid extraction and real-time PCR systems used on site. Good laboratory practices and adherence to the Instructions for Use are required to minimize contamination and ensure reliable assay performance .

This assay has been evaluated for use with EDTA plasma specimens. Analytical performance with EDTA plasma does not establish clinical diagnostic performance or clinical utility. Use of specimen types other than EDTA plasma has not been established and may yield inaccurate or uninterpretable results.

A negative result does not exclude the possibility that ebolavirus RNA may be absent from the sample, below the assay detection limit, or affected by specimen quality, handling, storage, transport, extraction efficiency, or amplification interference. The Internal Control and Sample Adequacy Control are included in PANDAA Ebola to help identify specimens containing substances that may interfere with nucleic acid isolation and PCR amplification. These controls do not eliminate the possibility of interference or other causes of invalid or non-representative results.

# General Guidelines

## Shipping, Storage and Handling

PANDAA Ebola is shipped on dry ice, and kit components arrive frozen. Store all components at **−15 °C to −25 °C** upon receipt, protected from light. **Stop-Start RT enzyme contains glycerol and may remain liquid** at −15 °C to −25 °C. Keep reagents in the original packaging when not in use and return them promptly to recommended storage conditions.

Inspect all kit components upon receipt. Do not use reagents if they appear compromised (e.g., cracked or leaking vials, illegible labels, or evidence of thawing). If a temperature excursion, shipping damage, or a discrepancy between the kit labels and this IFU is suspected, quarantine the kit and contact Technical Support (support@aldatubio.com) before use.

Ensure all reagents are fully thawed and mixed before use. Keep Stop-Start RT enzyme on ice during handling. Perform the PANDAA Ebola assay in environmental conditions at 20 °C to 25 °C and 30% to 70% relative humidity.

## Real-time PCR Best Practices

Since real-time PCR is a sensitive technique and the dynamic range of this assay extends to a very low template copy number, it is critical to perform accurate liquid handling to produce reliable results, and precaution must be taken when executing this protocol.

- Handle reagents and RNA templates at separate / dedicated laboratory areas to prevent cross contamination.
- Always ensure reagents and samples are fully thawed, thoroughly mixed and spun/centrifuged briefly before use.
- Enzymes (Stop-Start RT enzyme and PRxB III buffer) and thawed viral RNA should be kept on ice while being used.
- Ensure that a new pipette tip is used for each step in the protocol. Cross contamination between the samples and controls will affect the accuracy of the results.
- Good laboratory practice should be observed at all times to avoid contamination of reagents, samples, consumables, pipettes, and work areas.

## Safety

Handle all specimens, extracted nucleic acids, and used consumables as potentially infectious and in accordance with applicable institutional biosafety procedures and local regulations. Wear appropriate personal protective equipment (PPE) (e.g., gloves, lab coat, and eye protection) and perform work in facilities suitable for molecular testing.

To minimize contamination risk, follow good molecular biology practices, including physical separation of pre-amplification and post-amplification areas, use of aerosol-resistant pipette tips, and routine decontamination of work surfaces and equipment. Dispose of waste according to institutional procedures and local regulations.

Chemical hazards associated with extraction reagents are specific to the extraction method used; follow the extraction kit manufacturer's IFU and Safety Data Sheets (SDS).

# Appendix A: Materials Required but Not Included

## All Workflows

The following materials are required for nucleic acid extraction (if performed by the user) and for real-time PCR setup and run on a compatible instrument.

| Category | Components, Materials, or Reagents |
|---|---|
| Personal protective equipment | • Appropriate PPE (e.g., gloves, lab coat, eye protection) per institutional requirements. |
| Liquid handling | • Calibrated pipettes capable of dispensing 1 µL to 1000 µL<br>• Aerosol-resistant (filter) pipette tips |
| Consumables | • RNase-free microcentrifuge tubes (for sample extraction and/or aliquoting)<br>• Real-time PCR instrument consumables appropriate to the platform (e.g., plates or tubes/strips, optical seals/caps) |
| Mixing and centrifugation | • Vortex<br>• Plate spinner / plate centrifuge (for brief spin-down of PCR plates, if using plate format)<br>• Benchtop microcentrifuge (for brief spin-down of tubes/strips, as applicable) |
| Temperature control and storage | • Cold block or ice<br>• Refrigerator, 2 °C to 8 °C (for short-term storage, as applicable)<br>• Laboratory freezer, −15 °C to −25 °C (for kit storage) |

# Appendix B: Real-time PCR Instrument Setup and Analysis

- **ABI / Thermo Fisher:** ABI 7500 / 7500 Fast *, QuantStudio 5 *, QuantStudio 6 Flex/Pro †, QuantStudio 7 Flex/Pro †, QuantStudio 12K Flex †, and ViiA 7. †
- **Qiagen:** Rotor-Gene Q5/6 Plex *
- **Bio Molecular Systems:** Mic *
- **Roche:** LightCycler 96 †, LightCycler 480 II †
- **Bio-Rad:** CFX96 †

* Validated instruments.

† Compatible real-time PCR instruments that have not been validated with PANDAA Ebola by Aldatu Biosciences.

**CAUTION**

Ensure that your real-time PCR instrument has been installed, calibrated, and maintained according to the manufacturer's instructions and recommendations.

Use the real-time PCR run template provided by Aldatu Biosciences, available at www.aldatu.bio/qpcr-templates. If your real-time PCR instrument is not listed then contact Technical Support (support@aldatubio.com) about your real-time PCR instrument requirements and configuration, and to obtain a run template file with the correct cycling conditions.

## Instrument Instructions


| Instrument | Page |
|---|---|
| ABI / Thermo Fisher | Page 29 |
| CFX96 (Bio-Rad) | Page 30 |
| LightCycler 96 and LightCycler 480 II (Roche) | Page 32 |
| Mic (Bio Molecular Systems) | Page 34 |
| Rotor-Gene Q (Qiagen) | Page 36 |

## ABI / Thermo Fisher

**Instruments:** ABI 7500 / 7500 Fast, QuantStudio 5, QuantStudio 6 Flex/Pro, QuantStudio 7 Flex/Pro, QuantStudio 12K Flex, and ViiA 7. Templates (file extension *.edt*) provided by Aldatu Biosciences can be downloaded from www.aldatu.bio/qpcr-templates.

| Target Name | Channel | Reporter | Quencher |
|---|---|---|---|
| Ebolavirus (PAN EBOV) | Blue | FAM | NFQ-MGB |
| Internal Control (IC) | Red | Cy5 | NFQ-MGB |
| Sample Adequacy Control (SAC) | Yellow | TAMRA | NFQ-MGB |

### ABI / Thermo Fisher Data Analysis

Template files provided by Aldatu Biosciences are programmed with the analysis settings below.

| Target Name | Threshold | Baseline |
|---|---|---|
| Ebolavirus (PAN EBOV) | 0.05 | Auto |
| Internal Control (IC) | 0.02 | Auto |
| Sample Adequacy Control (SAC) | 0.02 | Auto |

1. **Adjust threshold (PAN EBOV):** Adjust the threshold for the PAN EBOV target so that the mean Ct of the Positive Control (PC) replicates is **~19 cycles (target range: 18 – 20 cycles)**.
2. **Analyze:** Ensure that data are reanalyzed after any threshold adjustments.
3. **Save:** Ensure that the analyzed *.eds* file is saved.
4. **Analysis:** Proceed to data analysis with the *PANDAA VHF Interpretation Software* (page 14) using either the *.eds* run file or an exported Excel file.

| Using the *.eds* File | Using the Exported Excel File |
|---|---|
| Import the analyzed .eds file directly into the *PANDAA VHF Interpretation Software*. | **Export settings:** From the *Export* menu, ensure that, at a minimum, the following content is selected for export.<br>• *Results*<br>• *Amplification Data*<br>• *Multicomponent Data*<br>**Export:** From the *Export* menu, choose **.xls* or **.xlsx* as the *File Type* and then export all data to one file. |

## CFX96 (Bio-Rad)

Two separate template files are required for the CFX96, and can be downloaded from www.aldatu.bio/qpcr-templates:

1. **Protocol file:** *PANDAA Ebola - Cycling Conditions - CFX96.prcl*
2. **Plate file:** *PANDAA Ebola - Plate Template - CFX96.pltd*

### Using the *.prcl* Protocol File and *.pltd* Plate File

1. **Import protocol file:** After starting a new user-defined run, import the *.prcl* template cycling conditions using *Select Existing* under the *Protocol* tab.
2. **Import plate file:** Under the *Plate* tab, import the *.pltd* plate template containing the assay targets using *Select Existing*.

| Target Name | Channel | Fluorophore |
|---|---|---|
| Ebolavirus (PAN EBOV) | 1 | FAM |
| Internal Control (IC) | 4 | Cy5 |
| Sample Adequacy Control (SAC) | 2 | Cal Orange 560 |

### CFX96 Data Analysis

1. **Show amplification cycles:** After the run is completed, select the *Step Number* dropdown in the *Data Analysis* window and select 8 to show the data collected during the amplification cycles.
2. **Adjust settings:** Under the *Settings* menu ensure that the following settings are selected:
    a. *Cq Determination Mode* ► *Single Threshold*
    b. *Baseline Setting* ► *Baseline Subtracted Curve Fit*
    c. *Analysis Mode* ► *Target*
3. **Adjust threshold (PAN EBOV):** Adjust the threshold for the PAN EBOV target so that the mean Ct of the Positive Control (PC) replicates is **~19 cycles (target range: 18 – 20 cycles)**.
4. **Fluorescence cutoff:** Under the *End Point* tab, edit the following settings for the PAN EBOV target:
    a. *End Cycles to Average*: 5
    b. *Percent of Range*: 2.0
    c. Record any wells that are not called *(+) Positive* by endpoint RFU.

5. **Analyze and save:** Ensure that data are reanalyzed after any adjustments and save the *.pcrd* file.
6. **Analysis:** Proceed to data analysis with the *PANDAA VHF Interpretation Software* (page 14) using either the *.pcrd* run file or an exported Excel file.

| Using the .pcrd File | Using the Exported Excel File |
|---|---|
| Import the analyzed .pcrd file directly into the *PANDAA VHF Interpretation Software*. | **Export settings:** From the *Export* menu, select *Custom Export*.<br>• Choose Excel 2007 (*.xlsx) as the *Export Format*<br>• Select all options for *Sample Description* and *Quantification*. Ensure that *End RFU* is selected as an export column. |

## LightCycler 96 and LightCycler 480 II (Roche)

**Instruments:** A template file for the LightCycler 96 or LightCycler 480 II can be downloaded from www.aldatu.bio/qpcr-templates.

**Detection format:** Hydrolysis probes.

| Target Name | Channel | Dye |
|---|---|---|
| Ebolavirus (PAN EBOV) | 1 | FAM |
| Internal Control (IC) | 4 | Cy5 |
| Sample Adequacy Control (SAC) | 2 | Yellow555 |

### LightCycler 96 Data Analysis

It is not possible to save analysis settings in the *.lc96* template file provided by Aldatu Biosciences.

1. **Adjust threshold (PAN EBOV):** Adjust the threshold for the PAN EBOV target so that the mean Ct of the Positive Control (PC) replicates is **~19 cycles (target range: 18 – 20 cycles)**.
2. **Adjust thresholds (IC and SAC):** Place the IC and SAC thresholds in the exponential (log-linear) region above baseline and below plateau; apply the same setting to all samples and controls.
3. **Save:** Ensure that the analyzed *.lc96* run file is saved.
4. **Analysis:** Proceed to data analysis with the *PANDAA VHF Interpretation Software* (page 14) using either the *.lc96* run file or an exported text file.

| Using the *.lc96* File | Using the Exported Excel File |
|---|---|
| Import the analyzed *.lc96* file directly into the *PANDAA VHF Interpretation Software*. | Ensure that the endpoint fluorescence (*EPF*) column is visible in the *Results Table* before exporting. If the EPF column is hidden, right click on the *Results Table* column headings ► *Column Selector* ► *EPF*<br>**Export:** Right click on the *Results Table* ► *Export to File* ► Save as file type *Text files (*.txt)*. |

## LightCycler 480 II Data Analysis

**Color compensation (multiplex):** Apply an appropriate Color Compensation (CC) setting/object for multicolor assays before analysis.

LightCycler 480 software reports the crossing point as Cp, which is treated as equivalent to Ct for reporting. To obtain Ct-equivalent values using a threshold-based method, **use Absolute Quantification – Fit Points** (threshold line method).

1. **Select analysis method:** In the analysis modules, select *Absolute Quantification* ► *Fit Points*. (Fit Points uses a threshold line approach.)
    a. Select *Analysis* ► *Abs Quant/2nd Derivative Max*
    b. Under *Method*, select *Fit Points* (threshold line method)
2. **Adjust threshold (PAN EBOV):** Adjust the threshold for the PAN EBOV target so that the mean Cp (Ct-equivalent) of the Positive Control (PC) replicates is **~19 cycles (target range: 18 – 20 cycles)**.
3. **Save:** Ensure that the analyzed *.ixo* run file is saved (*.ixo* is a supported export format on LC480).
4. **Analysis:** Proceed to data analysis with the *PANDAA VHF Interpretation Software* (page 14) using either the *.ixo* run file or an exported text file.

| **Using the *.ixo* File** | **Using the Exported Excel File** |
|---|---|
| Import the analyzed *.ixo* file directly into the *PANDAA VHF Interpretation Software*. | **Export:** Ensure that the endpoint fluorescence (EPF) column is visible in the Results Table.<br>Right click on the Results Table ►*Export to File* ► Save as file type *Text files (*.txt)*. |

## Mic (Bio Molecular Systems)

The Mic Real-Time qPCR Cycler (Mic) from Bio Molecular Systems uses either an *Assay* definition file (file extension *.micassay*) containing cycling conditions, assay targets, and analysis settings, or a *Template* definition file (file extension *.mictemplate*), which additionally contains sample information. Templates provided by Aldatu Biosciences can be downloaded from www.aldatu.bio/qpcr-templates.

1. **Assay file:** *PANDAA Ebola.micassay*
2. **Template file:** *PANDAA Ebola.mictemplate*

| Target Name | Channel | Reporter Dye |
|---|---|---|
| Ebolavirus (PAN EBOV) | Green | FAM |
| Internal Control (IC) | Red | Cy5 |
| Sample Adequacy Control (SAC) | Yellow | NED |

### Storing the Assay and Template Definition Files

The *.micassay* and/or *.mictemplate* must be saved in the local user document folder on your PC e.g., C:\Users*username*\Documents\Bio Molecular Systems\micPCR\Templates. This folder should have been automatically created when installing the micPCR software. If this folder is missing, contact Aldatu Technical Support (support@aldatubio.com).

### Using the *.micassay* Assay Definition File

In the micPCR software select *Run* from the *New* menu. In the new run file, click on the + icon next to *Assays* on the left-hand bar. *PANDAA Ebola* can be selected from *My Assays*. If it is not visible then select *Browse*. Locate and select the *PANDAA Ebola.micassay* file, which will now be loaded into your new run.

### Using the *.mictemplate* Template Definition File

The *PANDAA Ebola.mictemplate* contains the same cycling conditions, assay targets, and analysis settings as the *.micassay* file. It is also pre-loaded with the Negative Control in sample position 1 and the Positive Control in sample position 48.

Select *Run from Template* from the *New* menu in the micPCR software. Choose *PANDAA Ebola* and click *OK*. If *PANDAA Ebola* is not visible in the list of templates, confirm that it has been stored in the correct folder on your computer. Alternatively, start a new run using the *.micassay* file.

## Mic Data Analysis

Template files provided by Aldatu Biosciences are programmed with the analysis settings below.

| Target Name | Method | Exclusion | Cutoff | Threshold |
|---|---|---|---|---|
| Ebolavirus (PAN EBOV) | Dynamic | Simple | N/A | Auto |
| Internal Control (IC) | Dynamic | None | N/A | Auto |
| Sample Adequacy Control (SAC) | Dynamic | None | N/A | Auto |

1. **Adjust threshold:** Adjust the threshold for the PAN EBOV target so that the mean Ct of the Positive Control (PC) replicates is **~19 cycles (target range: 18 – 20 cycles)**.
2. **Save:** Ensure that the analyzed *.micrun* file is saved.
3. **Analysis:** Proceed to data analysis with the *PANDAA VHF Interpretation Software* (page 14) using either the *.micrun* run file or an exported Excel file.

| Using the *.micrun* File | Using the Exported Excel File |
|---|---|
| Import the analyzed *.micrun* file directly into the *PANDAA VHF Interpretation Software*. | **Export:** From the *Save As* menu, select *Excel Workbook* and save the exported *.xlsx* file. |

## Rotor-Gene Q (Qiagen)

The Rotor-Gene Q uses template files with the file extension *.ret*. Start a new run in the Rotor-Gene Q software by selecting *New* ► *Open a template in another folder*, and select the ***PANDAA Ebola - RGQ Template.ret*** file. The template file provided by Aldatu Biosciences contains the assay cycling conditions, targets, and gain settings and can be downloaded from www.aldatu.bio/qpcr-templates.

| Target Name | Channel | Source | Detector |
|---|---|---|---|
| Ebolavirus (PAN EBOV) | Green | 470nm | 510nm |
| Internal Control (IC) | Red | 625nm | 660nm |
| Sample Adequacy Control (SAC) | Yellow | 530nm | 555nm |

### Gain and Auto-Gain Settings

Auto-gain optimization is performed before the 1st acquisition. The Rotor-Gene Q template provided by Aldatu Biosciences, *PANDAA Ebola - RGQ Template.ret*, is pre-programmed with this setting. *Do not* perform gain optimization at 60 °C at the beginning of the run.

### Rotor-Gene Q Data Analysis

1. **Set global analysis settings for all three assay targets:**
   - *Dynamic Tube* and *Slope Correct* should be selected for consistent results across all runs.
2. **Set normalization options (PAN EBOV):** In the analysis window for the PAN EBOV target, Outlier Removal should be set at 2% for the Ebolavirus (PAN EBOV) target.
3. **Adjust threshold (PAN EBOV):** Adjust the threshold for the PAN EBOV target so that the mean Ct of the Positive Control (PC) replicates is **~19 cycles (target range: 18 – 20 cycles)**.
4. **Adjust thresholds (IC and SAC):** Place the IC and SAC thresholds in the exponential (log-linear) region above baseline and below plateau; apply the same setting to all samples and controls.
5. **Save:** Ensure that the analyzed *.rex* file is saved.
6. **Analysis:** Proceed to data analysis with the *PANDAA VHF Interpretation Software* (page 14) using either the *.rex* run file or an exported Excel file.

| Using the *.rex* File | Using the Exported Excel File |
|---|---|
| Import the analyzed *.rex* file directly into the *PANDAA VHF Interpretation Software*. | **Export:** Select File ►Save As ► Excel Analysis Sheet and save the *.csv* file. |

## Customer and Technical Support

Email: support@aldatubio.com

Address: Aldatu Biosciences
313 Pleasant Street
Watertown, MA 02472
USA

## Disclaimers

This kit is for Research Use Only and is not intended for use in diagnostic procedures.

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## Explanation of Symbols

| | |
|---|---|
| RUO | For Research Use Only |
| REF | Catalog number |
| LOT | Batch number |
| | Storage temperature range |
| | Use-by date |
| | Contents sufficient for n reactions |
| | Consult Instructions for Use |
| Cont. | Contents of the PANDAA® Ebola kit |
| | Manufacturer |

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al • da • tu [ɒl - də - tu]: **to become something different**