

# Effective Chemical Inactivation of Ebola Virus

## Technical Appendix

### Materials and Methods

#### Ethics and Safety Statement

All work involving infectious material was performed in the Biosafety Level 4 (BSL4) laboratory at Rocky Mountain Laboratories (RML), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of RML and performed following the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC), by certified staff in an AAALAC-approved facility.

#### Viruses and Infected Cells

Testing involved  $10^7$  FFU/ mL stock virus of wild-type EBOV expressing enhanced green fluorescent protein (EBOV-eGFP) or  $10^7$  FFU/ mL stock virus of mouse-adapted EBOV (MA-EBOV). Virus stocks were grown in VERO E6 cells and titrated using a focus forming unit (FFU) assay. Infected cells were produced by infecting VERO E6 cells at a multiplicity of infection of 0.01. Cells were harvested at CPE of  $\approx 75\%$ , pelleted, resuspended in 6 mL Dulbecco's phosphate-buffered saline (DPBS) and 1 mL aliquots were stored at  $-80^\circ\text{C}$  until use ( $1 \times 10^7$  cells/ mL). Negative control cells were made of similarly treated, uninfected cell monolayers. For those tests involving blood or tissues, BALB/c mice were infected with MA-EBOV and blood and liver were collected at the height of disease. Blood and liver from uninfected control animals were used as negative control samples.

#### Inactivation Reagents

Buffers AVL and RLT (Qiagen), TRIzol (Life Technologies), 10% neutral-buffered formalin (Leica Biosystems), paraformaldehyde (Electron Microscopy Science) and glutaraldehyde (Sigma Aldrich) were tested as supplied for RNA and fixation samples. A sodium

dodecyl sulfate (SDS) 4× loading buffer (200 mmol/L Tris, pH 6.8; 4% SDS; 35% glycerol; 0.05% bromophenol blue; 20% 2-mercaptoethanol (added at time of use)) and an enzyme-linked immunosorbent assay (ELISA) buffer (DPBS with 5% skim milk; 0.5% Triton X-100; 0.5% Tween-20) were produced in-house for protein samples.

### **Dialysis**

For those tests requiring dialysis, samples were dialyzed with a 10kDa molecular weight cutoff. DPBS was used as a dialysis buffer at >500-fold exchange volumes over five changes and 32-48 hours, at 4°C over a stir plate, before samples were collected and used to infect for testing. All samples were dialyzed using Spectra/Por Float-A-Lyzer G2 tubing (Spectrum Laboratories) with the exception of those involving TRIzol, which was dialyzed using Slide-A-Lyzer cassettes (Fisher Scientific).

### **Detergent Removal**

DetergentOUT GBS10-5000 columns (G-Biosciences) were utilized to remove detergent from samples, per manufacturer's recommended protocol. Columns were equilibrated twice with DPBS before the detergent-containing sample was incubated on the column for two minutes, spun through the column, collected and used to infect for testing.

### **Validation Protocol, Cell Culture Model**

Virus-infected samples (in triplicate unless otherwise noted) were treated according to the specific testing parameters and dialyzed or run over detergent-removal columns to remove inactivating reagents. Each of the treated samples was then increased in volume to 3 mL as necessary and equally divided to infect triplicate 25 cm<sup>2</sup> flasks of fresh VERO E6 cells at 80% confluency. Following an infection time of 60 minutes at 37°C, inoculum was removed and 6 mL/flask DMEM with 2% FBS was added. Unless otherwise noted, cells were not washed before addition of fresh medium. Cells were incubated at 37°C for an additional 14 days and monitored regularly for CPE (MA-EBOV) or CPE and fluorescence (EBOV-eGFP). Positive and negative samples were included in every validation, subjected to the same mechanical treatments (e.g. dialysis, spin columns) as the test samples, and tested on 3 flasks of fresh cells each. Negative control samples were DPBS or uninfected cells /tissue homogenates from animals which were not infected; positive control samples were virus stock/infected cells/infected tissue homogenates

that were not treated. A DPBS mock infection of 3 flasks was included in each experiment to control for residual inactivating reagent.

#### **Validation Protocol, Animal Model**

Six- to eight-week old female BALB/c mice (Charles River Laboratories) were housed in microisolator cages and allowed to acclimatize prior to use in experiments. Three virus-infected samples were treated according to the specific testing parameters and dialyzed or run over detergent-removal columns to remove inactivating reagents. Each of the treated samples was then increased in volume to 1 mL as necessary and equally divided to infect 5 mice intraperitoneally. Mice were assessed daily according to approved protocol for weight loss and/or other clinical signs of illness for 28 days post infection. Positive and negative samples were included in every validation, subjected to the same mechanical treatments (e.g. dialysis, spin columns) as the test samples, and tested in groups of 5 animals each. Negative control samples were DPBS or uninfected cells; positive control samples were virus stock/infected cells that were not treated. A DPBS mock infection of 5 animals was included in each experiment to control for residual inactivating reagent.

#### **Protocols**

##### **Buffer AVL Testing**

140  $\mu$ L Liquid virus stock ( $1.4 \times 10^6$  TCID<sub>50</sub>)

+ 560  $\mu$ L Buffer AVL

10 minute contact time in 2 mL tube at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

##### **Or**

140  $\mu$ L Liquid virus stock ( $1.4 \times 10^6$  TCID<sub>50</sub>)

+ 560  $\mu$ L Buffer AVL

10 minute contact time in 2 mL tube at 20°C. Then transferred to 2 mL tube containing 560  $\mu$ L

100% ethanol for 20 minutes at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

##### **Or**

100  $\mu\text{L}$  Liquid virus stock ( $1.0 \times 10^6$  TCID<sub>50</sub>)

+ 600  $\mu\text{L}$  Buffer AVL

10 minute contact time in 2 mL tube at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

100  $\mu\text{L}$  Liquid virus stock ( $1.0 \times 10^6$  TCID<sub>50</sub>)

+ 600  $\mu\text{L}$  Buffer AVL

10 minute contact time in 2 mL tube at 20°C. Then transferred to 2 mL tube containing 560  $\mu\text{L}$  100% ethanol for 20 minutes at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

140  $\mu\text{L}$  Liquid virus stock ( $1.4 \times 10^6$  TCID<sub>50</sub>)

+ 560  $\mu\text{L}$  Buffer AVL

Overnight contact time in 2 mL tube at 4°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

140  $\mu\text{L}$  Liquid virus stock ( $1.0 \times 10^6$  TCID<sub>50</sub>)

+ 560  $\mu\text{L}$  Buffer AVL

7 day contact time in 2 mL tube at -80°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

All samples were removed from dialysis tubing, raised to 3 mL final volume with DPBS, and split evenly to infect three flasks of VERO E6 cells. Infection contact time was 1 hour; inoculum was removed and replaced with medium (DMEM). Cells were not washed. For those methods tested in mice, samples were raised to an equal volume ( $\approx 1.4$  mL) with DPBS and split equally to infect 5 mice.

#### **Buffer RLT Testing**

Infected cells pelleted and supernatant removed ( $5 \times 10^6$  infected cells,  $\approx 5 \times 10^6$  TCID<sub>50</sub>)

+ 600  $\mu\text{L}$  Buffer RLT

10 minute contact time in 2 mL tube at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

Infected cells pelleted and supernatant removed ( $5 \times 10^6$  infected cells,  $\approx 5 \times 10^6$  TCID<sub>50</sub>)

+ 600  $\mu$ L Buffer RLT

10 minute contact time in 2 mL tube at 20°C. Then transferred to 2 mL tube containing 600  $\mu$ L 70% ethanol for 20 minutes at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

Infected cells pelleted and supernatant removed ( $5 \times 10^6$  infected cells,  $\approx 5 \times 10^6$  TCID<sub>50</sub>)

+ 800  $\mu$ L Buffer RLT

10 minute contact time in 2 mL tube at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

Infected cells pelleted and supernatant removed ( $5 \times 10^6$  infected cells,  $\approx 5 \times 10^6$  TCID<sub>50</sub>)

+ 800  $\mu$ L Buffer RLT

10 minute contact time in 2 mL tube at 20°C. Then transferred to 2 mL tube containing 600  $\mu$ L 70% ethanol for 20 minutes at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

30 mg infected liver ( $\approx 3 \times 10^5$  TCID<sub>50</sub>)

+ 600  $\mu$ L Buffer RLT

A larger piece ( $\approx 100$  mg) was homogenized in 600  $\mu$ L Buffer RLT at 30Hz with a stainless steel bead for 10 minutes in a 2 mL tube at 20°C. The equivalent volume of 30 mg (180  $\mu$ L) was transferred to a clean 2 mL tube and a volume of Buffer RLT (320  $\mu$ L) added to bring volume back to 600  $\mu$ L total. This was followed by a 10 minute contact time at 20°C, after which the entire sample was transferred to 2 mL tube containing 600  $\mu$ L 70% ethanol for 20 minutes at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

All samples were removed from dialysis tubing, raised to 3 mL final volume with DPBS, and split evenly to infect three flasks of VERO E6 cells. Infection contact time was 1 hour;

inoculum was removed and replaced with medium (DMEM). Cells were not washed. For those methods tested in mice, samples were raised to an equal volume ( $\approx 1.4$  mL) with DPBS and split equally to infect 5 mice.

#### **TRIzol Testing**

Infected cells pelleted and resuspended in 250  $\mu$ L DPBS ( $\approx 5 \times 10^6$  infected cells,  $5 \times 10^6$  TCID<sub>50</sub>)

+ 750  $\mu$ L TRIzol

10 minute contact time in 2 mL tube at 20°C. Dialyzed in Slide-A-Lyzer tubes.

**Or**

250  $\mu$ L infected blood ( $\approx 2.5 \times 10^5$  TCID<sub>50</sub>)

+ 750  $\mu$ L TRIzol

10 minute contact time in 2 mL tube at 20°C. Dialyzed in Slide-A-Lyzer tubes.

**Or**

50 mg infected liver ( $5 \times 10^5$  TCID<sub>50</sub>)

+ 1 mL TRIzol

A larger piece ( $\approx 100$  mg) was homogenized in 1 mL TRIzol at 30Hz with a stainless steel bead for 10 minutes in a 2 mL tube at 20°C. The equivalent volume of 50 mg (0.5 mL) was transferred to a clean 2 mL tube and a volume of TRIzol (0.5 mL) added to bring volume back to 1 mL total. This was followed by a 10 minute contact time in 2 mL tube at 20°C. Dialyzed in Slide-A-Lyzer tubes.

All samples were removed from dialysis tubing, raised to 3 mL final volume with DPBS, and split evenly to infect three flasks of VERO E6 cells. Infection contact time was 1 hour; inoculum was removed, cells were washed to remove any traces of TRIzol and medium (DMEM) was added. TRIzol samples were not tested in mice.

#### **Formalin Testing**

250  $\mu$ L infected cells ( $2.5 \times 10^6$  infected cells,  $\approx 2.5 \times 10^6$  TCID<sub>50</sub>)

+ 750  $\mu$ L 10% formalin (7.5% final concentration)

Overnight contact time in 2 mL tube at 4°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

One-half infected liver lobe

+ 10 mL 10% formalin

7 day contact time in 15 mL tube at 4°C

This larger piece was dissected following contact time for a smaller internal piece (150 mg,  $\approx 1.5 \times 10^6$  TCID<sub>50</sub>) which was homogenized in 1 mL DPBS at 30Hz with a stainless steel bead for 10 minutes in a 2 mL tube at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

One infected liver

+ 10 mL 10% formalin

30 day contact time in 15 mL tube at 4°C

This larger piece was dissected following contact time for a smaller internal piece (150 mg,  $\approx 1.5 \times 10^6$  TCID<sub>50</sub>) which was homogenized in 1 mL DPBS at 30Hz with a stainless steel bead for 10 minutes in a 2 mL tube at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

All samples were removed from dialysis tubing, raised to 3 mL final volume with DPBS, and split evenly to infect three flasks of VERO E6 cells. Infection contact time was 1 hour; inoculum was removed and replaced with medium (DMEM). Cells were not washed. For those methods tested in mice, samples were split equally to infect 5 mice.

#### **Glutaraldehyde and Paraformaldehyde Testing**

330  $\mu$ L infected cells ( $3.3 \times 10^6$  infected cells,  $\approx 3.3 \times 10^6$  TCID<sub>50</sub>)

+ 1.3 mL 2.5% Glutaraldehyde (2% final concentration)

overnight contact time in 2 mL tube at 4°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

330  $\mu$ L infected cells ( $3.3 \times 10^6$  infected cells,  $\approx 3.3 \times 10^6$  TCID<sub>50</sub>)

+ 1.3 mL 2.5% Paraformaldehyde (2% final concentration)

overnight contact time in 2 mL tube at 4°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

One-half infected liver lobe

+ 10 mL 2% Glutaraldehyde

7 day contact time in 15 mL tube at 4°C

This larger piece was dissected following contact time for a smaller internal piece (150 mg,  $\approx 1.5 \times 10^6$  TCID<sub>50</sub>) which was homogenized in 1 mL DPBS at 30Hz with a stainless steel bead for 10 minutes in a 2 mL tube at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

One-half infected liver lobe

+ 10 mL 2% Paraformaldehyde

7 day contact time in 15 mL tube at 4°C

This larger piece was dissected following contact time for a smaller internal piece (150 mg,  $\approx 1.5 \times 10^6$  TCID<sub>50</sub>) which was homogenized in 1 mL DPBS at 30Hz with a stainless steel bead for 10 minutes in a 2 mL tube at 20°C. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

All samples were removed from dialysis tubing, raised to 3 mL final volume with DPBS, and split evenly to infect three flasks of VERO E6 cells. Infection contact time was 1 hour; inoculum was removed and replaced with medium (DMEM). Cells were not washed. For those methods tested in mice, samples were split equally to infect 5 mice.

### **Heat Testing**

1 mL 1:10 dilution of infected cells ( $1 \times 10^6$  infected cells,  $\approx 1 \times 10^6$  TCID<sub>50</sub>)

Heat 5 or 10 minutes in AccuBlock heater at 100°C or 120°C in 2 mL tubes

**Or**

1 mL 1:10 dilution of liquid stock virus ( $\approx 1 \times 10^6$  TCID<sub>50</sub>)

Heat 15 or 30 minutes in non-shaking water bath at 60°C, 65°C or 70°C in 2 mL tubes

All samples were raised to 3 mL final volume with DPBS, and split evenly to infect three flasks of VERO E6 cells. Infection contact time was 1 hour; inoculum was removed and replaced with medium (DMEM). Cells were not washed. Heat samples were not tested in mice.

### **Detergent Testing**

#### **ELISA Buffer Testing**

40  $\mu\text{L}$  Liquid virus stock ( $4 \times 10^5$  TCID<sub>50</sub>)

+ 960  $\mu\text{L}$  ELISA Buffer (with 0.5% Triton X-100 and 0.5% Tween-20)

10 minute contact time in 2 mL tube at 20°C. Spun through pre-equilibrated (DPBS)

DetergentOUT GBS10-5000 column.

**Or**

40  $\mu\text{L}$  Liquid virus stock ( $4 \times 10^5$  TCID<sub>50</sub>)

+ 960  $\mu\text{L}$  ELISA Buffer (with 0.5% Triton X-100 and 0.5% Tween-20)

15 or 30 minute contact in non-shaking water bath at 60°C, 65°C or 70°C in 2 mL tubes. Spun through pre-equilibrated (DPBS) DetergentOUT GBS10-5000 column

#### **SDS Buffer Testing**

250  $\mu\text{L}$  infected cells ( $2.5 \times 10^6$  cells,  $\approx 2.5 \times 10^6$  TCID<sub>50</sub>)

500  $\mu\text{L}$  DPBS

+ 250  $\mu\text{L}$  4 $\times$  SDS Loading Buffer (with 4% SDS and 20% 2-ME)

10 minute contact time in 2 mL tube at 20°C. Spun through pre-equilibrated (DPBS)

DetergentOUT GBS10-5000 column.

**Or**

250  $\mu\text{L}$  infected cells ( $2.5 \times 10^6$  cells,  $\approx 2.5 \times 10^6$  TCID<sub>50</sub>)

500  $\mu\text{L}$  DPBS

+ 250  $\mu\text{L}$  4 $\times$  SDS Loading Buffer (with 4% SDS, no 2-ME)

10 minute contact time in 2 mL tube at 20°C. Spun through pre-equilibrated (DPBS)  
DetergentOUT GBS10-5000 column.

**Or**

150 mg infected liver ( $\approx 1.5 \times 10^6$  TCID<sub>50</sub>)

750  $\mu$ L DPBS

+ 250  $\mu$ L 4 $\times$  SDS Loading Buffer (with 4% SDS and 20% 2-ME)

Tissue was homogenized in 1 mL buffer with 1% SDS and 5% 2-ME at 30Hz with a stainless steel bead for 10 minutes in a 2 mL tube at 20°C. Spun through pre-equilibrated (DPBS) DetergentOUT GBS10-5000 column.

All samples were raised to 3 mL final volume with DPBS, and split evenly to infect three flasks of VERO E6 cells. Infection contact time was 1 hour; inoculum was removed, cells washed and medium (DMEM) was added. After incubation for 24 hours, half the medium was removed and replaced with an additional full volume to avoid a pH drop occasionally seen after use of detergent removal columns. For those methods tested in mice, samples were split equally to infect 5 mice.