# Effective Chemical Inactivation of Ebola Virus

**Elaine Haddock, Friederike Feldmann, Heinz Feldmann**

Reliable inactivation of specimens before removal from high-level biocontainment is crucial for safe operation. To evaluate efficacy of methods of chemical inactivation, we compared in vitro and in vivo approaches using Ebola virus as a surrogate pathogen. Consequently, we have established parameters and protocols leading to reliable and effective inactivation.

The safe operation of high-level biocontainment laboratories throughout the world is of highest importance. These laboratories are under stringent national oversight and must adhere to international guidelines. Laboratories in the United States that handle select agents are further regulated by the US Centers for Disease Control and Prevention's Division of Select Agents and Toxins and the US Department of Agriculture's Animal and Plant Health Inspection Service.

Proper and reliable inactivation of specimens destined for removal from high-level biocontainment is a critical aspect for laboratory certification and operation. Standard operating procedures (SOPs) are approved by institutional biosafety committees in most cases and additionally by state and/or national regulatory authorities in other cases. In the past, specimens were commonly inactivated on the basis of operational experiences rather than well-documented protocols (*1*–*3*).

To evaluate the efficacy of chemical inactivation procedures for specimen removal, we used the US prime select agent and Tier-1 pathogen (*4*) *Zaire ebolavirus* (EBOV) as a surrogate model for enveloped high-level containment viruses with single-strand, negative-sense RNA genomes, such as arenaviruses, bunyaviruses, filoviruses, orthomyxoviruses, and paramyxoviruses. These viruses share certain biologic, biochemical, and structural features, making them sensitive to the same chemical inactivation methods. Furthermore, EBOV is currently a prominent example as the causative agent of an unprecedented epidemic in West Africa (*5*,*6*).

## The Study

Standard biologic specimens containing infectious EBOV commonly generated in high-level biocontainment operations were inactivated by several methods of chemical treatment (Figure; Table, http://wwwnc.cdc.gov/EID/article/22/7/16-0233-T1.htm; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/16-0233-Techapp1.pdf). For in vitro testing, we used wild-type EBOV expressing enhanced green fluorescent protein (EBOV-eGFP) (*7*), which allows for cytopathic effect (CPE) and fluorescence as simple readout parameters. For in vivo testing, we used mouse-adapted EBOV (MA-EBOV) (*8*) infection of BALB/c mice. Virus stocks were grown in Vero E6 cells and titrated by using a 50% tissue culture infectious dose ($TCID_{50}$) assay (*9*). Infected cells were produced by infecting Vero E6 cells at a multiplicity of infection of 0.01. Cells were harvested at CPE of ≈75%, pelleted, and resuspended in 6 mL Dulbecco's phosphate-buffered saline (DPBS); 1 mL aliquots were stored at −80°C. Samples were chemically treated according to the specific testing parameters and dialyzed or run over detergent-removal columns to remove inactivating reagents. In brief, samples were dialyzed by using a 10-kDa molecular weight cutoff (Spectrum Laboratories, Lawrenceville, GA, USA, or Fisher Scientific, Pittsburgh, PA, USA) and using DPBS over a stir plate at 4°C (>500-fold exchange volumes, 5 changes over 32–48 h); detergent was removed by using DetergentOUT GBS10–5000 columns (G-Biosciences, St. Louis, MO, USA).

Negative control samples included DPBS and noninfected Vero E6 cells and tissue homogenates (mouse); positive control samples included untreated virus stocks and infected Vero E6 cells and mouse tissues. For in vitro testing, all samples were increased in volume to 3 mL and equally divided to infect Vero E6 cells (80% confluency) in triplicates. Cells were incubated at 37°C for 14 days and monitored regularly for CPE or fluorescence. For in vivo testing, samples were increased in volume to 1 mL and equally divided to infect 5 mice intraperitoneally. BALB/c mice (female, 6–8 weeks old; Charles River Laboratories, Wilmington, MA, USA) were housed in microisolator cages and were monitored daily for 28 days. Because in vitro and in vivo safety testing correlated well, we discontinued mouse infections for ethical reasons.

Nucleic acid extraction is often carried out with commercial guanidinium isothiocyanate buffers. We used Buffer AVL and Buffer RLT (QIAGEN, Valencia, CA, USA) and TRIzol (Life Technologies, Grand Island, NY, USA) according to manufacturers' recommendations. AVL was mixed with stock virus at different ratios, and infected cells were resuspended in RLT (Table). Samples were either

Author affiliation: National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA

DOI: http://dx.doi.org/10.3201/eid2207.160233

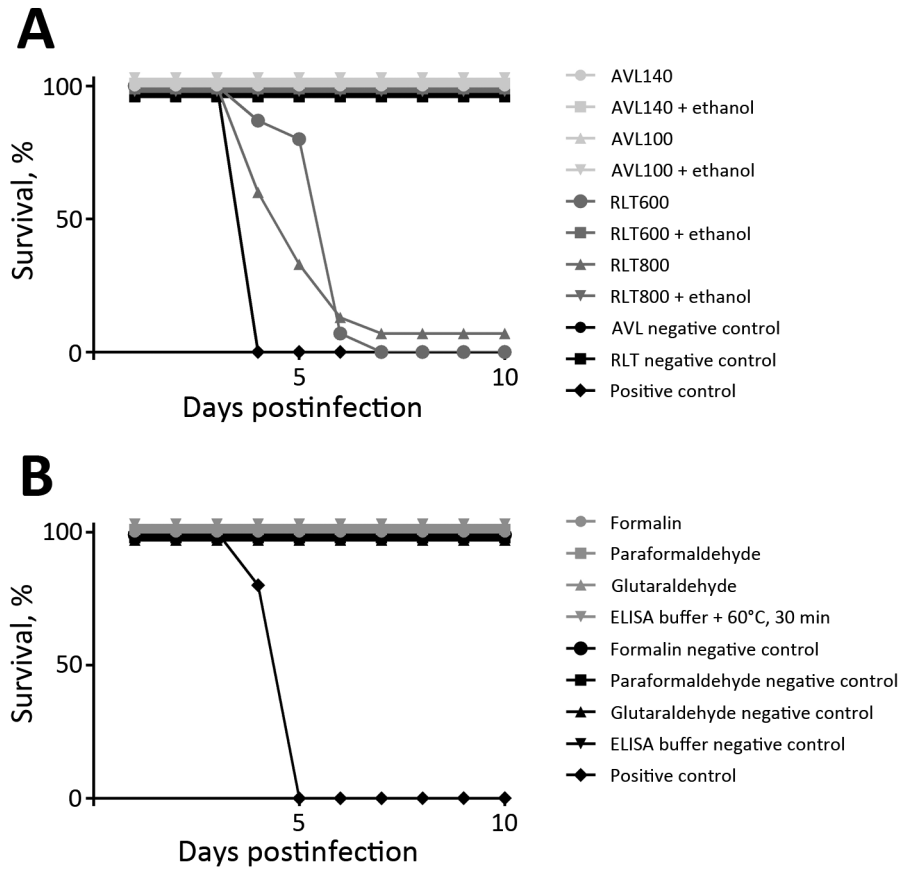


**Figure.** Ebola virus inactivation results as tested in BALB/c mouse model. A) Survival in animal groups tested with samples inactivated by guanidinium isothiocyanate buffers. AVL140, 140 µL Buffer AVL (QIAGEN, Valencia, CA, USA) + 560 µL sample; AVL100, 100 µL Buffer AVL + 600 µL sample; RLT600, 600 µL Buffer RLT (QIAGEN) treatment of cells; RLT800, 800 µL Buffer RLT treatment of cells; + ethanol, after a Buffer AVL or Buffer RLT inactivation contact time of 10 min, addition of 100% or 70% ethanol, respectively, for an additional 20 min of contact time. B) Survival in animal groups tested with samples inactivated by fixative or detergent buffers. For all test groups, n = 15; for all control groups, n = 5.

immediately dialyzed or treated with ethanol (AVL, 100% ethanol, 560 µL; RLT, 70% ethanol, 600 µL). Infected liver tissue was homogenized in RLT with a stainless steel bead (10 min at 30 Hz). A soluble aliquot (≈30 mg) was transferred to a new tube, and fresh RLT was added, followed by 70% ethanol (600 µL). After dialysis, samples were used to infect Vero E6 cells and mice. Similar to a results in a previous study (*10*), AVL and RLT treatment alone for 10 minutes at either ratio did not fully inactivate EBOV; however, the addition of ethanol (the next step of the manufacturer's protocol) rendered all samples completely noninfectious. AVL alone resulted in complete inactivation with longer contact times (i.e., refrigerated overnight or frozen for 7 days) (Table; Figure).

Infected cells were resuspended and treated with TRIzol (1:4 vol/vol). Infected liver samples were homogenized in 1 mL TRIzol as described in the previous paragraph. After centrifugation, an aliquot of tissue homogenate (≈50 mg) was transferred to a new tube, and fresh TRIzol was added. Additionally, blood from infected animals was mixed (1:4 vol/vol) with TRIzol. After dialysis, Vero E6 cells were inoculated and monitored for CPE or fluorescence. In all cases, virus growth was not detected (Table), indicating complete inactivation.

Formalin, paraformaldehyde, and glutaraldehyde can be used to fix cells or tissues for histologic or microscopic studies. Infected cells were diluted 1:4 in 10% neutral-buffered formalin (7.5% fixative) or 1:5 in either 2.5% glutaraldehyde or 2.5% paraformaldehyde (2% fixative). Samples were dialyzed and used to infect Vero E6 cells or mice. Monitoring of cell culture and animals resulted in the absence of CPE or fluorescence and clinical signs, respectively, indicating complete inactivation of EBOV (Table; Figure).

Infected liver segments were incubated in 10% neutral-buffered formalin, 2% glutaraldehyde, or 2% paraformaldehyde (10 mL) for a period of 7 days (<1-$cm^3$ piece) or 30 days (>1-$cm^3$ piece) at 4°C. Subsequently, a small section of tissue (≈150 mg) was dissected, homogenized in DPBS with a stainless steel bead (10 min at 30 Hz), and then dialyzed. After dialysis, samples were used to infect Vero E6 cells. All samples were completely inactivated (Table).

Samples for protein assays are often inactivated by a combination of detergent and heat. We tested the parameters of 60°C for 30 min, 65°C for 15 or 30 min, and 70°C for 15 min in conjunction with a buffer containing 0.5% Triton X-100 and 0.5% Tween-20 (both from Sigma-Aldrich,

St. Louis, MO, USA); this mixture is commonly used for ELISA. Stock virus was diluted 1:25 in this buffer and heated for the appropriate times before samples were clarified of detergent and used to infect Vero E6 cells or mice. All samples were completely inactivated as indicated by lack of CPE or fluorescence in cells and clinical signs in mice (Table; Figure).

Boiling (at 100°C for 10 min or 120°C for 5 min) might be sufficient to inactivate EBOV (Table) (*11*) but is often used in conjunction with sodium dodecyl sulfate (SDS)–containing buffers for protein analysis. Aliquots of infected cells were diluted in DPBS and 4× loading buffer (1% SDS final). Infected liver tissue (≈150 mg) were placed in DPBS and 4× loading buffer (1% SDS final). The samples were then homogenized with a stainless steel bead (10 min at 30 Hz). After detergent removal, samples were used to infect Vero E6 cells; all treated cells and tissue homogenates were negative for infectious EBOV (Table).

## Conclusions

Our study establishes inactivation procedures for EBOV that can be safely applied to distinct specimen types and research purposes and might also apply to other enveloped, single-strand, negative-sense RNA viruses. Our findings should help to improve and approve SOPs for inactivation without the need for safety testing each individual sample, an unfeasible and unwarranted task in current diagnostic and research operations in high-level biocontainment settings. However, any changes to inactivation SOPs make further safety testing essential. Safety testing for inactivation, at least for EBOV, can rely on cell culture only because this seems to be as sensitive as in vivo testing.

### Acknowledgments

The authors are grateful to the Rocky Mountain Veterinary Branch of the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH) for assistance with animal work.

The study was supported by the Intramural Research Program of NIAID at NIH.

Dr. Haddock is a biologist in the Laboratory of Virology, Division of Intramural Research, NIAID, NIH. Her interests include high-level biocontainment operations and emerging zoonotic pathogens.

### References

1. Mitchell SW, McCormick JB. Physicochemical inactivation of Lassa, Ebola, and Marburg viruses and effect on clinical laboratory analyses. J Clin Microbiol. 1984;20:486–9.
2. Elliott LH, McCormick JB, Johnson KM. Inactivation of Lassa, Marburg, and Ebola viruses by gamma irradiation. J Clin Microbiol. 1982;16:704–8.
3. Lupton HW. Inactivation of Ebola virus with 60Co irradiation. J Infect Dis. 1981;143:291. http://dx.doi.org/10.1093/infdis/143.2.291
4. US Centers for Disease Control and Prevention, US Department of Agriculture. Select agents and toxins list [cited 2016 Mar 29]. http://www.selectagents.gov/SelectAgentsandToxinsList.html
5. World Health Organization. Ebola virus disease outbreak: Ebola is no longer a public health emergency of international concern [cited 2016 Mar 29]. http://www.who.int/csr/disease/ebola/en
6. US Centers for Disease Control and Prevention. 2014 Ebola outbreak in West Africa [cited 2016 Mar 29]. http://www.cdc.gov/vhf/ebola/outbreaks/2014-west-africa/index.html
7. Ebihara H, Theriault S, Neumann G, Alimonti JB, Geisbert JB, Hensley LE, et al. In vitro and in vivo characterization of recombinant Ebola viruses expressing enhanced green fluorescent protein. J Infect Dis. 2007;196(Suppl 2):S313–22. http://dx.doi.org/10.1086/520590
8. Bray M, Davis K, Geisbert T, Schmaljohn C, Huggins J. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J Infect Dis. 1998;178:651–61. http://dx.doi.org/10.1086/515386
9. Marzi A, Feldmann F, Hanley PW, Scott DP, Günther S, Feldmann H. Delayed disease progression in Cynomolgus macaques infected with Ebola virus Makona strain. Emerg Infect Dis. 2015;21:1777–83. http://dx.doi.org/10.3201/eid2110.150259
10. Smither SJ, Weller SA, Phelps A, Eastaugh L, Ngugi S, O'Brien LM, et al. Buffer AVL alone does not inactivate Ebola virus in a representative clinical sample type. J Clin Microbiol. 2015;53:3148–54. http://dx.doi.org/10.1128/JCM.01449-15
11. Chong YK, Ng WY, Chen SP, Mak CM. Effects of a plasma heating procedure for inactivating Ebola virus on common chemical pathology tests. Hong Kong Med J. 2015;21:201–7. http://dx.doi.org/10.12809/hkmj144373

Address for correspondence: Heinz Feldmann, Rocky Mountain Laboratories, 903 S 4th St, Hamilton, MT 59840, USA; email: feldmannh@niaid.nih.gov

Article DOI: http://dx.doi.org/10.3201/eid2207.160233

# 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 ≈75%, pelleted, resuspended in 6 mL Dulbecco's phosphate-buffered saline (DPBS) and 1 mL aliquots were stored at –80ºC 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-mercaptoe thanol (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 4ºC 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 $cm^2$ flasks of fresh VERO E6 cells at 80% confluency. Following an infection time of 60 minutes at 37ºC, 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 37ºC 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 µL Liquid virus stock ($1.4 \times 10^6$ $TCID_{50}$)

\+ 560 µL Buffer AVL

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

**Or**

140 µL Liquid virus stock ($1.4 \times 10^6$ $TCID_{50}$)

\+ 560 µL Buffer AVL

10 minute contact time in 2 mL tube at 20ºC. Then transferred to 2 mL tube containing 560 µL 100% ethanol for 20 minutes at 20ºC. Dialyzed in Spectra/Por Float-A-Lyzer tubes.

**Or**

100 µL Liquid virus stock ($1.0 \times 10^6$ $TCID_{50}$)

+ 600 µL Buffer AVL

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

**Or**

100 µL Liquid virus stock ($1.0 \times 10^6$ $TCID_{50}$)

+ 600 µL Buffer AVL

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

**Or**

140 µL Liquid virus stock ($1.4 \times 10^6$ $TCID_{50}$)

+ 560 µL Buffer AVL

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

**Or**

140 µL Liquid virus stock ($1.0 \times 10^6$ $TCID_{50}$)

+ 560 µL 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 (≈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, $\approx 5 \times 10^6$ $TCID_{50}$)

+ 600 µL 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, $\approx 5 \times 10^6$ $TCID_{50}$)

+ 600 µL Buffer RLT

10 minute contact time in 2 mL tube at 20°C. Then transferred to 2 mL tube containing 600 µL 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, $\approx 5 \times 10^6$ $TCID_{50}$)

+ 800 µL 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, $\approx 5 \times 10^6$ $TCID_{50}$)

+ 800 µL Buffer RLT

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

**Or**

30 mg infected liver ($\approx 3 \times 10^5$ $TCID_{50}$)

+ 600 µL Buffer RLT

A larger piece (≈100 mg) was homogenized in 600 µL Buffer RLT at 30Hz with a stainless steel bead for 10 minutes in a2 mL tube at 20°C. The equivalent volume of 30 mg (180 µL) was transferred to a clean 2 mL tube and a volume of Buffer RLT (320 µL) added to bring volume back to 600 µL 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 µL 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 (≈1.4 mL) with DPBS and split equally to infect 5 mice.

#### TRIzol Testing

Infected cells pelleted and resuspended in 250 µL DPBS (≈$5 \times 10^6$ infected cells, $5 \times 10^6$ $TCID_{50}$)

\+ 750 µL TRIzol

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

**Or**

250 µL infected blood (≈$2.5 \times 10^5$ $TCID_{50}$)

\+ 750 µL TRIzol

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

**Or**

50 mg infected liver ($5 \times 10^5$ $TCID_{50}$)

\+ 1 mL TRIzol

A larger piece (≈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 µL infected cells ($2.5 \times 10^6$ infected cells, ≈$2.5 \times 10^6$ $TCID_{50}$)

+ 750 µL 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, ≈$1.5 \times 10^6$ $TCID_{50}$) 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, ≈$1.5 \times 10^6$ $TCID_{50}$) 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 µL infected cells ($3.3 \times 10^6$ infected cells, ≈$3.3 \times 10^6$ $TCID_{50}$)

+ 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 µL infected cells ($3.3 \times 10^6$ infected cells, ≈$3.3 \times 10^6$ $TCID_{50}$)

+ 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, ≈$1.5 \times 10^6$ $TCID_{50}$) 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, ≈$1.5 \times 10^6$ $TCID_{50}$) 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, ≈$1 \times 10^6$ $TCID_{50}$)

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 (≈$1 \times 10^6$ $TCID_{50}$)

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 µL Liquid virus stock ($4 \times 10^5$ $TCID_{50}$)

\+ 960 µL 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 µL Liquid virus stock ($4 \times 10^5$ $TCID_{50}$)

\+ 960 µL 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 µL infected cells ($2.5 \times 10^6$ cells, ≈$2.5 \times 10^6$ $TCID_{50}$)

500 µL DPBS

\+ 250 µL 4× 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 µL infected cells ($2.5 \times 10^6$ cells, ≈$2.5 \times 10^6$ $TCID_{50}$)

500 µL DPBS

\+ 250 µL 4× 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 (≈$1.5 \times 10^6$ $TCID_{50}$)

750 µL DPBS

\+ 250 µL 4× 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 and 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.