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Inactivation and safety testing of Middle East Respiratory Syndrome Coronavirus

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Abstract

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is a recently emerged virus that has caused a number of human infections and deaths, primarily in the Middle East. The transmission of MERS-CoV to humans has been proposed to be as a result of contact with camels, but evidence of human-to-human transmission also exists. In order to work with MERS-CoV in a laboratory setting, the US Centers for Disease Control and Prevention (CDC) has determined that MERS-CoV should be handled at a biosafety level (BSL) 3 (BSL-3) biocontainment level. Many processes and procedures used to characterize MERS-CoV and to evaluate samples from MERS-CoV infected animals are more easily and efficiently completed at BSL-2 or lower containment. In order to complete experimental work at BSL-2, demonstration or proof of inactivation is required before removal of specimens from biocontainment laboratories. In the studies presented here, we evaluated typical means of inactivating viruses prior to handling specimens at a lower biocontainment level. We found that Trizol, AVL buffer and gamma irradiation were effective at inactivating MERS-CoV, that formaldehyde-based solutions required at least 30 minutes of contact time in a cell culture system while a mixture of methanol and acetone required 60 minutes to inactivate MERS-CoV. Together, these data provide a foundation for safely inactivating MERS-CoV, and potentially other coronaviruses, prior to removal from biocontainment facilities.

Highlights

- Standard cell culture fixation protocols are sufficient to inactivate MERS-CoV
- The use of Trizol®, AVL or similar reagents is completely effective at inactivating MERS-CoV
- A dose of 2 Mrad using a ^{60}Co source inactivates MERS-CoV
- Positive-sense RNA isolated from MERS-CoV infected cells does not contain viable virus and is not infectious

1.0 Introduction

In the fall of 2012 the emergence and identification of a novel SARS-like coronavirus in the Middle East raised international concerns for a possible viral epidemic or pandemic. Subsequently, a significant amount of effort was put towards developing vaccines, antivirals and animal models for what became known as Middle East Respiratory Syndrome-Coronavirus (MERS-CoV), an enveloped virus with a positive-sense RNA genome. Since its initial emergence in Jordan in April 2012, the virus has become a significant health concern throughout the Middle East, particularly in Saudi Arabia where the virus was isolated and first identified in June 2012 (Corman et al., 2012; Hijawi et al., 2013; Zaki et al., 2012). As of 24 July 2014 there have been over 830 laboratory confirmed cases of MERS-CoV infection with at least 290 deaths associated with infection with this virus (Global Alert and Response Website: http://www.who.int/csr/don/en/), with the majority of cases occurring in Saudi Arabia.

Since its discovery, considerable effort has been put toward determining the animal reservoir for MERS-CoV and also for development of experimental animal models that mimic human disease. These efforts have identified the camel as a potential reservoir for MERS-CoV (Azhar et al., 2014; Hemida et al., 2014). Evaluation of potential animal models has found that mice and hamsters are not ideal models (Coleman et al., 2014; de Wit et al., 2013a) and that macaques develop a relatively mild, self-limiting infection (de Wit et al., 2013b). In order to complete many of the field and model development studies, inactivation of MERS-CoV was a critical activity that allowed safe testing of material potentially containing MERS-CoV.

The United States Centers for Disease Control and Prevention (CDC) determined that propagation and laboratory characterization of MERS-CoV should be performed in a biosafety level (BSL)-3 laboratory due to a risk for aerosol transmission and the apparently high case fatality rate. These guidelines add a number of restrictions on the movement of and subsequent work with material generated in a BSL-3 facility. Typically, many of the downstream studies performed on highly pathogenic viruses are completed in BSL-2 or BSL-1 laboratory spaces due to the availability of equipment and to reduce stress on staff. In order to complete this work safely, viruses must be inactivated prior to removal from high containment (BSL-3 or BSL-4) laboratories. Previous studies with the closely related SARS-Coronavirus have established that treatment of virus with heat, ultraviolet light, chemical inactivants and a variety of detergents are

effective at inactivating beta-Coronaviruses (Darnell et al., 2004; Darnell and Taylor, 2006; Kariwa et al., 2006; Leclercq et al., 2014; Rabenau et al., 2005a; Rabenau et al., 2005b) while Leclercq et al have shown that heat effectively inactivates MERS-CoV (Leclercq et al., 2014).

In the studies described here, we tested five typical virus inactivation methods that would allow downstream analysis of MERS-CoV infection outside of the high containment laboratory. These methods include inactivation with Trizol® or AVL buffer for RNA analyses, formalin/paraformaldehyde treatment for fixation of cells in cell culture plates, a mixture of methanol:acetone (1:1) for fixation/permeabilization of cells and gamma irradiation for inactivation of virus in cell culture or tissue samples. We found that while gamma irradiation and Trizol® or AVL inactivation methods were consistently effective, fixation of cell culture samples was clearly time dependent and varied between fixation methods.

2.0 Materials and Methods

2.1 Virus and Cells

The MERS-CoV Jordan strain (courtesy of Dr. Kanta Subbarao, NIAID, Bethesda, MD and Gabriel Defang, Naval Medical Research Unit-3, Cairo, Egypt) was used for all studies presented here. The virus was cultivated on Vero (ATCC #CCL-81) and Vero E6 (ATCC# CRL-1586) which were each maintained in DMEM (Lonza) supplemented with 5% FBS. MRC-5 cells (ATCC# CCL-171) were maintained in EMEM (Lonza) + 5% FBS. All cells were kept in a 37°C, 5% CO_2 incubator and neither antibiotics nor antimycotics were used during the maintenance of cells.

2.2 MERS-CoV plaque assay

Vero E6 cells were plated in 6-well plates at a density of $1x10^6$ cells/well to ensure at least 90% confluence the following day. Ten-fold serial dilutions of MERS-CoV were added, in duplicate, to individual wells and the virus was allowed to infect the cells for 1h at 37ºC, 5% CO_2 with rocking every 15 minutes. The cells were then overlaid with 1.6% Tragacanth diluted (1:1) (f/c 0.8%) in 2x EMEM (Quality Biological) containing 4% FBS and incubated at 37ºC, 5% CO_2 for 4 days. The overlay was removed and the cells fixed with 0.25% crystal violet in 10% NBF for 1h at room temperature (RT). The plates were washed with water and the plaques enumerated.

2.3 Irradiation Methods

Virus stock tubes containing either $1x10^6$ or $1.6x10^{10}$ pfu/ml in 0.5 ml volumes and irradiated at specified doses using a JL Shepherd 484-R2 ^{60}Cobalt (^{60}Co) source using a rotating stage to ensure even irradiation. Virus stocks were maintained on dry ice during the entire course of the irradiation process. Determination of viability was completed by plaque assay in duplicate samples.

2.4 Trizol® Inactivation of Cell Culture Supernatants

Cell culture supernatants with a titer of $3.25x10^9$ pfu/ml was inactivated with 3X volume of Trizol LS according to the manufacturer's instructions. The mixture was incubated for 10 min at room temperature before removal of the Trizol using Amicon Ultra 50,000 KDa NMWL centrifuge concentrators. The Trizol:virus solution (500 µl) was added to the concentrator and centrifuged at 14,000xg for 10 min. The concentrate was diluted with 500 µl PBS and centrifuged again at 14,000xg for 10 min. The concentrate was diluted with 500 µl DMEM and centrifuged for 14,000xg for 3 minutes. The concentrator was inverted into a clean tube and centrifuged for 2 min at 1000xg to collect the concentrate. The concentration/dialysis procedure was also performed with virus stock that was not treated with Trizol to determine the loss of virus during the concentration/dialysis procedure.

The concentrated material from above was added to Vero E6 cells in a T-25 flask and incubated 37ºC, 5% CO_2 for 3 days. Positive and negative control flasks were also inoculated. Following the incubation period, the cell culture supernatant was removed, clarified and added to a fresh flask of Vero E6 cells. The cells were subsequently incubated at 37ºC, 5% CO_2 for 5 days. The cell culture supernatants were collected, clarified and subjected to an immunofluorescence assay (IFA) on fresh Vero E6 cells using the method described below.

2.5 AVL Inactivation of Cell Culture Supernatants

Duplicate samples of cell culture supernatants with an initial titer of $3.25x10^9$ pfu/ml were treated with AVL buffer (Qiagen) at a 4:1 ratio (400 µl AVL:100 µl supernatant) and incubated at room temperature for 10 min. Ethanol (>95%) was added to a final volume of 900 µl and

vortexed. The mixture was transferred to Amicon centrifuge concentrators and processed as above. Residual titers were determined by plaque assay.

2.6 Loss of infectivity of Trizol® Purified MERS-CoV RNA

On the day before inoculation, T-25 flasks were seeded with Vero E6 cells or MRC-5 cells to ensure 85% confluence on the day of inoculation. On the day of inoculation, media was removed from the flasks and cells were infected with MERS-CoV at a multiplicity of infection (MOI) of 1 in 1ml of complete media. A mock sample was also prepared for each cell type with media only. All samples were incubated at 37°C, 5% CO_2 and rocked every 15 minutes for one hour. After the one hour incubation, 3ml of complete media was added to the flasks and incubated at 37°C, 5% CO_2 for 24 hours.

At 24 hours post infection, media was collected from each flask and transferred to a 15ml conical. One infected flask from each cell type served as the positive control sample, and was diluted 1:10 for inoculation of a control flask in the next passage. Media from a second infected flask was combined with Trizol® LS (Life Technologies) in a 1:3 ratio as recommended by the manufacturer and incubated for 10 min at RT to inactivate the virus. Total RNA was subsequently extracted following the manufacturer's protocol (http://tools.lifetechnologies.com/content/sfs/manuals/trizol_ls_reagent.pdf).

RNA extracted from MERS-CoV infected Vero E6 and MRC-5 cells was brought up to 1ml with media and was used to inoculate a T-25 flask for each cell type. Positive (1:10 MERS-CoV stock dilution) and negative (media only) controls were included for both Vero E6 and MRC-5 cells. Following inoculation, flasks were incubated at 37°C for 30 minutes, with a rock at 15 minutes. After 30 minutes, 3ml media was added to each flask and subsequently incubated at 37ºC, 5% CO_2 for 96 hours when positive control cells began to show cytopathic effects (cpe).

2.7 Aldehyde and Methanol:Acetone Fixation

On the day before inoculation, 24x60mm dishes were seeded with $2.0x10^6$ Vero E6 cells per dish to ensure 90% confluence on the day of inoculation. On the day of inoculation, the estimated cell count used for calculation was $4.0x10^6$ cells per 60mm dish. Media was removed from the dishes

and cells were infected with MERS-CoV at a MOI of 5 in 440ul of inoculum in duplicates. A mock infected sample was also prepared in duplicate with media only (Lonza DMEM + 5% FBS). All samples were incubated at 37°C, 5% CO_2 and rocked every 15 minutes for one hour. After the one hour incubation, 1.5ml media was added to the dishes and incubated at 37°C, 5% CO_2 for 24 hours.

At 24 hours post infection, media was removed from each dish. The positive (unfixed) and negative (uninfected) control dishes were washed three times with 1X PBS, resuspended by scraping in 1ml 1X PBS and aliquoted to a clean tube. Cells in the remaining dishes were fixed as follows (in duplicates): 10% neutral buffered formalin (NBF) (Fisher Scientific) for 10 minutes, 20 minutes or 30 minutes; 4% paraformaldehyde (PFA) (freshly made from a 20% PFA stock concentrate (Electron Microscopy Sciences)) for 10 minutes, 20 minutes or 30 minutes; 1:1 Methanol:Acetone (M/A) for 15 minutes, 30 minutes, 60 minutes or 120 minutes. After each time point, the fixative was removed and the cells were washed 3X in 1X PBS before resuspending by scraping in 1ml 1X PBS.

2.8 Serial passaging of fixative inactivated material

In order to demonstrate inactivation of virus, virus samples were blind passaged twice on Vero (CCL-81) cells with supernatants from the final passage being tested for the presence of viable virus using a cell culture based immunofluorescence assay (IFA).

For the first serial passage, T-25 flasks were seeded with $9x10^5$ Vero cells to ensure 70% confluence on the day of inoculation. All media was removed from the flasks and replaced with 1ml of the test inoculum. The positive (a 1:10 dilution of virus stock) and negative controls were also used to inoculate T-25 flasks seeded at the same density. All flasks were incubated at 37°C for 30 minutes, with a rock at 15 minutes. After 30 minutes, 3ml media was added to the flasks and left to incubate at 37°C, 5% CO_2 for 3 days.

On day 3, all media from each of the flasks was removed and centrifuged at 1000xg for 10 min to remove cell debris, and the supernatant transferred to a fresh T-25 flask seeded the day prior. Flasks were incubated at 37°C, 5% CO_2 for 5 days. The cells were photographed at the end of

each passage to document any visible cpe (Figure 1). The cell culture supernatant was removed and clarified by centrifugation at 500xg for 10 minutes. The supernatant was removed and tested for the presence of virus using an IFA (below).

2.9 Immunofluorescence Assay

Test samples were serially diluted ten-fold from $1x10^{-1}$ to $1x10^{-7}$ for the IFA inoculum. In addition to the blind passaged samples, we included an IFA negative control (complete media only) and an IFA positive control (1:10 dilution of virus stock). One-hundred microliters of the specified inoculum was added to each well of a 96 well plate (Greiner BioOne) seeded the day before with $4x10^4$ Vero (CCL-81) cells per well and incubated for 48 hours at 37°C, 5% CO_2.

At 48 hrs, the plates were fixed by adding 100ul of 20% NBF for a final concentration of 10% NBF and incubated for 30 minutes at RT. The NBF was removed and replaced with fresh 10% NBF and incubated 24 hours at 4°C following previously established safety protocols.

The fixed plates were washed four times with 1X PBS and then permeabilized with permeabilization solution (0.25% Triton X-100 in 1X PBS) (Sigma) for 5 minutes at RT. The cells were washed four times and then blocked with 10% normal goat serum (Vector Labs) in 1X PBS for 1 hour at RT. Thirty microliters of rabbit polyclonal antibody to novel coronavirus Spike protein (Sino Biological) diluted 1:1000 in 1.5% normal goat serum in 1X PBS was added to each well. Plates were rocked for 1 hour at RT then washed five times with 1X PBS. Anti-rabbit AlexaFluor 594 (Invitrogen) was diluted 1:2500 in 1X PBS and 100ul was added to each well. The plates were covered with foil and rocked for 30 minute at RT followed by four washes with 1X PBS. For nuclear counterstaining, the cells were incubated with 100ul/well of Hoechst 33342 (Life Technologies) diluted 1:2500 in 1X PBS for 10 min at RT and then washed twice with 1X PBS. 100ul of 1X PBS was added to each well to prevent the cells from drying out.

Data were collected on an Operetta high content imaging system (Perkin Elmer). All images were taken using a 20X long WD objective, nine fields per each well of a 96-well plate were accounted for. The main components of quantitative analysis using the Harmony Imaging and Analysis Software were the total number of cells based on nuclear staining and the number of

cells infected with MERS-CoV based on the intensity of Alexa 594 fluorescence above the threshold in the cytoplasmic region. The ratio between the total number of cells and the fluorescent cells was determined as a percentage of cells positive for MERS-CoV.

3.0 Results

3.1 Gamma Irradiation

While the use of gamma irradiation (^{60}Co source) is not common for all BSL-3 facilities due to the expense of the gamma source, these instruments are available in most BSL-4 laboratories and a number of newer BSL-3 facilities. Lower energy ^{137}Cs sources are often found within hospital settings for irradiation of blood or blood products, but their lower energy does not provide a practical time-frame for inactivation of viruses. Gamma irradiation also provides a means of translating inactivation protocols between institutions as the effective dose (rad) will be consistent between instruments and is not necessarily user dependent. The kinetics of MERS-CoV inactivation by gamma irradiation was evaluated using a virus stock with typical titer (~5 $\log_{10}$) and a concentrated virus stock (~10 $\log_{10}$). Virus stocks were irradiated to a specified dose and the resulting titer quantified. These studies found that 1 Mrad was sufficient to reduce titers by 4-5 $\log_{10}$ and that 2 Mrad was sufficient to completely inactivate the virus as determined by plaque assay (Figure 1). These data suggest that a radiation dose of at least 3 Mrad should be sufficient to inactivate MERS-CoV in most laboratory cell culture or tissue-based assays.

3.2 Trizol® Treatment

The use of Trizol® or Trizol® LS is typical for the isolation and purification of RNA or DNA from virus-infected cells or cell culture supernatants. The combination of guanidine isothiocyanate and phenol has proven to be effective at inactivating enveloped viruses (Blow et al., 2004) and has been used for many years for isolation of viral DNA or RNA for both biological and virological analysis. In these studies virus stock ($2x10^6$ pfu/ml) was mixed with Trizol® LS at a ratio of 1:3 following manufacturer recommended procedures. Given the toxicity of Trizol® LS for cultured cells, RNA was extracted from the Trizol® LS treated material prior to inoculating fresh cells. Following blind passaging of treated material, it was found that treatment with Trizol® LS was completely effective at inactivating MERS-CoV (Figure 2) following a 10 minute RT incubation. The use of concentrators for the removal of residual Trizol® LS is

effective and more efficient than using standard dialysis approaches, but there is a loss of about 1 log of virus as seen in Figure 2 in untreated control samples. In addition, the fact that there was no evidence of virus production also indicates that the purified RNA from MERS-CoV (a positive-sense RNA virus) is not infectious.

3.3 AVL buffer treatment

The use of AVL buffer has replaced the use of Trizol® LS in many applications where the isolation of viral RNA is desired. AVL-based extraction systems are more efficient, can be automated and eliminates the need use of organic solvents. In order to demonstrate that treatment of MERS-CoV containing cell culture supernatant was effective at inactivating the virus, a slightly modified version of the protocol distributed with the QIAamp viral RNA isolation kits was used. Following inactivation and serial passage of the cell culture supernatant, no virus was evident in the test material while positive controls treated in a similar manner less the use of AVL, had titers $>10^7$ pfu/ml (Table 1A).

3.4 Formaldehyde-based Inactivants

The use of NBF or PFA is a common practice for the fixation of virus infected cells or tissues for various assays including plaque assays, focus forming assays and histology studies. In order to determine the effectiveness of NBF and PFA at inactivating MERS-CoV in a cell culture setting, infected cells were fixed for specified periods of time, washed and then used to infect fresh cells in two serial blind passages. Cell culture supernatants from the second passage were then tested for the presence of virus using a cell-based immunofluorescence assay that was established for evaluation of MERS-CoV infection (Dyall et al., 2014). In duplicate samples we found that incubation with either new commercially provided 10% NBF or freshly prepared 4% PFA for 10 minutes was ineffective at completely inactivating MERS-CoV. We found that incubation with 10% NBF for 20 and 30 minutes completely inactivated MERS-CoV, but that a 30 minute treatment with 4%PFA was required (Table 1B). Given that 10% NBF and 4%PFA are chemically similar except that NBF contains 1% methanol, a minimum 30 minute incubation time at RT with either of these reagents would be prudent to ensure complete inactivation of MERS-CoV in a cell culture setting. Inactivation of MERS-CoV in tissues was not determined.

3.5 Methanol-Acetone Inactivation

A mixture of methanol and acetone (1:1) (M/A) is frequently used for the fixation and permeabilization of cells for immunostaining and other techniques in an effort to avoid the cross-linking of proteins and antigenic epitope loss that can occur using aldehyde-based fixatives (Thavarajah et al., 2012). Here we tested the ability of M/A to inactivate MERS-CoV infected cultured cells that would be typical of an IFA-type assay. In these studies we found that M/A was only partially effective using a 15 or 30 minute fixation, but that it was effective at longer fixation times (Table 1B). These data suggest that a minimum 60 minute fixation time should be used when inactivation MERS-CoV with M/A in infected cell culture systems.

4.0 Discussion

In many academic, government or industrial settings, the positive demonstration of virus inactivation is becoming a more common requirement prior to removal of material from biocontainment laboratories, for shipment of material to collaborators or for testing purposes. The Division of Select Agents and Toxins has also indicated that the demonstration of virus inactivation or provision of a clearly defined method for demonstrating virus inactivation be incorporated into safety programs for BSL-3 and BSL-4 pathogens. In this light, we tested the recently emerged MERS-CoV using various typical inactivation methods to validate that our standard procedures were sufficient for inactivation of MERS-CoV. Here we found that the frequently used inactivation methods of NBF, PFA or Trizol® LS performed as expected with complete inactivation of MERS-CoV within 30 (NBF/PFA) or 10 (Trizol® LS/AVL) minutes. The use of gamma irradiation was shown to render 10 $\log_{10}$ MERS-CoV undetectable by plaque assay following a dose of 2Mrad.

Published penetration rates for formalin suggest a rate of 1mm/ hour (Medawar, 1941), however, the rate of penetration into tissues or cells is not a simple linear process and is dependent upon tissue composition or cell type. In addition, the necessary aldehyde cross-linking that is required to inactivate a virus particle is independent of chemical penetration rates. Our evaluation process of blind passaging harvested cells requires that all virus particles in a preparation were adequately inactivated. As NBF and freshly prepared PFA are similar chemically, we were surprised to find that one replicate of 4% PFA treatment did not completely inactivate MERS-

CoV after a 20 minute incubation while one replicate of 4% PFA and both replicates of NBF were completely effective. Treatment for 30 minutes was shown to be universally effective for both NBF and PFA and has been adopted as our minimum standard for inactivation of MERS-CoV infected cultured cells.

The use of a mixture of methanol and acetone, typically 1:1, is often used to fix and permeabilize cells for immunoassays such as IFA or other microscopy techniques. We found that this mixture was sufficient to inactivate MERS-CoV infected cells after a room temperature incubation of at least 60 minutes. While this technique is effective at inactivating virus, its use in evaluation of cellular morphology should be carefully considered due to the generation of cellular artifacts (Moloney et al., 2004).

Trizol® inactivation of viruses for the purpose of extracting viral DNA or RNA has been standard methodology for many years. The combined activity of the phenol component to disrupt membranes and denature proteins in addition to the powerful denaturing activity of guanidine isothiocyanate makes this reagent effective at inactivating enveloped viruses. Despite the likelihood that Trizol® inactivates virus in cell culture supernatant or infected cells within seconds, our standard methodology dictates a minimum 10 minute room temperature incubation to ensure inactivation. Reagents such as AVL buffer, which rely primarily on guanidine isothiocyanate to denature and inactivate viruses, are now used frequently in research labs and in field work due to its ease of transport, ability to automate and easier use and disposal. In the work shown here, we have clearly demonstrated that treatment of MERS-CoV infected material with Trizol® LS or AVL buffer renders MERS-CoV, and likely other coronaviruses, inactive. We have also shown that RNA purified from MERS-CoV is not naturally infectious in a cell culture system. The ability of positive strand viral RNA to infect cultured cells has been a point of contention for a number of years with the NIH Office of Biotecnology Activities (OBA) and the Recombinant DNA Advisory Committee suggesting that purified fully intact RNA from positive strand viruses be handled at the same biocontainment level as the pathogen in question. The data for inactivation of MERS-CoV with Trizol® LS and subsequent addition to cultured cells demonstrates that purified RNA from this virus does not infect cultured cells suggesting that the guidance from OBA is not appropriate for this virus.

The use of gamma irradiation has likewise been used for many years to inactivate viruses. While there is no doubt that irradiation will inactivate the viruses, the required "kill" dose is valuable information as irradiation times can get long, particularly as the age of the gamma source increases. There is also a question of thermal impact on virus stocks or experimental samples as the heat generated within the gamma cell is significant and can adversely affect material if not carefully controlled. Here we found that complete inactivation from a 10 $\log_{10}$ cell culture stock was achieved following a 2Mrad dose (^{60}Co source) with samples held on dry ice in a 2 L Nalgene beaker through the course of the irradiation. While clearly effective at 2 Mrad and appreciating that 3 Mrad should be sufficient to inactivate MERS-CoV, a prudent level of caution in our facility recommends that we utilize a minimum of 4 Mrad for the inactivation of MERS-CoV in cell culture supernatants under these irradiation conditions, a dose which should also be sufficient for virus collected from other biological systems, including tissues.

In the work described here, we have tested a series of procedures that are frequently used for the inactivation of enveloped viruses in a laboratory setting in their ability to render MERS-CoV non-infectious. These procedures are used for the fixation of infected cells, preparation of material for DNA or RNA analysis or irradiation of samples for assays such as antibody or cytokine analysis. Each of the techniques shown here will inactivate MERS-CoV, but most have a critical time component that must be met. The data provided here can serve as a guideline for other researchers, but demonstration of virus inactivation should be determined empirically by individual investigators, particularly in regards to the use of fixatives for inactivating viruses as these procedures a heavily dependent upon the user and requires quality reagents.

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460 **Figure Legends**

461

462 **Figure 1:** Irradiation inactivation curve for MERS-CoV. Two different stocks of MERS-CoV
463 were irradiated with up to 6 Mrad from a ^{60}Co gamma source. Aliquots from each stock were
464 titrated by plaque assay to determine the residual virus titer. Each data point is the average of
465 duplicate aliquots.

466

467 **Figure 2:** Trizol® LS inactivation of MERS-CoV. A. Demonstration of a lack of viable virus
468 following inactivation of virus stocks and two blind passages on Vero E6 cells. Columns
469 "Passage+ Filter and Passage+" are untreated virus controls. "Mock" columns are virus negative
470 controls. B. Demonstration of a lack of cytopathic effects following incubation of Trizol® LS
471 inactivated and purified RNA from MERS-CoV infected cells at 96 hpi. Negative control cells
472 are mock infected while positive control cells were infected with approximately 1x10^5 pfu in a
473 T-25 flask.

474

475 **Table 1:** Inactivation of MERS-CoV with AVL buffer (A) or fixatives (B).

476

477

478

479 **Table 1**

A

	Titer (log_{10})
Negative Control	0
AVL treated	0
Positive Contol-processed	8.48
Plaque Control	7.18

B

	% Positive Cells				
	Untreated	10 min	15 min	20 min	30 min
Media	0	NT	NT	NT	NT
10%NBF	NA	27.25	NT	0	0
4% PFA	NA	12.85	NT	0	0
M/A*	NA	NT	7.5	NT	0
Assay Control	98.5				
Passage Control	12.3				

*Methanol:Acetone (50:50)

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