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Methods for successful inactivation of Rift Valley fever virus in infected mosquitoes

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Summary: Ensuring the successful inactivation of select agent material is critical for maintaining compliance with federal regulations and safeguarding laboratory personnel from exposure to dangerous pathogens. Rift Valley fever virus (RVFV), naturally transmitted by mosquitoes, is classified as a select agent by the CDC and USDA due to its potential to cause significant economic losses to the livestock industry and its demonstrated potential to emerge into naïve geographic areas. Herein we describe several effective inactivation procedures for RVFV infected mosquito samples. We also demonstrate the vaccine strain MP-12 can be used as an appropriate analog for inactivation testing and describe a method of validating inactivation using Amicon filters. Briefly, we show the following inactivation methods are all effective at inactivating RVFV and MP-12 by following the manufacturers'/established protocols: 4% paraformaldehyde, Trizol LS (ThermoFisher Scientific), MagMAX™-96 Viral RNA Isolation Kit (ThermoFisher Scientific), and Mag-Bind® Viral DNA/RNA 96 Kit (Omega Bio-Tek).

Rift Valley fever virus (RVFV) (order: *Bunyavirales*, family: *Phenuiviridae*, genus: *Phlebovirus*) is an enveloped, single-stranded, negative sense RNA virus with a tripartite genome (Bouloy and Weber, 2010). The virus is mosquito-transmitted, causes severe epidemics among ruminants and humans, and is endemic to eastern and southern Africa (Daubney, Hudson, and Garnham, 1931; Linthicum, Britch, and Anyamba, 2016; Weaver and Reisen, 2010). RVFV has also demonstrated an ability to emerge into non-endemic areas due to the wide disbursement of vectors capable of transmitting the virus; this is highlighted by the recent introduction of RVFV to the Arabian Peninsula (Al-Afaleq and Hussein, 2011), a recent outbreak on Mayotte island (Touze, 2019), and other introductions to islands located in the Indian Ocean (Balenghien et al., 2013). Furthermore, importation of infectious mosquito vectors and viremic travelers present a realistic threat for virus introduction into North America (Golnar, Kading, and Hamer, 2018; Konrad and Miller, 2012). Because of the potential for RVFV to cause severe disease in both human and domestic animal species, its ability to emerge into naïve geographic locations, its history as a bioterrorism agent (Borio et al., 2002), and its potential to cause significant economic losses to the livestock industry, the Centers for Disease Control and Prevention (CDC) and United States Department of Agriculture (USDA) have classified RVFV as an overlap select agent (Federal Select Agents Program, 2018). The term "select agent" refers to a group of biological agents for which the United States has deemed to have the potential to pose a severe threat to public health and safety.

Several gaps exist in our current understanding of RVFV including its disease mechanisms, ecology, and epidemiology (Bird and McElroy, 2016). Understanding how human genetic factors contribute to disease outcome; why human maternal-to-fetal transmission is so rare when ungulate maternal to fetal transmission is so prevalent; the underlying factors of RVFV induced

retinitis and encephalitis; and how the virus is maintained during inter-epidemic periods are all not well understood. Notably, many mosquito vectors are competent for horizontally transmitting RVFV in the laboratory, but more work must be completed to understand the risk of RVFV establishment and transmission in different geographic areas (Brustolin et al., 2017; Turell et al., 2013a; Turell, Byrd, and Harrison, 2013b; Turell, Wilson, and Bennett, 2010). Another critical aspect of RVFV transmission in East Africa is the transovarial transmission (TOT) of the virus from adult female *Aedes* (*Neomelaniconion*) *macintoshi* mosquitoes to their offspring (Linthicum, Davies, and Kairo, 1985). TOT is a common phenomenon among the *Bunyavirales* (Bergren and Kading, 2018), and the potential contribution of this transmission mechanism to RVFV establishment in North American mosquito vectors has not yet been adequately assessed. Modeling efforts to estimate the establishment potential of RVFV in North America have either assumed exceptionally low TOT rates due to the lack of experimental data (Barker et al. 2013), or not incorporated this parameter at all (Golnar et al., 2014).

In order to effectively mitigate virus transmission in the field, experimental studies with virulent RVFV must be conducted in a containment laboratory to understand the particular vectors to target and vulnerabilities in transmission that can be blocked. Furthermore, laboratory studies can generate data to parameterize risk models and more accurately assess the potential for RVFV establishment in regions such as the United States. Many laboratory studies have contributed to our understanding of virus transmission with other arboviruses and can focus on various aspects of arbovirus transmission including assessing the competency of different strains of virus (Weger-Lucarelli et al., 2016) and local vectors (Gendernalik et al., 2017), comparing the transmission of different mosquito species (Hurk et al., 2010) and different populations within the same species (Bennett et al., 2002), assessment of transmission dynamics under

different experimental/environmental conditions (Muturi, Blackshear Jr., and Montgomery, 2012), exploration of mechanisms that drive transmission efficiency for the vector (Bennett et al., 2005) and virus (Tsetsarkin et al., 2007), and evaluation of transmission blocking strategies (Magalhaes et al., 2019).

Downstream analysis of inactivated samples is often required to accomplish the studies outlined above. These analyses include various molecular and immunological techniques such as RT-qPCR, Sanger and next-generation sequencing, and immunofluorescence assay. Efficient and effective inactivation of infectious biological material is critical for the safety of laboratorians and their communities particularly when working with select agents.

In 2017, stringent regulations went into effect to address gaps that were discovered regarding inactivation of infectious biological material, specifically biological select agents. The Division of Select Agents and Toxins (DSAT) mandated that all inactivation procedures be validated in-house and approved by each institution's Select Agent Responsible Official. Further, verification of complete inactivation is required upon each occasion an inactivation method is used and inactivated select agent material is removed from select agent registered space. Guidelines for the inactivation of select agents from the CDC and the USDA- Animal and Plant Health Inspection Services allow for the use of a surrogate virus to validate inactivation procedures so long as the virus has the same or greater resistance to the inactivation procedure (7 CFR Part 331, 9 CFR Part 121.3, 42 CFR Part 73.3, CDC and USDA, 2017). Compliance with the above regulations becomes complicated when studying RVFV in mosquitoes as these experiments usually require biosafety level 3 (BSL3) and arthropod containment level 3 (ACL3) facilities, large sample sizes, and high throughput methodologies to process the samples.

In order to facilitate compliance with federal select agent regulations when conducting critical transmission experiments involving this high consequence emerging virus, we present several methods of inactivating mosquito samples infected with an epidemic strain of RVFV as well as the vaccine stain MP-12. MP-12 is a live attenuated vaccine of RVFV generated by passing the ZH548 strain 12 times in MRC-5 cells in the presence of 5-fluorouracil (Caplen, Peters, and Bishop, 1985). Specifically, we show the effectiveness of Trizol LS™ (Thermo Fisher Scientific), MagMAX™ Viral RNA Isolation Kit (Thermo Fisher Scientific), Mag-Bind® Viral DNA/RNA 96 Kit (Omega Bio-Tek), and 4% paraformaldehyde fixation at inactivating infectious RVFV particles and an avirulent surrogate, MP-12. MagMAX™ and Mag-Bind® are both magnetic bead-based RNA extraction methods that allow for high throughput of samples (an attribute that is extremely valuable when conducting vector competence studies). Furthermore, these bead-based systems allow for automation which further increases throughput. We also chose to test Trizol LS™ as it is commonly used and has been well documented to efficiently inactivate samples (Blow et al., 2004; Patterson et al., 2018). Four percent paraformaldehyde fixation was chosen because of a well-established methodology for its utilization to visualize RVFV antigen in infected mosquitoes (Kading, Crabtree, and Miller, 2013). These methods will be beneficial to the research community in providing methods of inactivation and validation that can be used in-house.

To test the effectiveness of virus inactivation by lysis buffers commonly used for nucleic acid isolation, which is a common requirement in vector competence experiments, we inoculated *Culex tarsalis* mosquitoes via the intrathoracic (IT) route as described previously (Rosen and Gubler, 1974). Mosquitoes were injected with 138nL of an epidemic strain of RVFV (Kenya128B-15) at a titer of $1.09x10^7$ plaque forming units (PFU)/mL (approximately 1,500

PFU inoculated) or the MP-12 vaccine strain at a titer of $2.16x10^7$ PFU/mL (approximately 2,980 PFU MP-12 inoculated), using a Nanoject III (Drummond Scientific). A total of 60 mosquitoes were required for the subsequent analyses for each virus (120 total), thus we injected 70 mosquitoes per virus to allow for attrition. Mosquitoes were held at 26ºC with 75% relative humidity and a 16:8h light:dark cycle for one week. Mosquitoes were also provided water and sugar *ad libitum*. After one week, mosquitoes were collected (1 per tube) into 2mL gasketed tubes (Sarstedt) containing two 5mm glass beads (Millipore) and 250µL mosquito diluent. Mosquito diluent consisted of Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 20% fetal bovine serum (FBS) (Atlas Biologicals), 50 U/mL Penicillin-Streptomycin (P-S) (Gibco), 0.05 mg/mL gentamycin (Sigma), and 0.5 mg/mL Amphotericin B (Gibco). Mosquitoes were stored at -80ºC prior to inactivation method assessment. A graphical flowchart of the experimental design is provided in Figure 1.

To ensure mosquitoes were uniformly infected with wild-type RVFV or MP-12, plaque assays were conducted on a subset of ten mosquitoes, of the total 120 (%), for each virus. Mosquitoes were homogenized in a MagNA Lyser (Roche) at 6,000 rotations per minute (rpm) for 30 seconds. Mosquito homogenate was then clarified via centrifugation at 12,000xg for 2 minutes in a MiniSpin centrifuge (Eppendorf). Plaque assays were conducted on Vero cells as previously described and allowed to incubate for 72 hours prior to fixation (Beaty, Calisher, and Shope, 1995). After the seven-day incubation, we found every mosquito was infected with mean titers $3.44x10^5$ and $1.63x10^5$ PFU/mosquito for RVFV/Kenya128B-15 and RVFV/MP-12, respectively (Figure 2).

To assess the effectiveness of Trizol LS™, the MagMAX™ Viral RNA Isolation Kit, and the Mag-Bind® Viral DNA/RNA 96 Kit, mosquito samples were homogenized and clarified as

described above (20 mosquitoes per extraction method). For reference, previous studies exploring the inactivation of RVFV-infected mosquitoes utilized three mosquitoes per group over various time points (Kading et al., 2013). Inactivation protocols were then followed for each reagent as per manufacturer's instructions. For Trizol LS™, 200µL of clarified homogenate was added to 750µL Trizol LS™ reagent followed by mixing via vortex for 30 seconds. For MagMAX™, the Lysis/Binding Solution was prepared as instructed by adding 63µL 100% isopropanol (Sigma) to 77µL Lysis Binding Solution Concentrate for a total of 140µL. Thirty microliters of clarified mosquito homogenate was then applied to 140µL Lysis/Binding Solution, samples were then mixed by pipetting. For Mag-Bind®, lysis mastermix was prepared as instructed by adding 70µL 100% isopropanol to 60µL TNA Lysis Buffer for a total of 130µL. Fifty microliters of clarified mosquito homogenate was then applied to 130µL lysis mastermix, samples were then mixed by pipetting. Time to inactivation is not listed on any of the protocols tested. To account for this we kept all samples at room temperature for five minutes prior to testing for inactivation.

MagMAX™, Mag-Bind®, and Trizol LS™ all utilize guanidinium thiocyanate, a chaotrope, to inactivate samples by solubilizing lipid bilayers and denaturing proteins. Because applying inactivation media directly to cells would result in cell death, samples were washed twice with 15mL DPBS (Gibco) in centrifugal concentrators (Amicon Ultra, MWCO: 10kDa; Millipore) at 4,000xg for 20 minutes. A final wash of 15mL DMEM supplemented with 2% FBS and 50 U/mL P-S was conducted so that the resulting ~200µL could be directly transferred to Vero cell monolayers on 12-well plates. Plates were incubated as described above for one hour, followed by adding 1mL of DMEM with 2% FBS and 50 U/mL P-S and incubating for a further three days. After incubation, plates were inspected for cytopathic effect (CPE) and recorded.

Regardless of if CPE was found 200µL was passaged onto new Vero cell monolayers on 12-well plates and processed exactly as the first passage. Mosquitoes that had not been subjected to an inactivation method were used as positive controls to provide validation of inactivation and use of centrifugal concentrators. These positive control mosquitoes were processed identically to the experimental samples (filtration and subsequent washing through centrifugal concentrators, etc.) to ensure comparability. Additionally, an uninfected well was included for all CPE assays for comparison to positive control and experimental wells and to control for potential contamination. All control mosquitoes showed CPE for first and second passages; Trizol LS™, MagMAX™, and Mag-Bind® protocols all consistently inactivated RVFV derived from infected mosquitoes, both virulent and vaccine strains (Figure 3). Mosquito infections were conducted under institutional biosafety approved protocol 16-078B.

To assess the effectiveness of 4% paraformaldehyde at inactivating both RVFV strains we IT-injected the infected mosquitoes, described above, with 276nL 4% paraformaldehyde (12% paraformaldehyde diluted in DI water, Electron Microscopy Sciences) (20 mosquitoes for each virus) in order to perfuse them with the fixative (Kading et al., 2013). Following injection, legs and wings were removed and discarded, mosquitoes were briefly submerged in 70% ethanol to break their hydrophobicity, and submerged in 1mL 4% paraformaldehyde for 24 hours. After 24 hours mosquitoes were transferred to 70% ethanol for another 24 hours. Samples were held at room temperature for all parts of this protocol. Mosquitoes were then transferred to homogenization tubes with mosquito diluent and two glass beads. Mosquitoes were processed as described above. Because of the residual paraformaldehyde in the sample, 200µL of the resulting clarified mosquito homogenate was applied to the centrifugal concentration and passage protocol

as described above. All samples showed complete inactivation of infectious virus particles on both passages (Figure 3).

All four methods successfully inactivated both wild-type RVFV and the MP-12 vaccine strain. The inactivation methods explored in this study represent the most relevant methods for inactivating virus derived from infected mosquito tissues due to their ability to be used in a high throughput manner and the downstream relevance in laboratories studying mosquito-virus interactions. We have also shown that MP-12 is an appropriate surrogate strain with which to validate inactivation procedures before these procedures are used with wild-type RVFV, as the analysis conducted will satisfy the select agent requirements in that this potential surrogate strain "possesses equivalent properties as the wild-type strain with respect to inactivation" (7 CFR Part 331, 9 CFR Part 121.3, 42 CFR Part 73.3) (Figure 2). While only one biological replicate was conducted, the inactivation methods tested were conducted under rigorous conditions and manufacturer's protocols and storage recommendations were followed exactly. The select agent inactivation regulations require that the inactivation procedures be validated "using a viability testing protocol" (7 CFR Part 331, 9 CFR Part 121.3, 42 CFR Part 73.3), for which we selected assessment of CPE on cell monolayers. Given the rapid replication of RVFV and inclusion of positive and uninfected controls for visual comparison, assessing CPE three days post infection provided appropriate sensitivity to determine whether or not infectious virus was present. Alternatively, plaque assays could also have been performed as an appropriate viability assessment method. While each individual lab will have to validate these protocols in-house, our goal was for this study to provide a framework to efficiently choose and validate the methods best fit for their specific research aims.

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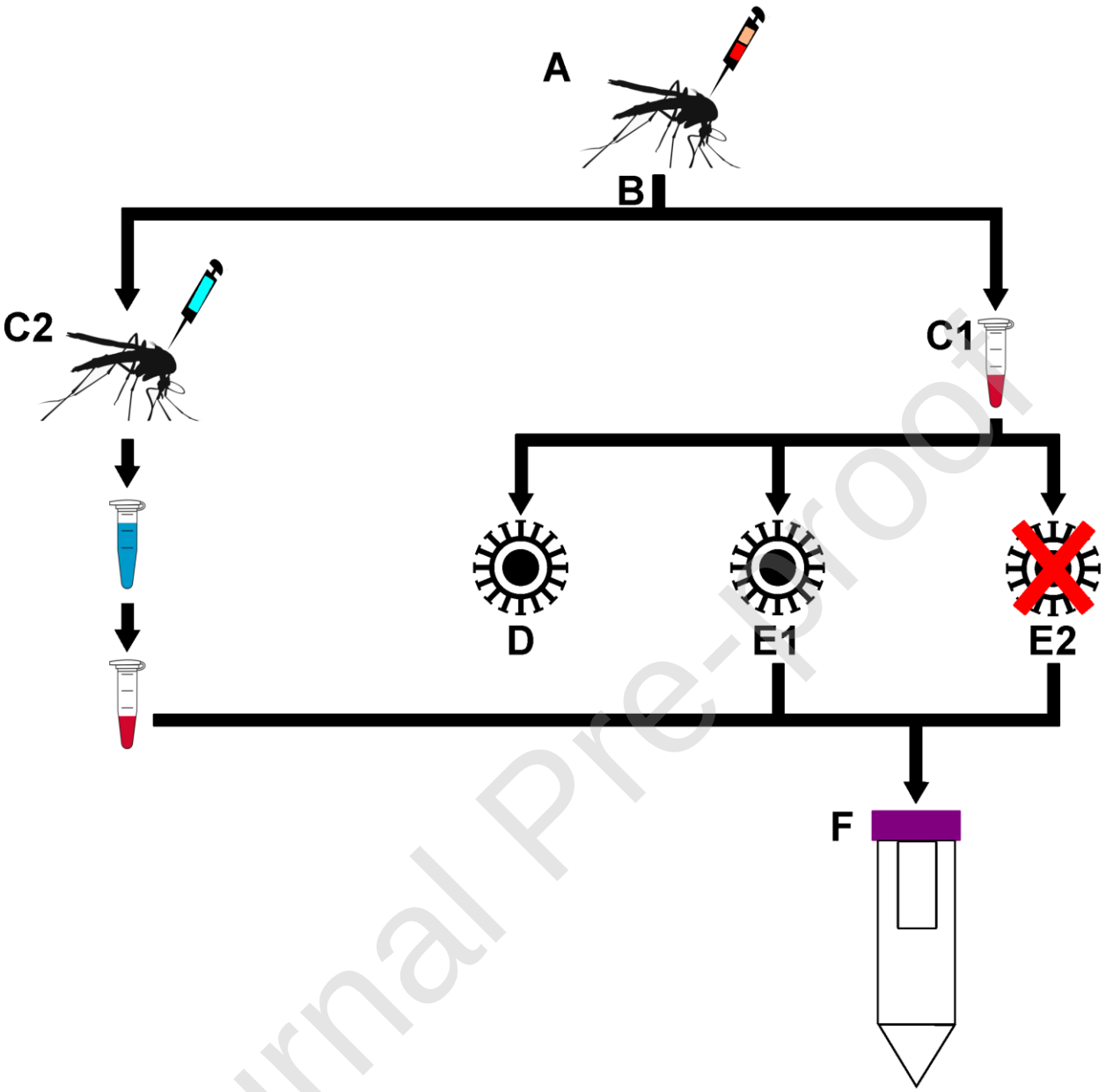

Figure 1. Graphical flowchart of experimental design with sample numbers. A) *Culex tarsalis* IT inoculated with RVFV/Kenya128B-15 or RVFV/MP-12, n=70 per virus (extra 10 to allow for attrition). B) 1 week incubation: 26°C, 75% relative humidity, 16:8 light dark cycle, C1) harvest infected mosquitoes in 2ml screwcap tubes with 250µl diluent and two 5mm glass beads, n=50 per virus. C2) 4% paraformaldehyde IT inoculated, dipped in 70% EtOH, legs & wings removed, submerged in 4% paraformaldehyde for 24 hours, transferred to 70% EtOH 24 hours, transfer to storage tubes with diluent, n=10 per virus. D) Samples for plaque assay, n=10 per group. E1) CPE positive controls, n=10 per group. E2) Run inactivation assays: Trizol LS, MagMAX-96, Mag-Bind, n=10 per group. F) Run samples through Amicon filter protocol, followed by CPE assay (2 passages), n=10 per group.

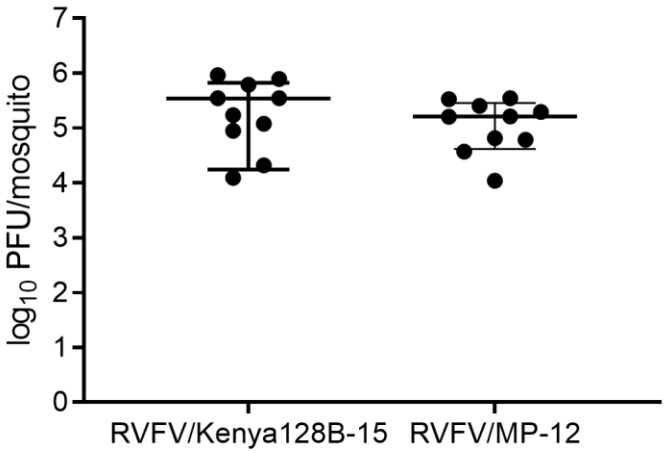

Figure 2. Titers of RVFV in individual positive control mosquitoes (not inactivated) for each virus tested (n=10 mosquitoes per virus) after IT injection, seven-day incubation, and collection.

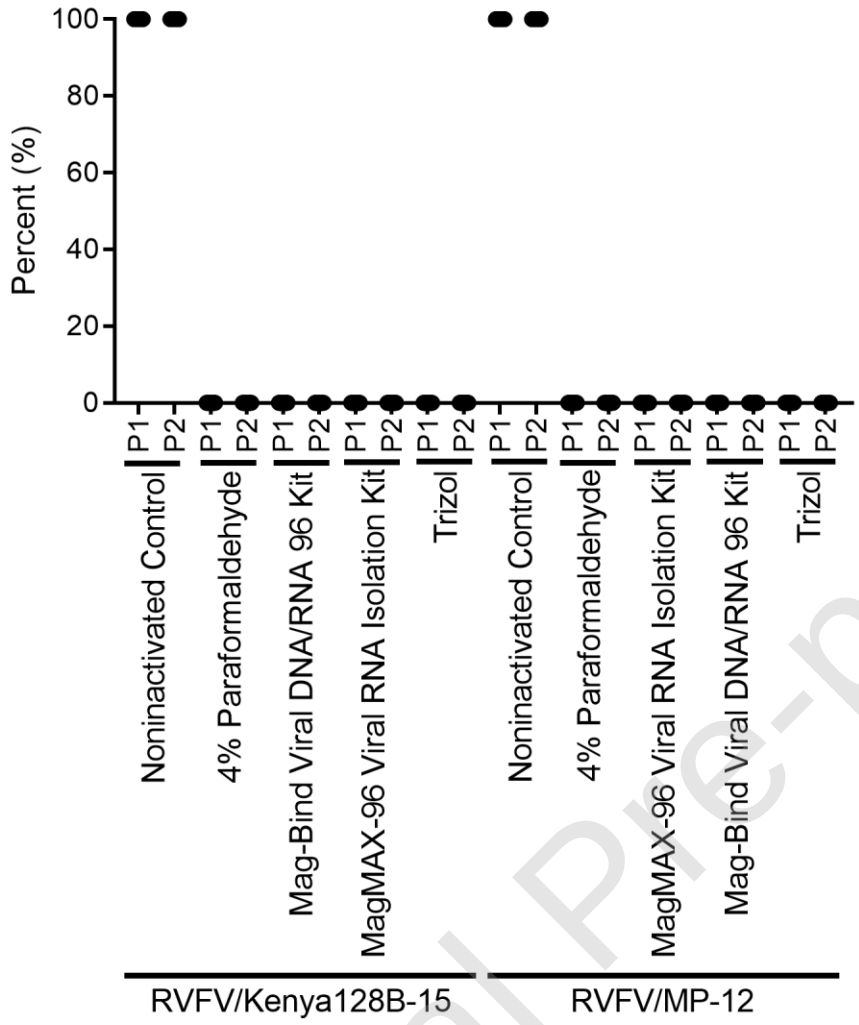

Figure 3. The percent of wells with visible CPE for each inactivation method for RVFV/Kenya128B-15 and RVFV/MP-12 (data represents one biological replicate). Every well was observed for CPE, the percentage reflects the amount of CPE present in the well as determined by the investigator.