Test for Infectivity of Inactivation Procedures for Positive-Strand RNA Viral Genomes

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Abstract

The United States Federal Select Agent Program (FSAP) oversees the possession, use and transfer of 68 biological select agents and toxins (BSAT), which have the potential to pose a severe threat to public, animal, or plant health. Among these agents are viruses that possess a positive single-stranded RNA (+ssRNA) genome, capable of serving as mRNA in cells to initiate virus replication, and therefore regulated by FSAP. For many laboratories, access to these agents is only possible if they are rendered inactive and no longer regulated. This can be achieved by complete physical, radiological or chemical inactivation of the agent (genome) followed by confirmatory testing using viability test and infectivity test protocols to ensure the treated nucleic acids are incapable of producing infectious viruses.

Using surrogate viruses for the select agents, here we report on infectivity assay assessments for three virus families with different-size +ssRNA genomes, namely the enterovirus EVD-68 (Picornaviridae, RNA size ~7.4kB), Venezuelan Equine Encephalitis virus (TC-83) (VEEV; Alphaviridae, RNA size ~12kB) and human NL63 (Coronaviridae, RNA size ~31kB) viruses. These viruses were inactivated by gamma irradiation followed by successful viability tests using two passages in a permissive cell line and observation for cytopathic effect (CPE). For infectivity testing, RNA extracted from treated viruses was transfected into cells using two independent methods (polyethyleneimine (PEI)-based and electroporation) to assess their ability to produce infectious viruses alongside control virus RNA from viable virus stocks. The results indicated that electroporation was much more efficient (>10-100X) than PEI for transfection of EVD-68 and VEEV TC-83 RNA controls, and ideal for transfection studies with viral RNA genomes. However, neither method of transfection was successful for the transfection of the large genome and reconstitution of viable coronavirus.

Study Plan

Virus Growth	Inactivation	Safety Testing	Transfection Testing
Growth of virus in susceptible host cells Clarification of virus	Virus inactivated: γ-irradiation (2 Mrad) Samples taken for inactivation testing	Incubate virus for 2 passages. 14 days each with appropriate controls Sampling at Day 0 and 14 post-inoculation	Pilot testing: PEI based and electroporation transfection Test samples: extracted viral RNA (vRNA): NR-50240: Zika Virus (PRVABC59) NR-470: HCoV (NL63)
QC Testing: Titer only	Virus frozen until testing	Confirmation of inactivation by CPE on permissive host cells and qPCR or IFA	Irradiated sample testing by electroporation Test Samples: extracted vRNA from irradiated VEEV TC-83 and EVD-68

Methods and Results

Pilot Testing:

Objective: Determine a protocol to use for both electroporation and PEI transfection and the lowest concentration of vRNA necessary for a successful transfection.

Procedure: Both viruses were individually tested by PEI and electroporation using extracted vRNA at various concentrations.

I. Transfection via Polyplus jetMessenger® PEI:

ZIKV vRNA: Successful transfection with 0.5µg (kit recommendation) and 1µg vRNA.

HCoV vRNA: Unsuccessful transfection as evidenced by lack of CPE including a second blind passage to confirm lack of virus propagation.

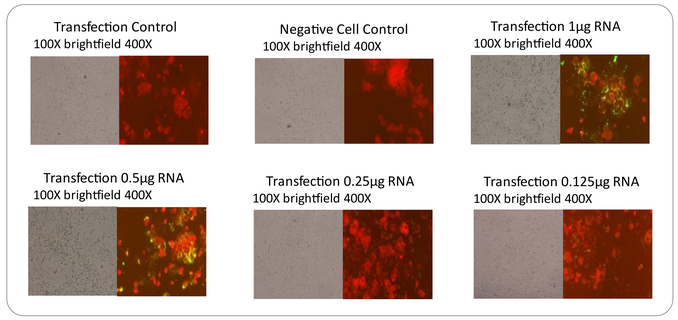

Figure 1. *Transfection of Zika Virus PRVABC59 RNA using jetMessenger® PEI. CPE (rounding and sloughing) and IFA positive somple are 0.5 and 1µg RNA in (CCL-81 ATCC®).*

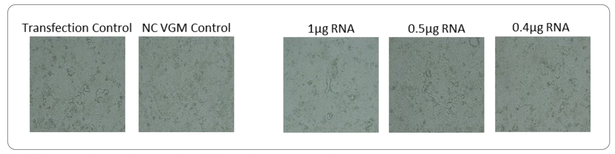

Figure 2. *CPE (cell rounding and sloughing) from HCoV NL63 is not detected in cells following jetMessenger® PEI* transfection in concentrations 1 µg, 0.5 µg, 0.4 µg RNA.

II. Transfection via Electroporation:

ZIKV vRNA: For Zika vRNA, a protocol of Square Wave transfection with a pulse field of 25 msec, voltage 120V, and a single pulse in a 0.2 cm electroporation cuvette with 300 ng, 200 ng, 100 ng, and 50 ng of vRNAs were electroporated into Vero (CCL-81 ATCC®) cells. Successful transfection all vRNA with CPE (cell rounding and sloughing) evident within 3 days of electroporation.

HCoV vRNA: Unsuccessful transfection as evidenced by lack of CPE for various square wave and exponential decay protocols tested. Exponential protocols had the most potential with an example provided below that appeared to have CPE detected after day 1 but with no progression upon subsequent incubation (Figure 4.) and complete recovery of the monolayer.

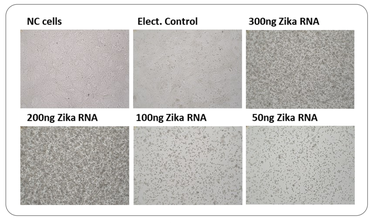

Figure 3. *Electroporation of ≥50ng Zika Virus RNA in Vero (CCL-81 ATCC®) cells. CPE was seen in all vRNA tested.*

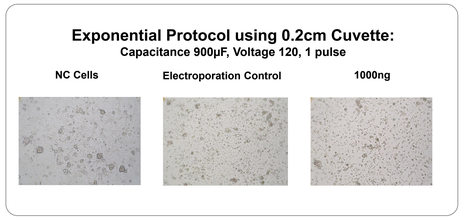

Figure 4. *Electroporation of 1000ng HCoV NL63 Virus RNA in LLC-MK2 (CCL-7.1 ATCC®) cells. CPE is not detected.*

Conclusion: The results indicated that electroporation was much more efficient (at least 10X) than the recommended protocol for jetMessenger® PEI mediated transfection, and was determined to be the best method for future transfection studies for vRNA genomes confirmation inactivation testing. Large vRNA genomes were not successfully transfected by either method so additional testing will be necessary.

Infectivity testing of γ-irradiatied extracted vRNA by electroporation:

Procedure: Electroporation parameters were Square Wave protocol, pulse time 25 msec, voltage 120V, and one pulse using 0.2 cm gap length sterile cuvettes.

Negative Controls: *Electroporation control:* Vero for VEEV TC-83 (CCL-81 ATCC®) or LLC-MK2 for EVD-68 (CCL-7.1 ATCC®) host cells prepared and electroporated with no viral RNA. *Cell Control:* cells prepared in electroporation buffer with no electroporation.

Positive Control: Extracted vRNA from VEEV TC-83 or EVD-68 at 1000ng, 200ng, 100ng, 50ng, 20ng and 10ng electroporated into Vero (CCL-81 ATCC®) or LLC-MK2 (CCL-7.1 ATCC®) cells.

Test Samples: Clarified γ-irradiated (2mRAD) extracted vRNA from 10 replicates of irradiated VEEV TC-83 or 6 replicates of irradiated EVD-68 for transfection.

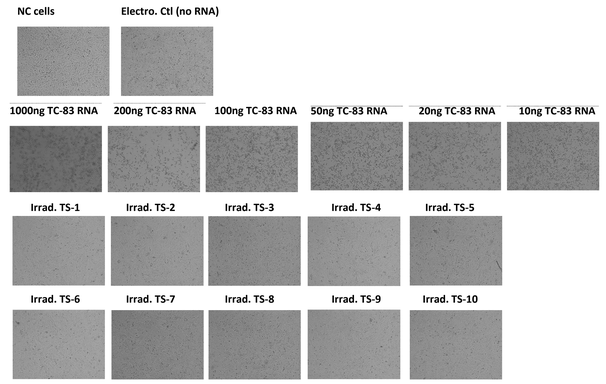

Figure 4. *Transfection by electroporation of control VEEV TC-83 (top 7 panels) and irradiated (irr.) VEEV TC-83 RNAs (bottom 10 panels). 1000ng of RNA were transfected in Vero cells in (CCL-81 ATCC®) and cell monolayers examined for CPE up to 14 days post-transfection.*

CPE was indicated in all the Positive Control samples (10ng to 1000ng RNA) and not seen in any of the irradiated samples tested.

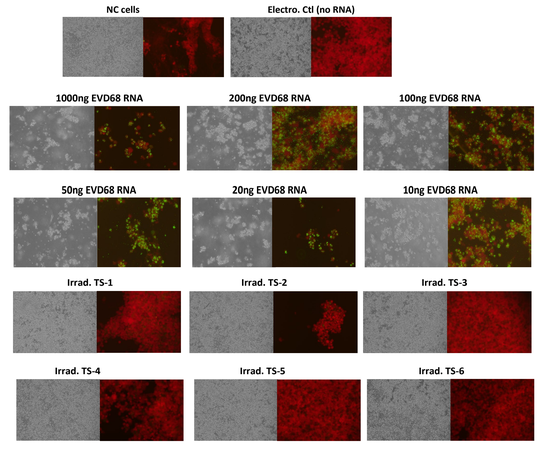

Figure 5. *Irradiation testing by electroporation of control (top 16 panels) EVD-68 RNAs (bottom 12 panels). 1000-10ng of RNA were transfected in RD (CCL-136 ATCC®) cells and cell monolayers examined for CPE up to 7 days post-transfection for positive controls and 12 days for all other samples.*

CPE was indicated in all the Positive Control samples (10ng to 1000ng RNA) and not seen in any of the irradiated samples tested.

Conclusions

The results indicate that electroporation could be an effective tool to assess the infectivity potential of small (EV-D68) and mid-size (VEEV-TC83) positive vRNA from irradiated viral material. However, this method was not successful for large genomes such as HCoV. Further studies will need to be completed to develop a protocol that would work for large genomes.

Future studies using these transfection approaches for testing inactivation treatments should also take into consideration the efficiency of the transfection method to monitor the potential presence of low-abundance intact RNA genomes and determination of methods to test large genome virus.

Acknowledgements

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