Determined Requirements to Ensure the Effectivity of Inactivated Venezuelan Equine Encephalitis

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Abstract

To ensure Biological Select Agents and Toxins (BSAT) are effectively inactivated, it is imperative to employ best practices in the development, validation, production, authentication, inactivation, traceability, and ultimate disposition of the material. Inactivated BSAT should be subject to the highest level of oversight and confirmation testing possible due to the potential risk of incompletely inactivated pathogens in downstream use under reduced containment. Implementing inactivation provisions for diverse agents and inactivation methods (e.g. heat, chemical, irradiation) has proven challenging since the effectiveness of the inactivation procedure and the viability testing approach used differs greatly between agents and test sample matrices.

In this project, we determined inactivation protocols (for inactivation by heat, chemical or y-irradiation) and pinpoint critical inactivation parameters for Venezuelan equine encephalitis virus (VEEV), TC-83 using pilot studies. Inactivation method parameters for VEEV were then validated by treatment of multiple replicate samples. To incorporate a safety margin, parameter set points were selected above effective pilot study parameters such as increased dose exposure time, elevated temperature or chemical concentration. The validated inactivation methods were then tested for effectiveness on a select agent strain (VEEV, Beck/Wycoff).

Using the surrogate method validation/verification test approach, we have identified parameters for three different methods of inactivation of VEEV. The validated heat inactivation method parameters determined for the surrogate VEEV, TC-83, were successfully transferred and verified on the select agent strain VEEV, Beck/Wycoff. Formalin inactivation of VEEV, TC-83 was accomplished using centrifugal filter units for buffer exchange of formaldehyde with PBS following treatment, and the inactivation method was successfully applied to VEEV, Beck/Wycoff. Finally, y-irradiation doses were validated with VEEV, TC-83 and effective parameters were successfully transferred and verified on the select agent VEEV, Beck/Wycoff.

Method

Set points for temperature, time, and concentration were established using published literature and internal data (Table 1). Inactivation was determined through CPE observation in permissible host cells, plaque formation in culture, and qPCR.

Virus production/titration/innocuity: For the pilot and validation study, surrogate VEEV, TC-83, an attenuated subtype IA/B isolate (BEI Resources NR-63) was grown on Vero cells (ATCC© CCL-81), clarified after harvest and frozen in aliquots. Titration in the same cell line yielded a titer of 8.91 x 10^9PFU/mL.
For verification study, VEEV, Beck/Wycoff (BEI Resources NR-21720), was grown on Vero cells (ATCC© CCL-81), clarified after harvest and frozen in aliquot. Titration in the same cell line yielded a titer of 2.81 x 10^8 $TCID_{50}$/mL.

Infectivity detection by CPE: Inactivated material was passaged on host cells and observed for 14 days. CPE (syncytia, cell rounding) was graded on a 0 to 4+ ranking system and was used as a means of detecting viral infection. Pilot study material was passaged once, and validation/verification material was passaged twice.

Infectivity detection by plaque assay: Inactivated material was serially diluted and plated on host cells. Following adsorption, an overlay was added, and plates were incubated for 2 days prior to staining. Plaques were counted and recorded to determine the limit of detection.

Confirmation of inactivation by qPCR: Each replicate was sampled on day 0 and day 14, and extracted (QIAGEN, QIAamp® Viral RNA Mini Kit). Increasing Ct values from day 0 to day 14, as evidence of non-replicative virus, were used to confirm viral inactivation (Invitrogen, SuperScript™ III Platinum™One-Step qRT-PCR, primers and probe developed internally).

Table 1: Inactivation Parameters.

Study	Heat Inactivation		Chemical Inactivation		y-irradiation
	Temperature (˚C)	Time (minutes)	Concentration (%)	Time (hour)	Dose (Mrad)
Pilot	55	0, 10, 20, 30, 60	0	2	0.2
	60	0, 10, 20, 30, 60	0.5	2	0.5
			1.0	2	1
			1.5	2	2
			2.0	2	control
Validation/ Verification	**65**	**30**	**2.0**	**2**	**2**

VEEV, TC-83 Validation Study/VEEV, Beck/Wycoff Verification Study:

Validation of surrogate VEEV, TC-83 inactivation was confirmed using 6 replicates for heat inactivation and 10 replicates each for chemical and y-irradiation (Table 1). Two passages of each treated material were performed to allow for delayed amplification of virus and to increase detection sensitivity to ensure recovery of any viable virus. Detection was determined by CPE and PCR for both passages and plaque infection for passage 1.

Ten independent replicates of VEEV, Beck/Wycoff were inactivated per method using the same parameters as the VEEV, TC-83 validation (Table 1).

Results

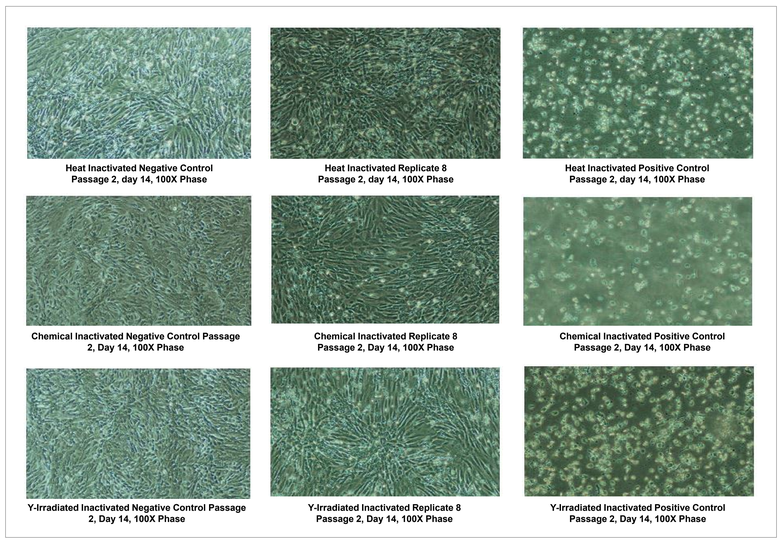

Figure 1. *Representative photographs of negative controls, sample replicates, and positive controls for each method of inactivation of VEEV, Beck/Wycoff.*

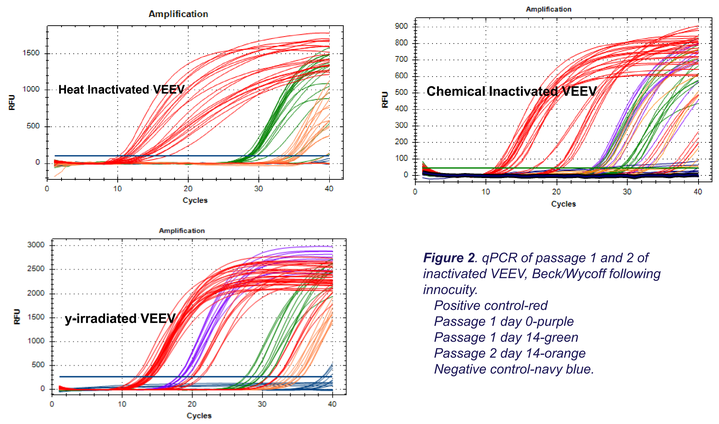

Figure 2. *qPCR of passage 1 and 2 of inactivated VEEV, Beck/Wycoff following innocuity.*
Positive control-red
Passage 1 day 0-purple
Passage 1 day 14-green
Passage 2 day 14-orange
Negative control-navy blue.

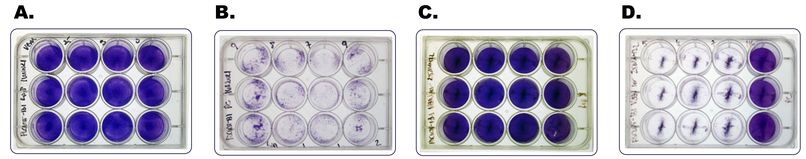

Figure 3. *VEEV, Beck/Wycoff plaque assay (A) following heat inactivation, (B) controls for heat inactivation, (C) following y-irradiation, (D) controls for y-irradiation. Treated material dilutions: neat (undiluted), 1:5, 1:25, NC. Control dilutions: 1:100,000, 1:1,000,000, 1:10,000,000.*

Conclusions

Inactivation methods determined during surrogate (VEEV, TC-83) validation studies were successfully used to inactivate select agent (VEEV, Beck/Wycoff) with no modifications to the parameters and protocols.

The sensitivity of methods used to determine inactivation must be considered when developing protocols for validation purposes. Interpretation of CPE alone can be highly subjective due to degradation of cell monolayer over time, which may lead to false-positive. These studies require confirmatory methods, such as qPCR, to affirm the results.

Multiple passages of treated material with prolonged incubations should be used to identify low levels of virus that may survive inactivation methods.

Future studies would include screening inactivated material using applicable molecular, antigenic, or biochemical tests to determine retention of sufficient molecular integrity for research use.

Acknowledgements

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