Summary of Validated and Verified Viral Inactivation Methods

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Disclaimer

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Outline

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• Background on requirements
• Buffer AVL Validation
• SDS Buffer Validation
• Formalin Validation
• Trizol LS Validation
• Conclusions
In July 2016, Army Directive 2016-24 was published:
- Required validation of any BSAT inactivation process with in house testing data
- Any inactivated material movement from registered to unregistered space could not occur without Biosafety, RO and Command approval

Many requirements in AD 2016-24 are now found in latest 42 CFR updated regulations

USAMRIID needed to develop validation testing data for inactivation protocols to continue operations
Background

• Many impediments to developing validation datasets
  – Time
  – Resources (monetary and personnel)
  – Methods to remove toxic chemicals affecting cell culture
  – Selection of viruses to serve as surrogates
Background

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• Three methods to remove toxic chemicals for virus cell culture
  – Concentrating columns
  – Desalting columns
  – Dilution
VALIDATION STUDIES
Buffer AVL Methodology

• TC83, a RG2 enveloped virus, was grown and concentrated with an Amicon Ultra-15 Centrifugal Filter Unit with a molecular weight cutoff size of 100kDa
• TC83 selected for high viral titer potential and quick propagation relative to other viruses
• Non-concentrated viral titer was 9.8E9 pfu/mL
• Concentrated viral titer was 4.4E11 pfu/mL
• NHP plasma was spiked with the concentrated virus at a 3:1 ratio to create plasma containing 1.1E11 pfu/mL
Buffer AVL Methodology

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37.5µl NHP Plasma + 12.5uL of concentrated TC83 in three replicates

↓

Add 200uL AVL

↓

Apply 125uL to 145cm² dishes of Veros

↓

Incubate 24 hours

↓

Transfer >50% of supernatant to new dish of Veros

↓

Incubate 24 hours

↓

Detect virus by IFA

↓

37.5µl NHP Plasma + 12.5uL of concentrated TC83 in three replicates

↓

Add 200uL AVL

↓

Heat for 30 minutes by submersion in a 56°C water bath

↓

Apply 125uL to 145cm² dishes of Veros

↓

Incubate 24 hours

↓

Transfer >50% of supernatant to new dish of Veros

↓

Incubate 24 hours

↓

Detect virus by IFA
Buffer AVL Methodology

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- Supernatent was collected from the dishes and applied to Hela cells in a 96 well plate format for 18 hours before fixing in 10% neutral buffered formalin for one hour.
- Formalin was washed from the plates twice with PBS
- Plates were blocked with 3% BSA in PBS for 1 hour at ambient temperature
- Plates were incubated with mouse 1A4A anti-VEE antibody at a 1:1000 dilution in 3% BSA in PBS for 1 hour at ambient temperature
- Plates were washed 3x with PBS
- Secondary antibody of DyLight goat anti-mouse 488 (ThermoFisher catalog #35502) was added to the plates at a 1:1000 dilution in 3% BSA in PBS and incubated for 1 hour at ambient temperature
- Plates were washed 3x with PBS and Hoescht and Cell Mask Deep Red were added at a 1:10000 dilution in PBS to stain cell nuclei and cytoplasm
- Image analysis was obtained using an Opera confocal reader (model 5025-Quadruple Excitation High Sensitivity [QEHS])
Buffer AVL Results

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Percent Positive Cells after Passaging Inactivated Samples

- AVL Sample 1
- AVL Sample 2
- AVL Sample 3
- AVL Heat Sample 1
- AVL Heat Sample 2
- AVL Heat Sample 3
- Mock Passage
- 50 pfu Passed
- 500 pfu Passed
- 5000 pfu Passed
- Unprocessed Sporadic Passed
- Pulsed Virus Passed
- IFN Mock
- MOI 0.1
- MOI 1
Buffer AVL Results

Cell Number after Passaging Inactivated Samples

- AVL Sample 1
- AVL Sample 2
- AVL Sample 3
- AVL Heat Sample 1
- AVL Heat Sample 2
- AVL Heat Sample 3
- Mock Passage
- 50,000 pfu Passed
- 50,000 pfu Passed
- IFA Mock
- MDI 0.1
- MDI 1

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Buffer AVL Results (IFA)

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TC 83
Infected
Cells stain green

Multiplicity of Infection 0.1 IFA Control
Buffer AVL Results (IFA)

Blue cells indicate absence of infection

Mock Infection Control
Buffer AVL Results (IFA)

Green cells indicate presence of live TC83

AVL Buffer alone is insufficient to inactivate virus
Buffer AVL Results (IFA)

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AVL Buffer with heat sufficiently inactivates virus
**SDS Buffer Methodology (JUNV)**

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6 well plates + MOI of 0.4 JUNV in three replicates

- Add 350uL 1.15x SDS
- Apply 500uL to 145cm² dishes of Veros
  - Incubate 72 hours
  - Transfer >66% of supernatant to new dish of Veros
    - Incubate 72 hours
    - Detect virus by IFA

6 well plates + MOI of 0.00005 JUNV in three replicates

- Apply 125uL to 145cm² dishes of Veros
  - Incubate 24 hours
  - Apply 500uL to 145cm² dishes of Veros
    - Incubate 72 hours
    - Transfer >66% of supernatant to new dish of Veros
      - Incubate 72 hours
      - Detect virus by IFA

Detect virus by IFA
SDS Buffer Results (JUNV)

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Cell Number for Validation of SDS Inactivation of Junin Infected Cells

- Heat 10' 1
- Heat 10' 2
- Heat 10' 3
- No Heat 10' 1
- No Heat 10' 2
- No Heat 10' 3
- Untreated Cells 10' 1
- Untreated Cells 10' 2
- Untreated Cells 10' 3
- S0001 treated 10' 1
- S0001 treated 10' 2
- S0001 treated 10' 3
- S0001 treated 10' 4
- S0001 treated 10' 5
- Mock

Cell Number
SDS Buffer Results (JUNV)

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Percent Antibody Positive Cells after Passaging SDS Inactivated Junin Infected Cell Samples
SDS Buffer Results IFA (JUNV)

50 pfu/well JUNV Infection Control
SDS Buffer Results (JUNV)

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SDS buffer is effective at inactivating JUNV with or without heat.
SDS Buffer Methodology (MERS)

6 well plates+ MOI 5 MERS

- Incubate 72 hours and remove 150 uL supernatant
- Add 50uL 4x SDS with reducing agent
- Apply 500uL to 145cm² dishes of Veros
- Incubate 72 hours
- Heat for 3 minutes at 95°C
- Apply 500uL to 145cm² dishes of Veros
- Incubate 72 hours
- Transfer >66% of supernatant to new dish of Veros
- Incubate 72 hours
- Transfer >66% of supernatant to new dish of Veros
- Incubate 72 hours
- Detect virus by IFA

6 well plates+ MOI of 1.5 or 0.000005 MERS in three replicates

- Apply 125uL to 145cm² dishes of Veros
- Incubate 72 hours
- Transfer >66% of supernatant to new dish of Veros
- Incubate 72 hours
- Detect virus by IFA
SDS Buffer Results (MERS)

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SDS Buffer Results (MERS)

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MOI 1.5 MERS Infection Control
SDS Buffer Results IFA (MERS)

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SDS with 95°C Heat

SDS without 95°C Heat

SDS buffer is effective at inactivating MERS with or without heat
Formalin Methodology (Cells)

6 well plates+ MOI 5 (MERS/EBOV)

- Incubate 48 hours
- Washed once with PBS
- Submersion in formalin
- Incubate 24 hours
- Washed 3x with PBS
- Cells scraped and transferred to 500 uL PBS

Apply 500uL to 145cm² dishes of Veros
- Incubate 72 hours
- Transfer >66% of supernatant to new dish of Veros
- Incubate 72 hours
- Detect virus by IFA

6 well plates+ MOI of 1.5 or 0.000005 (MERS/EBOV) in three replicates

- Apply 125uL to 145cm² dishes of Veros
- Incubate 72 hours
- Transfer >66% of supernatant to new dish of Veros
- Incubate 72 hours
- Detect virus by IFA
Formalin Results (Cells)

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Percent Antibody Positive Cells from
Formalin Inactivated MERS Infected
Cell Monolayer Supernatant Infection

Percent Antibody Positive Cells from
Formalin Inactivated Ebola Infected Cell
Monolayer Supernatant Infection
Formalin Results (Cells)

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Infection from sample of 5 pfu/well MERS at beginning of passaging

Infection from sample of 5 pfu/well EBOV at beginning of passaging
Formalin Results (Cells)

MOI 5 Infected MERS cells following 24 h exposure to formalin

MOI 5 Infected EBOV cells following 24 h exposure to formalin
Formalin Methodology (Tissue)

Liver removed from NHPs succumbing to EBOV infection (titers between $10^7$ and $10^8$ pfu/mL)
- Fixed in formalin for 30d
- ~1cm$^3$ taken from liver
- Washed in PBS 2X
- Homogenization
- Placed in 5mL EMEM with 10% FBS, 1% Pen Strep, and 1% Fungizone
- 15% of total volume applied to T75 flasks of Vero Cells
- Incubate for 7 days
- Transfer >50% of volume to new dish of Veros
- Incubate for 7 days
- Transfer 100uL to HeLa cells
- Incubate 48 hours
- Detect virus by IFA

Liver removed from NHPs succumbing to EBOV infection (titers between $10^7$ and $10^8$ pfu/mL)
- Homogenization
- Placed in 5mL EMEM with 10% FBS, 1% Pen Strep, and 1% Fungizone
- 15% of total volume applied to T75 flasks of Vero Cells
- Incubation and IFA conditions same as formalin fixed samples
Formalin Results (Tissue)

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Infection from sample of 5 pfu/well EBOV at beginning of passaging
Unfixed, unfiltered EBOV liver samples demonstrating infection
Formalin Results (Tissue)

Fixed, unfiltered EBOV liver samples demonstrating successful inactivation
Trizol LS Methodology (Version 1)

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75 µl NHP Plasma + 25uL of concentrated TC83 in three replicates

↓

Add 300uL Trizol LS

↓

Dilute to 50mL in PBS

↓

Spin/concentrate to ~200-400uL

↓

Spin/wash/concentrate 3x with 12mL PBS each wash

↓

Apply 100% of final contents (~200-400uL) to Vero cells for viral amplification

↓

Incubate 24 hours, passage >50% supernatant, incubated 24 hours, then detect virus by IFA
Trizol LS Methodology (Version 2)

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Add TRIzol LS treated Zika (R4577T) at 1:1000 in fresh media to a HYPRflask (0.56 ml per flask, 0.14 ml of which is virus and 0.42 ml is TRIzol LS)

↓

Incubate 48 hours

↓

Pour off all media and replace with fresh media

↓

Incubate 5 days

↓

Harvest passage one and use 50% for passage two.

↓

Incubate 7 days

↓

Plaque assay analysis
Take 4 mL of TRIzol treated Zika (R4577T) and use a Zebra column to buffer exchange out the TRIzol LS and into completed media (MEM + 10% FBS).

Add the product (100%) of the Buffer exchange into one HYPRflask, ~4mL.

Incubate 7 days

Take 50% of passage 1 for passage 2

Incubate 7 days

Plaque assay analysis
Trizol LS Results (Version 1)

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Percent Positive Cells after Passaging Inactivated Samples

- Trizol LS Sample #1
- Trizol LS Sample #2
- Trizol LS Sample #3
- Mock Passaged
- 5,000 pfu Passaged
- 50,000 pfu Passaged
- Unprocessed Supernatant
- Filtered Vivos
- IRA Mock
- MOI 0.1
- MOI 1
Trizol LS Results (Version 1)

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Cell Number after Passaging Inactivated Samples
Trizol LS Results (Version 1)

MOI 1.0 TC83 Infection Control
Efficacy of Trizol LS inactivation confirmed across three replicates
## Trizol LS Results (Versions 2 and 3)

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### Version 2 using dilution method

<table>
<thead>
<tr>
<th>Sample + 560 mL media</th>
<th>Media Exchanged on Day 2?</th>
<th>Passage 1, Day 7 Observed CPE</th>
<th>Titer on D7 by Plaque Assay</th>
<th>Passage 2, Day 7 Observed CPE</th>
<th>Titer on D7 by Plaque Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM Only</td>
<td>No</td>
<td>None</td>
<td>None Detected</td>
<td>None</td>
<td>None Detecte</td>
</tr>
<tr>
<td>Target 100 PFU Zika</td>
<td>Yes</td>
<td>1+</td>
<td>1.08E+7</td>
<td>4+</td>
<td>1.60E+7</td>
</tr>
<tr>
<td>Actual = 60</td>
<td></td>
<td></td>
<td>5.75E+7</td>
<td></td>
<td>1.65E+7</td>
</tr>
<tr>
<td>Target 10 PFU Zika</td>
<td>No</td>
<td>Minimal (+/-)</td>
<td>8.00E+7</td>
<td>4+</td>
<td>9.50E+6</td>
</tr>
<tr>
<td>Actual = 4.4</td>
<td></td>
<td></td>
<td>2.30E+7</td>
<td></td>
<td>1.13E+7</td>
</tr>
<tr>
<td>Target 1 PFU Zika</td>
<td>No</td>
<td>None</td>
<td>1.50E+6</td>
<td>4+</td>
<td>5.75E+5</td>
</tr>
<tr>
<td>Actual = 0.5</td>
<td>Yes</td>
<td>None</td>
<td>2.53E+7</td>
<td>4+</td>
<td>3.53E+6</td>
</tr>
<tr>
<td>Target 0.1 PFU Zika</td>
<td>No</td>
<td>None</td>
<td>None Detected</td>
<td>None</td>
<td>None Detected</td>
</tr>
<tr>
<td>Actual = 0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Version 3 using desalting columns

<table>
<thead>
<tr>
<th>Sample + 560 mL media</th>
<th>Media Exchanged on Day 2?</th>
<th>Passage 1, Day 7 Observed CPE</th>
<th>Titer on D7 by Plaque Assay (PFU/mL)</th>
<th>Passage 2, Day 7 Observed CPE</th>
<th>Titer on D7 by Plaque Assay (PFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>Yes</td>
<td>None</td>
<td>None Detected</td>
<td>None</td>
<td>None Detected</td>
</tr>
<tr>
<td>0.56mL R4577T (Flask A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method 1</td>
<td>Yes</td>
<td>None</td>
<td>None Detected</td>
<td>None</td>
<td>None Detected</td>
</tr>
<tr>
<td>0.56mL R4577T (Flask B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method 1</td>
<td>Yes</td>
<td>None</td>
<td>None Detected</td>
<td>None</td>
<td>None Detected</td>
</tr>
<tr>
<td>0.56mL R4577T (Flask C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method 1</td>
<td>Yes</td>
<td>None</td>
<td>None Detected</td>
<td>None</td>
<td>None Detected</td>
</tr>
<tr>
<td>4mL of R4577T after Zebra buffer Exchange into MEM.</td>
<td>No</td>
<td>None</td>
<td>None Detected</td>
<td>None</td>
<td>None Detected</td>
</tr>
</tbody>
</table>

Version 3 using desalting columns
Conclusions

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• USAMRIID has been able to verify efficacy of different toxic chemicals against viruses through dilution, concentration and desalting columns
• Dilution methods are effective for validation of both Trizol LS and formalin based inactivation assays but sample volume is limiting factor
• Both IFA and plaque assays are effective at verifying absence of virus with low LODs (≤5pfu/mL)
• Extensive resources are required at the Institute level to carry out these studies
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**Mission**
Provide leading edge medical capabilities to deter and defend against current and emerging biological threat agents

**Vision**
To be the leader in the advancement of medical biological defense with world renowned experts dedicated to protecting our military forces and the nation

**Medical Biological Defense Insurance Policy for the Nation**