Summary of Validated and Verified Viral Inactivation Methods

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USAMRIID
United States Army Medical Research Institute of Infectious Diseases
Biodefense solutions to protect our nation

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Outline

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- Background on requirements
- Buffer AVL Validation
- SDS Buffer Validation
- Formalin Validation
- Trizol LS Validation
- Conclusions

Background

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- 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

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- 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

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- 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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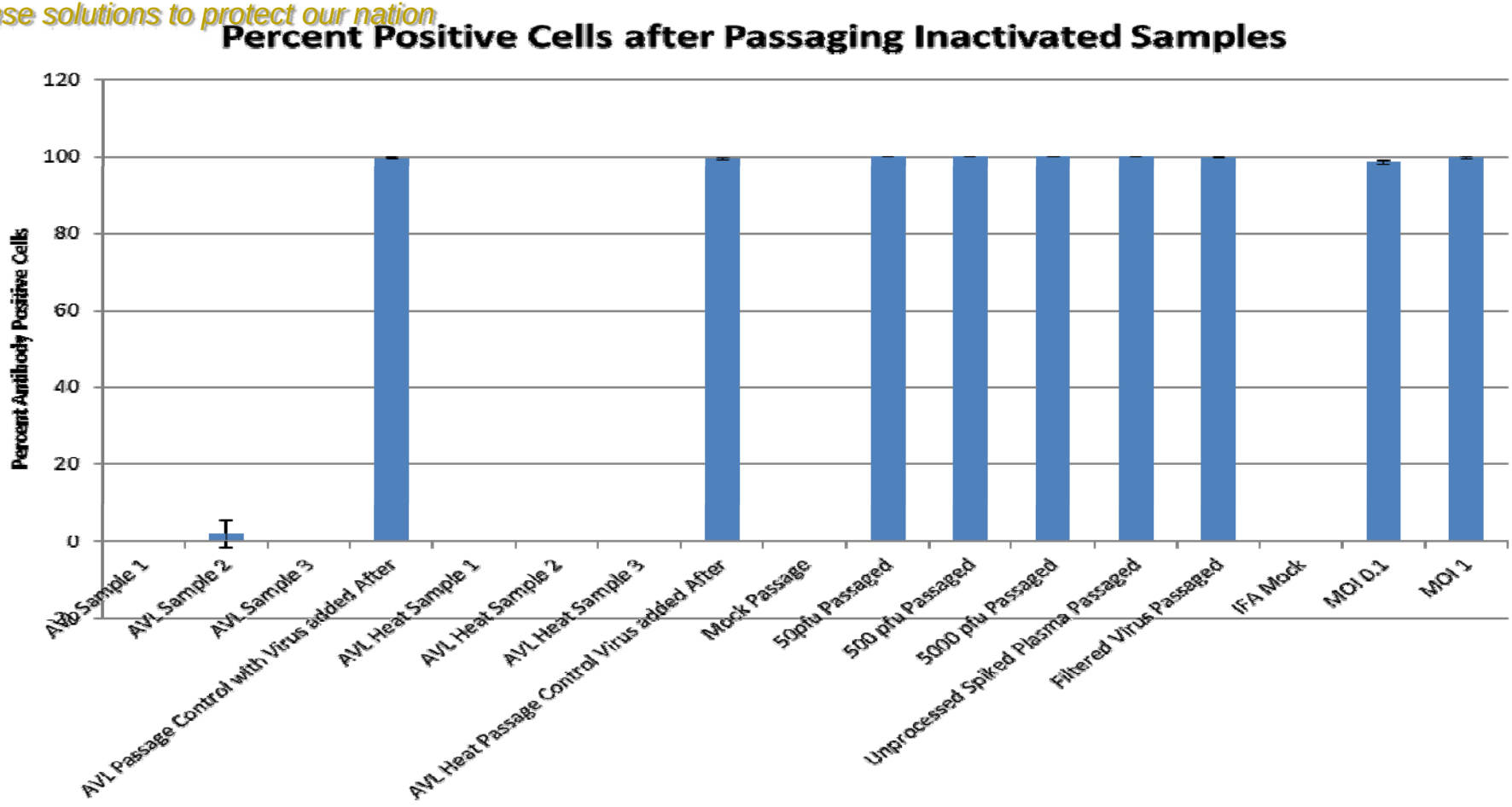

Buffer AVL Results

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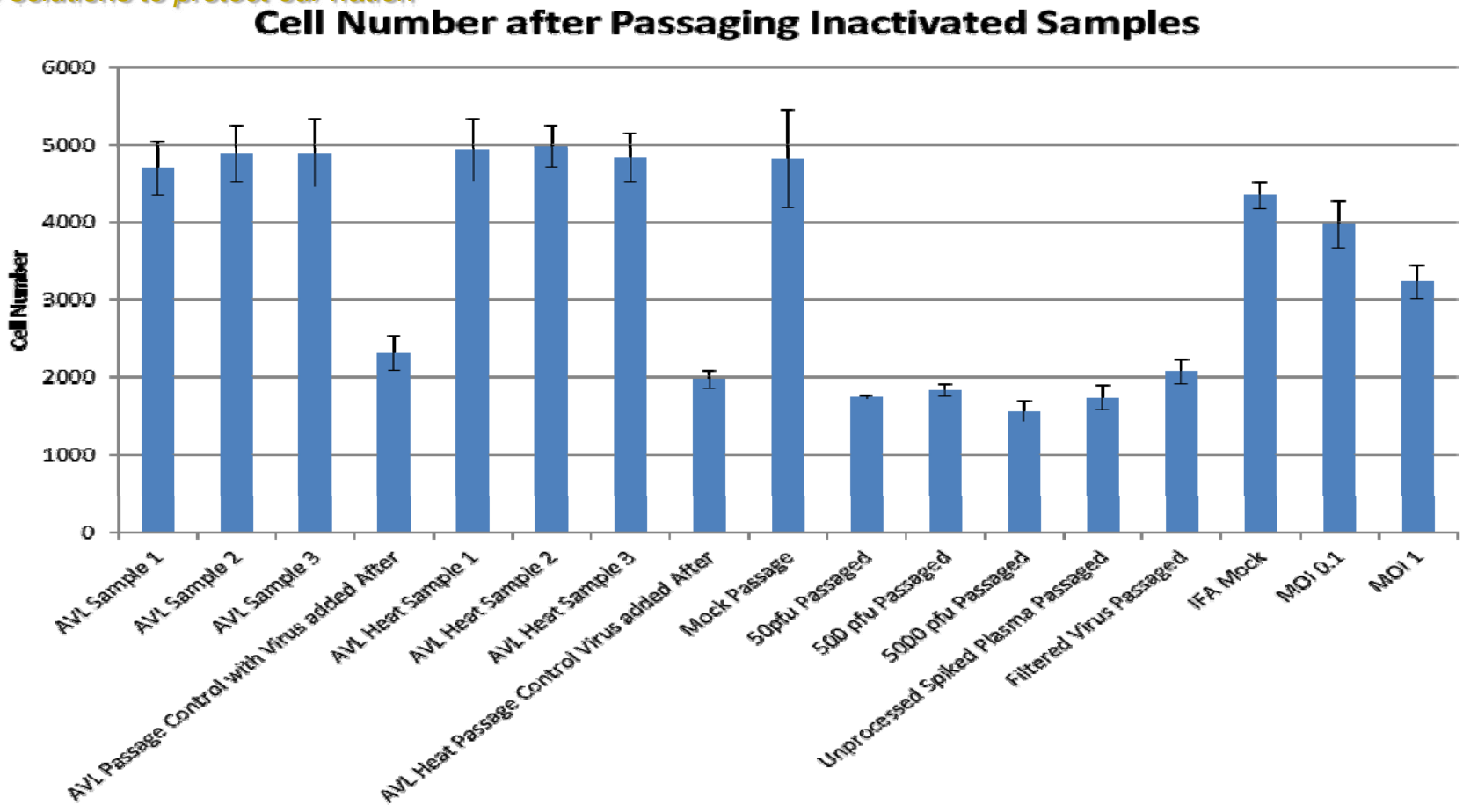

Buffer AVL Results (IFA)

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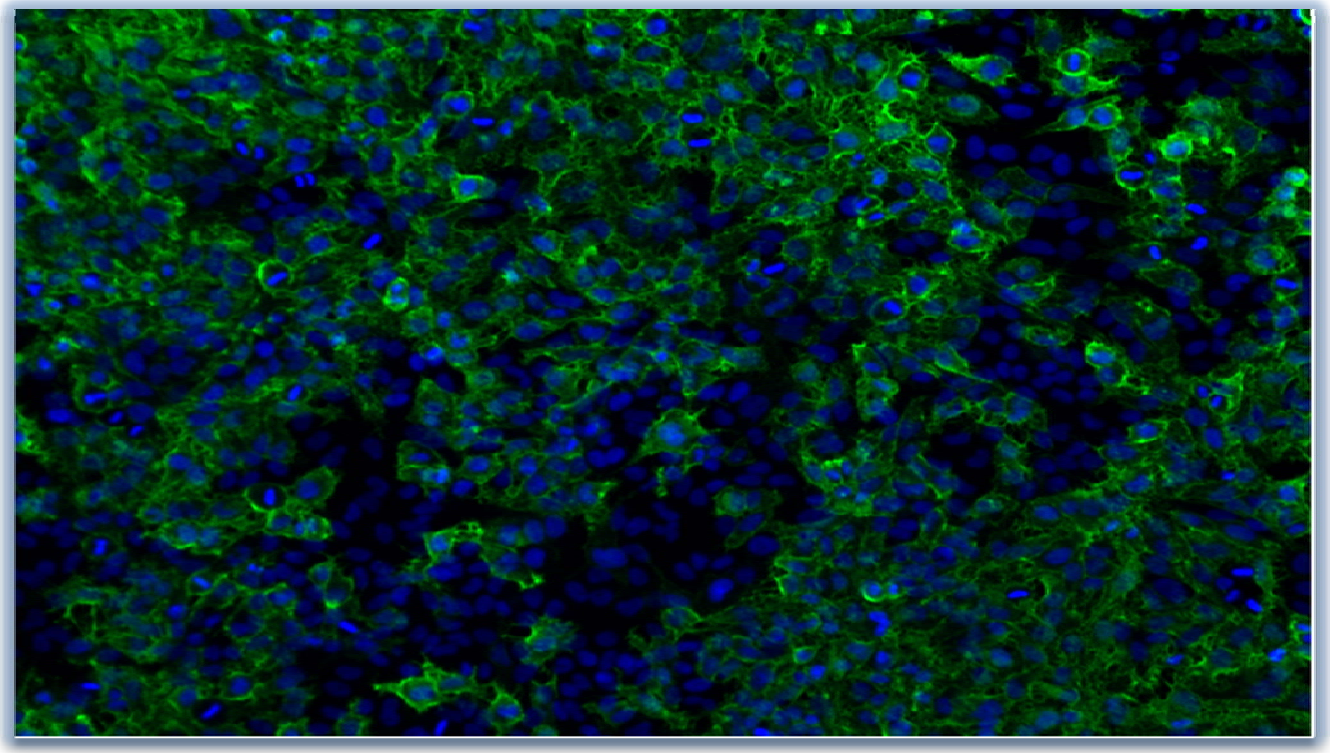
TC 83 Infected Cells stain green



Multiplicity of Infection 0.1 IFA Control

Buffer AVL Results (IFA)

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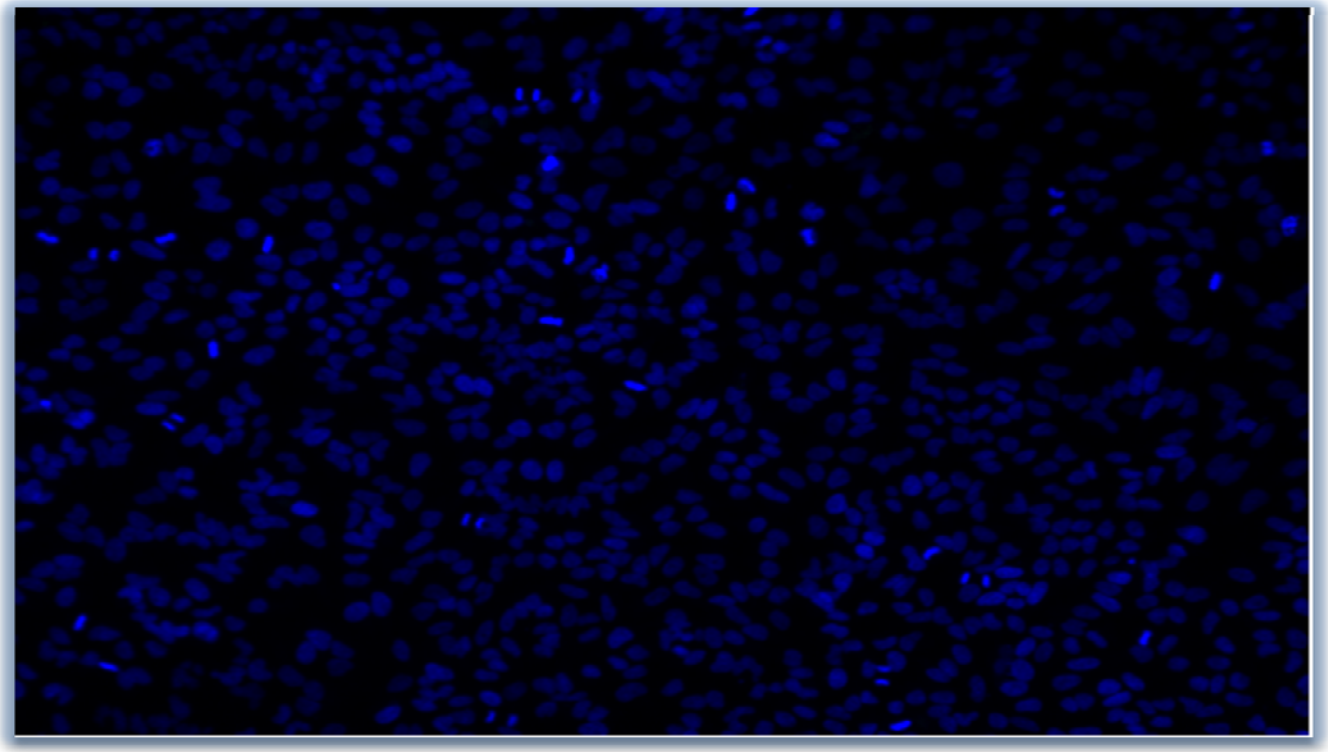
Blue cells indicate absence of infection



Mock Infection Control

Buffer AVL Results (IFA)

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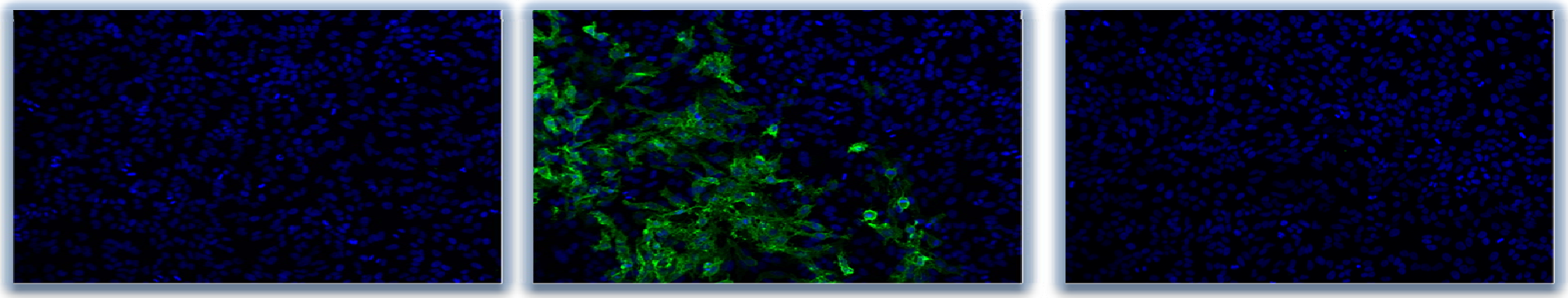

Green cells indicate presence of live TC83

AVL Buffer alone is insufficient to inactivate virus

Buffer AVL Results (IFA)

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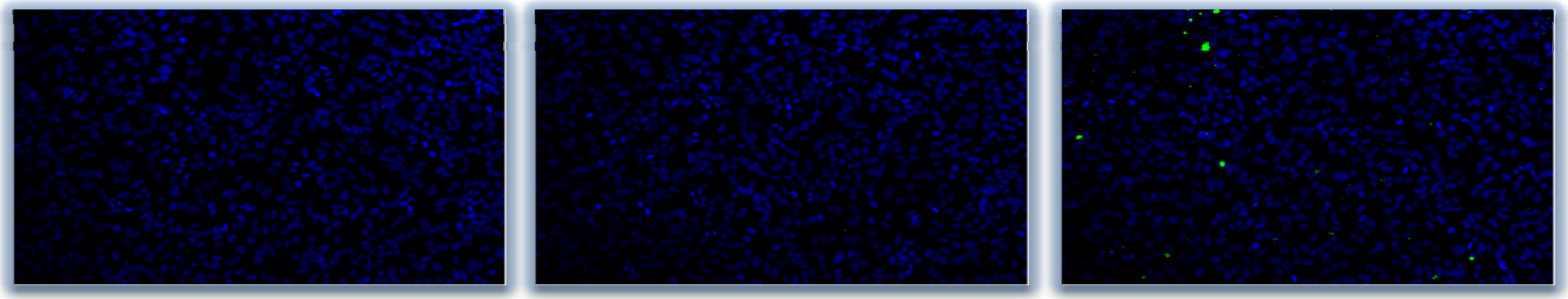

AVL Buffer with heat sufficiently inactivates virus

SDS Buffer Methodology (JUNV)

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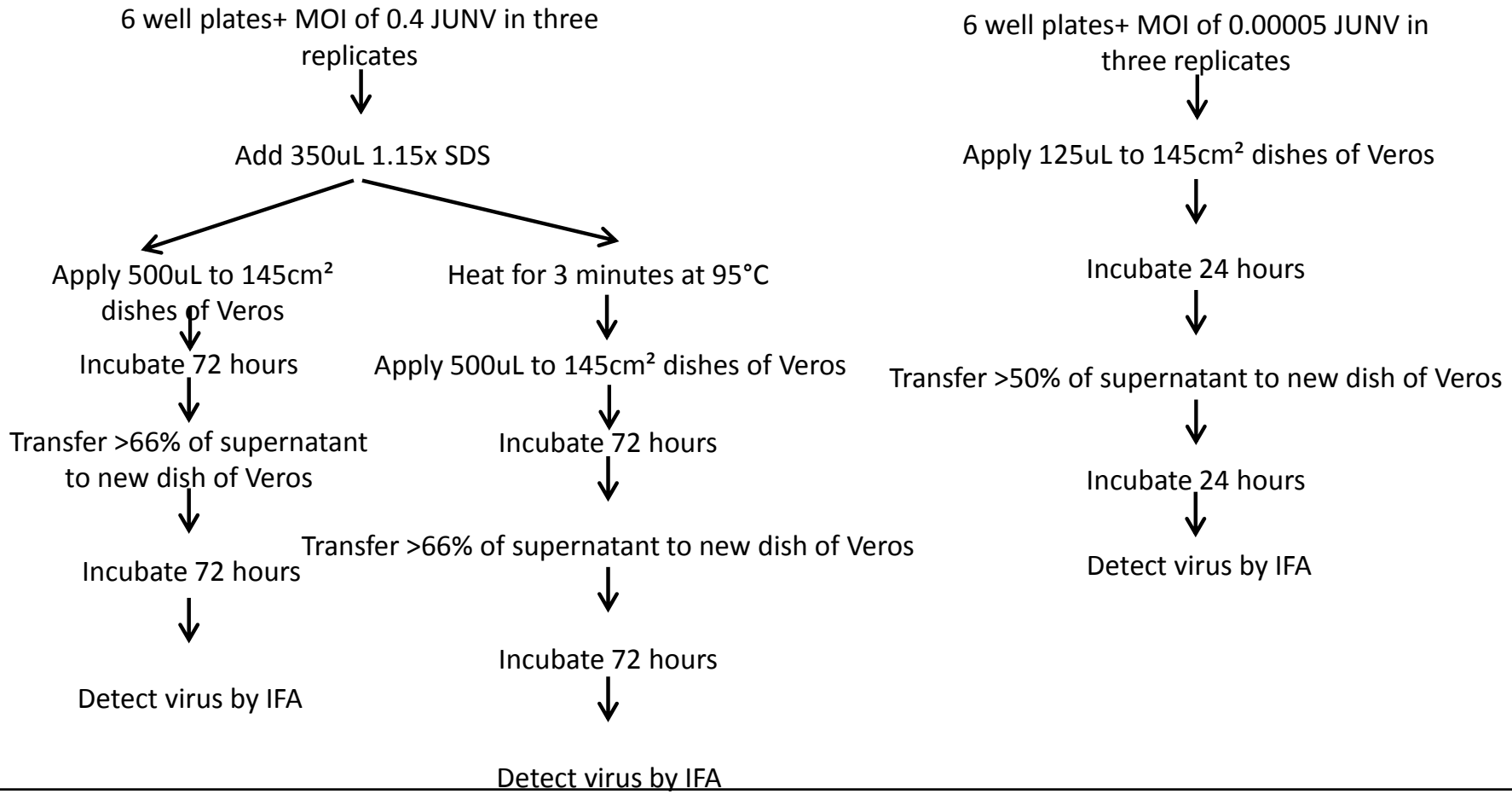

SDS Buffer Results (JUNV)

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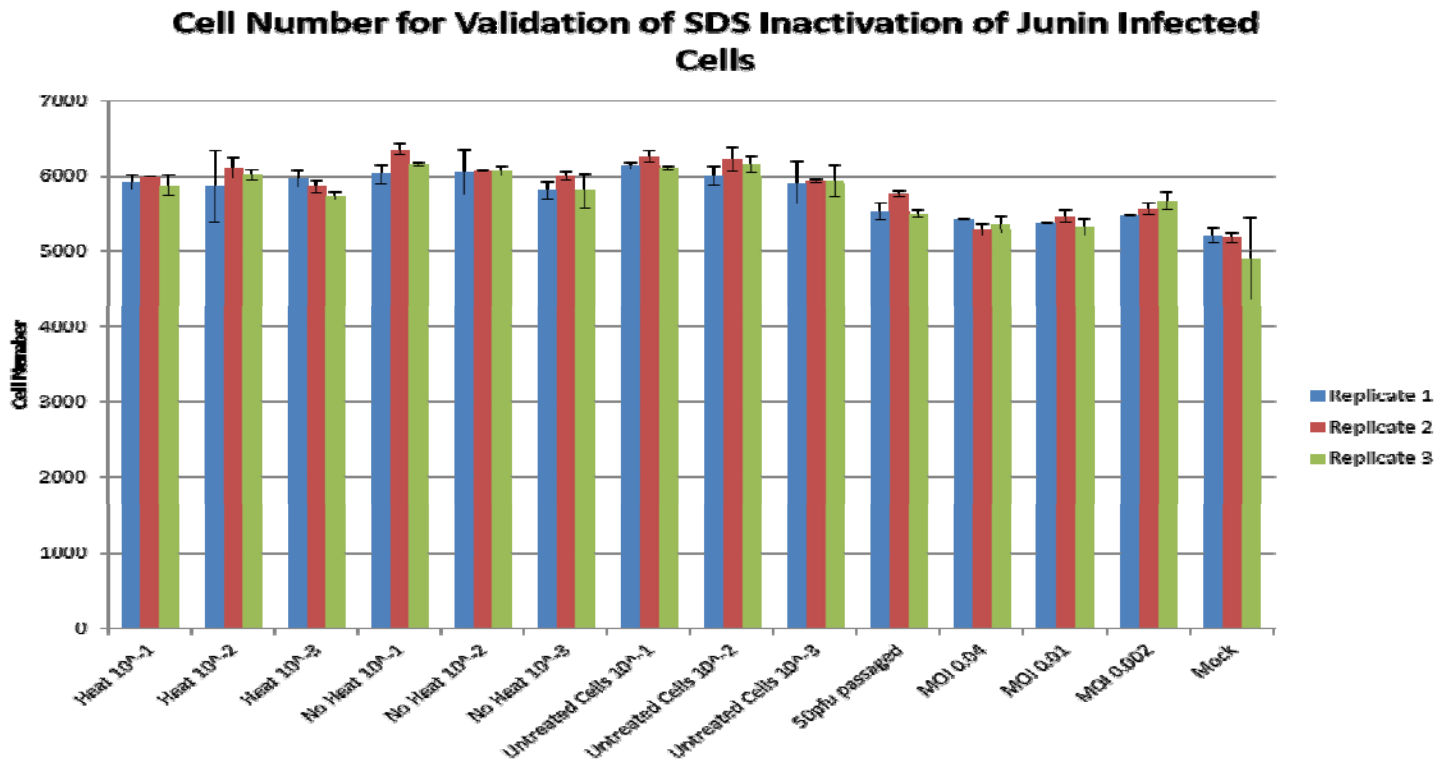

SDS Buffer Results (JUNV)

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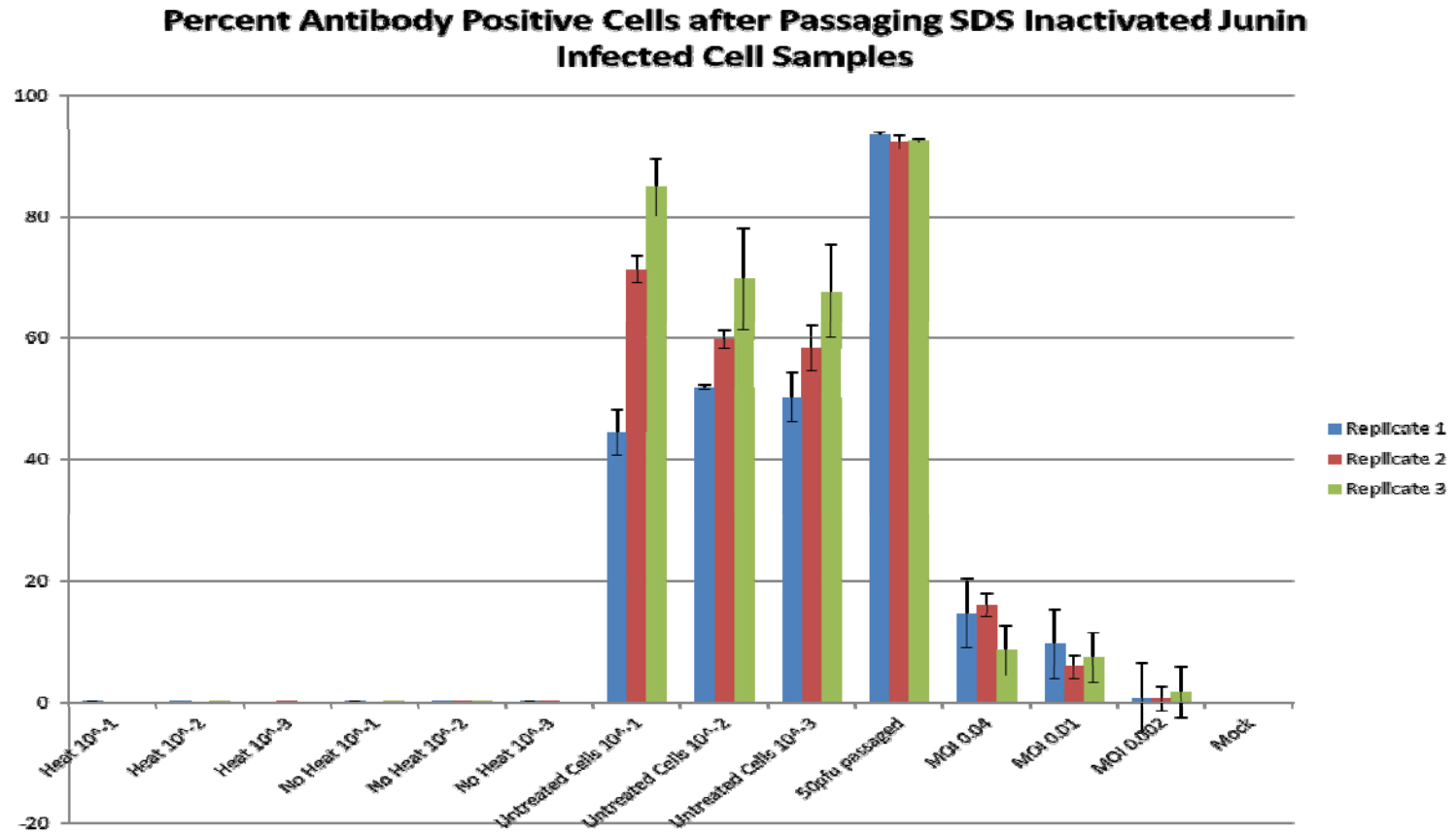

SDS Buffer Results IFA (JUNV)

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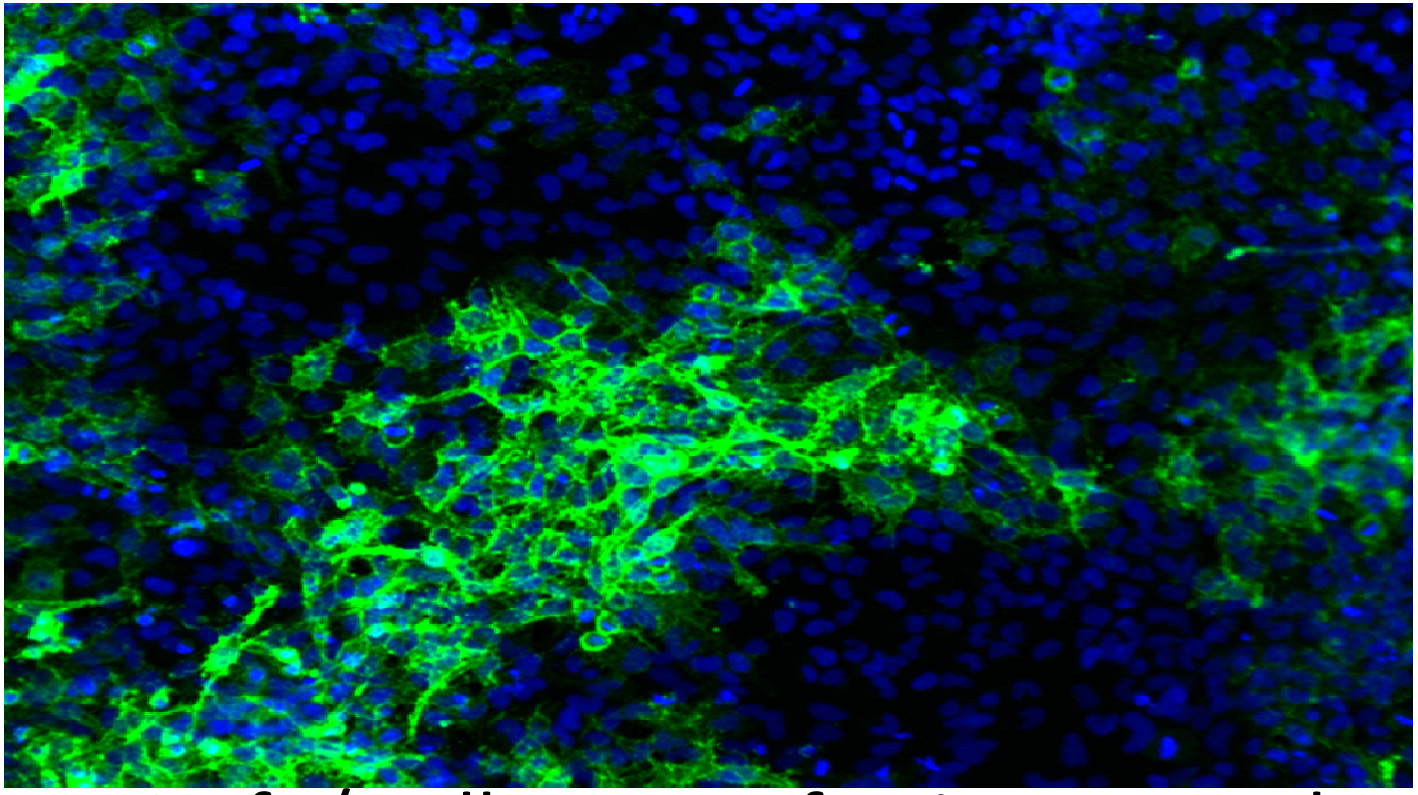

50 pfu/well JUNV Infection Control

SDS Buffer Results (JUNV)

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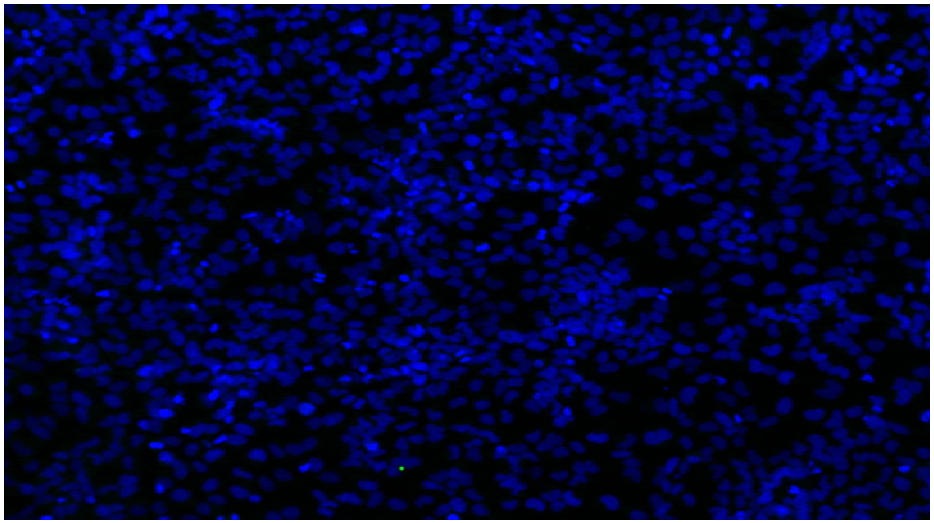
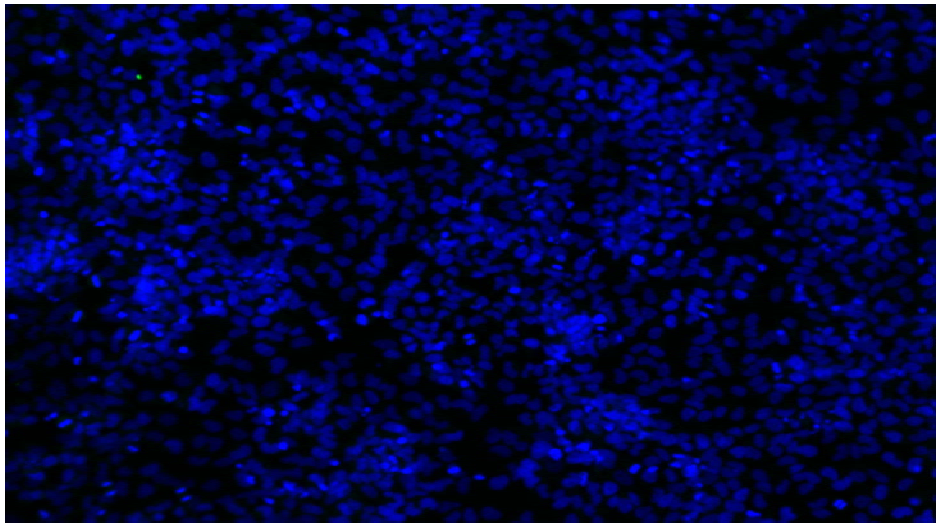


SDS with 95°C Heat



SDS without 95°C Heat

SDS buffer is effective at inactivating JUNV with or without heat

SDS Buffer Methodology (MERS)

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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

↓

Transfer >66% of supernatant to new dish of Veros

↓

Incubate 72 hours

↓

Detect virus by IFA

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

↓

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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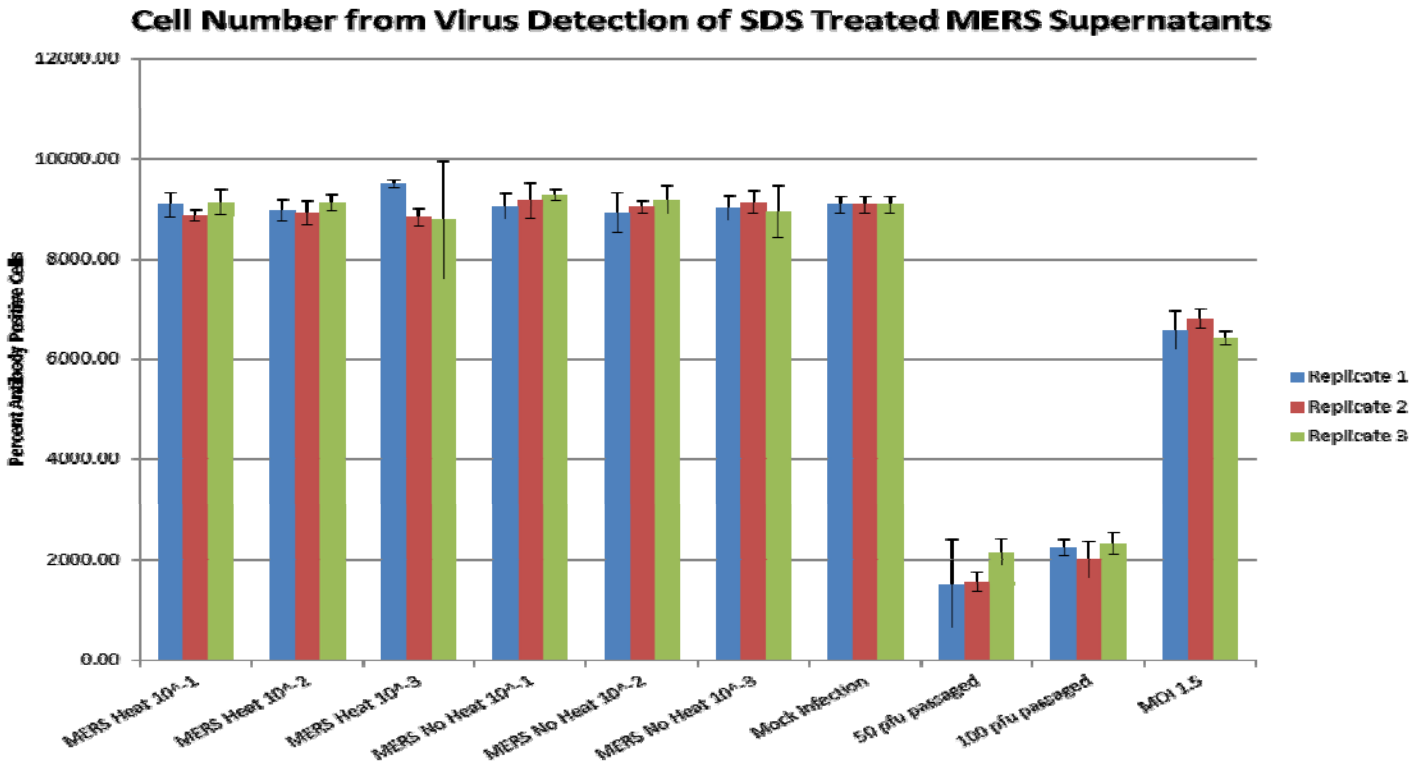

SDS Buffer Results (MERS)

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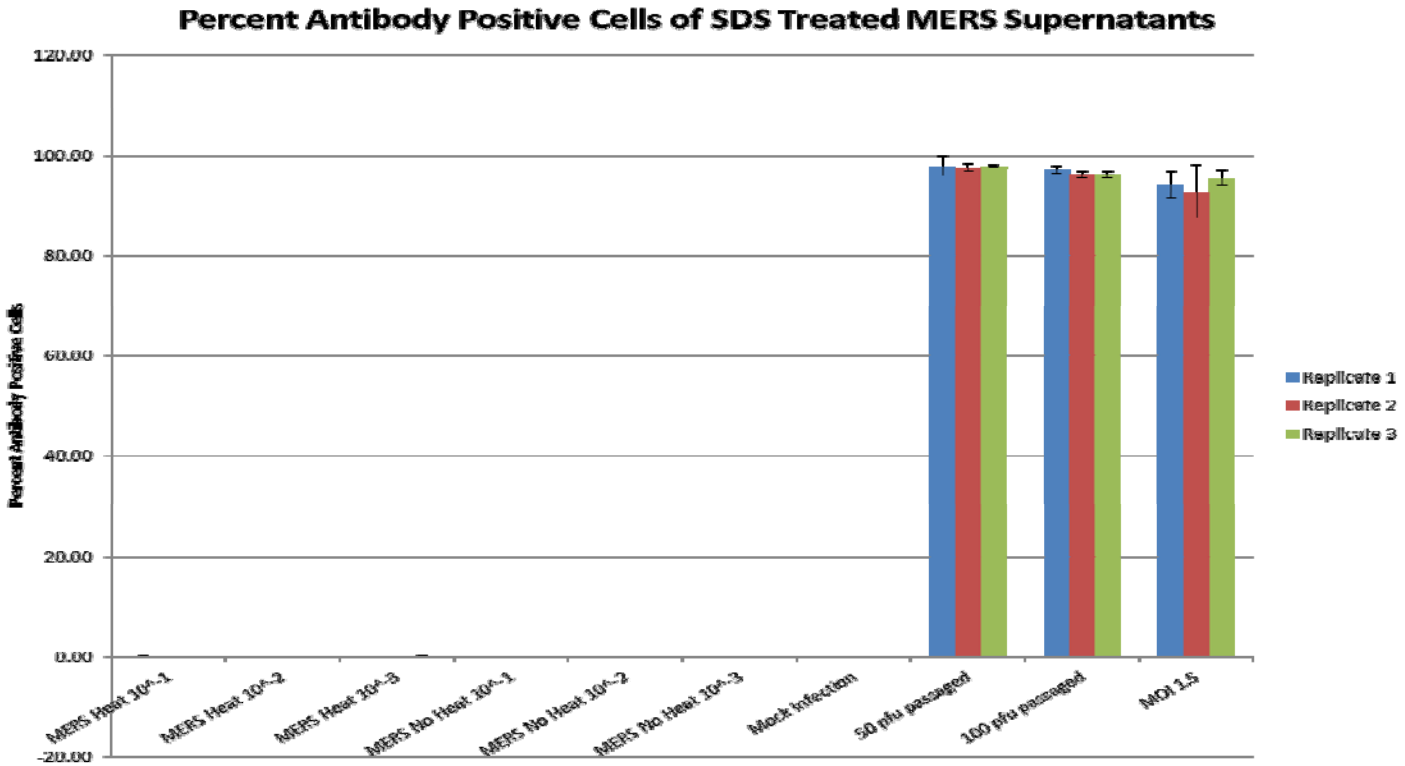

SDS Buffer Results IFA (MERS)

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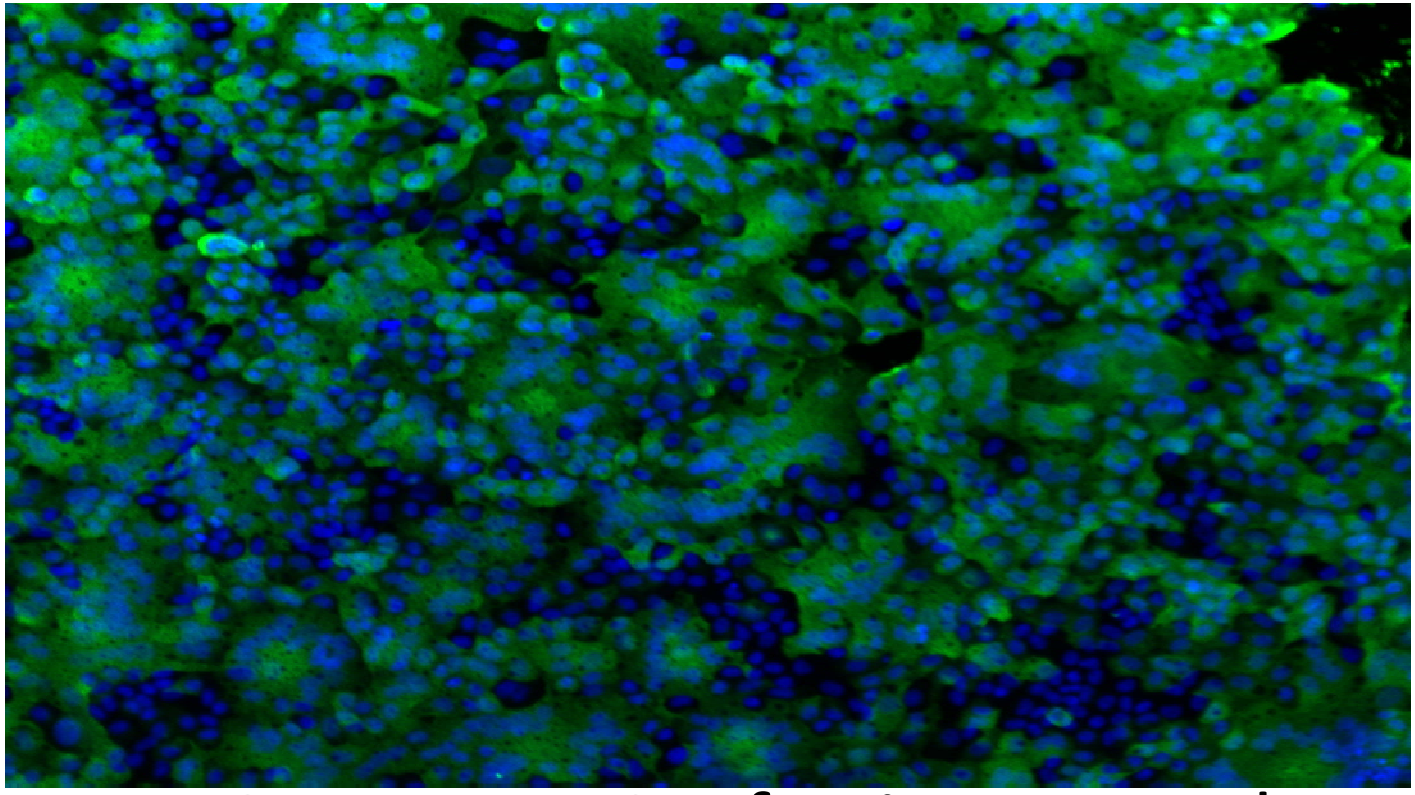

MOI 1.5 MERS Infection Control

SDS Buffer Results IFA (MERS)

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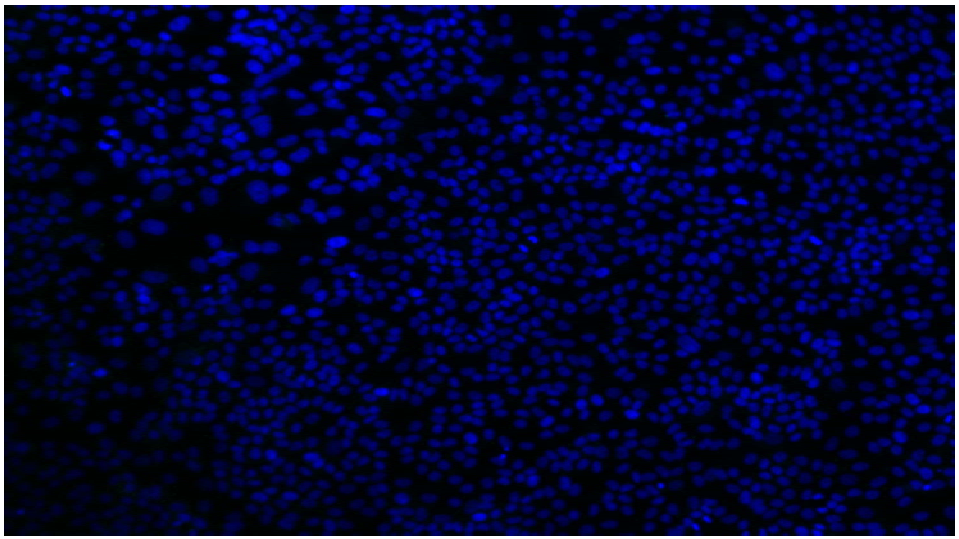
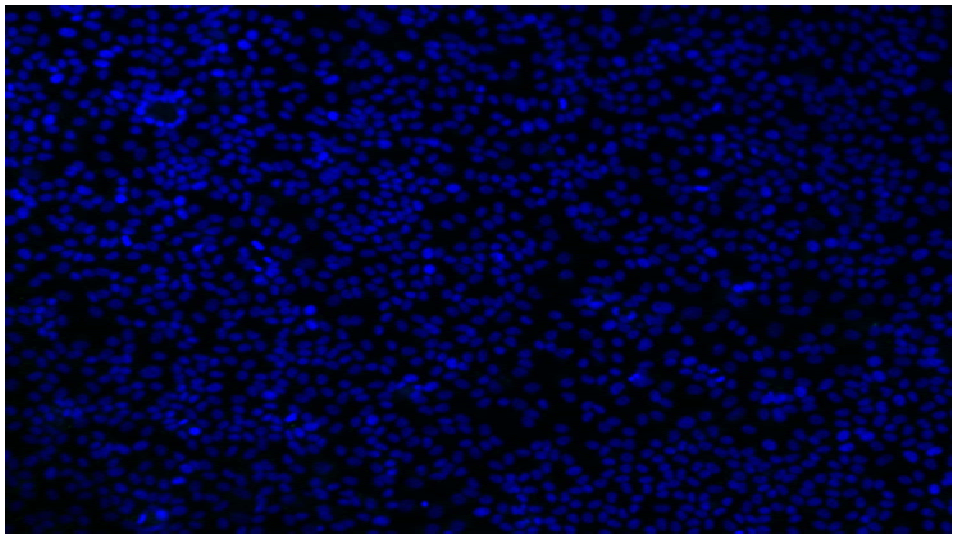


SDS with 95°C Heat



SDS without 95°C Heat

SDS buffer is effective at inactivating MERS with or without heat

Formalin Methodology (Cells)

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6 well plates+ MOI 5 (MERS/EBOV)

↓

Incubate 48 hours

↓

Washed once with PBS

↓

Submersion in formalin

↓

Incubate24 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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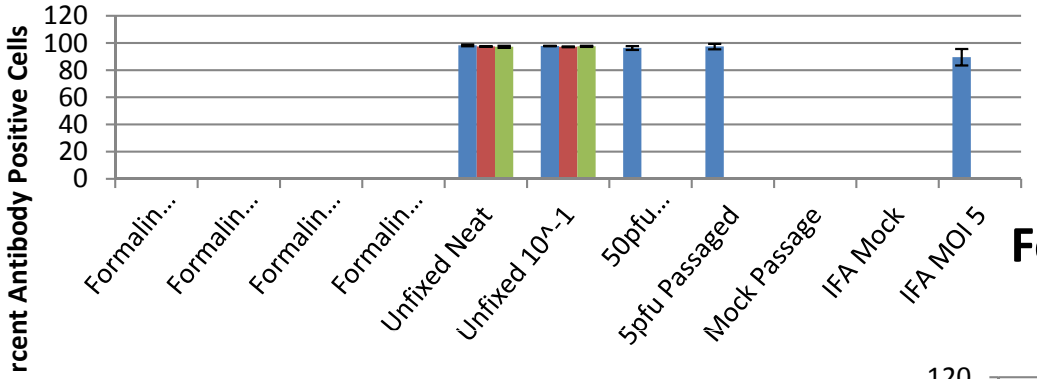
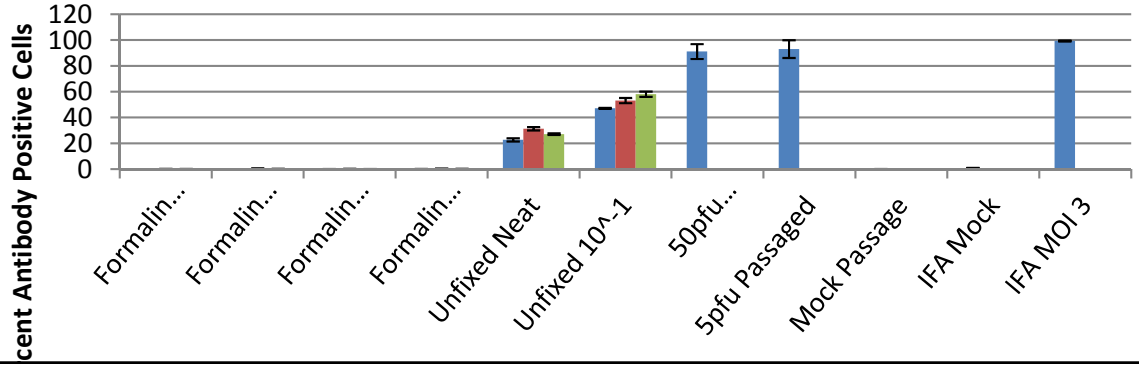
Percent Antibody Positive Cells from Formalin Inactivated MERS Infected Cell Monolayer Supernatant Infection



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



Formalin Results (Cells)

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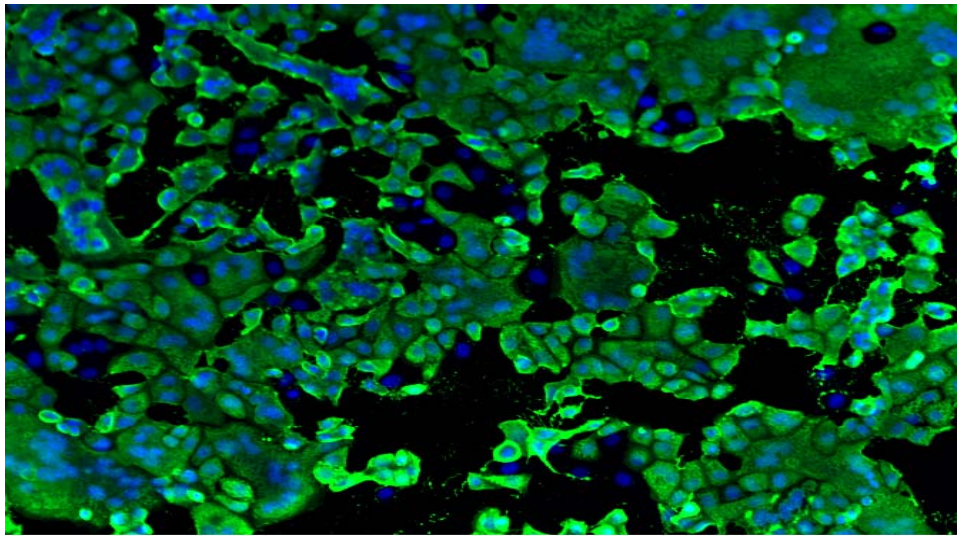
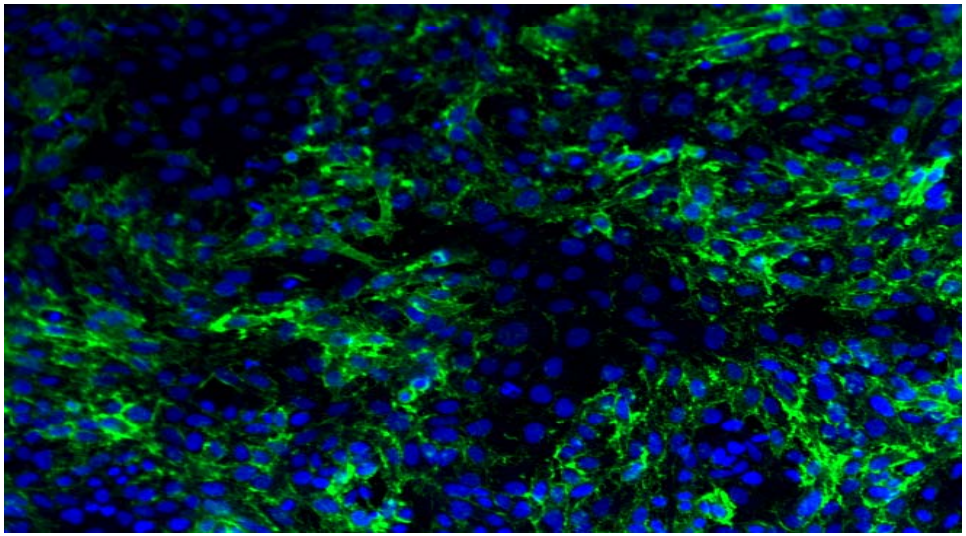


Infection from sample of 5 pfu/well MERS at beginning of passaging



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

Formalin Results (Cells)

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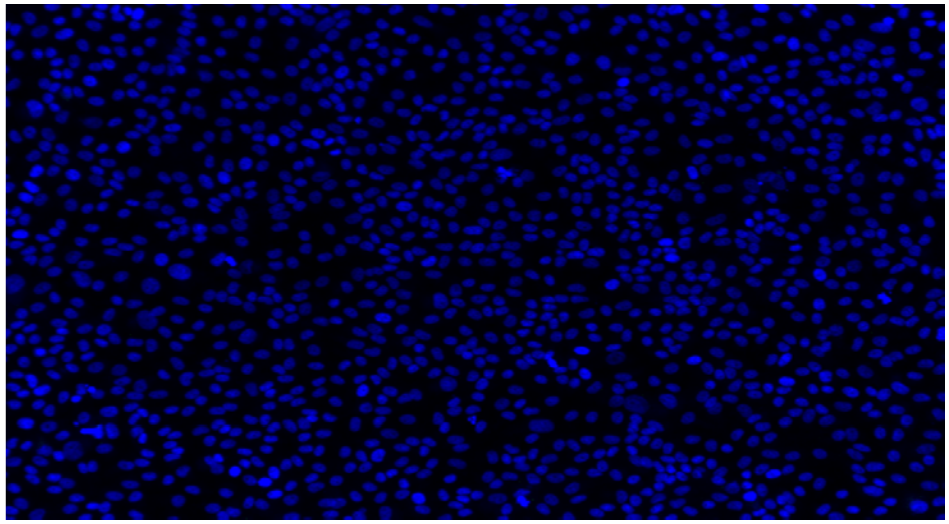
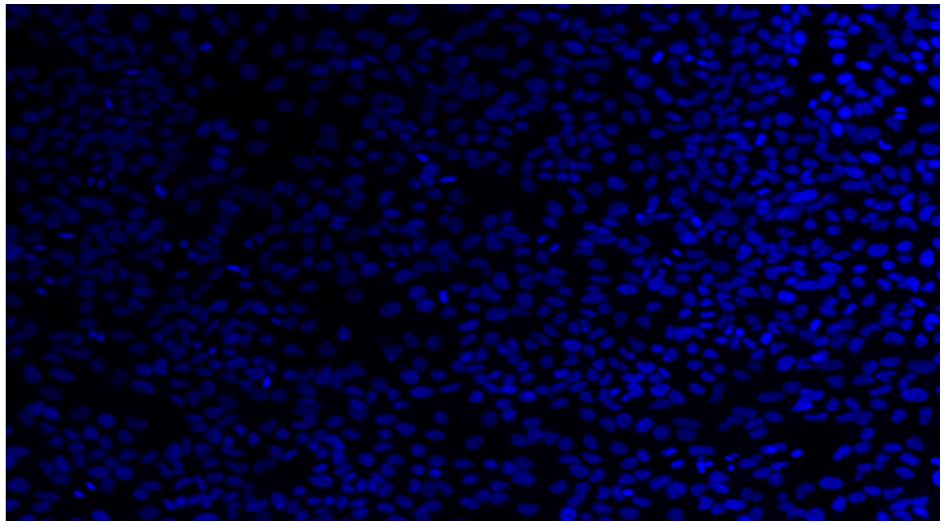


MOI 5 Infected MERS cells following 24 h exposure to formalin



MOI 5 Infected EBOV cells following 24 h exposure to formalin

Formalin Methodology (Tissue)

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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

Added to 10mL PBS and spun down in Amicon Ultra-15 Centrifugal Filter Unit

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

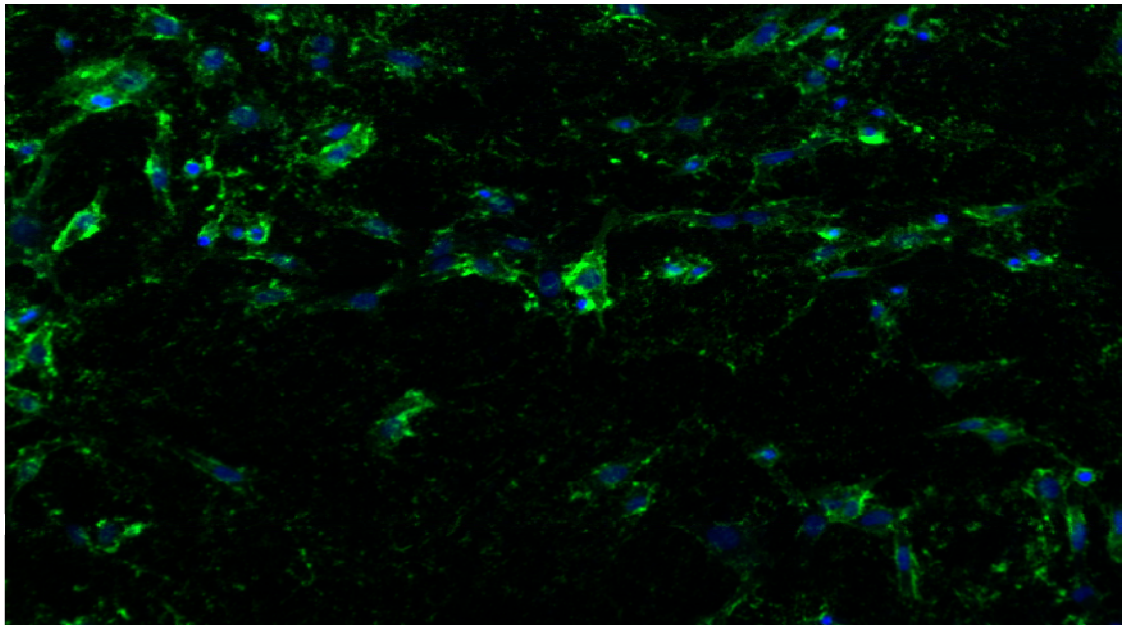
Placed in 5mL EMEM with 10% FBS, 1% Pen Strep, and 1% Fungizone

15% of total volume applied to T75 flasks of Vero Cells

Incubation and IFA conditions same as formalin fixed samples

Formalin Results (Tissue)

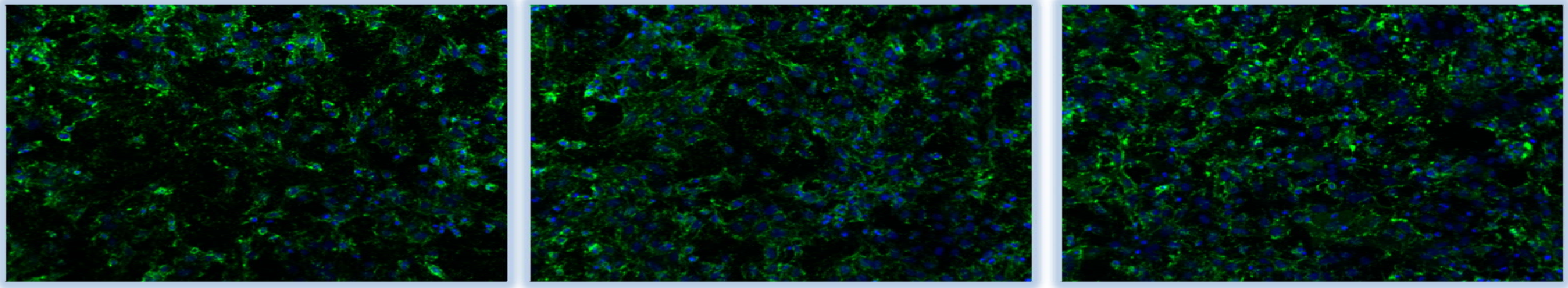
Biodefense solutions to protect our nation



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

Formalin Results (Tissue)

Biodefense solutions to protect our nation



Unfixed, unfiltered EBOV liver samples demonstrating infection

Formalin Results (Tissue)

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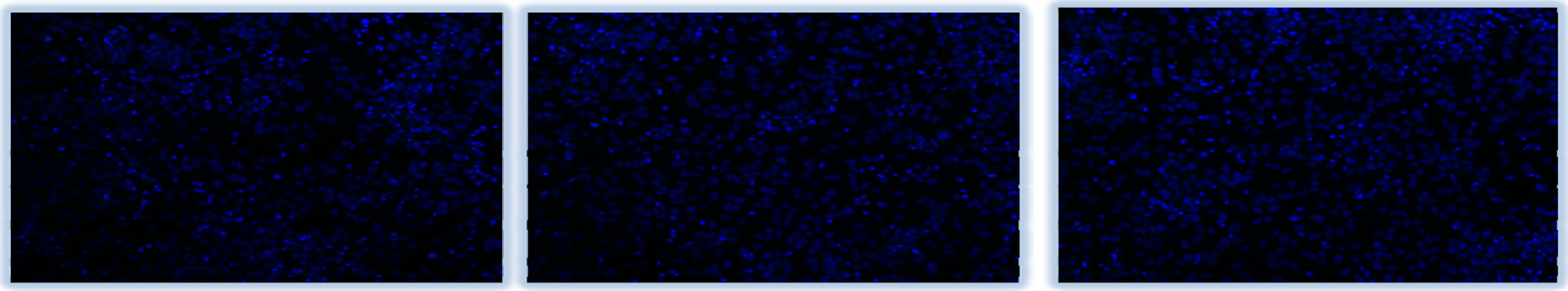


Fixed, unfiltered EBOV liver samples demonstrating successful inactivation

Trizol LS Methodology (Version 1)

Biodefense solutions to protect our nation

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)

Biodefense solutions to protect our nation

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

↓

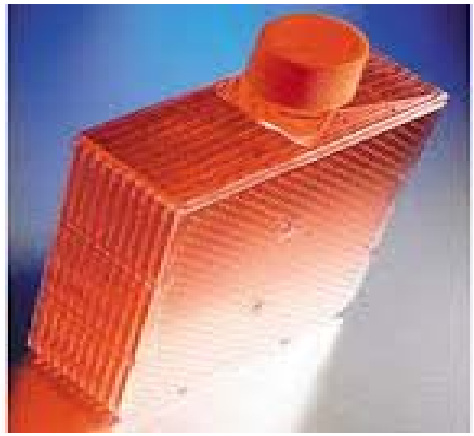
Harvest passage one and use 50% for passage two.

↓

Incubate 7 days

↓

Plaque assay analysis



Trizol LS Methodology (Version 3)

Biodefense solutions to protect our nation

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

↓

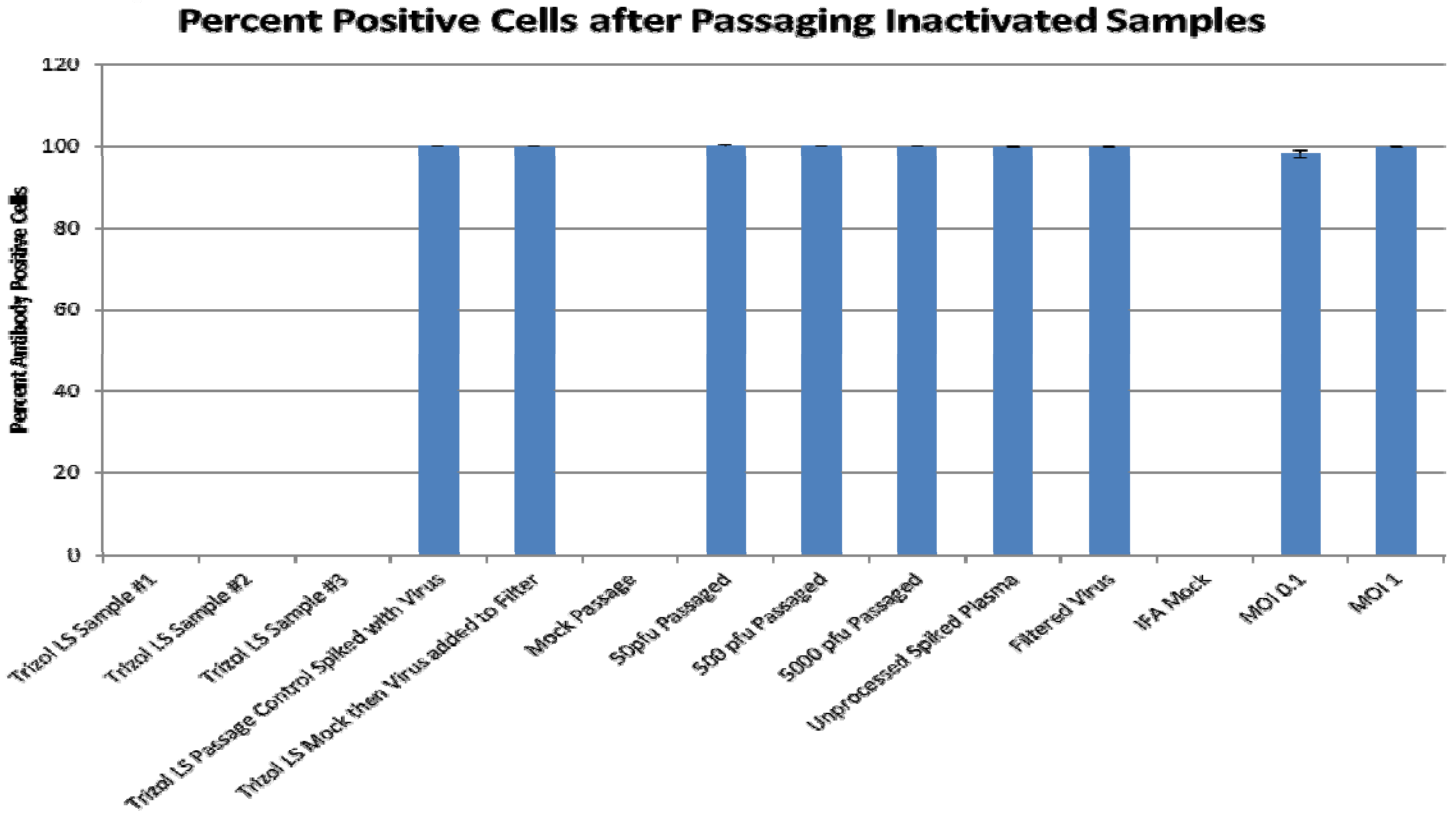
Incubate 7 days

↓

Plaque assay analysis

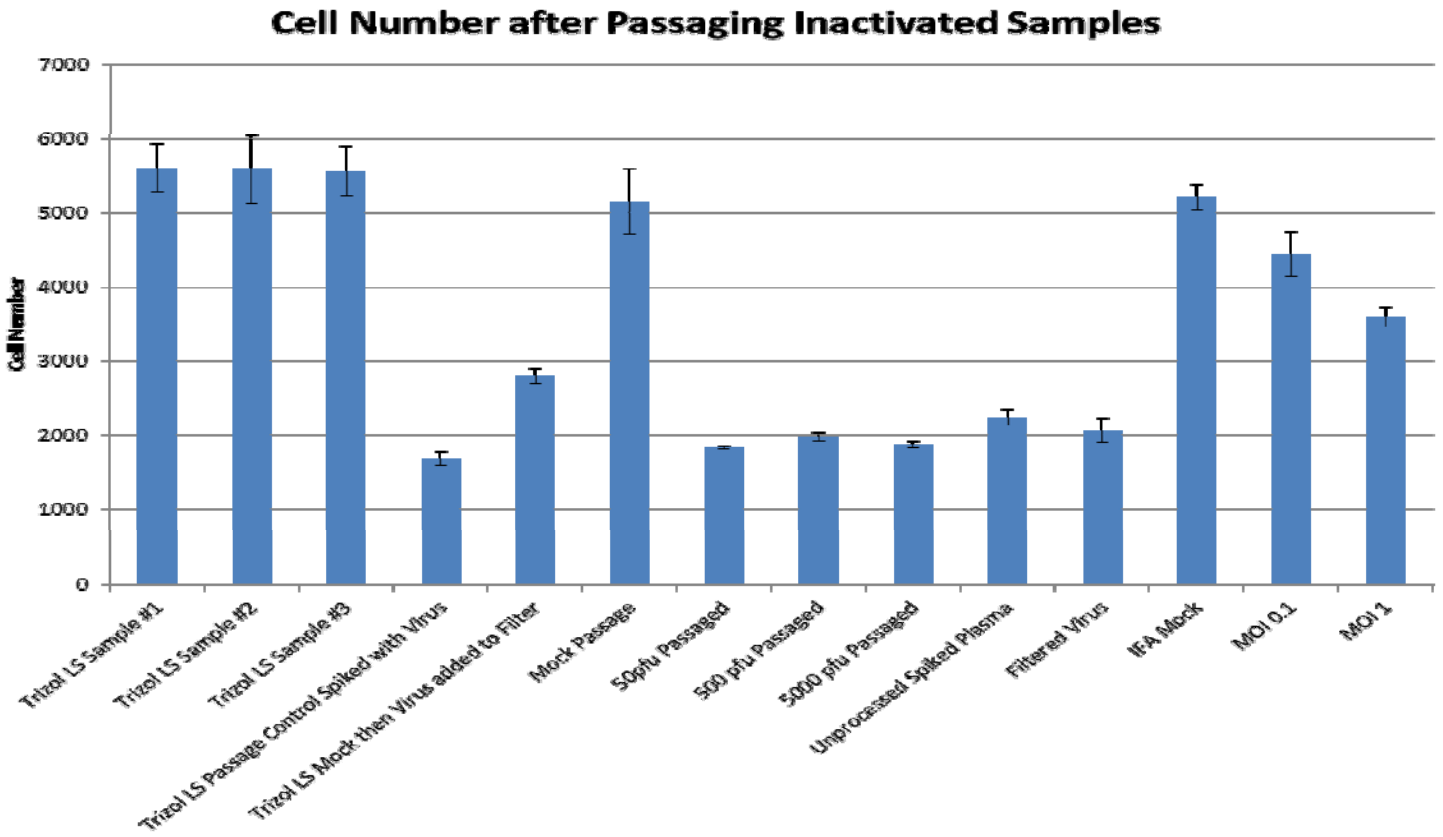
Trizol LS Results (Version 1)

Biodefense solutions to protect our nation



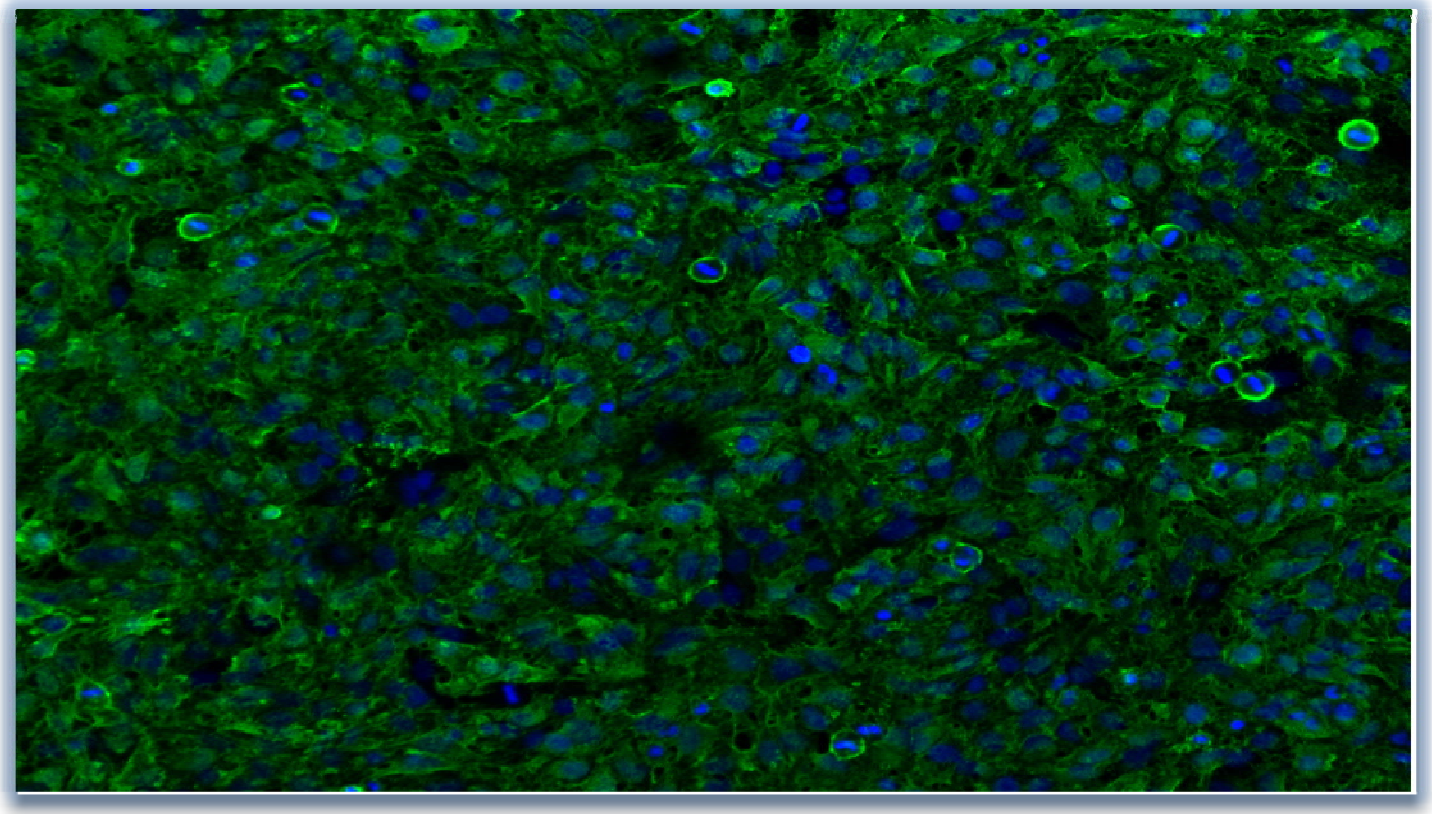
Trizol LS Results (Version 1)

Biodefense solutions to protect our nation



Trizol LS Results (Version 1)

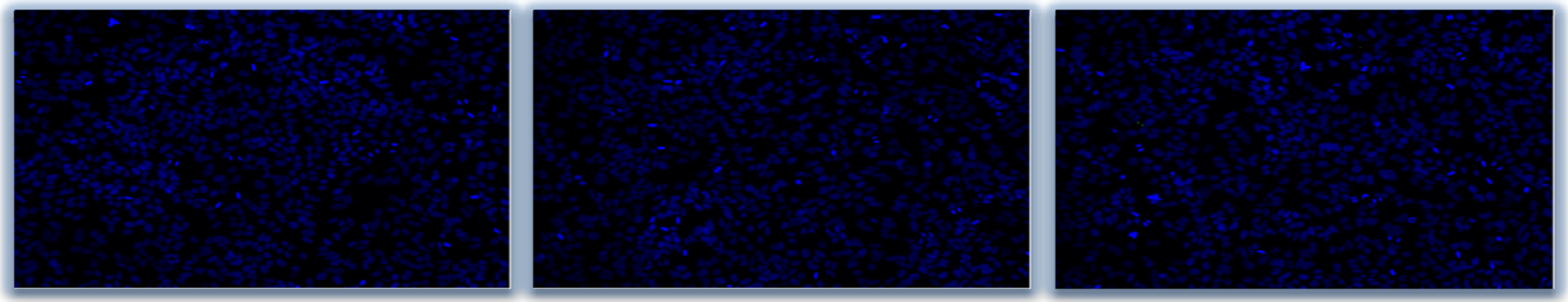
Biodefense solutions to protect our nation



MOI 1.0 TC83 Infection Control

Trizol LS Results (Version 1)

Biodefense solutions to protect our nation



Efficacy of Trizol LS inactivation confirmed across three replicates

Trizol LS Results (Versions 2 and 3)

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Sample + 560 mL media	Media Exchanged on Day 2?	Passage 1, Day 7 Observed CPE	Titer on D7 by Plaque Assay	Passage 2, Day 7 Observed CPE	Titer on D7 by Plaque Assay
MEM Only	No	None	None Detected	None	None Detected
Target 100 PFU Zika Actual = 60	No	1+	1.08E+7	4+	1.60E+7
	Yes	1+	5.75E+7	4+	1.65E+7
Target 10 PFU Zika Actual = 4.4	No	Minimal (+/-)	8.00E+7	4+	9.50E+6
	Yes	+/-	3.20E+7	4+	1.13E+7
Target 1 PFU Zika Actual = 0.5	No	None	1.50E+6	4+	5.75E+5
	Yes	None	2.53E+7	4+	3.53E+6
Target 0.1 PFU Zika Actual = 0.05	No	None	None Detected	None	None Detected

Version 2 using dilution method

Version 3 using desalting columns

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

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

Acknowledgements

Biodefense solutions to protect our nation

- Cary Retterer
- Tara Kenny
- Rouzbeh Zamani
- Dr. Lou Altamura
- Cindy Rossi
- Brian Kerney
- Jim Jaissle
- Susan Coyne

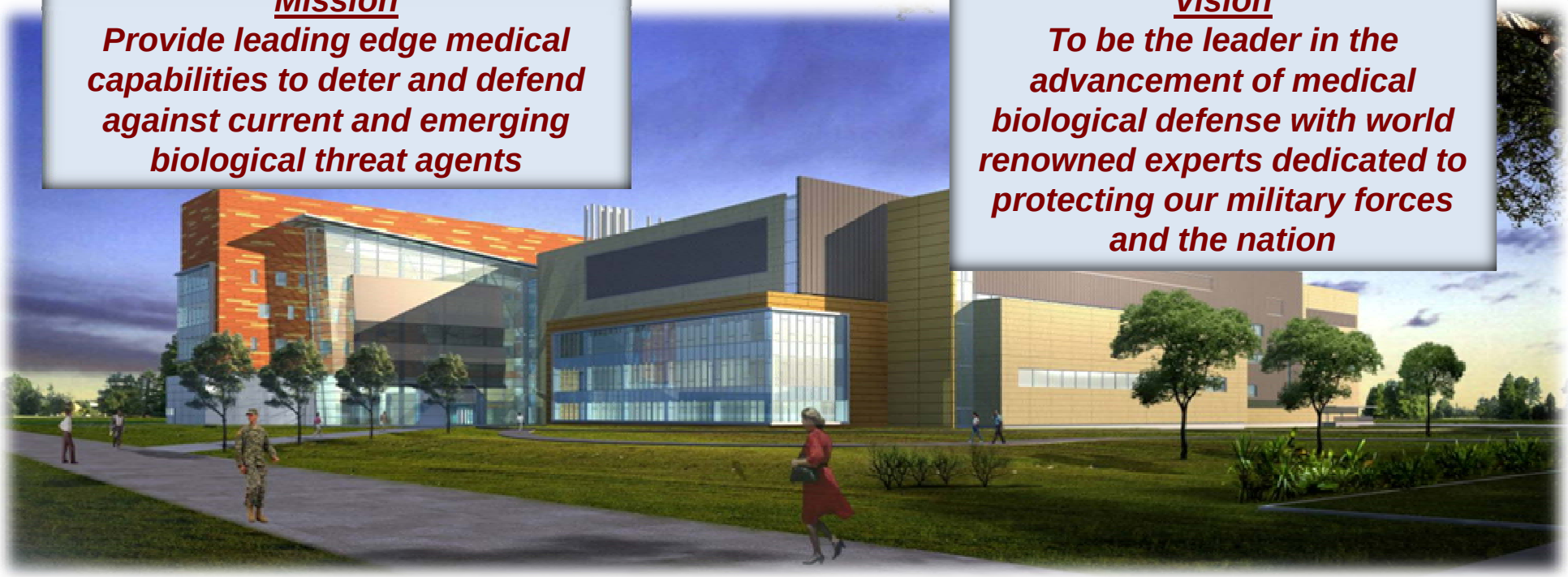
US Army Medical Research Institute of Infectious Diseases

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