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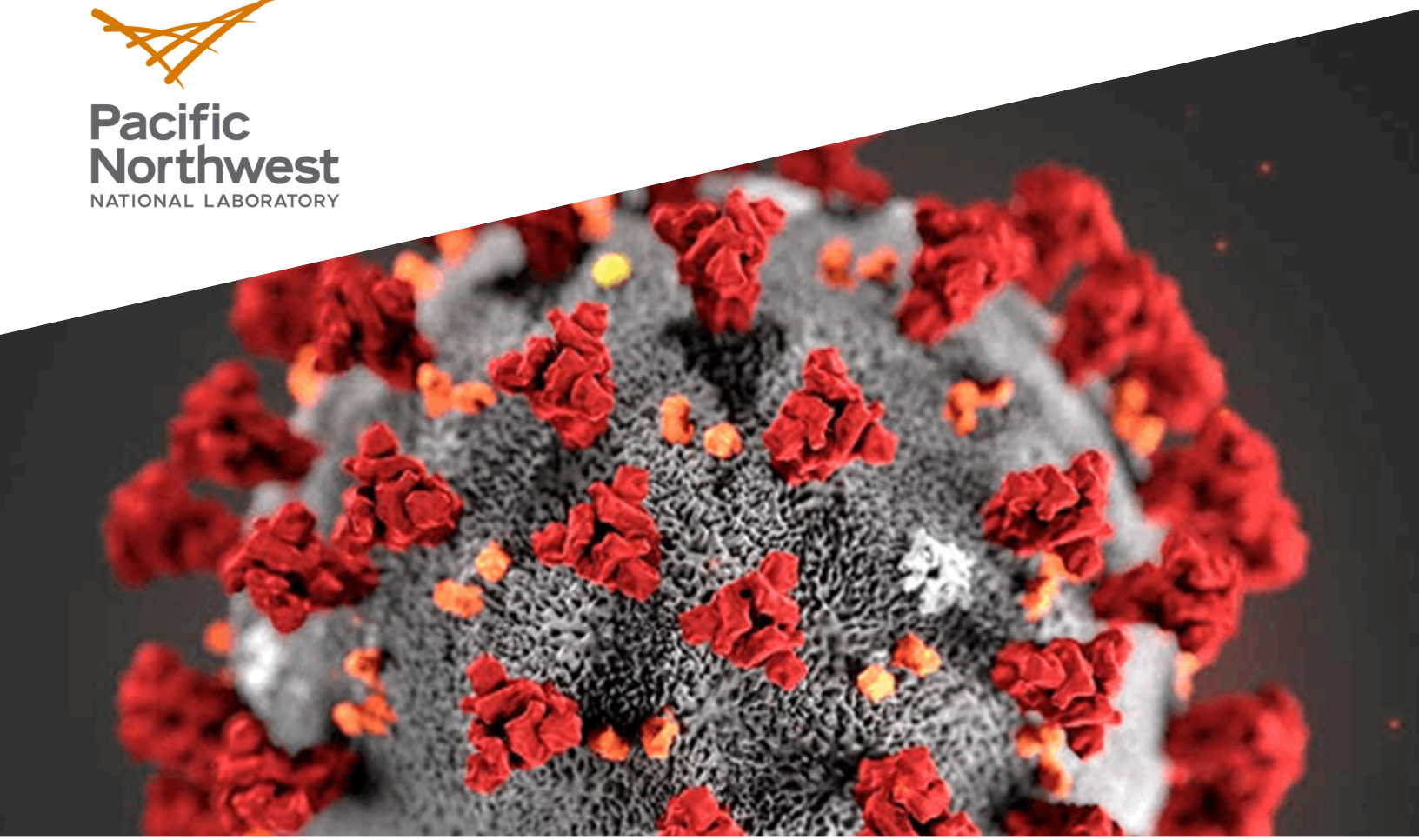

PNNL-30085

Evaluation of Three RNA Extraction Kits with a 2019-nCoV Assay

June 2020

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U.S. DEPARTMENT OF ENERGY | Prepared for the U.S. Department of Energy under Contract DE-AC05-76RL01830

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Pacific Northwest National Laboratory
Richland, Washington 99354

Summary

The following characteristics of three RNA extraction kits (Norgen Biotek Total RNA Purification Kit, #17200; Bioneer AccuPrep Viral RNA Extraction Kit, #K-3033; and Promega Maxwell HT Viral TNA Kit, #AX2340) were evaluated:

- Limits of detection for quantitative synthetic RNA using the Centers for Disease Control and Prevention's (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel and the Applied Biosystems 7500 Fast Dx system;
- Limits of detection for positive clinical specimen using the Centers for Disease Control and Prevention's (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel and the Applied Biosystems 7500 Fast Dx system;
- Ability of the lysis buffers in each kit to inactivate the virus as measured by a cell-based infectivity assay.

The QIAGEN QIAamp Viral RNA Mini Kit (#52906) and Buffer AVL (which is part of the kit) were used as reference. All kits were run using the manufacturer's instructions.

A summary of the limit of detection for each kit with clinical specimen is shown below.

Table S-1. Performance of the Norgen Biotek Total RNA Purification Kit (#17200), Bioneer AccuPrep Viral RNA Extraction Kit (#K-3033), Promega Maxwell HT Viral TNA Kit (#AX2340) and Qiagen QIAamp Viral RNA Mini Kit (#52906) at approximately 0.3 copy, 1 copies and 3 copies per microliter of virus in positive clinical specimen.

Kit	Concentration copies/uL	Positive Replicates / Total Replicates	N1 Avg Ct	N1 SD	N2 Avg Ct	N2 SD
Norgen	0.3	20/20	35.8	1.0	36.4	0.7
Bioneer	0.3	18/20	36.4	1.0	36.6	0.7
Promega	0.3	20/20	32.0	1.2	31.4	2.1
Qiagen*	0.3	24/24	33.7	1.9	34.4	2.2
Norgen	1	20/20	31.9	1.3	32.2	0.6
Bioneer	1	20/20	34.5	0.8	35.2	0.6
Promega	1	20/20	31.8	0.6	31.5	0.3
Qiagen*	1	24/24	31.7	1.1	32.7	1.2
Norgen	3	20/20	32.3	0.8	33.3	0.4
Bioneer	3	20/20	33.0	0.4	33.8	0.4
Promega	3	20/20	30.0	0.6	30.2	0.5
Qiagen*	3	24/24	30.6	0.9	31.7	2.5

*Results shown are the average and standard deviation of eight replicates performed on three separate days

All four lysis buffers inactivated SARS-CoV-2 USA-WA1/2020 at the concentrations recommended in the kit instructions. All four demonstrated similar patterns of cytopathic effect (CPE) at high concentrations of lysis buffer followed by decreasing CPE at lower concentrations, followed by a return of CPE at the point of failure where virus was no longer inactivated. A summary of the point of failure for each of the buffers is presented in Table S-2.

Table S-2. Points of failure for inactivation of SARS-CoV-2 by Norgen Buffer RL, Bioneer VB Buffer, Promega Lysis Buffer MC5018 and Qiagen Buffer AVL.

Manufacturer	Point of failure Virus : Buffer	Kit Instructions Sample : Buffer	Fold-increase of Buffer Above Point of Failure
Norgen	2 : 1	100 uL : 350 uL (1 : 3.5)	7
Bioneer	1 : 1	200 uL : 300 uL (1 : 1.5)*	1.5*
Promega	2 : 1	200 uL : 200 uL (1 : 1)	2
Qiagen	2 : 1	140 uL : 560 uL (1 : 4)	8

*Only lysis buffer was tested in this experiment. The Bioneer kit instructions include a 60°C 10 minute incubation which was not performed and could change the results.

Contents

1.0 Introduction

The COVID-19 pandemic and associated increase in clinical testing have produced a sharp rise in demand for RNA extraction kits. Laboratories have reported issues receiving extraction kits in a timely manner, resulting in subsequent delays in test results. To help alleviate these issues, the Department of Energy provided CARES Act funding to PNNL to assist the HHS/FEMA COVID-19 Diagnostics Task Force by evaluating alternative extraction kits.

PNNL evaluated the following characteristics of three RNA extraction kits (the Norgen Biotek Total RNA Purification Kit; Bioneer AccuPrep Viral RNA Extraction Kit; and Promega Maxwell HT Viral TNA Kit) identified by the Task Force:

- Limits of detection for quantitative synthetic RNA using the Centers for Disease Control and Prevention's (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel and the Applied Biosystems 7500 Fast Dx system;

- Limits of detection for positive clinical specimen using the Centers for Disease Control and Prevention's (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel and the Applied Biosystems 7500 Fast Dx system;

- Ability to inactivate the virus as measured by a cell-based infectivity assay.

The QIAGEN QIAamp Viral RNA Mini Kit and Buffer AVL (which is part of the kit) were used as reference. All kits were run using the manufacturer's instructions.

To evaluate limits of detection, first, quantitative synthetic RNA from SARS-Related Coronavirus 2 was spiked into a diluent consisting of a suspension of human A549 cells and viral transport medium (VTM).[1] The limit of detection of the CDC assay was evaluated by performing 20 extraction replicates using each kit.

Next, positive clinical specimen was diluted to concentrations that approximated the Ct values obtained with synthetic RNA at 3, 1 and (estimated) 0.3 genome copies per microliter. The limit of detection of the CDC assay was evaluated by performing 20 extraction replicates with each kit.

To evaluate inactivation efficiency, the lysis buffers from each kit were mixed with SARS-CoV-2 USA-WA1/2020 at various ratios in serum-free media. Suspensions were incubated at room temperature for 10 minutes then added to confluent Vero cell (ATCC CCL-81) monolayers in 96-well plates. The monolayers were incubated at 37°C for four days then scored for cell death by microscopic observation.

During the planning phase of the inactivation experiments, colleagues at other national laboratories requested that the inactivation efficiency of Zymo DNA/RNA Shield (#R1100) and a heat inactivation protocol developed by the University of California at Berkeley be evaluated in parallel. The data for these experiments are included for information purposes in Appendix A and B.

[1] VTM was prepared using the Center for Disease Control and Prevention Standard Operating Procedure, "Preparation of Viral Transport Medium," #DSR-052-03.

2.0 Methods

Prior to evaluation of the alternative extraction kits, the performance of the IDT 2019-nCoV CDC qPCR Probe Assay (#10006606, lot #0000510344) with the Qiagen Viral RNA Mini Kit (#52906) was verified in the Pacific Northwest National Laboratory (PNNL) Medical Test Site laboratories (CLIA #50D2180995). The performance of the assay in the PNNL laboratories was comparable to the performance reported in CDC-006-0019, Revision: 03, effective 3/30/2020.

2.1 Limits of detection with quantitative synthetic RNA

Quantitative Synthetic RNA from SARS-Related Coronavirus 2 (BEI Resources, NR-52358) was spiked into a diluent consisting of a suspension of human A549 cells and viral transport medium (VTM)[1] to mimic clinical specimen. The suspension was extracted using the QIAGEN QIAamp Viral RNA Mini Kit (#52906), Norgen Biotek Total RNA Purification Kit (#17200), Bioneer AccuPrep Viral RNA Extraction Kit (#K-3033) and Promega Maxwell HT Viral TNA Kit (#AX2340). Kits were run using the manufacturer's instructions.

The IDT 2019-nCoV CDC qPCR Probe Assay (#10006606, #0000510344), with the ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix, CG (#A15299), was run on the Applied Biosystems 7500 Fast Dx to detect the extracted RNA. The IDT 2019-nCoV_N_Positive Controls (#10006625, lot #0000512083) were used as positive controls. The concentration of the positive control (PC) for each assay was 60 copies/uL. A suspension of A549 cells in VTM was used as a human specimen control (HSC) with each batch. The concentration of A549 cells was set at a concentration that consistently produces amplification between 34 and 35 Ct using the Qiagen Viral RNA Mini Kit and IDT CDC assay. Nuclease-free water was used as the negative control. The suggested plate set up from the CDC Instructions for Use, CDC-006-00019, Revision: 03, effective 3/30/2020, was used for all plates.

To allow direct comparison of the kits, on each day of testing, one initial suspension was prepared and split to generate sets of samples for extraction on both the Qiagen kit and the kit under evaluation. Extracts from the Qiagen kit and the kit under evaluation were run in parallel on the Applied Biosystems 7500 Fast Dx using the procedure described in CDC-006-00019, Revision: 03, effective 3/30/2020.

The extraction efficiency of the kits was evaluated first by extracting three replicate specimens and serially diluting to multiple concentrations. Based on those results, a threefold dilution series (9, 3 and 1 genome copies per microliter) was identified for confirmatory LOD. 20 replicates were extracted with each of the alternative kits and amplified for confirmatory LOD. 20 replicates were not always performed with the Qiagen kit. The number of replicates performed on each day with the Qiagen kit is presented with the data for each test kit. In the summary tables, results for the Qiagen kit were averaged over all days of testing.

2.2 Limits of detection with positive clinical specimen

Positive clinical specimen (as determined by prior analysis using the CDC assay with the Qiagen kit) was spiked into a diluent of pooled negative specimen (as determined by prior

[1] VTM was prepared using the Center for Disease Control and Prevention Standard Operating Procedure, "Preparation of Viral Transport Medium," #DSR-052-03.

analysis using the CDC assay with the Qiagen kit) to concentrations that approximated the Ct values obtained at 3, 1 and (an estimated) 0.3 viral genome copies per microliter using the CDC assay and the QIAGEN QIAamp Viral RNA Mini Kit. The suspension was extracted using the QIAGEN QIAamp Viral RNA Mini Kit, Norgen Biotek Total RNA Purification Kit, Bioneer AccuPrep Viral RNA Extraction Kit and Promega Maxwell HT Viral TNA Kit. Kits were run using the manufacturer's instructions.

20 replicates were extracted with each of the test kits and amplified using the IDT 2019-nCoV CDC qPCR Probe Assay, with the ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix, on the Applied Biosystems 7500 Fast Dx. The IDT 2019-nCoV_N_Positive Controls were used as PCs. The concentration of the PCs for each assay was 60 copies/uL. A suspension of A549 cells in VTM was used as an HSC with each batch. The concentration of A549 cells was set at a concentration that consistently produces amplification between 34 and 35 Ct using the Qiagen Viral RNA Mini Kit and IDT CDC assay. Nuclease-free water was used as the negative control. The suggested plate set up from the CDC Instructions for Use, CDC-006-00019, Revision: 03, effective 3/30/2020, was used for all plates.

To allow direct comparison of the kits, on each day of testing, one initial suspension was prepared and split to generate sets of samples for extraction on both the Qiagen kit and the kit under evaluation. Extracts from the Qiagen kit and the kit under evaluation were run in parallel on the Applied Biosystems 7500 Fast Dx using the procedure described in CDC-006-00019, Revision: 03, effective 3/30/2020. 20 replicates were not always performed with the Qiagen kit. The number of replicates performed on each day with the Qiagen kit is presented with the data for each test kit. In the summary tables, results for the Qiagen kit were averaged over test days.

2.3 Inactivation

Inactivation was determined using an established procedure[1] provided by the HHS/FEMA COVID-19 Diagnostics Task Force. Briefly, SARS-CoV-2 USA-WA1/2020 (BEI Resources, NR-52281) was grown to a titer of 10^6 TCID50 / mL in Vero-CCL81 cells then mixed with serum-free media (SFM) and each lysis buffer (Qiagen Buffer AVL; Norgen Buffer RL; Bioneer VB Buffer; Promega Lysis Buffer MC5018) according to Table 1. The virus/lysis buffer suspension was incubated at room temperature for 10 minutes.

Table 1. Ratios of virus-to-lysis-buffer incubated.

	Virus	Lysis Buffer	SFM	final volume
virus : buffer	50	350	0	400
virus : buffer 002	50	200	150	400
virus : buffer 003	50	100	250	400
virus : buffer 004	50	50	300	400
virus : buffer 005	50	25	325	400
virus : buffer 006	50	10	340	400
virus only	50	0	350	400
buffer only	0	350	50	400

[1] Centers for Disease Control and Prevention, "Report – SARS-CoV-2 inactivation by guanidinium-containing lysis buffer," RVB.RVI.LABOP.Draft.

Media was aspirated from confluent Vero monolayers in 96-well plates. The virus/lysis buffer mixtures were diluted with SFM as shown in Table 2. For dilutions 1:5 through 1:200 the volume of virus : lysis buffer was diluted directly into the volume of SFM as listed on the table. For dilutions 1:500 through 1:16,000, 2-fold serial dilutions were performed by transferring 500 ul from the previous dilution to 500 ul of SFM media, leaving a final volume of 500 ul in all tubes except the final tube. Dilutions were added to the monolayers and incubated at 37°C 5% CO_2 for four days. After four days, cell death was scored as + / - and the minimum proportion of virus : lysis buffer required to inactivate the virus was determined.

Table 2. Dilutions of inactivated virus added to confluent Vero monolayers.

	Virus : Lysis Buffer Volume (ul)	SFM (ul)	Final vol (ul)
1 : 5	80	320	400
1 : 10	40	360	400
1 : 50	8	392	400
1 : 100	4	396	400
1 : 200	2	398	400
1 : 500	2	998	500
1 : 1000	500	500	500
1 : 2000	500	500	500
1 : 4000	500	500	500
1 : 8000	500	500	500
1 : 16,000	500	500	1000

3.0 Limits of detection

3.1 Verification of the CDC 2019-nCoV assay with the Qiagen Viral RNA Mini Kit

Performance characteristics for the CDC assay using the Qiagen Viral RNA Mini Kit are reported in CDC-006-00019, Revision: 03, effective 03/30/2020. These performance characteristics were verified by the PNNL Medical Test Site (PNNL-MTS). The PNNL-MTS limit of detection are presented in Table 3 in comparison to those reported in CDC-006-00019. The CDC reports that the limit of detection (LOD) of their assay with the Qiagen Viral RNA Mini Kit is 1 RNA copy per microliter.

Table 3. Performance verification results for the CDC 2019-nCoV assay using the Qiagen Viral RNA Mini Kit and quantitative synthetic RNA.

	Concentration	Positive Replicates /	N1		N2	
Lab	copies/uL	Total Replicates	Avg Ct	SD	Avg Ct	SD
PNNL	0.3	20/20	34.3	0.8	35.9	0.6
CDC	0.3	6/20	NA	NA	36.2	1.9
PNNL	1	20/20	33.1	1.0	34.3	0.7

Lab	Concentration copies/uL	Positive Replicates / Total Replicates	N1 Avg Ct	N1 SD	N2 Avg Ct	N2 SD
CDC	1	20/20	32.8	0.8	35.4	0.9
PNNL	3	20/20	31.3	0.8	32.5	0.6
CDC	3	20/20	32.0	0.7	33.0	1.4

3.2 Norgen Biotek Total RNA Purification Kit

A summary of the performance of the Norgen Biotek Total RNA Purification Kit (#17200) with quantitative synthetic RNA is shown in Table 4, along with the performance of the Qiagen Viral RNA Mini Kit (#52906). All replicates were positive for both kits at all concentrations.

Table 4. Performance of the Norgen 17200 and the Qiagen 52906 at 1 copy, 3 copies and 9 copies per microliter of quantitative synthetic RNA.

Kit	Concentration copies/uL	Positive Replicates / Total Replicates	N1 Avg Ct	N1 SD	N2 Avg Ct	N2 SD	RP Avg Ct	RP SD
Norgen	1	20/20	32.2	0.5	34.2	0.7	34.3	1.1
Qiagen	1	20/20	32.8	0.4	35.7	1.2	34.2	0.5
Norgen	3	20/20	31.1	0.1	33.3	0.3	34.4	0.4
Qiagen	3	20/20	30.9	0.3	33.5	1.1	33.6	0.4
Norgen	9	20/20	29.5	0.1	31.2	0.3	34.4	0.4
Qiagen	9	20/20	30.3	0.2	32.9	1.0	34.3	0.5

A summary of the performance of the Norgen Biotek Total RNA Purification Kit (#17200) with positive clinical specimen is shown in Table 5, along with the performance of the Qiagen Viral RNA Mini Kit (#52906). All replicates were positive for both kits at all concentrations.

Table 5. Performance of the Norgen 17200 and the Qiagen 52906 at approximately 0.3 copy, 1 copies and 3 copies per microliter of virus in positive clinical specimen.

Kit	Concentration copies/uL	Positive Replicates / Total Replicates	N1 Avg Ct	N1 SD	N2 Avg Ct	N2 SD	RP Avg Ct	RP SD
Norgen	0.3	20/20	35.8	1.0	36.4	0.7	31.0	0.5
Qiagen	0.3	8/8	35.1	1.0	36.2	0.4	30.4	0.4
Norgen	1	20/20	31.9	1.3	32.2	0.6	29.2	0.6
Qiagen	1	8/8	30.6	0.5	31.8	0.3	28.7	0.7
Norgen	3	20/20	32.3	0.8	33.3	0.4	31.5	0.5
Qiagen	3	8/8	31.1	0.1	32.8	0.3	31.0	0.2

3.3 Bioneer AccuPrep Viral RNA Extraction Kit

A summary of the performance of the Bioneer AccuPrep Viral RNA Extraction Kit (#K-3033) with quantitative synthetic RNA is shown in Table 6, along with the performance of the Qiagen Viral RNA Mini Kit (#52906). All replicates were positive for both kits at all concentrations.

Table 6. Performance of the Bioneer K-3033 and the Qiagen 52906 at 1 copy, 3 copies and 9 copies per microliter of quantitative synthetic RNA.

Kit	Concentration copies/uL	Positive Replicates / Total Replicates	N1 Avg Ct	N1 SD	N2 Avg Ct	N2 SD	RP Avg Ct	RP SD
Bioneer	1	20/20	33.5	0.8	35.1	0.5	36.8	0.7
Qiagen	1	3/3	34.5	0.3	37.0	1.1	37.2	1.0
Bioneer	3	20/20	31.5	0.6	32.4	0.7	35.4	1.0
Qiagen	3	3/3	32.3	0.4	33.1	1.0	34.8	0.6
Bioneer	9	20/20	29.7	0.3	30.9	0.3	36.4	1.1
Qiagen	9	3/3	29.4	0.8	30.6	0.9	35.8	1.0

A summary of the performance of the Bioneer AccuPrep Viral RNA Extraction Kit (#K-3033) with positive clinical specimen is shown in Table 7, along with the performance of the Qiagen Viral RNA Mini Kit (#52906). All replicates were positive for both kits at 3 and 1 copies per microliter. 18/20 copies were positive for the Bioneer at 0.3 copies per microliter.

Table 7. Performance of the Bioneer K-3033 and the Qiagen 52906 at approximately 0.3 copy, 1 copies and 3 copies per microliter of virus in positive clinical specimen.

Kit	Concentration copies/uL	Positive Replicates / Total Replicates	N1 Avg Ct	N1 SD	N2 Avg Ct	N2 SD	RP Avg Ct	RP SD
Bioneer	0.3	18/20	36.4	1.0	36.6	0.7	29.4	0.5
Qiagen	0.3	8/8	34.0	0.7	35.4	0.5	28.6	0.8
Bioneer	1	20/20	34.5	0.8	35.2	0.6	29.4	0.6
Qiagen	1	8/8	33.1	0.4	34.3	0.3	28.9	0.5
Bioneer	3	20/20	33.0	0.4	33.8	0.4	29.9	0.6
Qiagen	3	8/8	31.2	0.2	32.5	0.2	29.4	0.4

3.4 Promega Maxwell HT Viral TNA Kit

A summary of the performance of the Promega Maxwell HT Viral TNA Kit (#AX2340) with quantitative synthetic RNA is shown in Table 8, along with the performance of the Qiagen Viral RNA Mini Kit (#52906). All replicates were positive for both kits at all concentrations.

Table 8. Performance of the Promega AX2340 and the Qiagen 52906 at 1 copy, 3 copies and 9 copies per microliter of quantitative synthetic RNA.

Kit	Concentration copies/uL	Positive Replicates / Total Replicates	N1		N2		RP	
			Avg Ct	SD	Avg Ct	SD	Avg Ct	SD
Promega	1	20/20	31.7	0.3	33.4	0.3	34.7	0.7
Qiagen	1	3/3	33.6	0.5	36.8	0.2	33.8	0.2
Promega	3	20/20	30.1	0.3	31.7	0.4	34.5	0.6
Qiagen	3	3/3	31.7	0.3	34.2	0.6	33.8	0.4
Promega	9	20/20	28.5	0.2	30.2	0.2	35.0	0.9
Qiagen	9	3/3	30.3	0.2	32.4	0.3	34.0	0.4

A summary of the performance of the Promega Maxwell HT Viral TNA Kit (#AX2340) with positive clinical specimen is shown in Table 9, along with the performance of the Qiagen Viral RNA Mini Kit (#52906). All replicates were positive for both kits at all concentrations.

Table 9. Performance of the Promega AX2340 and the Qiagen 52906 at approximately 0.3 copy, 1 copies and 3 copies per microliter of virus in positive clinical specimen.

Kit	Concentration copies/uL	Positive Replicates / Total Replicates	N1		N2		RP	
			Avg Ct	SD	Avg Ct	SD	Avg Ct	SD
Promega	0.3	20/20	32.0	1.2	31.4	2.1	26.1	0.5
Qiagen	0.3	8/8	32.0	2.3	31.6	1.6	24.7	0.8
Promega	1	20/20	31.8	0.6	31.5	0.3	28.4	0.5
Qiagen	1	8/8	31.4	0.5	32.0	0.5	26.8	0.9
Promega	3	20/20	30.0	0.6	30.2	0.5	26.0	0.4
Qiagen	3	8/8	29.6	0.6	29.9	3.8	24.9	0.3

4.0 Inactivation

All four buffers inactivated SARS-CoV-2 USA-WA1/2020 at the concentrations recommended in the kit instructions. All four demonstrated similar patterns of cytopathic effect (CPE) at high concentrations of lysis buffer followed by decreasing CPE at lower concentrations, followed by a return of CPE at the point of failure where virus was no longer inactivated. A summary of the point of failure for each of the buffers is presented in Table 10.

Table 10. Points of failure for inactivation of SARS-CoV-2 by Norgen Buffer RL, Bioneer VB Buffer, Promega Lysis Buffer MC5018 and Qiagen Buffer AVL.

Manufacturer	Point of Failure Virus : Lysis Buffer	Kit Instructions Sample : Buffer	Fold-increase of Buffer Above Point of Failure
Norgen	2 : 1	100 uL : 350 uL (1 : 3.5)	7
Bioneer	1 : 1	200 uL : 300 uL (1 : 1.5)*	1.5*
Promega	2 : 1	200 uL : 200 uL (1 : 1)	2

Manufacturer	Point of Failure Virus : Lysis Buffer	Kit Instructions Sample : Buffer	Fold-increase of Buffer Above Point of Failure
Qiagen	2 : 1	140 uL : 560 uL (1 : 4)	8

*Only lysis buffer was tested in this experiment. The Bioneer kit instructions include a 60°C 10 minute incubation which was not performed and could change the results.

4.1 Norgen Buffer RL

The inactivation data for Norgen Buffer RL are shown in Figure 1. In the controls, virus alone killed cells out to 1 : 8000 and Buffer RL alone killed cells out to 1 : 1000.

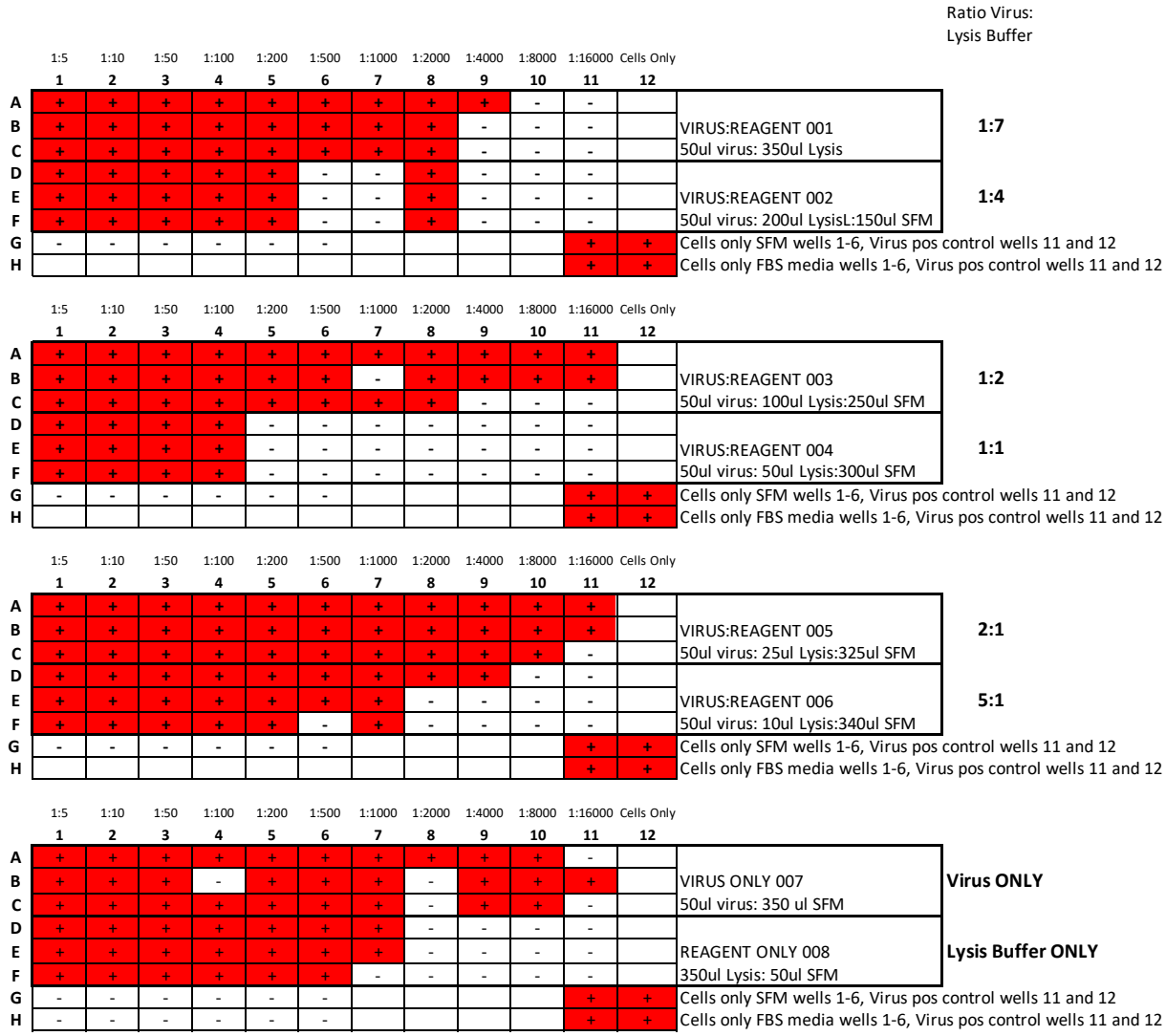

Figure 1. Inactivation results for Norgen Buffer RL.

The point of failure for Norgen Buffer RL was determined to be 2 : 1 or 50 ul virus : 25 ul buffer : 325 ul serum-free media (SFM). The Norgen kit instructions recommend using 100 uL of

specimen and 350 uL of buffer, which is a ratio of 1 : 3.5, or a 7-fold excess of lysis buffer above the point of failure.

4.2 Bioneer VB Buffer

The inactivation data for Bioneer VB Buffer are shown in Figure 2. In the controls, virus alone killed cells out to 1 : 4000 and VB Buffer alone killed cells out to 1 : 2000.

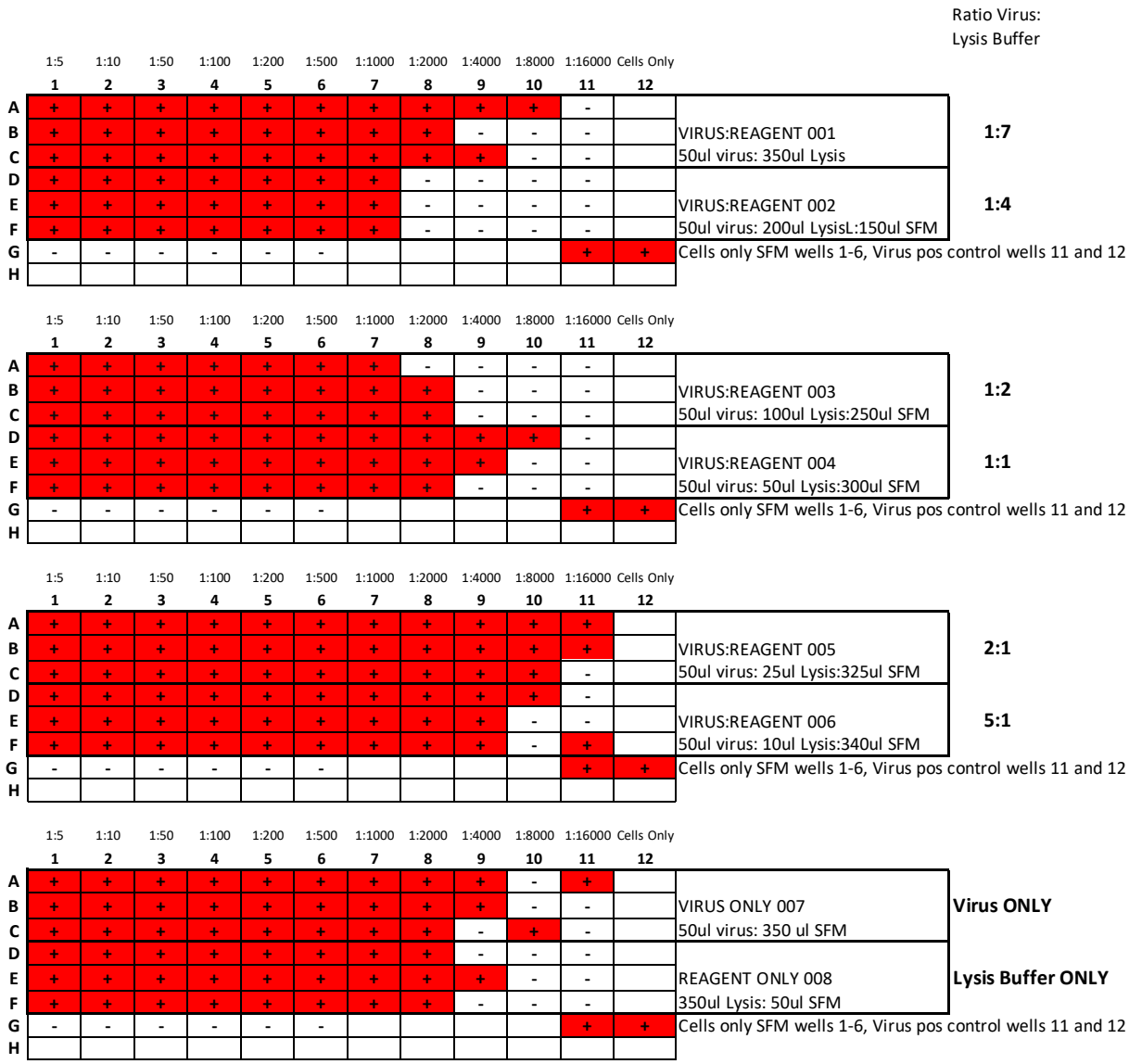

Figure 2. Inactivation results for Bioneer VB Buffer. Only lysis buffer was tested in this experiment. The Bioneer kit instructions include a 60°C 10 minute incubation which was not performed and could change the results.

The point of failure for Bioneer VB Buffer was determined to be 1 : 1 or 50 ul virus : 50 ul buffer : 300 ul serum-free media (SFM). The Bioneer kit instructions recommend using 200 uL of

specimen and 300 uL of buffer, which is a ratio of 1 : 1.5, or a 1.5-fold excess of lysis buffer above the point of failure. The Bioneer kit instructions also include a 60°C 10 minute incubation which was not performed in this experiment and could change the results.

4.3 Promega Lysis Buffer MC5018

The inactivation data for Promega Lysis Buffer MC5018 are shown in Figure 3. In the controls, virus alone killed cells out to 1 : 8000 and Buffer MC5018 alone killed cells out to 1 : 2000.

	1:5 (1)	1:10 (2)	1:50 (3)	1:100 (4)	1:200 (5)	1:500 (6)	1:1000 (7)	1:2000 (8)	1:4000 (9)	1:8000 (10)	1:16000 (11)	Cells Only (12)		Ratio Virus: Lysis Buffer
A	+	+	+	+	+	+	+	+	-	-	-			
B	+	+	+	+	+	+	+	+	-	-	-		VIRUS:REAGENT 001	**1:7**
C	+	+	+	+	+	+	+	+	-	-	-		50ul virus: 350ul Lysis	
D	+	+	+	+	+	+	+	-	-	-	-			
E	+	+	+	+	+	+	+	-	-	-	-		VIRUS:REAGENT 002	**1:4**
F	+	+	+	+	+	+	+	-	-	-	-		50ul virus: 200ul LysisL:150ul SFM	
G	-	-	-	-	-	-					+	+	Cells only SFM wells 1-6, Virus pos control wells 11 and 12	
H														

	1:5 (1)	1:10 (2)	1:50 (3)	1:100 (4)	1:200 (5)	1:500 (6)	1:1000 (7)	1:2000 (8)	1:4000 (9)	1:8000 (10)	1:16000 (11)	Cells Only (12)		
A	+	+	+	+	+	+	-	-	-	-	-			
B	+	+	+	+	+	+	-	-	-	-	-		VIRUS:REAGENT 003	**1:2**
C	+	+	+	+	+	+	-	-	-	-	-		50ul virus: 100ul Lysis:250ul SFM	
D	+	+	+	+	+	+	-	-	-	-	-			
E	+	+	+	+	+	-	-	-	-	-	-		VIRUS:REAGENT 004	**1:1**
F	+	+	+	+	+	-	-	-	-	-	-		50ul virus: 50ul Lysis:300ul SFM	
G	-	-	-	-	-	-					+	+	Cells only SFM wells 1-6, Virus pos control wells 11 and 12	
H														

	1:5 (1)	1:10 (2)	1:50 (3)	1:100 (4)	1:200 (5)	1:500 (6)	1:1000 (7)	1:2000 (8)	1:4000 (9)	1:8000 (10)	1:16000 (11)	(12)		
A	+	+	+	+	-	+	-	+	-	-	-			
B	+	+	+	+	+	+	+	+	-	+	+		VIRUS:REAGENT 005	**2:1**
C	+	+	+	-	+	-	+	+	-	-	-		50ul virus: 25ul Lysis:325ul SFM	
D	+	+	+	+	+	-	+	+	-	-	-			
E	+	+	+	+	+	+	-	-	-	-	-		VIRUS:REAGENT 006	**5:1**
F	+	+	+	+	+	+	+	+	-	-	-		50ul virus: 10ul Lysis:340ul SFM	
G	-	-	-	-	-	-					+	+	Cells only SFM wells 1-6, Virus pos control wells 11 and 12	
H														

	1:5 (1)	1:10 (2)	1:50 (3)	1:100 (4)	1:200 (5)	1:500 (6)	1:1000 (7)	1:2000 (8)	1:4000 (9)	1:8000 (10)	1:16000 (11)	Cells Only (12)		
A	+	+	+	+	+	+	+	+	+	+	-			
B	+	+	+	+	+	+	+	+	+	-	+		VIRUS ONLY 007	**Virus ONLY**
C	+	+	+	+	+	+	+	+	+	+	-		50ul virus: 350 ul SFM	
D	+	+	+	+	+	+	+	+	-	-	-			
E	+	+	+	+	+	+	+	+	-	-	-		REAGENT ONLY 008	**Lysis Buffer ONLY**
F	+	+	+	+	+	+	+	+	-	-	-		350ul Lysis: 50ul SFM	
G	-	-	-	-	-	-					+	+	Cells only SFM wells 1-6, Virus pos control wells 11 and 12	
H														

Figure 3. Inactivation results for Promega Lysis Buffer MC5018.

The point of failure for Promega Lysis Buffer MC5018 was determined to be 2 : 1 or 50 ul virus : 25 ul buffer : 325 ul serum-free media (SFM). The Promega kit instructions recommend using

200 uL of specimen and 200 uL of buffer, which is a ratio of 1 : 1, or a 2-fold excess of lysis buffer above the point of failure.

4.4 Qiagen Buffer AVL

The inactivation data for Qiagen Buffer AVL are shown in Figure 4. In the controls, virus alone killed cells out to 1 : 8000 and AVL alone killed cells out to 1 : 1000.

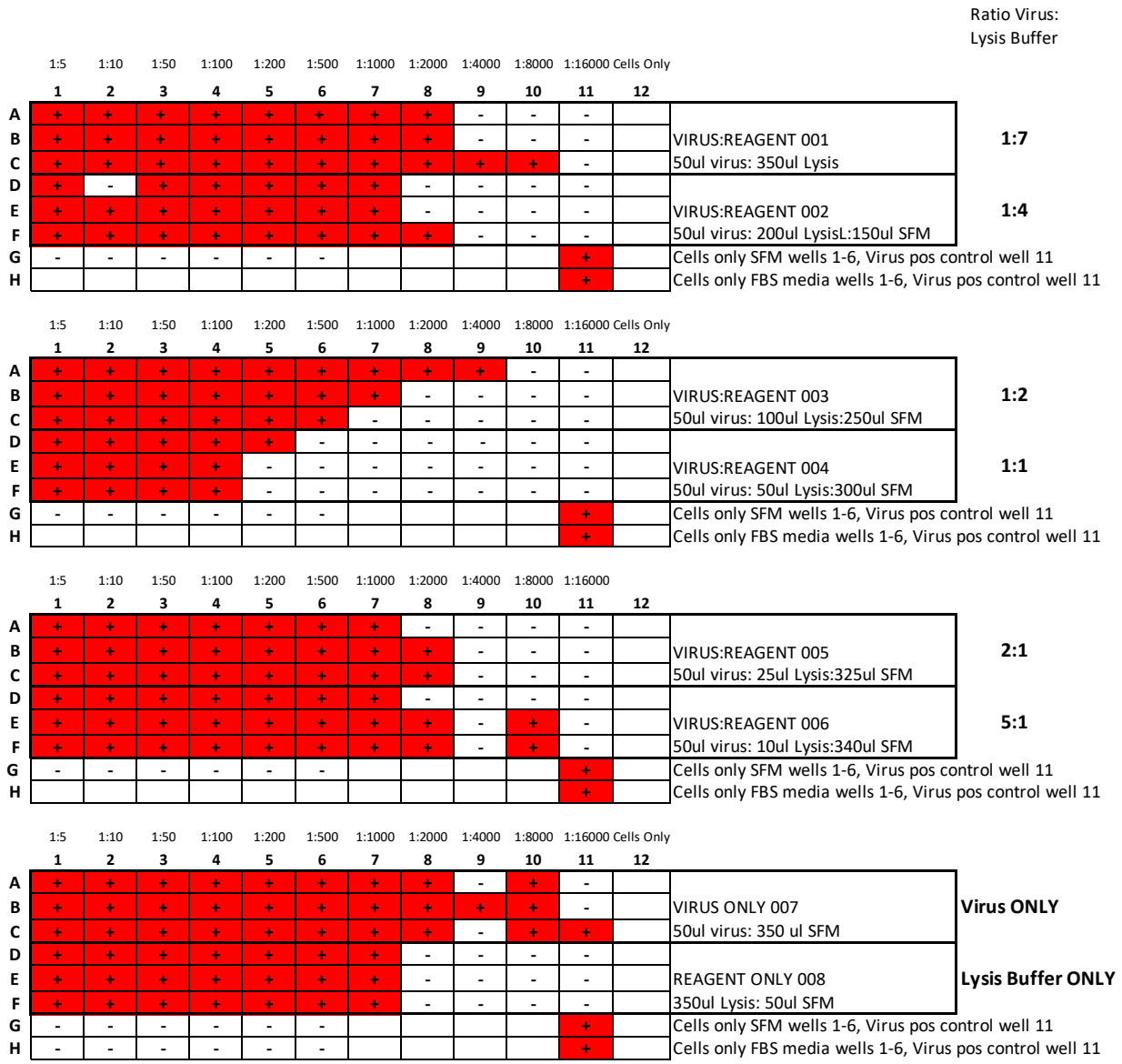

Figure 4. Inactivation results for Qiagen Buffer AVL.

The point of failure for Qiagen Buffer AVL was determined to be 2 : 1 or 50 ul virus : 25 ul buffer : 325 ul serum-free media (SFM). The Qiagen kit instructions recommend using 140 uL of

specimen and 560 uL of buffer, which is a ratio of 1 : 8, or an 8-fold excess of lysis buffer above the point of failure.

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Appendix A – Inactivation Data for Zymo RNA/DNA Shield

During the planning phase of the inactivation experiments, colleagues at other national laboratories requested that the inactivation efficiency of Zymo DNA/RNA Shield (#R1100) be evaluated. The inactivation data for Zymo DNA/RNA Shield are shown in Figure A-1. In the controls, virus alone killed cells out to 1 : 16000 and DNA/RNA Shield alone killed cells out to 1 : 4000.

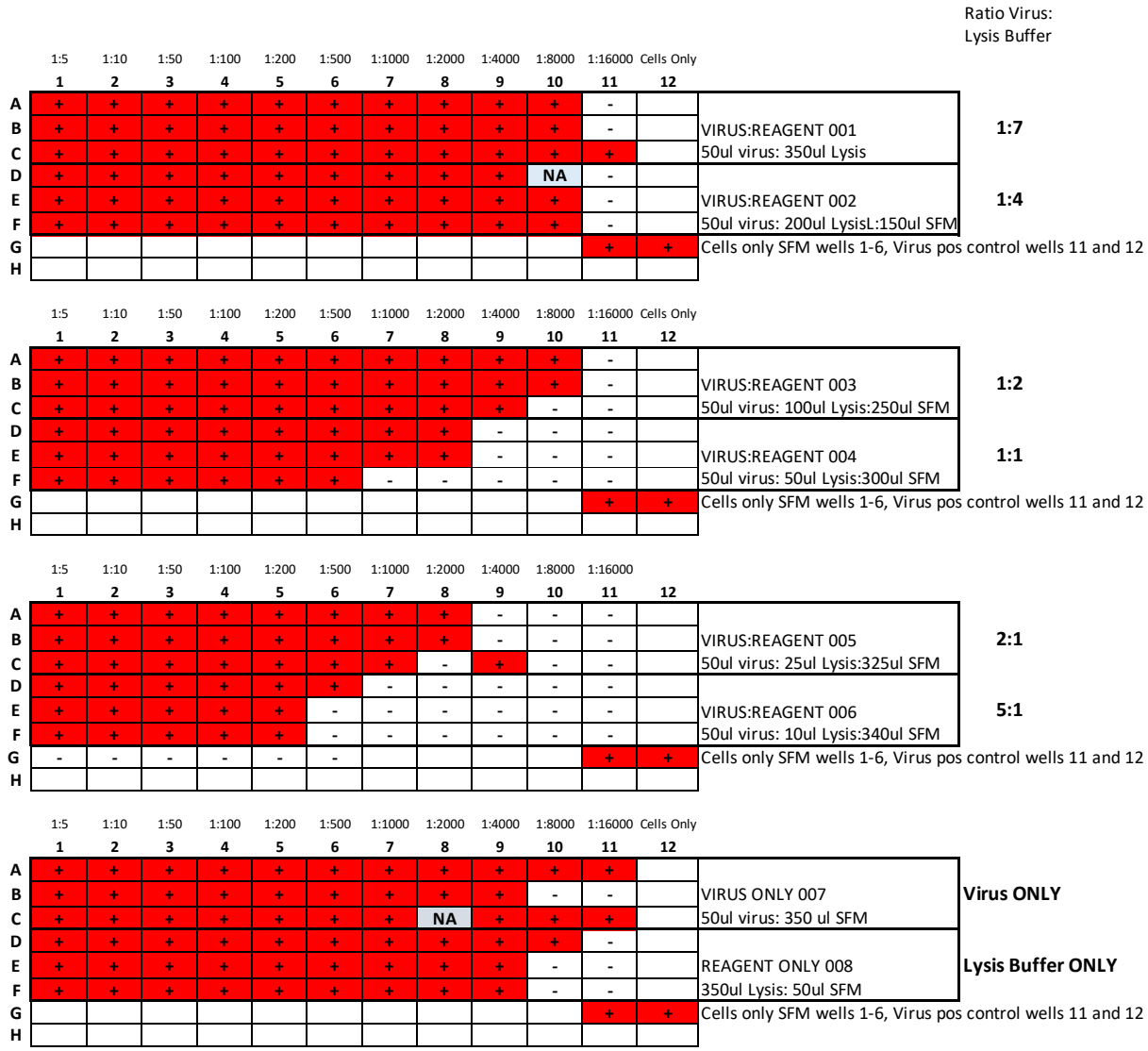

Figure A-1. Inactivation results for Zymo DNA/RNA Shield.

The point of failure for Zymo DNA/RNA Shield is difficult to determine as the cytotoxicity of DNA/RNA Shield only was greater than expected. However, the virus appears to be inactivated at a concentration of 5:1. If more information is desired, PNNL recommends repeating the experiment at ratios of 1 : 2, 1 : 1, 2 : 1, 5 : 1, 10 : 1 and 20 : 1.

Appendix B – Inactivation Data for a Modified Heat Inactivation Protocol

During the planning phase of the inactivation experiments, colleagues at other national laboratories requested that inactivation be assessed for a modification of a heat inactivation process developed by the Tjian-Darzacq laboratory at the University of California at Berkeley. Briefly, the process involves collecting a swab sample in a 200 ug/ml solution of proteinase K in ddH_2O; incubating the swab at 37°C for 30 min; then incubating the swab at 75°C for 30 min to inactivate both virus and proteinase K.

SARS-CoV-2 USA-WA1/2020 (BEI Resources, NR-52281) was grown to a titer of 10^6 TCID50 / mL in Vero-CCL81 cells. The virus was diluted into the appropriate matrix at the ratios shown in Table B-1. Human SARS-CoV-2 negative clinical nasopharyngeal specimen (NP) matrix was made with 1 part proteinase K (200 ug/mL) + 9 parts NP. Human saliva matrix was made with 1 part proteinase K + 1 part saliva. The dilutions were incubated at 37°C for 30 min; then at 75°C for 30 min.

Table B-1. Ratios of virus-to-matrix incubated.

Virus:Matrix	Virus (µL) or Previous Dilution	Matrix (µL)	Final Volume (µL)
1 : 2	250	250	400*
1 : 10	100	400	400*
1 : 50	100	400	400*
1 : 250	100	400	400*
1 : 1250	100	400	400*
1 : 6250	100	400	500
Virus only	400	0	400
Matrix only	0	400	400

* Final volume after 100 uL removed for next serial dilution

Media was aspirated from confluent Vero monolayers in 96-well plates. The virus/ matrix/proteinase K mixtures were added to the monolayers and incubated at 37°C 5% CO_2 for four days. After four days, cell death was scored as + / -. Results are shown in Figure B-1.

		Saliva heat inactivated			Saliva not heat inactivated			NP heat inactivated*			NP not heat inactivated*		
		1	2	3	4	5	6	7	8	9	10	11	12
1:2	A							-	-	-	+	+	+
1:10	B							-	-	-	+	+	+
1:50	C							-	-	-	+	+	+
1:250	D							-	-	-	+	+	+
1:1250	E							-	-	-	+	+	+
1:6250	F							-	-	-	+	+	+
Virus only SFM	G	-	-	-	+	+	+	-	-	-	+	+	+
Matrix (no virus)	H	-	-	-	-	-	-	-	-	-	+	+	-

Figure B-1. Inactivation results for heat inactivation with proteinase K. Row G is a positive control with virus + serum free media (SFM). Row H is a negative control where columns 1,2, 4, 5, 7, 8, 10, and 11 are matrix and wells 3, 6, 9, and 12 are SFM only.

Cells inoculated with saliva mixtures could not be scored due to the presence of bacterial overgrowth which obscured the monolayers.

Cells inoculated with dilutions of SARS-CoV-2 + 1 : 9 proteinase K/NP matrix that was heat inactivated (Column 7-9, Row A-F) did not exhibit CPE, indicating inactivation of the virus. These cells appeared similar to cells that received heat inactivated K/NP matrix without virus (Column 7-9, Row H). There did appear to be some effect from the matrix on the cells, as the cells with heat inactivated proteinase K/NP looked slightly different than the cells that received heat inactivated virus + media (Column 7-9, Row G). This could be due to the cellular material in the nasopharyngeal specimen, ,the proteinase K, or a chemical component of the viral transport media.

Cells inoculated with non-heat-treated virus/nasopharyngeal specimen mixtures were completely killed; the entire monolayer was no longer visible after 4 days of incubation. This CPE was different than the CPE observed for virus only, where many cells were dead or dying but the monolayer remained intact. Cells treated with non-heat-treated nasopharyngeal specimen and proteinase K, without virus, were also completely killed. This is likely due to the presence of the proteinase K.

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