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A SIMPLE MODIFICATION TO THE MOSQUITO HOMOGENIZATION PROTOCOL SAFELY INACTIVATES WEST NILE VIRUS AND ALLOWS VIRUS DETECTION BY THE RAPID ANALYTE MEASUREMENT PLATFORM (RAMP®) ASSAY

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Abstract

We evaluated the ability of the Rapid Analyte Measurement Platform (RAMP®, Response Biomedical Corp., Burnaby, British Columbia, Canada) mosquito grinding buffer to inactivate West Nile virus (WNV) by subjecting WNV-positive samples ground in RAMP buffer to incubation intervals ranging from 5–60 minutes. At each time point an aliquot was removed and serially diluted in Bovine Albumin (BA)-1 cell culture media to stop the inactivation process by RAMP buffer. Each BA-1 sample was tested for viable virus using Vero 6-well cell culture plaque assay and observed for plaques. We observed very limited inactivation of WNV (1–2 $\log_{10}$ PFU/ml) by RAMP buffer. Concerned for RAMP operators who may be using this assay in low level bio-containment facilities, we developed an alternate sample homogenization protocol using Triton X-100 detergent that ensures complete WNV inactivation without compromising the performance of the RAMP assay.

Keywords

West Nile virus; RAMP® test; virus inactivation; Triton X-100; mosquito

INTRODUCTION

The Rapid Analyte Measurement Platform (RAMP®, Response Biomedical Corp., Burnaby, British Columbia, Canada) West Nile virus (WNV) assay is a commercially available antigen detection lateral flow assay used to detect WNV in mosquito pools. Mosquito pools are ground in the proprietary RAMP buffer included in the test kit. An aliquot of the supernatant is mixed with a conjugated-antibody complex and applied to an immunochromatographic strip housed in a cartridge. After a 90-minute incubation period the RAMP reader reads the strip and produces results in RAMP units which are then interpreted to be WNV positive or negative. A RAMP score of 30 or higher is considered positive for

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WNV by the manufacturer, while the Centers for Disease Control (CDC) recommends a cutoff of ≥ 50 (Burkhalter et al. 2014).

WNV is a biosafety level (BSL)-3 agent (U.S. Department of Health and Human Services 2009). Procedures that use live virus must be performed in BSL-3 containment which includes but is not limited to the use of a biosafety cabinet, proper personal protective equipment (PPE) and controlled access to the BSL-3 laboratory. After inactivation of virus, nucleic acid detection methods such as RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR) and immunologic assays such as enzyme immunoassays (EIA) can be performed in low containment facilities as there is little to no risk for infection when performing these procedures. Samples that are tested using the RAMP assay are ground in RAMP buffer that exposes the antigen and facilitates the detection mechanism. It has been assumed by some workers that the RAMP buffer fully lyses the virus and that the assay may be performed under low biosafety containment conditions. However, inactivation of WNV by RAMP buffer has not been documented. Many mosquito abatement districts (MAD) use RAMP assay results to guide operational decisions but do not have access to sufficient biosafety containment facilities. Agencies that assume the RAMP buffer renders the virus inactive run the risk of manipulating infectious material outside containment. Even if the virus was inactivated after being exposed to RAMP buffer for some amount of time, the risk of exposure to infectious virus remains if the material is released during homogenization, while opening caps, or due to spills or tube breakage before the effective incubation period has passed.

Accordingly, we performed an evaluation to assess the WNV inactivating effect of RAMP buffer. When it was observed that RAMP buffer did not fully inactivate the virus even after incubation periods of up to 60 minutes, we then explored a modification to the protocol that would render WNV non-infectious. The modification had two requirements: that it fully inactivated WNV on contact, and that it did not interfere with the RAMP assay. We investigated adding a commonly used detergent, Triton X-100, to the RAMP buffer and used the modified buffer to grind the mosquitoes before proceeding with the RAMP assay as described in the kit insert. Triton X-100 is a non-ionic detergent extremely effective against enveloped viruses, such as WNV. It disrupts lipid-lipid and protein-lipid associations rendering the virus noninfectious, but it does not denature proteins making it suitable for use in EIAs. Triton X-100 has been demonstrated to quickly inactivate not only WNV (Kreil et al. 2003) but other lipid-enveloped viruses such as Chikungunya virus, Sindbis virus, vesicular stomatitis virus, human immunodeficiency virus, and hepatitis B and C viruses (Horowitz et al. 1992, Roberts et al. 2008, Song et al. 2010, Leydold et al. 2012).

METHODS

Evaluation of WNV inactivation by RAMP buffer

Aliquots of seed virus strain WNV NY99–35262-11 were used to spike pools containing 50 laboratory-reared uninfected *Cx. quinquefasciatus* Say mosquitoes to create high-titered virus samples of 6.2 and 7.3 $\log_{10}$ PFU/ml. These samples were ground in 1 ml RAMP buffer by vortexing for 1 min, centrifuged for 3 min at 4000 rpm, and incubated at room temperature (RT) for a total of 60 min. Aliquots were removed at the following time points:

5, 10, 15, 20, 25, 30 and 60 min. Each aliquot was serially diluted in chilled Bovine Albumin (BA)-1 and kept on ice to halt potential inactivation activity of the RAMP buffer at each time point, preserve any live virus that remained in the sample, and dilute out the potential cytopathic effect of the buffer. The seed virus was also serially diluted in BA-1 to determine the titer of the virus used to spike the RAMP buffer samples.

Virus infectivity was determined by plaque assay on monolayers of Vero cells in 6-well plates as described previously (Beaty et al. 1995). One hundred μl of each serially diluted BA-1 sample was applied in duplicate to wells of confluent Vero cells. A 1% agarose overlay was applied to each well after a 60 min incubation period at 37° C and 5% CO_2. On day 2 post-infection a second overlay containing neutral red was applied to each well. Wells were observed for WNV plaques on days 3–7. The differences between the titers calculated from the control BA-1 samples and RAMP buffer samples were used to estimate titer reduction of the samples incubated in RAMP buffer.

Evaluation of WNV inactivation and RAMP assay performance by RAMP buffer fortified with 1% Triton X-100

We added Triton X-100 (Sigma-Aldrich, St Louis, MO) to RAMP buffer in a final concentration of 1% as recommended previously (Hotta et al. 2010, Kreil et al. 2003) to create a modified grinding buffer (RB-TX). We used unmodified RAMP buffer as supplied in the kit and BA-1 as control buffers. In each of the three buffers, three types of samples containing the following were prepared: seed WNV referred to as “virus-only samples,” pools of 25 mosquitoes spiked with seed virus referred to as “virus-spiked mosquito pools,” and pools of 24 negative mosquitoes plus 1 WNV positive mosquito which had been infected via intrathoracic inoculation (Rosen and Gubler 1974), referred to as “ITI infected mosquito pools.”

To prepare virus-only and virus-spiked mosquito pool samples, we added 100 μl aliquots of WNV strain NY99–35262-11 to tubes containing 900 μl of each buffer type, creating 1 ml samples with titers of 6.5 and 7.5 $\log_{10}$ PFU/ml. Virus-spiked mosquito pools contained 25 laboratory-reared uninfected *Cx. quinquefasciatus* mosquitoes in addition to virus as prepared above. After the addition of WNV to each tube of buffer, a 100 μl aliquot was removed after briefly mixing ≈ 3 seconds. The samples were then processed by vortexing for 1 minute after which time another 100 μl aliquot was removed. Aliquots removed from the samples at each time point were immediately serially diluted in BA-1 and kept on ice as described above.

The ITI infected mosquito pools were created by adding one laboratory-reared *Cx. quinquefasciatus* mosquito that had been infected with WNV via ITI to pools containing 24 uninfected laboratory-reared mosquitoes. The pools were ground in each buffer type by vortexing for a total of 1 min; aliquots of 100 μl were taken after ≈ 3 seconds and 1 min and serially diluted in BA-1 as described above.

Virus infectivity was determined by plaque assay on monolayers of Vero cells in 6-well plates as described above for the evaluation of RAMP buffer. The virus seed used to spike

the virus-only and virus-spiked mosquito pools was also titrated on Vero cells to estimate the titer of those pools.

We tested all samples processed in RAMP buffer or RB-TX using the RAMP assay as directed by the manufacturer's protocol. Because the samples used to estimate virus inactivation had such high titers, all produced results > 640. To create samples that would produce a range of RAMP scores, several sets of 10-fold serial dilutions of WNV were made in unmodified RAMP buffer with titers ranging from 1.5 – 7.5 $\log_{10}$ PFU/ml. The RAMP assay was first performed on these RAMP buffer samples according to the manufacturer's protocol. We then added Triton X-100 to these same RAMP buffer samples in a 1% final concentration, mixed well, and performed the RAMP assay on the RB-TX samples according to the manufacturer's protocol. Pools containing 25 uninfected mosquitoes were also processed in RAMP buffer and RB-TX and tested with the RAMP assay to serve as negative controls.

Paired Student's t-tests and confidence intervals were used to compare the differences in mean RAMP units produced by RAMP buffer and RB-TX samples for titers of 2.5, 2.9, 3.5, and 3.9 $\log_{10}$ PFU/ml. We estimated the proportion of RAMP values expected to be ≥ 50 for RAMP buffer and RB-TX samples containing the titers listed above to determine the sensitivity of the RAMP assay when testing samples that have been processed in each buffer type. The sensitivity estimates were calculated by applying the method detailed in Burkhalter et al., 2014, where we used Student's t distribution for the distribution of the statistic W to determine RAMP assay sensitivity.

RESULTS

Virus Inactivation

The calculated WNV virus titers from samples after 5–60 min incubation in RAMP buffer are presented in Table 1. Samples processed in RAMP buffer showed a maximum titer reduction of 2 $\log_{10}$ PFU/ml after a 60-min incubation when compared to virus samples ground in BA-1 that served as positive controls and produced an expected number of plaques for each titer.

The calculated virus titers from virus-only and virus-spiked mosquito pool samples ground in RAMP buffer and RB-TX for ≈ 3 sec and 1 min are presented in Table 2. The average calculated virus titers from ITI-infected mosquito pool samples ground in each buffer type for ≈ 3 sec and 1 min are presented in Table 3. Samples processed in RAMP buffer showed titer reductions of ≈1 $\log_{10}$ PFU/ml after the ≈ 3 sec and 1 min time points when compared to virus samples ground BA-1 that served as positive controls and produced an expected number of plaques for each titer. No plaques were produced from any samples exposed to RB-TX and healthy cell sheets were observed under magnification in all wells to which the RB-TX samples were applied.

RAMP assay results

The three sample types (virus-only, virus-spiked mosquito pools, and ITI infected mosquito pools) ground in RAMP buffer and RB-TX produced RAMP scores > 640, the maximum

result that is displayed by the RAMP reader (Tables 2 and 3). RAMP assay results for the panels of serially diluted WNV samples processed in RAMP buffer and RB-TX are presented in Fig. 1. We used a positive cut-off value of ≥ 50 RAMP Units as recommended previously (Burkhalter et al. 2014). RAMP results produced by samples processed in RAMP buffer fell within the expected range based on titer (Burkhalter et al. 2014)

Samples processed in both buffer types containing < 2 $\log_{10}$ PFU/ml and > 4.5 $\log_{10}$ PFU/ml produced < 50 RAMP units or well over 50 RAMP units, respectively, rendering the comparison of RAMP Unit means for samples in these titer ranges unnecessary. The mean RAMP Units produced by RB-TX samples containing titers 2.5, 2.9, 3.5, and 3.9 $\log_{10}$ PFU/ml were statistically significantly higher (at α=0.05) than samples processed in RAMP buffer (Table 4). The estimated RAMP assay sensitivity when testing RB-TX samples was also higher when compared to RAMP buffer samples at the same titers (Table 4). None of the negative control mosquito pools processed in RAMP buffer or RB-TX produced scores ≥ 50 RAMP Units (Fig. 1).

DISCUSSION

Although the RAMP assay kit insert states that “the RAMP buffer is intended to facilitate the immunoreaction of the assay and is not intended to inactivate the virus,” this cautionary statement can be overlooked by RAMP operators that assume that it does inactivate virus, and many MADs perform the assay under lower containment than is required for working with WNV. We conducted this evaluation to assess whether the buffer does or does not inactivate WNV.

In the first evaluation, we observed a maximum titer reduction of only 2 $\log_{10}$ PFU/ml in samples incubated in the standard kit-supplied RAMP buffer for 60 min, and shorter incubations exhibited less reduction. Even if the results of this evaluation showed that RAMP buffer would inactivate WNV after a certain incubation period, until that incubation period had elapsed, the sample could still pose a biological hazard. Case reports of non-mosquito transmitted infections of WNV (Fonseca et al. 2005) and other arboviruses (Chen et al. 2004; Sewell et al. 1995; Hanson et al. 1967) indicate that droplets or aerosolized particles of the virus can enter the body through mucous membranes and cause disease. To harvest material from mosquitoes for arbovirus testing, the mosquitoes must first be homogenized, which is often done by adding BBs to polypropylene tubes containing mosquitoes and a grinding buffer and processing the sample using a vortexer or mechanized homogenizer. The resulting supernatant is then used for testing in various assays. If there are WNV-positive mosquitoes in the pool and the grinding buffer does not inactivate virus, this supernatant will likely contain infectious, live virus. During the homogenization process there is risk, albeit rare, of spills or aerosolization of the material as the vigorous shaking of tubes may cause them to break. Since homogenization is the first step in processing and usually takes between 1 and 4 minutes, any method that does not provide immediate inactivation would be insufficient to prevent potential aerosol exposure when the tube is opened, or in the event of a spill or splash.

Realizing the inability for many MADs to process and test their mosquito pools in the appropriate BSL containment required for WNV, but recognizing the need for this testing to continue, we investigated a protocol modification that would meet our requirement to render the virus noninfectious on contact. We modified the standard RAMP buffer by adding Triton X-100 and used it to process three types of samples. The virus-only samples, containing high titers of WNV in the buffer alone, allowed us to determine the precise time of virus inactivation. The virus-spiked mosquito pools contained the same amount of virus as the virus-only samples, but the addition of mosquitoes allowed us to determine if the presence of homogenized mosquitoes interfered with the detergent. Testing pools of mosquitoes spiked with one mosquito infected by ITI mimicked real-world testing of field collected mosquitoes, where the virus would be contained within the mosquito and exposed to the buffer during the grinding process.

All three sample types were affected by the RB-TX in the same way, in that no viable WNV was recovered after ≈ 3 sec homogenization nor after 1 min, when homogenization was deemed sufficient. This quick inactivation was expected based on the results of previous studies using pure virus (Kreil 2003) and we found that the presence of homogenized mosquitoes did not affect inactivation. The samples' RAMP results of > 640 indicated levels of virus that would produce plaques if viable, however the absence of plaques confirmed the inactivation of virus in the RB-TX samples.

Conversely, samples that were processed in RAMP buffer retained much of the original virus infectivity. The virus-only and virus-spiked mosquito pool samples ground in RAMP buffer showed a reduction of ≈1 $\log_{10}$ PFU/ml after the ≈ 3 sec and 1 min incubation periods. The ITI-infected mosquito pools produced slightly different results, in that more live virus was recovered after vortexing for 1 min than after vortexing for a few seconds. Apparently, the virus contained in the mosquito was not fully released into the buffer after ≈ 3 seconds of vortexing, and more virus was released by vortexing for 1 min.

Once complete virus inactivation was demonstrated, we evaluated the effect of Triton X-100 on the performance of the RAMP assay. While the virus titers of samples homogenized in RAMP buffer generated RAMP results that were consistent with previously determined RAMP score ranges (Burkhalter et al. 2014), the addition of Triton X-100 generated results that were consistently higher than the RAMP buffer samples at each dilution (Fig. 1). The reason for this not certain, but we surmise that the lysing effect of the added detergent liberates more viral antigen into the supernatant, which is detected by the RAMP assay and produces higher RAMP scores. The addition of Triton X-100 may slightly boost the ability of the RAMP assay to detect WNV positive samples but only for samples at the limit of detection, 3.5–3.9 $\log_{10}$ PFU/ml, as described previously using a positive cut-off of ≥ 50 RAMP Units (Burkhalter et al. 2014). Triton X-100 treated samples that contain titers at this limit of detection produce positive results, while some of the samples containing the same titers and processed in untreated RAMP buffer will produce < 50 RAMP units (Table 4). With the exception of this very narrow titer range at the RAMP assay's limit of detection, the qualitative results remain as expected at each titer (i.e., positive or negative results that are produced by sample titers are the same regardless of grinding buffer used) despite the overall increase in RAMP scores of samples processed in RB-TX. All negative controls

produced negative results (< 50 RAMP Units), which verifies that the addition of Triton X-100 to RAMP buffer will not produce false positives.

Triton X-100 is inexpensive and readily available from a number of commercial vendors, and only a very small amount is needed to make an effective WNV-inactivating buffer. This detergent is extremely viscous and requires meticulous pipetting techniques to accurately aspirate and dispense the proper amount; slow pipetting is key. Do not attempt to add aliquots of Triton X-100 to individual tubes of mosquitoes. To maximize efficiency and pipetting accuracy, we recommend preparing the RB-TX using large volumes of RAMP buffer supplied in the kit in a final concentration of 1%. When added to the RAMP buffer, Triton X-100 will initially dispense in a ribbon. Gentle mixing by inversion or pipetting is necessary to prevent the Triton X-100 from forming an impermeable clump at the bottom of the container, and after a few minutes the detergent will be completely dissolved. Long-term storage of the RB-TX does not reduce its effectiveness nor does the detergent precipitate (data not shown). Triton X-100 should be added to RAMP buffer that has been stored at RT because it will not dissolve in a cold medium. After the Triton X-100 has dissolved completely, the RB-TX is ready to be used following the manufacturer's protocol for homogenization and performing the assay.

Data from this study indicate that the addition of Triton X-100 detergent to RAMP buffer in a 1% final concentration inactivates WNV and allows the RAMP assay to be performed safely outside biosafety containment, without compromising RAMP assay results. We nevertheless recommend proper PPE such as lab coats, gloves and eye protection when processing mosquito pools regardless of grinding buffer used, and agencies that process pools within biosafety containment should continue to do so.

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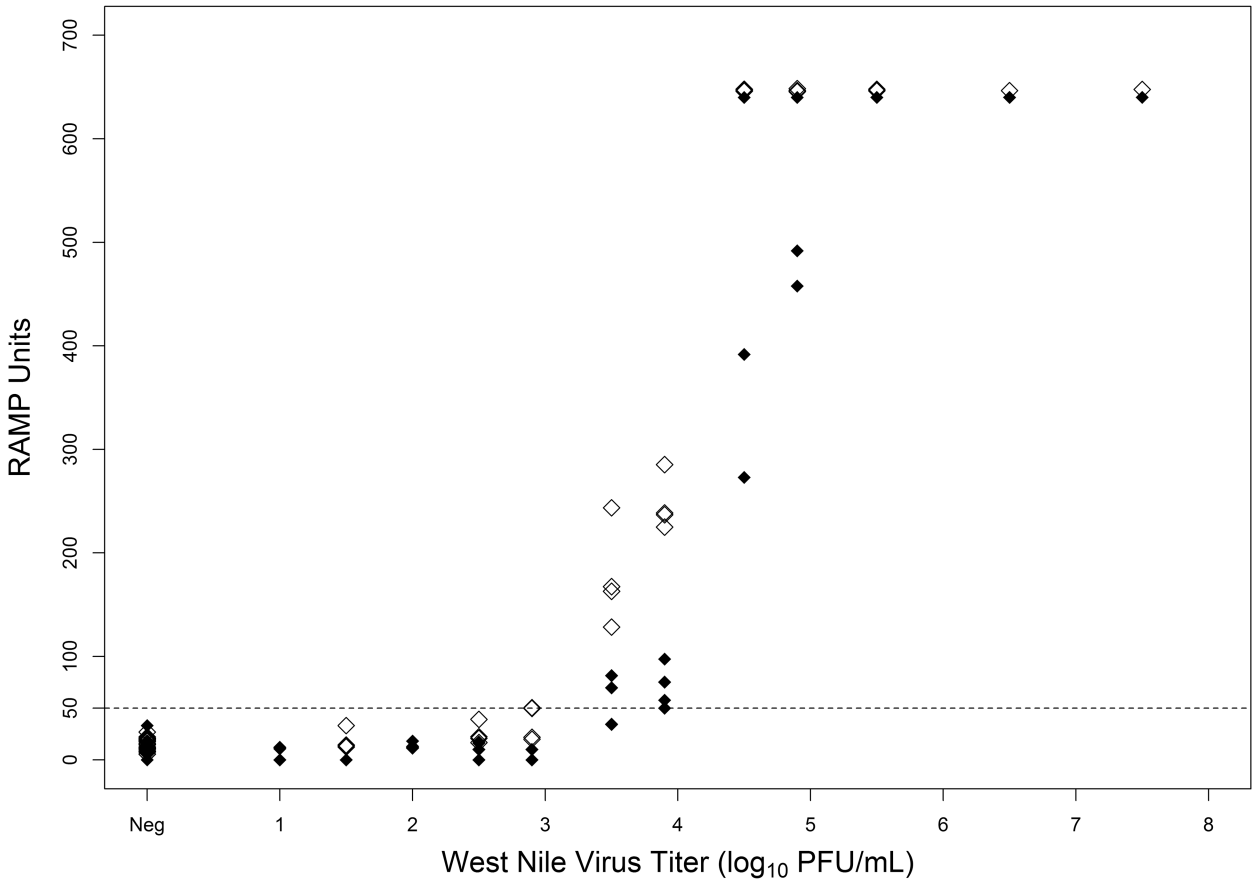

Fig. 1.

RAMP results from West Nile virus (WNV) positive samples processed in RAMP buffer (◆) and RAMP buffer fortified with 1% Triton X-100 (RB-TX; ◇). Samples producing RAMP Units ≥ 50 (represented by the horizontal line) are considered positive; > 640 is the maximum displayed result.

Table 1.

Calculated titers (log_{10} PFU/ml) of West Nile virus (WNV) incubated in RAMP buffer. Samples processed in BA-1 served as controls and indicate the expected titer for each sample.

	Titer of sample in BA-1	
	7.3	6.2
Time incubation (min)	**Titer of sample in RAMP Buffer**	
5	6.0	5.0
10	5.9	4.9
15	5.8	4.7
20	5.7	4.6
25	*5.7*	4.5
30	5.6	4.4
60	5.5	4.2
Maximum titer reduction	1.8	2.0

Table 2.

Calculated titers (log_{10} PFU/ml) of West Nile virus (WNV) virus-only and virus-spiked mosquito pools processed in RAMP buffer and RAMP Buffer fortified with 1% Triton X-100 (RB-TX). Samples processed in BA-1 served as controls and indicate the expected titer for each sample.

	Virus-only samples				**Virus-spiked mosquito pools**			
	Titer of sample in BA-1				**Titer of sample in BA-1**			
	7.5	6.5	7.5	6.5	7.5	6.5	7.5	6.5
Time incubation	**Titer of sample in RAMP Buffer**		**Titer of sample in RB-TX**		**Titer of sample in RAMP Buffer**		**Titer of sample in RB-TX**	
≈ 3 sec	6.8	5.6	0	0	6.8	5.7	0	0
1 min	6.7	5.5	0	0	6.8	5.5	0	0
	Max.[1] titer reduction		Max. titer reduction		Max. titer reduction		Max. titer reduction	
	0.8	1.0	~7.5	~6.5	0.7	1.0	~7.5	~6.5
RAMP Results[2] (RAMP Units)	> 640	> 640	> 640	> 640	> 640	> 640	> 640	> 640

[1] Max., Maximum

[2] RAMP assay results; ≥ 50 Units are considered positive; > 640 is the maximum displayed result.

Table 3.

Average calculated titers (log_{10} PFU/ml) and 95% Confidence Intervals (95% CI) of West Nile virus (WNV) positive mosquito pools processed in BA-1, RAMP Buffer, and RAMP Buffer fortified with 1% Triton X-100 (RB-TX). Pools contained 1 mosquito intrathoracically inoculated with WNV and 24 negative mosquitoes.

Time incubation	Ave. titer of sample in BA-1 N=6 (95% CI)	Ave. titer of sample in RAMP Buffer N=6 (95% CI)	Ave. titer of sample in RB-TX N=6
≈ 3 sec	ND[1]	3.9 (3.4 – 4.4)	0
1 min	5.5 (5.4 – 5.5)	4.3 (4.0 – 4.5)	0
Ave. RAMP Result[2] (RAMP Units)	ND	> 640	> 640

[1] ND = not done

[2] RAMP assay results; ≥ 50 Units are considered positive; > 640 is the maximum displayed result.

Table 4.

Mean RAMP assay results and 95% confidence intervals (95% CI) for samples containing the specified titers (log_{10} PFU/ml) processed in RAMP buffer or RAMP Buffer containing 1% Triton X-100 (RB-TX). Differences in mean RAMP Units and 95% CI were calculated by subtracting the mean RAMP Units of the RAMP buffer samples from the mean RAMP Units of the RB-TX samples. RAMP assay sensitivity (i.e., the probability that a positive sample will produce a positive result in the RAMP assay) and 95% CI were calculated for each buffer type and titer using a positivity cutoff of ≥ 50 RAMP units.

Titer (log_{10} PFU/ml)	Mean RAMP Units of RB-TX samples	Mean RAMP Units of RAMP buffer samples	Difference in mean RAMP Units	RB-TX Sensitivity %	RAMP Buffer Sensitivity %
2.5	24.8 (9.1 – 40.5)	10.9 (−1.7 – 23.6)	13.9 (0.9 – 26.9)	4.2 (0.1 – 21.9)	0.8 (0 – 4.4)
2.9	32.9 (18.0 – 47.8)	1.7 (−2.6 – 6.0)	31.2 (13.9 – 48.4)	14.1 (0.8 – 43.9)	0 (0 – 0)
3.5	175.6 (98.3 – 252.9)	55.0 (16.4 – 93.5)	120.6 (54.1 – 187.1)	95.9 (87.8 – 99.9)	54.5 (6.9 – 96.9)
3.9	245.9 (223.1 – 268.7)	70.3 (52.7 – 87.8)	175.6 (141.6 – 209.6)	100.0 (100.0 – 100.0)	86.0 (56.2 – 99.2)