**Immuoassay-Compatible Inactivation of SARS-CoV-2 in Plasma Samples for Enhanced Handling Safety**

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**ABSTRACT:** Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) inactivation is an important step toward enhanced biosafety in testing facilities and affords a reduction in the biocontainment level necessary for handling virus-positive biological specimens. Virus inactivation methods commonly employ heat, detergents, or combinations thereof. In this work, we address the dearth of information on the efficacy of SARS-CoV-2 inactivation procedures in plasma and their downstream impact on immunoassays. We evaluated the effects of heat (56 °C for 30 min), detergent (1−5% Triton X-100), and solvent−detergent (SD) combinations [0.3−1% tri-n-butyl phosphate (TNBP) and 1−2% Triton X-100] on 19 immunoassays across different assay formats. Treatments are deemed immunoassay-compatible when the average and range of percentage recovery (treated concentration relative to untreated concentration) lie between 90−110 and 80−120%, respectively. We show that SD treatment (0.3% TNBP/1% Triton-X100) is compatible with more than half of the downstream immunoassays tested and is effective in reducing SARS-CoV-2 infectivity in plasma to below detectable levels in plaque assays. This facile method offers enhanced safety for laboratory workers handling biological specimens in clinical and research settings.

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**INTRODUCTION**

The implementation of robust infection control and prevention strategies in both research and clinical settings is crucial in minimizing the exposure risks of laboratory personnel to the highly transmissible severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus. As of January 2021, the biosafety recommendations of the Centre for Disease Control and Prevention (CDC, USA) specifies biosafety level 3 (BSL-3) for work related to SARS-CoV-2 propagation and isolation where high concentrations of live viruses or large volumes of infectious material are involved, while BSL-2 laboratories may perform routine diagnostic testing with standard precautions in place. Given the arguable airborne transmissibility of SARS-CoV-2, diagnostic analyses of virus-positive clinical specimens on high-throughput analyzers using open tubes or sample cups raise safety concerns for personnel handling such samples. Several studies have shown that the positive nucleic acid detection rate of SARS-CoV-2 in blood from Covid-positive patients with mild to critical illness ranges between 1 and 41%. Although positive detection does not necessarily equate with the infectiousness of the sample, the potential severity of the Covid-19 disease and its ease of transmission mandate the development of virus inactivation protocols, especially in research-only settings where there is no urgency to provide results for immediate clinical management and there is time to further minimize risks. Furthermore, inactivated samples could be handled at a lower biocontainment level, thus increasing the capacity and reducing the costs for much needed Covid-related research.

Virus inactivation can be accomplished via physical (heat and ultraviolet light), chemical (detergents, fixatives, and denaturants), and energetic (sonication and ionizing radiation) methods and combinations thereof. The SARS-CoV-2 virus is an enveloped virus and has one of the hardest outer shells among the coronaviruses. This portends harsher treatment conditions required for complete inactivation. Heat inactivation at 56 °C for 30 min or less has been demonstrated to be effective against SARS-CoV-2 in cell culture media and nasopharyngeal and sera samples. These authors showed that the duration of treatment can be reduced with higher temperatures applied. However, heat treatment generally

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results in a significant reduction in the measured analyte levels in subsequent antibody-based assays due to thermal-induced protein denaturation and aggregation. Although a handful of soluble factors has been found to be essentially unaffected by heat treatment at 60 °C for up to 60 min, thermal inactivation schemes would be more suitably applied in molecular assays involving nucleic acid testing.

Detergents and solvent–detergents (SD) are commonly employed against enveloped viruses. These substances act on the viral envelope, whereby the irreversible disruption of the lipid/protein coat compromises its integrity and renders the virus noninfectious. A range of detergents, Trizol reagents, lipids/protein coats, and denaturants have been reported to be effective in inactivating SARS-CoV-2 in plasma and is immensely helpful.

Since its introduction in the mid-1980s, SD treatment has been the standard method used for process-scale inactivation of enveloped viruses in human-derived biologics. SD preparations typically comprise a combination of tri- n-butyl phosphate (TNBP) and Triton X-100 at 0.3 and 1%, respectively, although Tween 80 may also be used as the detergent component. The effects of SD treatment on plasma composition have been extensively studied, and the evidence indicates that this agent does not adversely impact the protein profile and quality, although some reduction in the levels and activity of coagulation factors and inhibitors has been observed. SD treatment has been shown to be highly effective in inactivating SARS-CoV-1, but there is currently a paucity of data on its effectiveness against SARS-CoV-2 in any matrix.

This study addresses an unmet gap in the investigations on SARS-CoV-2 inactivation methods by evaluating the effectiveness of heat, Triton X-100, and SD treatments in plasma and downstream compatibility of treated samples for antibody-based testing. Considering that Covid-19 has profound long- and short-term impacts on the cardiovascular status of infected people, the analytes selected for immunoassay across broad assay platforms (semi-automated immunoanalyzers, sandwich ELISAs, and microfluidic cartridge-based assays) are biomarkers that reflect cardiac injury, oxidative stress, inflammation, and endothelial dysfunction (Table 1). To demonstrate the lack of infectious virus after SD treatment with 0.3% TNBP/1% Triton X-100, serial dilutions of treated plasma after SD removal were inoculated into Vero cells and assessed for the absence of SARS-CoV-2 plaques at all dilutions. Schematicsof the study experimental design are illustrated in Figure 1. We show that 0.3% TNBP/1% Triton X-100 is effective in inactivating SARS-CoV-2 in plasma and is compatible with the immunoassay of a wide range of analytes on multiple assay platforms.

## RESULTS

The effects of SARS-CoV-2 inactivation procedures on immunoassay results are shown in Table 2. Of the three methods used, heat inactivation at 56 °C for 30 min had the most detrimental effects on immunoassays, whereby assay signals were reduced by 30–50% for hs-cTnI, ST2, and LGALS3 and completely abolished for ANGPT2. However,
NT-proBNP, hs-cTnT, and GDF15 assays were largely unaffected. Heat treatment in the presence of 1% Triton X-100 did not result in further signal reduction, except for hs-cTnI.

Recovery indicators show that immunoassays are more tolerant of Triton X-100 treatments compared with heat. More than half of the immunoassays met the acceptable limits defined for the average range of % recovery. The average % recovery was above 90% for all analytes tested, except for hs-cTnI (82%), renin (REN) (88.5%), and KITGL (76%). Triton X-100 treatment reduced the average recovery of KITGL, but not REN, in a concentration-dependent manner. Interestingly, the susceptibility of KITGL measurements to detergent concentration coincided with the relatively low plasma dilution (2× dilution) required for this assay compared with all other microplate-based assays (4×−200× dilution). Unexpectedly, the measured concentrations of SELP and hs-cTnT increased with the increasing concentration of Triton X-100, whereby the average recovery was slightly above 120% at the highest detergent concentration tested for these analytes. Average recovery of around 120% was also observed for EDN1 (only for the microplate-based sandwich ELISA assay), but this increase was not in a detergent concentration-dependent manner. By far, aldosterone levels showed the largest increase, with the average recovery between 250 and 280% across the whole range of Triton X-100 concentrations tested.

Approximately 60% of the immunoassays tested performed within the defined acceptable limits after SD (0.3% TNBP/1% Triton X-100) treatment. The overall trends largely recapitulate those observed with 1% Triton X-100. Increasing the concentration of TNBP and/or Triton X-100 beyond 0.3 and 1%, respectively, have detrimental effects on NT-proBNP and LGALS3, while ST2, GDF15, and leptin (LEP) are unaffected.

Prior to testing the efficacy of SD inactivation, we evaluated the effects of plasma alone on Vero cells. Cytotoxic effects and morphological changes were observed in cells after 1 h incubation with undiluted and twofold diluted plasma but not with fivefold diluted plasma (Figure 2A). Cells appear rounded and smaller in size when exposed to high plasma concentrations. Cell death and/or loss of substrate adherence were also observed as assessed by the marked reduction in cell coverage, especially in the neat plasma well. However, after
Table 2. Measured Concentrations [Mean (Range)] of the Investigated Biomarkers in Untreated Plasma and % Recovery after Virus Inactivation Treatment [Mean (Range)]

| analyte      | untreated (concentration) | heat | 1% Triton X-100 + heat | 1% Triton X-100 | 1.5% Triton X-100 | 2% Triton X-100 | 3% Triton X-100 | 5% Triton X-100 | 0.3% TNBP + 1% Triton X-100 | 0.3% TNBP + 1% Triton X-100 | 0.6% TNBP + 1% Triton X-100 |
|--------------|---------------------------|------|-------------------------|----------------|------------------|----------------|----------------|----------------|--------------------------|--------------------------|--------------------------|
| NT-proBNP (pg/mL) | 201 (60.0–6476)           | 96.1 | 96.7 (90.9–101)         | 102* (97.1–107)* | 97.4 (94.3–103) | 91.4 (87.3–95.1) | 89.5 (82.6–96.3) | 81.2 (73.8–83.2) | 94.2 (87.0–105)              | 74.6 (60.9–87.8)              | 81.0 (75.7–89.4)              |
| hs-cTnT (pg/mL)  | 23.1 (3.00–45.6)          | 90.4 | 102 (82.9–137)          | 101* (58.5–152)* | 105 (85.1–175) | 106 (79.6–177) | 115 (94.3–204) | 122 (92.5–247) | 103 (97.4–110)               | 105 (93.7–111)               | 105 (99.9–110)               |
| hs-cTnI (pg/mL)  | 31.6 (2.50–920)           | 70.8 | 41.5 (4.00–71.9)        | 82.0 (47.6–110) | ND               | ND             | ND             | ND             | ND                       | ND                       | ND                       |
| ST2 (ng/mL)     | 30.3 (22.3–441)           | 59.3 | 61.0 (50.0–73.3)        | 100* (90.0–115)* | 103 (96.5–108) | 104 (97.1–111) | 100 (96.4–109) | 99.3 (92.7–114) | 103 (97.4–110)               | 105 (93.7–111)               | 105 (99.9–110)               |
| REN (pg/mL)     | 62.0 (11.0–153)           | ND   | ND                      | 88.5* (68.7–106)* | 85.4 (69.0–118) | 91.3 (73.7–139) | 81.9 (61.0–125) | 85.0 (56.5–133) | 66.8 (59.3–74.6)              | 66.4 (48.5–116)              | 50.9 (40.0–67.2)              |
| aldosterone (pg/mL) | 82.5 (49.9–120)          | ND   | ND                      | 273* (228–340)* | 278 (229–328) | 281 (230–351) | 262 (220–323) | 251 (208–315) | 248 (221–316)               | 282 (256–357)               | 250 (225–310)               |
| ANGPT2 (pg/mL)  | 2006 (956–6125)           | 0.42 | 0 (0)                   | 91.7* (75.4–118)* | 85.7 (56.6–102) | 84.9 (64.5–96.0) | 83.8 (71.4–92.7) | 88.7 (81.6–97.4) | 77.4 (68.3–82.0)              | 62.9 (52.3–70.9)              | 70.2 (61.8–78.8)              |
| GDF15 (pg/mL)   | 2089 (548–4767)           | 101  | 104 (93.7–119)          | 101* (90.3–113)* | 100 (95.3–105) | 101 (96.3–109) | 99.9 (95.0–102) | 99.6 (95.9–97.7) | 95.6 (93.5–106)              | 98.2 (91.2–109)              | 98.0 (91.2–109)              |
| LGALS3 (ng/mL)  | 8.33 (2.44–144)           | 50.3 | 50.8 (34.5–59.4)        | 96.7 (91.0–102) | ND               | ND             | ND             | ND             | 90.5 (75.3–100)              | 84.7 (77.0–91.9)              | 88.6 (78.8–97.5)              |
| LEP (ng/mL)     | 15.1 (9.14–241)           | ND   | ND                      | 103* (77.5–116)* | 102 (83.6–109) | 102 (85.6–118) | 104 (80.1–120) | 99.7 (75.7–112) | 74.9 (71.0–78.4)              | 74.6 (71.2–77.8)              | 57.8 (50.7–62.7)              |
| KITLG (ng/mL)   | 30.3 (22.3–441)           | ND   | ND                      | 76.0* (66.9–82.5)* | 67.4 (64.1–71.5) | 56.8 (52.9–61.2) | 38.5 (30.0–42.0) | 38.7 (25.5–30.4) | 74.9 (71.0–78.4)              | 74.6 (71.2–77.8)              | 57.8 (50.7–62.7)              |
| SELP (ng/mL)    | 50.2 (29.5–663)           | ND   | ND                      | 117 (108–125)    | ND               | ND             | ND             | ND             | 108 (94.3–116)               | 109 (101–117)                | ND                       |
| #ICAM-1 [R] (ng/mL) | 231 (167–287)            | ND   | ND                      | 107 (101–113)    | ND               | ND             | ND             | ND             | 107 (101–113)                | ND                       | ND                       |
| #ICAM-1 [E] (ng/mL) | 318 (225–399)            | ND   | ND                      | 99.0 (87.0–114)  | ND               | ND             | ND             | ND             | 99.8 (89.8–113)              | ND                       | ND                       |
| #EDN1 [R] (pg/mL) | 1.07 (0.47–1.68)         | ND   | ND                      | 129 (105–163)    | ND               | ND             | ND             | ND             | 154 (125–196)                | ND                       | ND                       |
| #EDN1 [E] (pg/mL) | 1.97 (0.96–2.69)         | ND   | ND                      | 116 (100–130)    | ND               | ND             | ND             | ND             | 106 (87.9–114)               | ND                       | ND                       |
| d-dimer (ng/mL) | 995 (227–2589)            | ND   | ND                      | 92.4 (81.6–103)  | ND               | ND             | ND             | ND             | 95.7 (85.7–103)              | ND                       | ND                       |
| SELE (ng/mL)    | 30.2 (21.8–362)           | ND   | ND                      | 102 (93.8–111)   | ND               | ND             | ND             | ND             | 101 (93.9–110)               | ND                       | ND                       |
| VCAM-1 (ng/mL)  | 672 (351–994)             | ND   | ND                      | 99.3 (89.7–119)  | ND               | ND             | ND             | ND             | 96.9 (84.5–108)              | ND                       | ND                       |

*Abbreviations: ND, not done. Other abbreviations as in Table 1. # Analytes are measured by sandwich ELISA (R) or ELLA (E) cartridge-based assay. Recovery is the percentage of sample concentration after virus inactivation treatments relative to untreated ones. Recovery based on values obtained from two (‘) or three (‘) independent experiments.
subsequent washing and addition of fresh culture media, the residual live cells were able to recover morphologically and form a monolayer after 24 h (Figure S1). We investigated the effect of plasma concentration on the infectivity of SARS-CoV-2 in Vero cells. Plasma was spiked to an estimated titer of \(1 \times 10^5\) PFU/mL viral particles, and 10-fold serial dilutions were used to infect cells. Surprisingly, no plaques were formed in wells incubated with virus-spiked neat plasma, although plaques can be observed in wells containing 10- to 1000-fold dilutions of the same sample (Figure 2B). Taken together, while Vero cells are able to recover from the exposure to high concentrations of plasma, the initial cytotoxic effects resulted in a large reduction in cell number, perhaps by virtue of cell death and/or loss of substrate adherence properties. In addition, SARS-CoV-2 infection of the residual cells was not observed.

Next, we investigated the cytotoxic effects of SD-treated plasma on Vero cells and its mitigation using Pierce detergent removal columns for reagent removal. Without SD removal, 100-fold dilution is required to overcome chemical toxicity, as determined by comparable morphology (Figure 3A) and crystal violet staining (Figure 3B) with control cells not exposed to plasma. However, with SD removal, cells were able to retain their typical morphology at fivefold dilution and were strongly stained by crystal violet in all cases.

A further critical consideration for successful SD removal is that filtration through the detergent removal column should not result in a significant reduction of virus titer or infectivity of Vero cells. Virus recovery tests indicated a minimal loss of viral particles post-filtration. Average virus titers from unfiltered and filtered plasma were \(1.4 \times 10^5\) PFU/mL and \(0.94 \times 10^5\) PFU/mL, respectively, based on plaque counts at \(10^{-2}\) dilution (Figure 4).

Finally, we show that the SD treatment of plasma is highly effective in SARS-CoV-2 inactivation (Figure 5). In the case of filtered plasma (positive control), clear plaques are observed at \(10^{-3}\) and \(10^{-4}\) plasma dilutions, equivalent to \(10^2\) and \(10^4\) PFU/mL, respectively. No plaques were observed at \(10^{-5}\) dilution, indicating that the limit of detection of infectious SARS-CoV-2 was approximately 10 PFU/mL. On the other hand, no plaques were observed at all inoculum dilutions with SD-treated plasma, demonstrating effective SARS-CoV-2 inactivation.

**DISCUSSION**

This work represents a data resource to facilitate safe protein biomarker research for developing diagnostic and prognostic tools in the fight against SARS-CoV-2. We report the effects of heat, Triton X-100 (1–5%), and SD treatment on immunoassays across a variety of assay platforms. We define our acceptance criteria for treatment effects of the virus inactivation procedure on the basis of average % recovery (90–110%) relative to untreated concentration values and the range of observed % recovery for all 10 samples within each experimental set. The latter parameter provides a glimpse of whether all samples are more or less equally affected by the inactivation treatment. Setting the acceptable range to fall within 80–120% is in line with the accuracy and precision limits recommended for ligand-binding assays.31
Despite the stringent acceptance criteria used, we show that more than half of the immunoassays tested performed within the defined acceptable limits after plasma treatment with 1–3% Triton-X100 or 0.3% TNBP/1% Triton X-100. In fact, immunoassays for ST2 and GDF15 can tolerate plasma containing 5% Triton X-100, with assays for ANGPT2 and LEP performing just outside the defined acceptable limits. The tolerance of NT-proBNP and hs-cTnT Roche assays to heat inactivation as well as total signal abolishment for ANGPT2 is consistent with the observations reported in previous studies.\textsuperscript{15,32} Our data demonstrated that heat inactivation is generally incompatible with immunoassays, resulting in drastic reduction in plasma levels (ANGPT2, ST2, and LGALS3) and high variability in % recovery between samples (hs-cTnT, hs-cTnI, ST2, and LGALS3). Surprisingly, the R&D Systems
GDF15 assay is extremely robust and is not affected by heat, detergent, or SD treatments.

Virus plaque assays also provide evidence that plasma spiked with SARSCoV-2 to $10^5$ PFU/mL can be inactivated to below the limit of detection ($\sim 10$ PFU/mL) after SD treatment. Incubation of Vero cells with undiluted plasma resulted in cellular stress manifested morphologically by their rounding up in shape and shrinking in size as well as, possibly, cell death and/or loss of substrate adherence. The cytotoxic effect of undiluted plasma on Vero cells was also observed by other workers. After washing, residual cells recovered after 72 h of further culture in fresh media. As plaques could not be obtained from Vero cells exposed to neat plasma, undiluted plasma was not plated for the evaluation of the efficiency of SD treatment for SARSCoV-2 inactivation. SD removal by filtration through Pierce detergent removal columns allowed the evaluation of titer reduction at higher plasma concentrations, effectively improving the limit of detection of the plaque assays. In agreement with a previous report, the processing of virus-spiked plasma through these columns did not compromise titer recovery or virus viability. Overall, the data presented in this study indicate that SD treatment with 0.3% TNBP/1% Triton X-100 is a viable SARSCoV-2 inactivating method suitable for immunoassays of plasma samples. Intuitively, this treatment should also be applicable to samples in physiological buffers and cell culture media.

The presence of Triton X-100 alone or in combination with TNBP can result in an increase (hs-cTnT, aldosterone, SELP, and EDN1) or decrease (hs-cTnI, REN, and KITLG) in apparent plasma concentrations. The change in the analyte level may be dependent or independent of the detergent concentration. Extracellular vesicles are well known to be a rich source of candidate protein biomarkers, and detergents/SDs have been shown to exhibit differential ability in disrupting these membrane-enclosed structures. Hence, increased levels may be attributed to the detergent-mediated disruption of residual cellular components and/or extracellular vesicles present in plasma, resulting in the release of intracellular contents. In the case of aldosterone, an unexpected large increase in average recovery of 250–280% was observed for detergent or SD-treated plasma, and this effect was independent of the reagent concentration. Aldosterone is a mineralocorticoid that plays an important role in the regulation of blood volume, pressure, pH, and electrolyte balance. Aldosterone may be present in circulation in complex with other interacting components. Our data suggest that Triton X-100 at 1% is sufficient to disrupt this putative complex to render aldosterone more accessible to the capture antibody in the competitive assay used. This incidental finding has interesting practical implications for immunoassays in biomarker research. For analytes where natural levels hover below or near the lowest calibrator point of a standard curve, detergent-mediated increase in analyte accessibility to antibody binding may be exploited so that most samples become measurable, with the concentration values rising above the lowest calibrator of the standard curve. In the case of analytes that are present in both free and vesicle-/exosome-encapsulated forms in circulation, paired measurements of both untreated and detergent-treated plasma samples can be used to determine the concentration of total, encapsulated, and free analytes, providing greater delineation of the association of a biomarker with the disease state and clinical outcome.

The susceptibility of an assay to detergent or SD-treatment may be dependent on the assay platform used. Measurements of ICAM-1 and EDN1 plasma levels on the ELLA (microfluidic cartridge) platform gave higher readings compared with the R&D Systems Quantikine microplate-based assay. However, while the detergent or SD treatment did not impact...
Table 3. Experimental Schemes for SARS-CoV-2 Inactivation

| analytes tested | treatment regimes |
|-----------------|-------------------|
| NT-proBNP, hs-cTnT, ST2, ANGPT2, GDF15, hs-TnI, LGALS3. | untreated, 1% Triton X-100, heat, 1% Triton X-100 + heat |
| NT-proBNP, hs-cTnT, ST2, ANGPT2, GDF15, REN, LEP, KITLG, aldosterone. | untreated, 1% Triton X-100, 1.5% Triton X-100, 2% Triton X-100 |
| NT-proBNP, hs-cTnT, ST2, ANGPT2, GDF15, REN, LEP, KITLG, aldosterone. | untreated, 1% Triton X-100, 3% Triton X-100, 5% Triton X-100 |
| NT-proBNP, hs-cTnT, ST2, ANGPT2, GDF15, REN, LEP, KITLG, aldosterone, LGALS3. | untreated, 0.3% TNBP/1% Triton X-100, 1% TNBP/1% Triton X-100, 0.6% TNBP/2% Triton X-100 |
| SELP, ICAM-1, EDN1, VCAM-1, SELE, D-dimer. | untreated, 1% Triton X-100, 3% Triton X-100, 0.3% TNBP/1% Triton X-100 |

ICAM-1 assays on either platform, EDN1 measurements manifested disparity in assay sensitivity to detergent and SD treatments. The average and range of % recovery were outside acceptable limits with the microplate-based EDN1 assay after Triton X-100 and SD treatment. On the other hand, these recovery metrics fell within acceptable limits for 3% Triton X-100 and SD-treated samples when measured by ELLA. It is also noteworthy that the measured concentrations that are markedly reduced in Triton X-100- and SD-treated groups are associated with assays where low predilution of plasma samples is used (hs-cTnT, hs-cTnI, REN, and KITLG). Overall, these results indicate that immunoassay sensitivity to detergent-based virus inactivation procedures is dependent on both the analyte and assay platform in question. For immunoassays that are adversely impacted by the presence of detergent in the plasma sample, it may be possible to circumvent this by selecting an alternative assay platform which allows for a much higher sample predilution to mitigate any interference in antibody–antigen binding in the assay.

The limitation of the proposed chemical-based viral inactivation method is that further investigation is warranted to evaluate the effect of added solvent/detergent on long-term storage of treated samples. In this work, all assays were performed within 2 weeks of chemical treatment. Future work will investigate if analyte levels change over extended storage of SD-treated samples. In addition, the small sample size ($n = 10$) for each test warrants further validation using a larger number of samples. Also, the accuracy of immunoassays with icteric, lipemic, and hemolyzed plasma samples after SD treatment requires investigation to afford greater confidence in the clinical utility of this virus inactivation method for “real-world” samples. Another limitation is that not all immunoassays are impervious to SD treatment although only a minority of tested analytes (ANGPT2, REN, KITLG, and aldosterone) manifested notable deviations in measured concentrations relative to untreated samples. Future work will entail search for, and evaluation of, other immunoassay-compatible SARS-CoV-2 inactivating agents. One possible reagent is beta-propiolactone, a commonly used virus inactivation agent in vaccine preparations. This nucleic acid modifier has been shown to be highly effective in completely inactivating SARS-CoV-2 at a concentration of 0.5% while preserving viral structure and antigenicity, hence pottering immunoassay compatibility. Unlike detergents, beta-propiolactone does not exert cytotoxic effects on Vero cells so as not to interfere with cell viability in viral plaque assays. However, beta-propiolactone poses significant hazard risks not only in terms of its acute toxicity via multiple exposure routes involving inhalation, direct contact, and ingestion but also mounting evidence of its carcinogenic and genotoxic properties. Nevertheless, it would be interesting to assess the impact of this agent on immunoassays.

**CONCLUSIONS**

In summary, Triton X-100 and SD treatments for SARS-CoV-2 inactivation are more compatible with immunoassays compared with heat, although the latter can still be successfully applied for selected assays. The compatibility of up to 5% Triton X-100 with some immunoassays offers scope for adding high concentrations of this detergent to the toolbox of reagents suitable for SARS-CoV-2 inactivation in blood-derived matrices. Finally, SD treatment with 0.3% TNBP/1% Triton X-100 affords a simple immunoassay-compatible method for inactivating high titers of SARS-CoV-2 to below the detection limit in plasma samples. The method is highly amenable, especially in resource-limited and rural testing facilities, as there is no requirement for the use of sophisticated equipment, the inactivating agents employed are relatively benign and low-cost, and the inactivation procedure does not generate secondary risks associated with aerosol production. The findings in this study will provide a springboard for enhancing biosafety in Covid-19-related research and diagnostic testing.

**MATERIALS AND METHODS**

Sample Preparation and Immunoassays. Plasma EDTA samples were obtained from the Singapore Longitudinal Aging Study (community-dwelling adults with cardiovascular risk factors), Singapore Heart Failure and Phenotypes Study (patients with heart failure (HF)), and a commercial source comprising healthy individuals with no known health issues (BioReclamation LLC, Hicksville, NY, USA). Appropriate informed consent was obtained from all patients and control subjects, and the study protocol was approved by the National Health Group Domain Specific Review Board and Institutional Review Board of the National University of Singapore.

Plasma (0.9 mL) was mixed with Triton X-100 or TNBP/Triton X-100 stock solutions (0.1 mL) to obtain the final concentrations shown in Table 3. The samples were then incubated in the dark for 2 h at room temperature with shaking at 300 rpm. Untreated samples comprise 0.9 mL of plasma to which 0.1 mL of water was added. Heat was applied at 56 °C for 30 min to untreated samples or plasma containing 1% (v/v) Triton X-100. All samples were stored at minus 80 °C and assayed within 2 weeks after treatment.

All immunoassays were performed in accordance to manufacturers’ instructions. Residual plasma from routine assays in our ongoing biomarker discovery program was recovered and pooled. Samples comprising non-HF, HF, HF with reduced ejection fraction (HFrEF), HF with preserved ejection (HFrEF), and mixed pools of non-HF and HF plasma
were prepared to cover a range of analyte concentrations. All plasma samples (n = 10) were measured in duplicates, and results were accepted when intra-assay CV was less than 20%. The percentage (%) of recovery following treatment of each sample was computed as:

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\% \text{ Recovery} = \left( \frac{\text{Concentration of untreated sample}}{\text{Concentration of treated sample}} \right) \times 100
\]

The effect of a virus inactivation procedure on an immunoassay was deemed to be acceptable when the % recovery average (n = 10) lies between 90 and 110% and ranges between 80 and 120%, the latter metric indicating that treatment effects are relatively consistent for all samples.

**Cells and Viruses.** Cells. African green monkey kidney cells (Vero E6; ATCC CRL-1586) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum and buffered with 2 g sodium hydrogen carbonate at 37 °C in 5% CO₂.

**Virus.** SARS-CoV-2 was isolated from a nasopharyngeal swab of a COVID-19 patient from the National University Hospital System, Singapore. The isolate was validated by qRT-PCR and propagated in Vero E6 cells. All virus work was performed in a BSL-3 laboratory, and all protocols were approved by the BSL-3 Biosafety Committee and Institutional Biosafety Committee of the National University of Singapore.

**Quantification by Plaque Assay.** To determine virus titers, viral supernatants were 10-fold serially diluted in DMEM. 250 μL of each serially diluted supernatant was added to confluent Vero E6 cells. After 1 h of absorption, the inoculum was removed, and 500 μL of 0.5% agarose overlay was added to each well and incubated for 4 days at 37 °C, 5% CO₂. The cells were fixed with formalin overnight, and agarose was removed before staining with crystal violet. The number of plaques was counted, and the virus titer of individual samples was expressed in the logarithm of plaque-forming units (PFU) per milliliter.

**Evaluation of the Cytotoxicity of Plasma on Vero Cells.** Plasma samples, either undiluted or diluted with DMEM at 2X, 5X, and 10X, were added to confluent Vero E6 cells and examined by microscopy after 1 h of incubation. Cells were then washed with PBS and cultured in fresh media for 72 h to assess the recovery. To evaluate the effect of undiluted plasma on the infectivity of SARS-CoV-2, plasma was spiked to an estimated viral titer of 1 × 10⁵ PFU/mL, and 10-fold serial dilutions were used in plaque assays. Cells were stained with crystal violet, and the plaques were counted after 72 h.

**Removal of SD Reagent Cytotoxicity and Evaluation of Post-Filtration Virus Titer.** To remove the cytotoxicity of the inactivation reagent (0.3% TNBP/1% Triton X-100), SD-treated plasma was processed through Pierce detergent removal columns (2 mL; Thermo Scientific). Following the removal of the storage solution by centrifugation at 1000g for 2 min, each Pierce detergent removal column was equilibrated by three consecutive 2 mL washes with PBS. SD-treated plasma (0.5 mL) was added to each column and allowed to incubate in the resin bed for 2 min at room temperature. Filtered plasma samples were recovered by centrifugation at 1000g for 2 min. The filtered plasma samples (250 μL) were added undiluted or diluted at 2X, 5X, 10X, 100X, and 1000X with DMEM to confluent Vero E6 cells and examined by microscopy after 1 h of incubation. Cells were also stained with crystal violet after 72 h of culture to assess the residual cytotoxicity effects.

To evaluate the loss of virus titer after column filtration, SARS-CoV-2 stock (1 × 10⁶ PFU/mL) was prepared and quantified by the plaque assay. Test samples were prepared by adding 100 μL of the virus stock and 100 μL of the culture media to 800μL of plasma sample to achieve the final concentration of 1 × 10⁵ PFU/mL SARS-CoV-2. The filtered and nonfiltered plasma samples were diluted with DMEM, and plaque assays were performed to assess the virus recovery.

**Effect of 0.3% TNBP/1% Triton X-100 on the Viability of SARS-CoV-2.** Virus stock preparations (1 × 10⁶ PFU/mL) were added to commercial plasma as the test matrix. For the SD-treated samples, 100 μL of the virus stock and 100 μL of 3% TNBP/10% Triton X-100 solution were added to 800 μL of plasma sample for a final concentration of 1 × 10⁵ PFU/mL SARS-CoV-2. For the positive control, 100μL of the virus stock and 100μL of the culture media were added to 800μL of plasma sample, also for a final concentration of 1 × 10⁵ PFU/mL SARS-CoV-2. All samples were incubated at room temperature for 2 h, following which plaque assays were performed. Both the SD-treated plasma and positive control samples were filtered through Pierce detergent removal columns as described above prior to performing plaque assays. This procedure was performed in triplicate each time for a total of three independent experiments.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02585.

Microscope images showing the time course recovery of Vero cells after exposure to undiluted plasma (PDF)

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Notes
The authors declare no competing financial interest. All data are available from the corresponding author upon reasonable request.

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