Doherty Institute

THE UNIVERSITY OF MELBOURNE

The Royal Melbourne Hospital

A joint venture between The University of Melbourne and The Royal Melbourne Hospital

Report on TEST B: SARS-CoV-2 Viricidal Effectiveness Testing with ViaClean Products

Study Identification Number: 100620B

Protocol Number: 240420B

Product Identity: BioProtect DP RTU Lot No. 20200319DP

Product Shipping Date: 20200319, Ryerson University, Toronto

Carrier Identity: 2 cm SS304-2B Testing Disc EN13697-2001, Pegen Industries Inc, Ottawa

Carrier application date: 20200319, Ryerson University, Toronto

Test Microorganism:

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), patient isolate

(*Reference:* Caly L, Druce J, Roberts J, *et al*. 2020. Med J Aust 212:459-462).

The University of Melbourne acknowledges the support of Melbourne Health, through its Victorian Infectious Diseases Reference Laboratory at the Doherty Institute, in providing our laboratory with isolated SARS-CoV-2 material

Data Requirements:

U.S. EPA 40CFR § 160

Product Performance Guidelines OCSPP 810.2200

Testing Facility:

High Containment Laboratory (Physical Containment level 3 (PC3))

The Doherty Institute for Infection and Immunity at the University of Melbourne,

792 Elizabeth St, Melbourne VIC 3000, Australia

Relative temperature: 18-20^{o}C; Relative Humidity: 40%

Recipient: ViaClean Technologies LLC, Philadelphia, PA

Experimenter:
Dr. Julie McAuley

Results Verified by:
Prof. Damian Purcell

Date experiment performed:
24th April - 23rd May, 2020

Introduction: SARS-CoV-2 is an enveloped RNA virus. It was hypothesized that exposure of the virus to BioProtect will disrupt the virus envelope, thus rendering the virus non-infectious.

Aim: **Test B:** To dry SARS-CoV-2 on pre-treated stainless steel and fabric surfaces and investigate whether the pre-treated surface maintains viricidal effectiveness.

Protocols:

TEST B: BioProtect pre-treated stainless-steel samples exposed to SARS-CoV-2

Experiment date: 24th April, 2020. (34 days post-application of BioProtect to the surface of the stainless-steel disc)

In the PC3 Laboratory:

1. 50µL SARS-CoV-2 (=10^4 $TCID_{50}$) was added directly to the pre-treated stainless steel disc (2cm round) and allow to dry (Appendix 1).
2. The clock was started after all of the virus is visibly dry at 1 hour.
3. At 10min, 1mL infection media (MEM containing 1mM HEPES, 50U/mL Penicillin, 50µg/mL Streptomycin (Gibco)) was added to each well. Surfaces were washed eight times with vigorous pipetting (see Risk Reduction).
    - *Risk Reduction: Alternative method instead of vortexing to obtain samples: Given the use of high titres of SARS-CoV-2, we did not use a vortex to elute virus from the surface due to the risk of fomites (small droplets) containing virus potentially contaminating personal protective equipment and other surfaces, as well as potentially inducing formation of aerosolized infectious particles. As such, vortexing samples was too great of a risk of potential infection to the experimenter. Instead, after drying + 10min we aseptically placed each stainless steel disc in the single well each of a sterile tissue culture plate (12 well, Corning brand). We then added 1mL infection media and vigorously pipetted to agitate virions/virus particles from the surface. We washed the surface with this method 8times, prior to taking aliquots for processing samples. All care was taken not to scratch the surface.*
4. Eluate was collected and immediately serially diluted and assayed for quantitation via $TCID_{50}$.
    - TCID50 values were calculated using the method of Reed and Muench *(Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. The American Journal of Hygiene:3)*. Data is plotted mean ± standard deviation (Figure 1A). Raw Data is given in Appendix 2.
5. ***Quantitative Real-Time PCR (qRT-PCR):*** 140µL of sample was immediately added to AVL buffer for 10min, then 100% Ethanol to initiate RNA extraction for qPCR analysis. RNA purification was then completed using the QiaAmp Viral RNA mini kit, then samples dunked out of the PC3 facility and stored in our PC2 laboratory at -80°C until processed via RT-PCR. Standard curves were generated using known amounts of RNA extracted from SARS-CoV-2 stocks. Unknown sample concentrations were then interpolated from the standard curve and back calculated to give ng/µL present in the original sample. Data was plotted mean ± standard deviation (Figure 1 B). Raw Data is given in Appendix 3.
6. Left over sample aliquots were stored at-80°C in the PC3 facility.
7. To verify lack of presence of virus in samples yielding no detectable cytopathic effect (CPE) and thus $TCID_{50}$ was not ascertainable (step 4), a frozen aliquot of each sample was thawed, added to Vero cell monolayers and incubated for 3 days at 37°C, 5%CO_2. Evidence for CPE or lack thereof was then observed under the microscope.

8. To verify lack of low levels of replication competent SARS-CoV-2 in wells that had not yet induced CPE from samples generated in step 7, each well supernatant was passaged for a second time in Vero cells and incubated for 3 days at 37°C, 5%CO_2. Evidence for CPE or lack thereof was then observed under the microscope.

Quantitation Protocol: 50% Tissue Culture Infectious Dose ($TCID_{50}$)

1. In the PC2 laboratory, plates to establish ~95% monolayers of Vero cells were seeded 24h prior to assay
2. After verification of quality/density of monolayer, the plates were washed using infection media (to remove any cell debris), then transferred into the PC3 laboratory
3. In the PC3 laboratory, Samples were serially diluted and a known volume inoculated into each well (n=4 replicates/sample and up to 6 serial dilutions).
4. Plates were incubated for 45min to enable virus infection of monolayers, then 1mL MEM infection media +TPCK trypsin(1ug/mL, Trypsin Worthington) was added.
5. Plates were returned to incubator (37°C, 5%CO_2) and microscopically examined every 24 h (up to 72h) for cytopathic effect (CPE) on cells
6. The $TCID_{50}$ was then back calculated to determine the $TCID_{50}$/mL of infectious virus present in the original sample.

Quantitative Real-Time Polymerase Chain Reaction Assay (qRT-PCR)

Reaction solutions specific for detection of SARS-CoV-2 Envelope (E) gene containing known volumes of standards, samples and control will be set-up according to the following conditions:

MasterMix:	**Vol**	
H_2O (RNAse free)	2.6 µl	
2x Reaction mix*	12.5 µl	
$MgSO_4$(50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer E_Sarbeco_F1 (10 µM stock solution)	1 µl	ACAGGTACGTTAATAGTTAATAGCGT
Primer E_Sarbeco_R2 (10 µM stock solution)	1 µl	ATATTGCAGCAGTACGCACACA
Probe E_Sarbeco_P1 (10 µM stock solution)	0.5 µl	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ
SSIII/Taq EnzymeMix*	1 µl	
Total reaction mix	20 µl	
Template RNA, add	5 µl	
Total volume	25 µl	
* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase ** $MgSO_4$ (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit		

RT-PCR temperature cycling conditions:

55°C 10’
94°C 3’
94°C 15’’ }45x
58°C 30’’

Standard curves were then generated based on serial dilution of positive control samples and CT values. The amount of RNA present in the original sample was then determined by comparing CT value to standard curve and back-calculating. Data is plotted mean ± standard deviation. Raw Data is given in Appendix 2.

Results:

TEST B: BioProtect pre-treated stainless-steel samples exposed to SARS-CoV-2

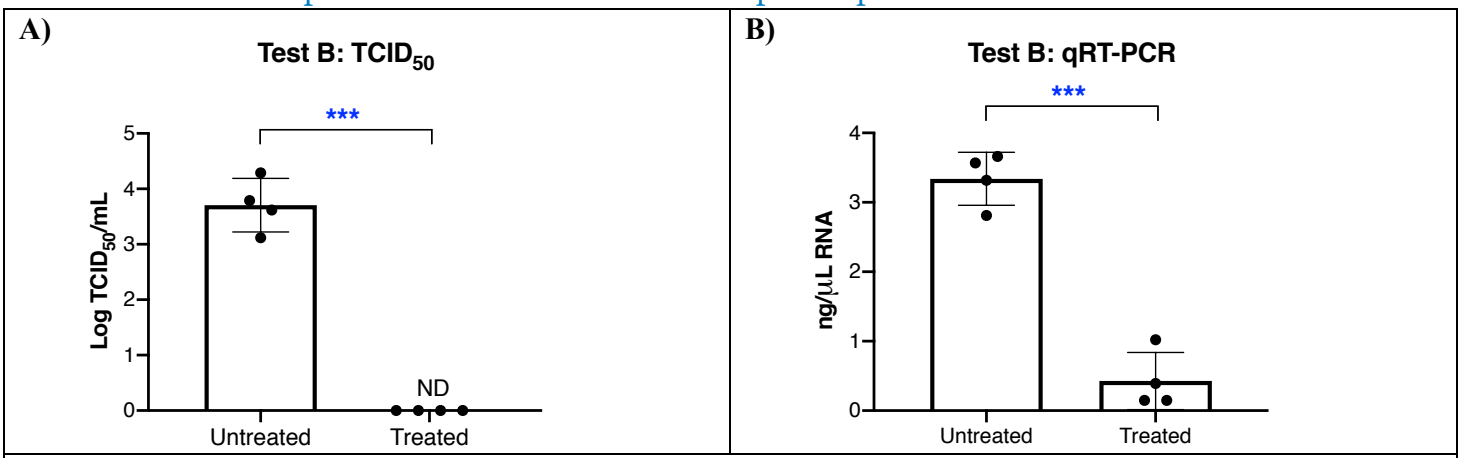


Figure 1: Samples (n=4) taken from BioProtect treated (47 days prior) and untreated stainless steel discs, exposed to SARS-CoV-2 inoculum (10^4 $TCID_{50}$) for 10min post-drying revealed viricidal activity by **A)** TCID50 **B)** qRT-PCR. Error bars = ± standard deviation. ***$p < 0.001$ Unpaired Student's T test ; ND = No CPE Detected

Conclusion:

- BioProtect reagent pre-coated on stainless steel discs 47 days prior to testing maintains significant viricidal activity against SARS-CoV-2. Based on lack of detection of virus induced cytopathic effects in our $TCID_{50}$ assay compared to a $TCID_{50}$ titre of $10^{3.705\pm0.48}$ for the untreated samples, we conclude that the treatment was completely effective at inactivating SARS-CoV-2 infectivity by this method.
- The BioProtect reagent on pre-coated stainless steel discs also maintained an ability to degrade virus RNA genome, as an 87.2% reduction in the amount of viral RNA in samples obtained from the treated discs compared to the untreated discs was determined.

Appendix 1:
Observations & Experimental Notes:

STAINLESS STEEL samples for Test B

(drying period = 1h, uncovered in the BSCII cabinet)

Time	Photo	Observations/notes
0min		Droplets on treated samples spread and came into contact with each other. Untreated sample droplets held their dome shape and did not spread.
~ 58min		Droplets on treated samples had dried. Untreated sample droplets were nearly completely dry.

Appendix 2: Raw data for $TCID_{50}$ values

$TCID_{50}$ assay notes: Performed in 96 well plate with 25µL inoculation volume. To calculate $TCID_{50}$ remaining in the original 'dried' sample:

1) Calculate $TCID_{50}$/25µL based on method of Reed and Muench:

$$\text{proportional distance formula} = \frac{[(\%\text{ positive value} > 50\%) - 50\%]}{[(\%\text{ positive value} > 50\%) - (\%\text{ positive value} < 50\%)]}$$

- Knowing the proportional distance between dilutions, the 50%-endpoint can be calculated using the exact dilutions used:

Log tissue culture infectious dose 50 = (log dilution >50%) + (proportional distance X log dilution factor)

- The reciprocal of this number is used to express the titre in infectious units per unit volume.

2) $TCID_{50}$/25µL x 1000/25 = TCD_{50}/mL (eluted sample)

3) TCD_{50}/mL (eluted sample) x 1050(final volume)/50 (original volume) = $TCID_{50}$/mL in original dried sample

Detection of CPE from Quantitative $TCID_{50}$ Assay of samples

Untreated discs:

Dilution:	Neat				10^{-1}				10^{-2}				10^{-3}				10^{-4}				10^{-5}			
Sample:	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4

CPE Detected No CPE detected

BioProtect Treated discs:

Dilution:	Neat				10^{-1}				10^{-2}				10^{-3}				10^{-4}				10^{-5}			
Sample:	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4

CPE Detected No CPE detected

<u>Log_{10} $TCID_{50}$/mL in original dried sample:</u>

Note: Treated samples yielded undetectable CPE,

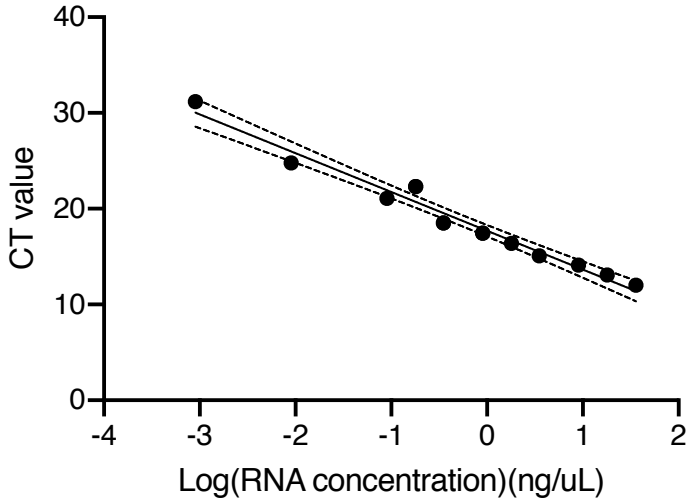
Therefore, <u>for treated samples Log_{10}=0 is incalculable</u>.

Untreated	Treated
3.12	0
4.29	0
3.62	0
3.79	0

Appendix 3: Raw data for qRT-PCR values

Standard Curve generation:

RNA concentration: Log_{10}(ng/µL)	Optical Density
1.56	12.03
1.26	13.09
0.95	14.10
0.54	15.08
0.26	16.39
-0.05	17.45
-0.46	18.51
-0.74	22.30
-0.05	17.45
-0.46	18.51
-0.74	22.30
-1.05	21.10
-2.05	24.78
-3.05	31.19



Sample	Optical Density	Interpolated Values	Sample Concentration (ng/µL)
Untreated 1	15.42	0.56	3.66
Untreated 2	15.59	0.52	3.32
Untreated 3	15.88	0.45	2.81
Untreated 4	15.46	0.55	3.57
Treated 1	21.06	-0.83	0.15
Treated 2	17.66	0.01	1.02
Treated 3	15.88	-0.40	0.39
Treated 4	15.46	-0.84	0.15
Positive Control	9.02	2.14	139.09
Negative Control	30.3392	-3.12	0.00