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## 1. HISTORICAL REVISION SUMMARY

**Table 1. Chronological Summary of Previous Versions**

Version #	Effective Date	Originator	Summary
3.0	Current	(b) (6)	Updated to improve quality assurance of data in the event LIMS is unavailable by completion, upload, and review of VR-TM-10298-FM02.
2.0	06-Jan-2021	(b) (6)	Updated settings of Cytation 7, manual steps on Day 2 involving the Cytation 7, and raw data and image export section
1.0	29-Jul-2020	Tyler Garretson	New

## 2. PURPOSE

This document describes the procedure for a manual 96-well SARS-CoV-2-mNG reporter virus microneutralization assay (SARS-CoV-2-mNG\_NT) designed to measure functional, neutralizing antibodies specific for SARS-CoV-2 in test serum. The assay can be performed utilizing LIMS or electronically filling in VR-TM-10298-FM02 (in case LIMS is down) while following the assay paperless guide VR-TM-10298-FM01. In the case of file-based (FB)-SAS assay runs, VR-TM-10298-FM03 should be completed electronically.

## 3. SCOPE

This procedure applies to all personnel responsible for executing VR-TM-10298 at Pfizer, Pearl River or Hackensack Meridian Health Center for Discovery and Innovation, Nutley, NJ.

**Table 2. Vaccine Research Functional Units**

Functional Units	Location
Pfizer, Vaccine Research & Development	Pearl River, NY and Nutley, NJ

**Table 3. Method Development, Qualification and Validation Status**

Project	Developed per	Qualified per	Validated per
General Use	N/A	VR-MQR-10214	N/A

#### 4. GLOSSARY

**Table 4. Terms and Definitions**

Term	Definition
Ab	Antibody
Batch	(b) (4)
BSC	Biological Safety Cabinet
BSL	Biosafety Level
CDTAS	Control Data Tracking and Analysis System
CMDP	Control Master Dilution Plate
Cytation 7	Cell imaging multi-mode reader manufactured by Biotek
DAP	Data Analysis Portal
FBS	Fetal Bovine Serum
Gen5	Software associated with the Cytation 7
GMT	Geometric Mean Titer
GPC	Green Positive Cells; SARS-CoV-2-mNG infected cells
HBSS	Hanks' Balanced Salt Solution
Hoechst 33342	Cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA
LIMS	Laboratory Information Management System
PPE	Personal Protective Equipment
PBS	Phosphate Buffered Saline
QCS	Quality Control Sample
QNS	Quantity Not Sufficient
ROP	Read Out Plate
SAP	Sample Assay Plate
SAPo	Sample Assay Plate output file produced by (b)(4) workstation
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SARS-CoV-2-mNG_NT	Manual 96-well microneutralization assay for the detection of functional antibodies to SARS-CoV-2
SARS-CoV-2-mNG	SARS-CoV-2-mNeonGreen reporter virus
SAS	Programming language and integrated software solution-set proprietary to SAS® (the company) that enables the coding of the various tasks associated with handling and analyzing data-sets.
SMDP	Sample Master Dilution Plate
SMDPo	Sample Master Dilution Plate output file produced by (b)(4) workstation
SOP	Standard Operating Procedure
TDV	Titer Determining Value. The threshold value in recovered viral focus forming units that is used to determine test serum titers in the microneutralization assay.
Titer	The reciprocal of the dilution of a test serum required to neutralize virus at a specified percentage of the viral input in a neutralization assay.

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**Table 4. Terms and Definitions**

Term	Definition
VI	Viral input is the number of recovered viral focus forming units (FFUs) or green positive cells (GPC) measured in neutralization assay reactions incubated without the presence of immune serum. The VI value is equal to 100% recovered virus for the purpose of calculating assay titers.
VSS	Viral Stock Solution
Vero	African green monkey kidney epithelial cells
(b) (4)	(b) (4) Handheld Electronic 96-Channel Pipette

## 5. GENERAL

The SARS-CoV-2-mNG-NT is a biofunctional assay designed to measure total serum antibody levels capable of neutralizing SARS-CoV-2 infection of cells in culture.

The SARS-CoV-2-mNG-NT is a three-day assay, performed manually. On Day 0, Vero cells are seeded into 96-well tissue-culture treated plates. On Day 1, serial dilutions of heat-inactivated immune sera in sample assay plates (SAPs) are incubated with SARS-CoV-2-mNG reporter virus to allow antigen-specific antibodies to bind to the virus. The reaction is then transferred onto the Vero cell monolayer read-out plates (ROPs) and allowed to incubate overnight; only non-neutralized virus is able to infect the cells. On Day 2, productive viral infection is detected in live cells using the mNeonGreen expressed by the reporter virus. Live cells are stained with the nuclear dye Hoechst 33342. Both viral foci and live cells are enumerated from the ROPs on a Cytation 7 reader.

A sample titer is calculated as the reciprocal serum dilution at which a specific percentage of the virus is neutralized (eg, 50% or 90% of the virus). The process for analysis and review of sample titer data generated during the execution of VR-TM-10298 is detailed in VR-SOP-LC-11293.

A qualified technician (ie, SARS-CoV-2-mNG-NT analyst) typically analyzes up to (b) (4) SAPs per run (batch). SAPs are produced using the (b) (4) workstation per VR-SOP-LC-11287. The SAP layout accommodates serum titrations of (b) (4) samples tested in replicate. A maximum of (b) (4) samples are tested in a (b) (4) -plate batch. (b) (4) (b) (4) Plates that contain the Vero monolayer (ROPs) are produced as per VR-SOP-LC-11299. Plating of Vero onto the ROPs occurs at least (b) (4) hours prior to the infection of the monolayer. This incubation time allows the cells to adhere to the plate surface and grow to a confluent monolayer to allow for consistent measurement of infection.

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## 6. RESPONSIBILITIES

- All personnel and positions referred to in this procedure are considered to have an alternate.
- An alternate must ensure that they are trained and knowledgeable with the process.

**Table 5. Roles and Responsibilities**

Role	Responsibilities
<b>SARS-CoV-2-mNG_NT Analyst</b>	<ul style="list-style-type: none"> <li>• Reads, understands, and completes training and qualification on this test method.</li> <li>• Reads, understands, and completes training on applicable equipment operating manuals, procedures and test methods that are required to execute the procedure described in this document.</li> <li>• If LIMS is used, logs in as Analyst role.</li> <li>• Executes this test method and complete steps in LIMS by following the paperless guide VR-TM-10298-FM01. If LIMS is unavailable, completes VR-TM-10298-FM02 and fills into LIMS when functional; attaches completed VR-TM-10298-FM02 as a batch comment. In case of FB-SAS or non-paperless runs, completes VR-TM-10298-FM03.</li> <li>• Consults with supervisor if there are any questions related to the procedure itself or serum samples, if an atypical event is observed or if a protocol deviation occurs.</li> </ul>
<b>SARS-CoV-2-mNG_NT Coordinator</b>	<ul style="list-style-type: none"> <li>• Provides detailed scheduling of all SARS-CoV-2-mNG_NT runs.</li> <li>• If LIMS is used, uses Lab Manager, Reviewer, and/or Analyst role.</li> <li>• Creates assay run batches in LIMS and corrects run information with QNS information from the annotated VR-SOP-LC-11287-FM01 printout or LIMS comments (Paperless) received from the (b)(4) Operator.</li> <li>• Coordinates with Vero analysts to ensure the correct numbers of ROPs are available for SARS-CoV-2-mNG_NT runs.</li> <li>• Coordinates with (b)(4) analysts to schedule production of SARS-CoV-2-mNG_NT assay plates.</li> </ul>
<b>(b)(4) Analyst</b>	<ul style="list-style-type: none"> <li>• Reads, understands and completes training on applicable equipment operating manuals, procedures and test methods that are required to operate (b)(4) workstations and produce serum dilution plates for SARS-CoV-2-mNG_NT.</li> <li>• Serially dilutes test sera and control sera in master dilution plates using the (b)(4) workstation according to VR-SOP-LC-11287.</li> <li>• Prepares SARS-CoV-2-mNG_NT assay plates from master dilution plates using the (b)(4) workstation according to VR-SOP-LC-11287.</li> <li>• Provides SARS-CoV-2-mNG_NT analysts with sample assay plates (SAPs) on the day of their assay runs.</li> <li>• Coordinates with SARS-CoV-2-mNG_NT Coordinator to schedule production of SARS-CoV-2-mNG_NT assay plates.</li> <li>• For non-paperless assays, provides the SARS-CoV-2-mNG_NT Analysts and SARS-CoV-2-mNG_NT Coordinator with appropriate run documentation (ie, SMDPo, QNS-Annotated LIMS run file, and VR-SOP-LC-11287 printouts) describing the processing of assay plates and QNS samples.</li> <li>• For Paperless assays, enters SMDP, SAP, and QNS data into LIMS.</li> <li>• If LIMS is used, uses Robotics role.</li> </ul>

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**Table 5. Roles and Responsibilities**

Role	Responsibilities
<b>Vero Analyst</b>	<ul style="list-style-type: none"> <li>Maintains and grows Vero cells.</li> <li>Plates the appropriate concentration of Vero cells onto ROPs according to VR-SOP-LC-11299.</li> <li>Provides an appropriate number of ROPs with a Vero monolayer to each SARS-CoV-2-mNG_NT analyst on the day of the assay.</li> <li>Provides worksheet VR-SOP-LC-11299-FM07 to each analyst.</li> </ul>
<b>SAS Operator, Data Analyst</b>	<ul style="list-style-type: none"> <li>Reads, understands and completes training on VR-SOP-LC-10336, VR-TM-10298, and VR-SOP-LC-11293.</li> <li>For Paperless assays, executes the steps of VR-TM-10298-FM01, Table 13.</li> <li>For FB-SAS and non-paperless assays, executes the steps of VR-TM-10298-FM03, Table 20.</li> <li>This person may be the same person as the SARS-CoV-2-mNG_NT analyst.</li> <li>This person may not be the Reviewer for the same batch.</li> </ul>
<b>Reviewer</b>	<ul style="list-style-type: none"> <li>Reads, understands and completes training on this test method.</li> <li>Reads, understands and completes training on VR-SOP-LC-11293.</li> <li>Review SARS-CoV-2-mNG_NT documentation to ensure completeness of data entry and compliance with Pfizer policies. If VR-TM-10298-FM02 is used during a paperless assay, review VR-TM-10298-FM02 in LIMS. Direct the SARS-CoV-2-mNG_NT Analyst to correct documentation errors.</li> <li>Applies data review rules according to VR-SOP-LC-11293.</li> <li>Consults with SARS-CoV-2-mNG_NT supervisor or management if there are any questions related to the procedure itself or serum samples, if an atypical event is observed or a protocol deviation occurs.</li> <li>Uploads SARS-CoV-2-mNG_NT data into LIMS, if applicable.</li> <li>If LIMS is used, uses Lab Reviewer role.</li> <li>This person must <u>not</u> be the SARS-CoV-2-mNG_NT analyst, SAS operator, or Data analyst for the same batch.</li> </ul>
<b>Lab Manager</b> (A role specific to LIMS)	<ul style="list-style-type: none"> <li>Verifies that sample data is correctly entered and amended in LIMS.</li> <li>Transfers quality control serum data into CDTAS.</li> <li>Verifies that quality control data is correctly entered and amended in CDTAS.</li> <li>This person may not be the SARS-CoV-2-mNG_NT analyst, SAS Operator, or Reviewer for the same batch.</li> <li>Lab authorizes batches.</li> <li>Releases data from LIMS to Data Management after QA authorization is obtained, where required.</li> </ul>
<b>SARS-CoV-2-mNG_NT Supervisors and Senior Managers</b>	<ul style="list-style-type: none"> <li>Reads and understands this test method.</li> <li>Reviews and approves this test method.</li> <li>Ensures that SARS-CoV-2-mNG_NT analysts executing this test method are trained in the appropriate procedures and test methods.</li> <li>Provides guidance to SARS-CoV-2-mNG_NT analysts and reviewers and answer any questions that arise during the execution of this procedure.</li> </ul>

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## 7. SAFETY

- Biosafety level 2 (BSL-2) precautions should be taken when handling human and non-human primate sera. Biosafety level 3 (BSL-3) precautions should be taken when handling live SARS-CoV-2-mNG. For current guidelines refer to the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories Manual (BMBL), 5th Ed., 2009: <http://www.cdc.gov/biosafety/publications/bmb15/index.htm>.
- Personnel must be trained on current laboratory safety procedures and made aware of the Safety Data Sheets for chemicals being used before performing this procedure.

### Prior to starting work at BSL3:

- Register as BSL3 worker and complete the training and access requirements for BSL3 facilities:
  - BSL3 training course
  - BSL3 facility specific training and access checklist form
  - BSL3 check off
  - BSL3 mentorship
- All laboratory work is to be carried out in compliance with Pfizer safety policies. For general laboratory safety guidelines consult the Pearl River Environmental Health and Safety Laboratory Manual located at:  
[\(b\) \(4\)](http://(b) (4))

## 8. MATERIALS/EQUIPMENT

### 8.1. Equipment

- (b) (4) channel handheld pipette, (b) (4), or equivalent
- (b) (4) Standard Plate Holder, (b) (4), or equivalent
- (b) (4) -Channel Pipetting Head 5 - 125  $\mu$ L, (b) (4), or equivalent
- Light Microscope, (b) (4) with fluorescence source, or equivalent
- Temperature and CO<sub>2</sub> Incubator (equilibrated to 37  $\pm$  2°C, 4-6% CO<sub>2</sub>), (b) (4) CO<sub>2</sub> Incubator or equivalent.
- Biosafety Cabinet, (b) (4), or equivalent



- Pipet-aid, (b) (4), or equivalent
- Temperature controlled water bath with (b) (4), set to  $37 \pm 2^\circ\text{C}$ , or equivalent
- Calibrated Single and Multi-Channel Pipettors, adjustable ranges  $5 \mu\text{L} - 1000 \mu\text{L}$ , (b) (4), or equivalent
- Calibrated freezer  $-80 \pm 15^\circ\text{C}$
- Calibrated Refrigerators or Refrigerated Cold Room,  $5 \pm 3^\circ\text{C}$
- Cytation 7 Cell Imaging Multi-Mode Reader, or equivalent, equipped with (b) (4) Software, and subsequent software updates.
- LabWare Laboratory Information Management System (LIMS) and PC workstations

## 8.2. Reagents

- Working bank of SARS-CoV-2-mNG. See LIMS and batch documentation for lot information
- Vero tissue culture cell monolayer
- Assay Media. (b) (4)  
(b) (4), prepared according to VR-SOP-LC-11299
- Quality control sera (QCS) appropriate for SARS-CoV-2-mNG
- Hoechst 33342 Solution, Thermo Cat. #62249
- Hanks' Balanced Salt Solution, (b) (4)
- 70% Ethanol, (b) (4), or equivalent
- Disinfecting agent, prepared according to product specifications

## 8.3. Supplies

- Sterile 96-well optical bottom plates with cell culture surface coating (hereafter referred to as "ROPs"), Greiner Bio-one™ 655090, or equivalent
- Sterile 96-well Microtiter plates with lids (hereafter referred to as "Assay plates" or "SAPs"), (b) (4), or equivalent
- Sterile, filter  $125 \mu\text{L}$  (b) (4) boxes, (b) (4)

- 150 mL Reservoirs, Individually Sealed, Sterile, Polystyrene, (b) (4), or equivalent
- Sterile pipette tips, (b) (4), 20 µL (b) (4), 200 µL (b) (4), 1000 µL (b) (4), or equivalent
- Sterile disposable serological pipettes, (b) (4) and 50 mL (b) (4), or equivalent
- Sterile polypropylene tubes, 50 mL, Falcon, (b) (4), or equivalent
- Sterile bottles, 250 mL, (b) (4), or equivalent
- (b) (4) Scientific Sealing Tape (b) (4), or equivalent
- (b) (4) wipes, (b) (4), or equivalent
- Timers, (b) (4), or equivalent
- (b) (4) Microplate Lids, (b) (4), or equivalent

## 9. PROCEDURE

### 9.1. General Points

- All procedures that involve active virus must be performed in a Type II Biological Safety Cabinet (BSC) within a BSL-3 environment while wearing appropriate PPE. All transfer steps outside of the BSC must be done in the appropriate secondary container.
- All procedures that involve sera and/or Vero cells must be performed in a Type II Biological Safety Cabinet (BSC) within a BSL-2 environment while wearing appropriate PPE including safety glasses, gloves and a lab coat. All transfer steps outside of the BSC must be contained in the appropriate secondary container.
- Qualified SARS-CoV-2-mNG\_NT analysts executing this test method will document all assay steps into LIMS and/or on the non-paperless assay worksheet, VR-TM-10298-FM02. Additional information such as execution under special circumstances, planned deviations, and atypical (unplanned) events are recorded in the appropriate comment sections.
- Paperless systems (ie, LIMS) may be used in place of or in conjunction with paper-based documentation (ie, VR-TM-10298-FM02). VR-TM-10298-FM01, Assay Guide for the SARS-CoV-2-mNG NT using Paperless LIMS, may be used to perform the test method by the assay analyst if not using VR-TM-10298-FM02. VR-TM-10298-FM02 is used to record assay information in the event that LIMS becomes unavailable during a Paperless assay. In the event LIMS is unavailable and VR-TM-10298-FM02 is completed, the

completed form must be attached to the batch as a LIMS comment. The reason why the form was completed should also be included in the LIMS comment section.

- The assay worksheet VR-TM-10298-FM03 is used during assay runs where FB-SAS processes are necessary.
- Assay plates containing test samples and QCS are provided by the (b)(4) group and are prepared as described in VR-SOP-LC-11287.
- The Vero cells are prepared by the Vero analyst as described in VR-SOP-LC-11299, “Thawing, Passaging and Harvesting of Vero Cells”. Generally, the SARS-CoV-2-mNG\_NT coordinator communicates plate requirements to the Vero coordinator at least 1 week in advance. Required plating densities are shown Table 6.

**Table 6. Vero Cell Concentration in ROP for SARS-CoV-2-mNG\_NT**

Virus	ROP Type	Cell concentration	Volume per well
SARS-CoV-2-mNG	Black µCLEAR flat-bottom 96-well plates (Greiner Bio-one 655090), or equivalent	(b) (4)	(b) (4)

- The SARS-CoV-2-mNG\_NT scheduler, in cooperation with (b)(4) analysts, coordinates the assessment of sample sera by SARS-CoV-2-mNG\_NT, including determining the number of SARS-CoV-2-mNG\_NT runs on each day and the number of assay plates in each SARS-CoV-2-mNG\_NT run.

## 9.2. Assay Set Up

1. If using paperless LIMS processes, move to Section 9.3. If using form VR-TM-10298-FM03, electronically place documents in batch folder received from the SARS-CoV-2-mNG\_NT Coordinator along with VR-TM-10298-FM03. This may include:
  - a. SAP to ROP barcode linkage file (heading is (b) (4) (b) (4)).
  - b. (b)(4) output file (heading is (b) (4) ).

## 9.3. Recording of Equipment and Reagent Information and Use of Barcode Scanner

1. Equipment and reagent information is recorded for each assay. This information includes the equipment barcode and calibration due date, reagent expiration dates and lot numbers.
2. Equipment and reagent information is recorded in one of two ways:
  - a. It can be typed or scanned into VR-TM-10298-FM02 or VR-TM-10298-FM03.

- b. It can be scanned with the use of a hand-held scanner or entered directly into fields in the batch electronically into LIMS. Complete all information requested by LIMS.

**Note:** Equipment conditions (temperature, CO<sub>2</sub>, etc.), Equipment Calibrations and Reagent Expirations should be visually inspected to be within tolerances prior to Performing the Assay.

#### 9.4. Preparation of ROPs

**Note:** Refer to Table 1 of VR-TM-10298-FM01 or Table 4 of VR-TM-10298-FM03.

1. Obtain ROPs from Vero analyst. Ensure ROPs have incubated at least (b)(4) hours prior to execution of [Section 9.10](#), Transfer of Neutralization Reaction onto ROPs.
2. If using Paperless LIMS:
  - a. Enter information from the cell plate distribution worksheet, VR-SOP-LC-11299-FM07, into LIMS.
3. If using form VR-TM-10298-FM03:
  - a. Fill in CWS # of Cell Plate Distribution Worksheet VR-SOP-LC-11299-FM07.
4. Inspect 6 wells at random of 2 ROPs per run under the light microscope to ensure the monolayer is healthy with no microbial contamination and has  $\geq 80\%$  confluency. A healthy Vero monolayer is evenly dispersed squamous cells adhered to the bottom of the ROP well. Record abnormalities in the Atypical Event(s) box in VR-TM-10298-FM03 or the Comment Field in Paperless LIMS.
5. In a BSC, label and number each plate appropriately (typically 1-20) with a marker and store in 37°C/5% CO<sub>2</sub> until transfer step ([Section 9.10](#)).

#### 9.5. Preparation of SAPs

1. SAPs are prepared by a (b)(4) workstation as described in VR-SOP-LC-11287.
2. SAPs are prepared according to a standard plate map as shown in [Table 7](#). The initial serum dilution for the SARS-CoV-2-mNG\_NT is 1:20, which, along with a 9-point 2-fold serum dilution series, enables a potential plate titer range of 20 to 5,120. Samples that receive titers greater than 5,120 (or the upper limit of quantitation as determined during qualification, whichever is the lower value) are retested after pre-dilution in assay buffer. Samples that fail to neutralize at the TDV when tested at the lowest serum dilution of 1:20 are reported to have a neutralizing titer of (b)(4).
3. Test samples are assayed in replicate and a typical SAP contains sample titrations in rows B through G. Each SAP typically contains 3 test sera. For example, a (b)(4)-plate assay contains (b)(4) slots for test samples, which translates to (b)(4) samples tested in replicate.

(b) (4)

**Table 7. Sample Assay Plate Map**

(b) (4)

### 9.6. Processing of SAPs

**Note:** Refer to Table 2 of VR-TM-10298-FM01 or Table 6 of VR-TM-10298-FM03.

1. Obtain sample assay plates (SAPs) prepared by a (b)(4) workstation.
2. If using Paperless LIMS:
  - a. Confirm the Batch number is correct for the plates received.
  - b. Confirm correct set of assay plates were received from the (b)(4) Analyst by scanning the assay plates into LIMS.
3. If using form VR-TM-10298-FM03:
  - a. Create batch folder in (b)(4) (b)(4) and select the subfolder for either (b)(4) (b)(4)
  - b. Upload documents from the (b)(4) Analyst to batch folder.
  - c. Confirm the Batch number is correct and matches batch files.
  - d. Confirm correct set of assay plates were received from the (b)(4) Analyst by:
    - o Confirm the assay plates received against the (b)(4) SAP output file.

4. Inspect plates to confirm that each well of each SAP contain approximately (b) (4)  $\mu\text{L}$  liquid and that volumes are consistent from well to well. Use the Atypical Event(s) box in VR-TM-10298-FM03 or the Comment Field in Paperless LIMS to record the location of any wells that have no liquid or appear to have a volume other than (b) (4)  $\mu\text{L}$ . Do not use a pipette to measure well volumes; this step is a visual inspection only.
5. Number each SAP with a marker. The hand-written number dictates the order in which the SAPs will receive a reagent and corresponds to its respective ROP.
6. Store SAPs at room temperature until addition of virus (Section 9.9). Do not let SAPs sit at room temperature longer than 8 hours.

### 9.7. Add Assay Media to Cell Control Wells in SAPs

1. Warm Assay media in 37°C water bath. Keep warm for activities in Section 9.8.
2. Perform all work below in BSC with a (b) (4) 8-channel pipette.
3. Fill reservoir with assay media.
4. Place SAPs in the BSC and one at a time remove the SAP lid and sealer.
5. Attach 8 pipette tips to the 8-channel pipettor and add (b) (4)  $\mu\text{L}$  of assay media to all wells in (b) (4) of SAP – as seen in Table 7.
6. Replace lid and repeat steps 4-5 until all SAPs have this assay media in (b) (4) .

### 9.8. Preparation of Viral Stock Solution

**Note:** If using Paperless LIMS, the bank information and volumes required to prepare the viral stock solution will be displayed in LIMS. Follow the instructions on Table 4 of VR-TM-10298-FM01. If using VR-TM-10298-FM03, refer to Table 9 of VR-TM-10298-FM03.

#### Calculations for Viral Stock Solution (VSS):

$$\text{Total Volume of VSS} = (4.0 \text{ mL} * \text{total number of SAPs in batch}) + 10 \text{ mL}$$

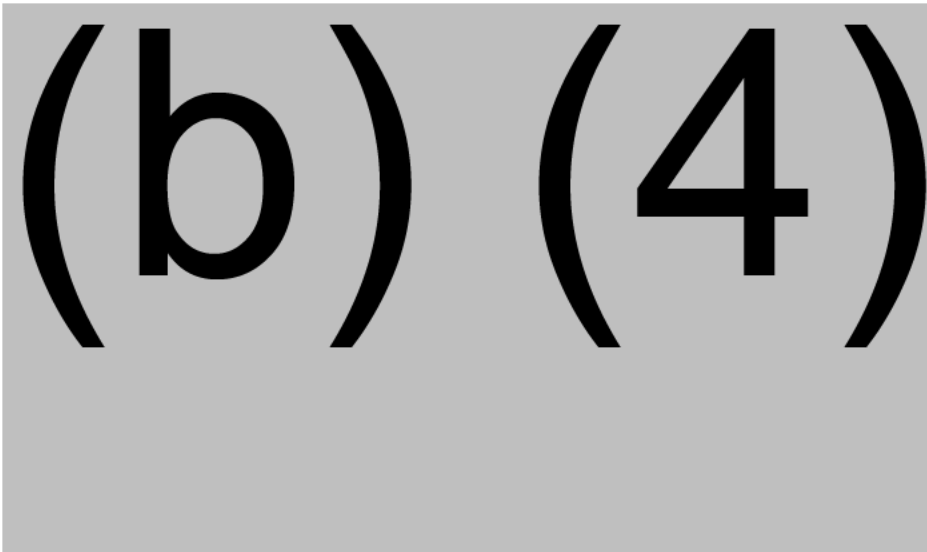
$$\text{Volume of Virus} = \text{Total Volume of VSS} \div \text{Viral Dilution}$$

$$\text{Volume of Assay Media} = \text{Total Volume of VSS} - \text{Volume of Virus}$$

1. Perform all manipulations of reagents and active virus in a BSC within the BSL-3 laboratory using sterile techniques.
2. Label a sterile bottle “VSS”.
3. If using VR-TM-10298-FM03, circle appropriate parameters needed to prepare viral stock solution in Table 9. If using VR-TM-10298-FM02, indicate parameters needed in Table 9.

4. Refer to Table 4 of VR-TM-10298-FM01 for specific virus dilution factor. Use the virus dilution factor that result in 30% infection rate at 24 hours post-infection.
5. Complete Viral Stock Solution (VSS) Table; verify with another analyst.
6. Remove the appropriate number of frozen viral stock vials from -80°C freezer. Place in secondary container and transport to BSC. Place vials in BSC.
7. Thaw vials at room temperature in BSC until there is a small piece of ice remaining in the vial.
8. If multiple vials are needed, pool all vials into a larger tube and pipette up and down 3 times to ensure proper mixing.
9. Add the appropriate volume of SARS-CoV-2-mNG into VSS bottle and gently swirl to mix.

**Figure 1.** (b) (4) (b) (4) Positions



### 9.9. Addition of Viral Stock Solution to SAPs and Neutralization of Virus

**Note:** Refer to Table 5 of VR-TM-10298-FM01 or Table 10 of VR-TM-10298-FM03. If not able to use the (b) (4), this process can be accomplished using a multi-channel pipettor.

1. Identify the appropriate size and quantity of tip boxes. Obtain one additional tip box for tip waste. Place tip boxes inside the BSC and appropriately label the tip boxes as “Clean” and “Waste” to avoid cross contamination.
2. Place appropriate clean tip box in (b) (4) on (b) (4) (b) (4).

3. Remove tips from column 12 (should not add virus to column 12 wells).
4. Select custom protocol “(b) (4).” (custom protocols outlined in (b) (4) SOP, VR-SOP-FE-11315).
5. Press action button to initialize preset (b) (4).
6. Place reservoir for VSS in (b) (4) on (b) (4) (b) (4). Carefully add VSS to reservoir.
7. Prepare reservoir with disinfectant and place to side in BSC.
8. Lower (b) (4) (b) (4) onto tips, press blue flashing button to secure tips, and raise (b) (4).
9. Remove lid of SAP and remove tip box in (b) (4).
10. Place SAP in (b) (4) on (b) (4) (b) (4).
11. Move (b) (4) (b) (4) over VSS in (b) (4).
12. Lower (b) (4) (b) (4) into VSS solution and aspirate (b) (4)  $\mu$ L of VSS.
13. Switch position of (b) (4) (b) (4) over SAP in (b) (4), lower (b) (4) into SAP volume, and dispense (b) (4)  $\mu$ L of VSS into each well of SAP.
14. Press action button to mix contents.
15. Raise (b) (4) (b) (4).
16. Remove SAP from (b) (4). Return lid to SAP.
17. Move disinfectant reservoir to (b) (4).
18. Lower (b) (4) (b) (4) into disinfectant and decontaminate tips by pressing action button.
19. Raise (b) (4) (b) (4) and remove disinfectant reservoir from (b) (4) (b) (4).
20. Add empty tip box (for tip waste) to (b) (4).
21. Lower (b) (4) (b) (4) to (b) (4) and eject tips into the waste tip box.
22. Remove the tip box waste from (b) (4) and replace with new clean tips (be careful to avoid cross contaminating waste and clean tips).
23. Repeat steps 5 – 22 for all remaining SAPs in batch.
24. Place stacks of 4 SAPs in secondary container in the BSC and place the SAPs in the secondary container. Latch the secondary container shut and disinfect the exterior.



25. Place the secondary container in the incubator and **open the secondary container lid and remove from incubator.**

26. Incubate SAPs for 60 minutes at 37°C/5% CO<sub>2</sub> in stacks of no more than 4 SAPs.

27. Using a serological pipette, add equal volume of disinfectant in VSS reservoir. Wait 15 minutes, then pour it down sink in BSL-3.

28. For Paperless LIMS, record the times in LIMS. For VR-TM-10298-FM03 assays, record the start and end times for this incubation in Table 10 in VR-TM-10298-FM03.

### 9.10. Transfer of Neutralization Reaction onto ROPs and Decontamination of SAPs

**Note:** If using Paperless LIMS, there will be prompts for scanning SAP and ROP barcodes. Follow the instructions on Table 6 of VR-TM-10298-FM01. If using VR-TM-10298-FM03, refer to Table 11 of VR-TM-10298-FM03.

1. Identify the appropriate size and quantity of tip boxes. Obtain one additional tip box for tip waste. Place tip boxes inside the BSC and appropriately label the tip boxes as “Clean” and “Waste” to avoid cross contamination.
2. Remove secondary container with SAPs from the incubator, transfer the container to the BSC, open the lid, and place SAPs inside the BSC.
3. Place appropriate clean tip box in (b) (4) on (b) (4) .
4. Select custom protocol (b) (4) (custom protocols outlined in (b) (4) SOP, VR-SOP-FE-11315).
5. Remove lid from ROP and place ROP in (b) (4) on (b) (4) (b) (4) .
6. Prepare reservoir with disinfectant and place to side in BSC.
7. Lower (b) (4) (b) (4) onto tips in (b) (4) press blue button to secure tips, and raise (b) (4)
8. Press action button to initialize (b) (4)
9. Remove lid of SAP and remove tip box at (b) (4)
10. Place SAP in (b) (4) on (b) (4) (b) (4)
11. Scan barcode on SAP and ROP.
12. Move (b) (4) (b) (4) over SAP in (b) (4)
13. Lower (b) (4) (b) (4) into SAP and aspirate (b) (4) μL of solution. Raise the (b) (4)

14. Switch position of (b) (4) (b) (4) over ROP in (b) (4) , lower (b) (4) into ROP wells, and dispense (b) (4)  $\mu$ L from SAP into each well of ROP.
15. Press action button to mix contents. Be careful to avoid touching/disturbing cell monolayer! Do not force the tips below the (b) (4) . Be careful to make sure pipette tips are in media to avoid introducing bubbles.
16. Raise (b) (4) (b) (4)
17. Remove ROP from (b) (4) Return lid to ROP.
18. Move disinfectant reservoir to (b) (4)
19. Lower (b) (4) (b) (4) into disinfectant in (b) (4) and press action button to aspirate and dispense (b) (4)  $\mu$ L of disinfectant.
20. Raise (b) (4) (b) (4) Add waste tip box (for tip waste) to (b) (4)
21. Switch position of (b) (4) (b) (4) to empty tip box at (b) (4) lower (b) (4) and eject tips into the empty tip box.
22. Remove the tip box waste from (b) (4) and replace with new clean tips (be careful to avoid cross contaminating waste and clean tips).
23. Remove disinfectant reservoir from (b) (4)
24. Repeat steps 4 – 23 until all remaining SAPs in the batch are transferred to ROPs.
25. Place secondary container in the BSC and place the ROPs in stacks of 4 ROPs in the secondary container. Latch the secondary container shut and disinfect the exterior.
26. Place the secondary container in the incubator and **remove the lid from the secondary container.**
27. Incubate ROPs at 37°C/5% CO<sub>2</sub> overnight for (b) (4) in stacks of no more than 4 ROPs.
28. For Paperless LIMS, record the time in LIMS. For VR-TM-10298-FM03 assays, record the time the ROPs were placed in incubator in Table 11 of VR-TM-10298-FM03.

### 9.11. Assay – Day 2

**Note:** Refer to Table 7-8 of VR-TM-10298-FM01 or Table 13-16 of VR-TM-10298-FM03.

1. Day 2 activities may be performed by the same analyst who performed day 1 activities or by another SARS-CoV-2-mNG NT analyst. For Paperless LIMS, the day 2 analyst will login to LIMS and select the correct batch and follow the instructions on VR-TM-10298-FM01. For VR-TM-10298-FM03, the individual who performed day 2 signs the “SARS-CoV-2-mNG\_NT analyst, day 2” line of Table 1 and enters the date these steps were completed.
2. Record the date and time that ROPs were removed from the incubator in LIMS, for Paperless assays, or in Table 15 of VR-TM-10298-FM03.
3. Carefully examine the instrument Cytation™ 7 and the workplace. Ensure no used plates or boxes are blocking the plate carrier. Power on the instrument, wait at least 5 minutes to warm up the instrument. Green light will display on the instrument when it is ready.

### 9.12. Preparation and Addition of Hoechst 33342 Solution to ROPs

Calculations for Hoechst 33342 Solution (HS):

$$\begin{aligned}\text{Total Volume of HS} &= (2.5 \text{ mL} * \text{total number of ROPs in batch}) + 20 \text{ mL} \\ \text{Volume of Hoechst 33342} &= \text{Total Volume of HS} \div \text{Hoechst 33342 Dilution} \\ \text{Volume of HBSS} &= \text{Total Volume of HS} - \text{Volume of Hoechst 33342}\end{aligned}$$

1. Prepare Hoechst 33342 solution by performing a 1:500 dilution of Hoechst 33342 to HBSS (1-part Hoechst 33342 stock to 499 parts HBSS).
2. Secure a lid to another secondary container and remove 5 ROPs from incubator.
3. Place ROPs inside the secondary container, close the lid, and transfer to the BSC. Open the lid and place the ROPs in the BSC.
4. Place reservoir for Hoeschst 33342 Solution in (b) (4) on (b) (4) (b) (4)
5. Carefully add Hoechst 33342 Solution to reservoir.
6. Select custom protocol (b) (4) (custom protocols outlined in (b) (4) SOP, VR-SOP-FE-11315).
7. Press action button to set (b) (4).
8. Place appropriate tip box in (b) (4) below (b) (4) (b) (4) lower (b) (4) and attach tips to (b) (4) by pressing the blue flashing button.
9. Remove tip box from (b) (4)

10. Remove lid of ROP and place ROP in (b) (4)
11. Lower (b) (4) (b) (4) into Hoeschst 33342 Solution in (b) (4) and aspirate 125 µL of solution.
12. Switch position of (b) (4) (b) (4) over ROP position, lower (b) (4) above ROP liquid, and dispense (b) (4) of Hoeschst 33342 Solution into each well of ROP.
13. Raise (b) (4) (b) (4), remove ROP from (b) (4) and return lid to ROP.
14. Repeat steps 11-13 for up to 4 more plates.
15. Repeat steps 6-14 for the rest of batch.
16. Raise (b) (4) (b) (4)
17. Prepare reservoir with disinfectant and place in (b) (4)
18. Lower (b) (4) (b) (4) and tips into disinfectant and mix 25 µL of disinfectant five times.
19. Remove the disinfectant reservoir.
20. Place waste tip box on (b) (4) (b) (4) in (b) (4)
21. Transfer (b) (4) over tip box waste. Eject tips.
22. Place the ROPs in a secondary container in stacks of no more than 4 ROPs. Latch the container and disinfect the exterior. Transport the container to the incubator.
23. Incubate ROPs at 37°C/5% CO2 for (b) (4) .
24. Record start and end time for Hoechst incubation.
25. At the end of the incubation, secure a lid to another secondary container and add one ROP from the incubator and place inside the BSC.
26. Remove lid from ROP and seal plate with (b) (4) Sealing Tape (b) (4) .
27. **Repeat steps 25 - 26 until no more plates remain.**
28. Using a serological pipette, add equal volume of disinfectant in Hoechst reservoir. Wait 15 minutes, then pour it down sink in BSL-3.

### 9.13. Image Acquisition and Counting on the Cytation 7

1. Instructions for image acquisition and FFU counting are given in Table 8 of VR-TM-10298-FM01 or Table 16 of VR-TM-10298-FM03. For Paperless LIMS, the Cytation analyst will login to LIMS and select the correct batch and follow the instructions on VR-TM-10298-FM01. For VR-TM-10298-FM03, the individual who performed scanning and counting completes the “Cytation Analyst” section of Table 1 and enters the date that these steps were completed.
2. Log on to Cytation PC using Pfizer NT ID and password.
3. Open the (b) (4) software on the Cytation desktop and confirm that the software is communicating with the instrument. Open the (b) (4) protocol “SARS-CoV-2-mNG\_NT”.
4. Create a data folder for the batch (located in the path – (b) (4) ). The folder should indicate the full LIMS batch name, as such (b) (4) .
5. Imaging parameters that are specific to the “SARS-CoV-2-mNG\_NT” protocol in (b) (4) are listed below:

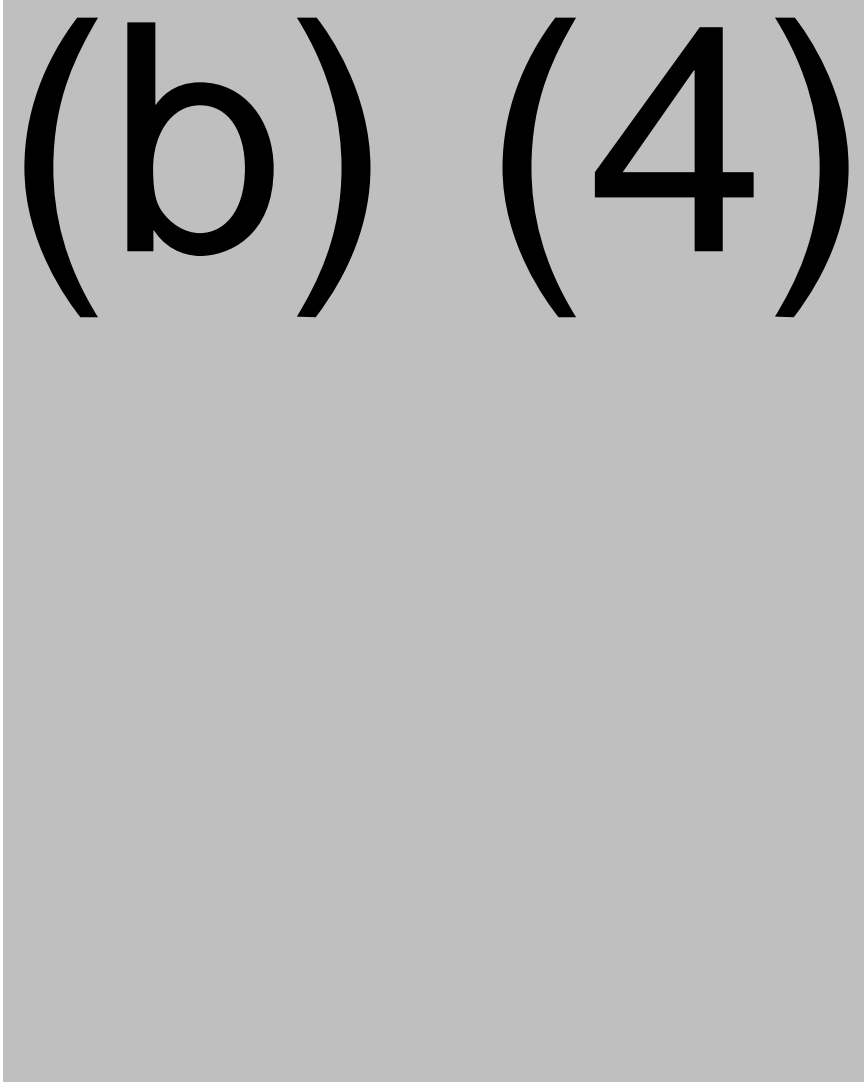
(b) (4)

- Counting and Data Reduction parameters that are specific to the SARS-CoV-2-mNG\_NT are listed below:

(b) (4)

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7. Press "Run Protocol in (b) (4) software.
8. Select option: (b) (4) and hit OK.



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(b)

(4)

(b)

(4)

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13. After reading the ROPs on the Cytation 7, place the ROPs in the BSC and remove the adherent film. Disinfect the ROPs by spraying disinfectant over the entire plate.

#### 9.13.1. Scanning Quality Check and Correction of Barcode Reader Errors

A large grey rectangular redaction box covering the majority of the page content. Inside the box, the text "(b) (4)" is printed in a large, bold, black font.

#### 9.14. Raw Data and Image Export

A smaller grey rectangular redaction box located below the previous one. Inside the box, the text "(b) (4)" is printed in a large, bold, black font.

(b) (4)

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(b) (4)

#### 9.15. Data Acquisition

(b) (4)

#### 9.16. Analysis of Data and Calculation of SARS-CoV-2-mNG<sub>NT</sub> Titers

(b) (4)

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(b) (4)

**9.17. SARS-CoV-2-mNG\_NT Data and Document Review**

(b) (4)

3. Review and processing of data are described in VR-SOP-LC-11293.

**10. TABLES, FIGURES, DIAGRAMS**

**Table 8. Assay Job Aid 1 - Comparison of Assay Runs Using Oracle-LIMS Based SAS and File-based SAS**

	<b>Runs Processed Through Oracle SAS</b>	<b>Runs Processed Through File-based SAS or VR-SOP-LC-11293-FM02</b>
Documentation	N/A	VR-SOP-LC-10601-FM30, VR-TM-10298-FM03, QNS Annotated File (if applicable), VR-SOP-LC-11287-FM01 (if applicable), VR-SOP-LC-11287-FM02 (if applicable), SAP <sub>o</sub> printout (if applicable), VR-SOP-LC-11299-FM07 (if applicable), Metadata and plate-count printouts, supplemental instructions from SARS-CoV-2-mNG_NT coordinator (if applicable) SAS printouts
Reagent and Equipment Information	Entered into LIMS and shown in the SAS printout	Entered into .Run file and shown in the SAS printout Entered by hand onto VR-TM-10216-FM01
Plate Identification	Barcoded plates	Barcoded plates Non-barcoded plates
Assay plate preparation	Using a (b)(4) Workstation	Using a (b)(4) Workstation Manual Preparation
Sample Testing	Replicate	Replicate

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**Table 8. Assay Job Aid 1 - Comparison of Assay Runs Using Oracle-LIMS Based SAS and File-based SAS**

	<b>Runs Processed Through Oracle SAS</b>	<b>Runs Processed Through File-based SAS or VR-SOP-LC-11293-FM02</b>
Data Analysis	Automated using the web-based SAS application found in Data Analysis Portal (DAP)	Automated using the web-based SAS application found in Data Analysis Portal (DAP). Semi-automated using form VR-SOP-LC-11293-FM02, if applicable
Quality control serum titer tracking	Automated using the web-based SAS application and LIMS to upload titers to the control database tracking and analysis system	Entered into CDTAS manually

## 11. REFERENCES

**Table 9. General References**

<b>Document</b>	<b>Title</b>
N/A	N/A

**Table 10. Form References**

<b>Form</b>	<b>Title</b>
VR-TM-10298-FM01	Manual 96-well SARS-CoV-2 Microneutralization Assay Using (b) (4) (paperless LIMS) Worksheet
VR-TM-10298-FM02	Manual 96-well SARS-CoV-2 Microneutralization Assay Using (b) (4) (LIMS metadata) Worksheet
VR-TM-10298-FM03	Manual 96-well SARS-CoV-2 Microneutralization Assay Using (b) (4) (FB-SAS) Worksheet
VR-SOP-LC-11287-FM01	Preparation of SMDPs for Sample Testing of SARS-CoV-2 in a Neutralization Assay Using the (b) (4) Head (b) (4) Workstation
VR-SOP-LC-11287-FM02	Preparation of Assay Plates from SMDPs for Sample Testing of SARS-CoV-2 in a Neutralization Assay Using the (b) (4) (b) (4) Workstation
VR-SOP-LC-11287-FM03	Preparation of 96 well CMDPs for Sample Testing of SARS-CoV-2 in a Neutralization Assay Using the (b) (4) (b) (4) Workstation
VR-SOP-LC-11299-FM07	Worksheet for Distribution of Vero and Vero E6 (V-E6) Cells
VR-SOP-LC-11293-FM02	Titer Calculation Worksheet for VR-SOP-LC-11293
VR-SOP-LC-10601-FM30	NT COV2 Data Package Chain of Custody
VR-SOP-FE-11315	Operation of (b) (4) Pipetting System

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**Table 11. Specific References**

Document	Title
VR-SOP-LC-11287	Preparation of Samples for SARS-COV-2 Neutralization Assay Using the (b) (4) Head (b)(4) Workstation
VR-SOP-LC-11293	Procedure for Data Review of the SARS-CoV-2-mNG Manual 96-well Microneutralization Assay
VR-SOP-LC-10336	Analyzing Bio-functional Assay Data Using the Web SAS
VR-SOP-LC-11299	Thawing, Passaging and Harvesting of Vero Cells
VR-RI-10072-RS01	Requirements Specification for COV2-mNG Neut
VR-SOP-FE-11315	Operation of (b) (4) Pipetting System

**12. DOCUMENT VERSION MODIFICATIONS****12.1. CRIF Number: VR-CRIF-21-14875****Table 12. Detailed Changes**

List detailed changes for document(s) Include section number(s) for each	List rationale for each change
Updated roles and responsibilities on <a href="#">Table 5</a> .	Updated to reflect procedure of using VR-TM-10298-FM02 in the event LIMS is unavailable.
Updated procedure for the event LIMS is unavailable in <a href="#">Section 9.1</a> .	Updated to improve quality assurance of data in the event LIMS is unavailable by completion and upload of VR-TM-10298-FM02.
Updated reviewer steps for the event LIMS is unavailable in <a href="#">Section 9.17</a> .	Updated procedure for data reviewers to include reviewing VR-TM-10298-FM02 in the event LIMS was unavailable for analyst.

## Document Approval Record

**Document Name:** VR-TM-10298  
**Document Title:** Manual 96-well Microneutralization Assay for the Detection of Functional Antibodies to SARS-CoV-2 in Test Serum

Signed By:	Date(GMT)	Signing Capacity
(b) (6)	03-Feb-2021 20:57:03	Author Approval
Scully, Ingrid	03-Feb-2021 21:31:49	Final Approval
Garretson, Tyler	03-Feb-2021 22:38:38	Final Approval
(b) (6)	04-Feb-2021 18:55:13	Quality Assurance Approval
(b) (6)	08-Feb-2021 14:22:51	Business Line Approver