MB-10-02	MB-29-02
Title	Quantitative Method for Evaluating Virucidal Activity of Antimicrobial Products on Hard, Non-Porous Surfaces
Revisions Made	• New SOP • This SOP is currently not a recommended method to support product registration. Please consult with the agency prior to utilizing for product registration.

SOP Number	MB-29-02
Title	Quantitative Method for Evaluating Virucidal Activity of Antimicrobial Products on Hard, Non-Porous Surfaces
Scope	The method provides a quantitative assessment of the performance of liquid antimicrobial substances against viruses on hard, non-porous surfaces. This SOP is currently not a recommended method to support product registration. Please consult with the agency prior to utilizing for product registration. This method can be used with a variety of viruses and cell lines. Refer to the Cell Line and Virus Supplement for MLB Virology SOPs ("Virology Supplement") for details (growth mediums, incubation conditions, etc.) about a specific test system.
Application	This method provides an estimate of log reduction (LR) in viable virus particles as the quantitative measure of efficacy for liquid disinfectants on a hard-non-porous surface.

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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. Virus stock suspension = frozen virus used for testing 2. Viral test suspension = the test suspension with the addition of the soil load 3. Cell line = the host cells that will allow for the recovery and cultivation of the test virus. 4. MPN = Most Probable Number 5. CPE = cytopathic effect. Morphological changes induced in cells by virus infection, which are recognizable under a light microscope. 6. LR = $\log_{10}$ reduction
2. Health and Safety	1. Follow procedures specified in SOP MB-01, Laboratory Biosafety. 2. Consult the Safety Data Sheet for specific hazards associated with the test substance or other potentially hazardous materials.
3. Personnel Qualifications and Training	1. Refer to SOP ADM-04, OPP Microbiology Laboratory Training. 2. A thorough knowledge of cell culture and virology methods are required to perform this assay.
4. Instrument Calibration	1. Refer to SOPs EQ-01 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-05 (timers), QC-05 (Rees system), and QC-19 (pipettes).
5. Sample Handling and Storage	1. Refer to SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	1. For quality control purposes, the required information is documented on the appropriate form(s) (see Section 14). 2. Test all new reagents and media in parallel with existing reagents and media to ensure that they perform as expected and are sterile. Quality control and sterility testing should be performed on the cell line to ensure there is no unexpected cytotoxicity.
7. Interferences	1. Inadequate neutralization may lead to errors in the measurement of test substance efficacy. Prior to efficacy testing, verify neutralizer effectiveness using the procedure outlined in **Appendix 1**. 2. The test substance or the neutralizer may interfere or be cytotoxic to the cell line. This should be experimentally determined prior to or concurrently with testing. See **Appendix 1.** 3. During testing, do not process carriers where the test substance runs off the

	carrier; replace and retest with new inoculated carrier(s) and vial(s). 4. Avoid touching the carrier surface with a pipette tip during the application of the test substance or the control substance. 5. Transparent vials are more desirable to facilitate the application of 50 µL test substance or control substance on inoculated carriers. 6. Avoid excess alkalinity of the base mediums and complete growth medium (CGM). Place medium in a CO_2 incubator with a loose cap to allow it to reach its normal pH if needed. pH is indicated by the color of the media. 7. Presence of atypical CPE for the test system virus being used may be indicative of a viral or mycoplasma contaminate. 8. Cytotoxicity of the cell line may compromise the test system. 9. If cells are not viable due to cytotoxicity past the 10^{-1} dilution, increased neutralizer volume (up to 20 mL), alternative neutralizers, or media changes may be utilized to reduce cytotoxicity. 10. Extended exposure of the cell line to the air during media removal can result in cell death.
8. Non-conforming Data	1. For an acceptable test, achieve mean control carrier counts of 5.0-6.0 logs MPN/carrier; each carrier should be within 5.0-6.0 logs MPN/carrier. 2. Any level of contamination which interferes with the recording and interpretation of results will result in invalid data.
9. Data Management	1. Archive data consistent with SOP ADM-03, Records and Archives. 2. Data is managed by use of statistical spreadsheets.
10. Cautions	1. Use of a different cell culture media, incubation conditions, or animal serum (ex: FBS) concentrations as specified in the Virology Supplement can produce inaccurate results. 2. Ensure that the cell monolayer in the 24 well plates is at the appropriate confluency before testing; do not use plates outside the recommended confluency for the test system. 3. Ensure that the cell line is free from *Mycoplasma* contamination. Test cells regularly with a *Mycoplasma* detection kit (e.g., MycoAlert Plus). 4. Freezing virus stock in aliquots of less than 1 mL will negatively affect the long-term viability of the virus. 5. For storage, ensure carriers are completely dry following sterilization.

11. Special Apparatus and Materials	1. Media and Reagents: a. *Base Mediums* (e.g., Eagle's Minimum Essential Media (EMEM)). Used to make complete growth medium. May be bought in liquid or powder form. b. *Complete Growth Medium* (CGM). Consisting of base medium and the appropriate concentration of FBS or other ingredients. Cell line CGM is used to grow cell lines. Virus CGM is used for virus growth. Refer to the Virology Supplement for details about a specific test system. Antibiotics and/or antifungals may be added to reduce potential contamination. c. *Neutralizer.* The default neutralizer for this test system is the virus CGM. Refer to the Virology Supplement for details about a specific test system. If the neutralization confirmation assay (see **Appendix 1**) demonstrates that the virus complete growth medium is ineffective, other neutralizers may be used. d. *Diluent (serial dilutions).* The virus CGM will be the default diluent. Refer to the Virology Supplement for details about a specific test system. e. *Dulbecco's Phosphate buffered saline* (DPBS) *or other equivalent buffer (e.g., PBS, Earle's Balanced Salt Solution).* Prepare per manufacturer's guidelines. f. *Soil load.* The 3-part soil load to be incorporated in the test suspension is a mixture of the following stock solutions in PBS: i. BSA: Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter membrane filter, aliquot (e.g., a minimum of 50 µL), and store at -20±2°C. ii. Yeast Extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter membrane filter, aliquot (e.g., a minimum of 70 µL) and store at -20±2°C. iii. Mucin: Add 0.04 g mucin (from bovine submaxillary gland or equivalent) to 10 mL of PBS, mix thoroughly until dissolved, and pass through a 0.2 µm pore diameter membrane filter, aliquot (e.g., a minimum of 125 µL) and store at -20±2°C. iv. The stock solutions of the soil load are single use only. Do not refreeze once thawed; store up to one year at -20±2°C. v. See section 12.4 for addition of soil load to inoculum.

g. *Test substance.* Antimicrobial test solution or product. If dilution is required, see section 11.1h for diluent.

h. *Test substance diluent.* Used for the preparation of dilutable products. See SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP MB-30, Preparation of Hard Water and Other Diluents.

i. Diluents and levels of water hardness will be specified in the study protocol.

i. *Water.* De-ionized (DI), distilled water or water with equivalent quality for making reagent solutions and culture media.

j. *Non-ionic detergent [e.g., Liquinox (1% solution)] or equivalent.* To clean carriers.

2. Apparatus

a. *Carriers*: Discs (1 cm in diameter) made of AISI Type 304 Stainless Steel with 150 grit unidirectional finish on one side. Only the top of the disc is visually screened and inoculated. Carriers are single use only. See **Appendix 2** for carrier specifications and photographs of screened carriers.

b. *Calibrated 10 µL positive displacement pipette* with corresponding 10 µL tips, for carrier inoculation.

c. *Calibrated micropipettes* (e.g., 200 µL, 1 mL) with 10-100 or 20-200 µL tips, for deposition of test substance on carriers and preparing dilutions.

d. *Filter paper.* Whatman No. 2, to line glass Petri plates during carrier sterilization.

e. *Water bath* to maintain cell culture media at the appropriate temperature.

f. *Sterile Forceps*. Straight or curved, non-magnetic, appropriate to pick up the carriers for placement in vials.

g. *Sterile vials (plastic or comparable).* Nalgene 2116-0030 or 2118-9050. Flat bottom with a wide mouth for holding inoculated carriers to be exposed to the test substance and for accommodating neutralizer/eluent. Suitable vials should be at least 25 mm in neck diameter and hold up to 20 mL of liquid.

h. *Desiccation unit* (with gauge to measure vacuum and maintain chamber pressure of 0.068 to 0.085 MPa) with fresh desiccant (e.g., anhydrous

	$CaCl_2$). For drying inoculated carriers. Change desiccant when moisture absorption is evident (e.g., desiccant clumping). i. Alternatively, an environmental chamber may be used to dry carriers; refer to section 12.5.f.i. i. *Vacuum source*. In-house line or suitable vacuum pump for drying inoculated carriers in desiccation unit and to remove media from plates or flasks. j. *Tissue culture plates (tissue culture treated)*. Appropriately sized flasks for propagation of the cell line and appropriate-number well plates (e.g., 24) for virus MPN determination. k. *Liquid nitrogen dewar* or comparable freezer capable of long-term storage of cell lines. l. *Certified timer*. Readable in minutes and seconds, for tracking of timed events and intervals. m. *Centrifuge* (with swinging bucket rotor). For preparing frozen virus stock. n. *Ultracentrifuge (capable of spinning approximately 100,000 x g).* For concentrating virus stock if needed. o. *Vortex-style mixer*. For vortex mixing of various solutions. p. *Inverted microscope.* For inspection of CPE and other cell characteristics. q. *Incubator* with 5% CO_2. r. *Hach digital titrator kit*. For measuring water hardness.
12. Procedure and Analysis	
12.1 Preparation and sterilization of carriers	a. Without magnification, visually check the brushed top surface of the carriers for abnormalities (e.g., rust, chipping, deep striations) and discard if observed; refer to Figures 1 and 2 of **Appendix 2** for examples of typical acceptable and unacceptable carriers, respectively. Record physical screening of carriers on Physical Screening of Carriers Record Form (See Section 14). b. Soak visually screened carriers in a suitable non-ionic detergent solution for 2-4 h to degrease and then rinse thoroughly in distilled or deionized water (e.g., place carriers in large beaker and flush beaker with DI water

	for 5-10 min), ensuring complete removal of detergent. Allow carriers to dry. i. Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting. c. Using gloved-hands or forceps, place up to 20 clean dry carriers on filter paper inside the bottom surface of a glass Petri dish (100 mm in diameter), ensure carriers were not damaged (scratched) during processing. Cover the Petri dish with its lid and sterilize at 121°C for 45 min. Ensure carriers are dry following sterilization. i. Screened, cleaned carriers may be stored dry in a clean, covered vessel prior to sterilization. d. Use sterilized carriers for up to six months. After six months, re-sterilize any remaining unused carriers and assign a new preparation number.
12.2 Preparation of cell lines	a. Refer to the Virology Supplement for details on the care and growth of the cell line being used. b. A minimum of six 24 well plates are needed for an efficacy test. c. Record the confluency of the cells in the plate(s) prior to use in the efficacy test.
12.3 Preparation of test viruses	a. Refer to the Virology Supplement for details on the care and growth of the test virus being used. b. Thaw a cryovial rapidly to avoid loss in the viability of the preserved virus (e.g., place in a waterbath). Each cryovial should be for single use only if practical. c. Use an appropriate dilution scheme to achieve control counts in the range of 5.0 to 6.0 logs (MPN/carrier). Dilute the virus stock (e.g., 1:10) in virus CGM prior to preparing the inoculum with the soil load. i. The virus may be concentrated by ultracentrifugation (100,000 x g for 4 hours at 4ºC) if necessary. d. Use the diluted virus suspension to prepare the final test suspension with the addition of the soil load per section 12.4.
12.4 Preparation of the final test suspension	a. Vortex the test suspension for 10-30 s. b. To obtain 500 µL of the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette (other volumes may be used proportionally):

	i. 25 µL BSA stock ii. 35 µL yeast extract stock iii. 100 µL mucin stock iv. Vortex soil suspension for 10 s prior to adding viral suspension v. 340 µL virus suspension c. Use final test suspension with soil load (at room temperature, 21±3°C) to inoculate carriers within 30 min of preparation. d. Impact of the three-part (section 12.4b) soil on the cell line must be determined in advance of testing. Refer to **Appendix 1**.
12.5 Inoculation and drying of carriers	a. Rescreen each carrier prior to inoculation. Place carriers screened side up inside an empty, sterile plastic Petri dish (no more than 20 carriers/dish). b. Vortex the final test suspension for 10 s following the addition of the virus suspension and immediately prior to use. c. Inoculate the number of carriers required for the evaluation of the test substance (3 controls and 3 treated) along with carrier(s) to serve as extras. d. Using a calibrated positive displacement pipette with a 10 µL tip, withdraw 10 µL of the final test suspension and deposit it at the center of each carrier (clean, screened, and sterile), keeping the pipette perpendicular to the carrier during deposition of the test suspension. Avoid contact of pipette tip with carrier and do not spread the final test suspension with the pipette tip. i. For consistency, vortex the inoculum frequently during inoculation of the carrier set. ii. The same pipette tip may be used to inoculate all carriers (unless the tip is compromised). iii. Discard any inoculated carrier where the final test suspension has run over the edge. e. Transfer the Petri dish(es) with the inoculated carriers into a desiccation unit (with desiccant) and completely remove the lid of the Petri dish. Close the desiccation unit door (or lid) and seal the unit. Apply vacuum to evacuate the desiccation unit. f. Maintain and monitor the vacuum level using a gauge. Achieve and

	maintain consistent level of vacuum (at 20-25 in of mercury, 508-635 torr, 677-847 mbar, or 68000-85000 Pascal, or 0.068-0.085 MPa) by leaving the vacuum on during the drying period with the desiccator stopcock opened or closed as necessary. i. Alternatively, place petri dish(es) into an environmental chamber set to 35±5% humidity and 21±3°C with the lid(s) ajar and allow to dry for 45 to 60 minutes. Document drying conditions in the test paperwork. g. Hold the inoculated carriers in the evacuated desiccation unit or environmental chamber at 21±3°C for 45 to 60 min. Visually inspect inoculated carriers to verify that they have completely dried and remove from desiccation unit or environmental chamber. Do not use carriers that are visibly wet for testing. Do not use carriers where the dried inoculum is near the edge of the carrier (See **Appendix 2**, Fig. 3). Do not use carriers whose dried inoculum spot cannot be completely covered by the 50 µL of disinfectant. i. If the carriers are not dry within the recommended time, continue to dry for up to an additional 10 min and record the time to dry on the paperwork. ii. If carriers dry very quickly (e.g., 5-10 min) or are not dry within 10 min beyond the specified time, check the desiccation unit and the vacuum system to ensure proper function (e.g., replace the desiccant if necessary and check the seal for leaks). h. Use dried inoculated carriers for testing within 30 min following removal from desiccation unit or environmental chamber; hold carriers in closed Petri dish at room temperature (21±3°C) until use.
12.6 Exposure of the dried inoculum to the test substance or control substance	a. Evaluate 3 untreated control carriers and 3 treated carriers for each test substance tested (one test organism and contact time/temperature combination) unless specified otherwise. **Note:** One set of control carriers may be used for evaluating multiple test substances against one virus on one test day (assuming the neutralizer is the same). b. Using sterile forceps, transfer each dried carrier with the inoculated side up to a flat-bottom vial and cap the vial. Repeat until all carriers are transferred. c. In a timed fashion with appropriate intervals, sequentially deposit 50 µL of the test substance (equilibrated to 21±3°C) with a calibrated micropipette (or positive displacement pipette) over the dried inoculum

	on each test carrier, ensuring complete coverage. i. Gently apply the antimicrobial test substance at a perpendicular angle to the inoculated carrier; do not forcefully deposit the disinfectant. d. Use a new tip for each carrier; do not touch the carrier surface with a pipette tip during the application of the test substance or the control substance; replace with new carrier(s) and vial(s) if this occurs. Do not cap the vials. i. For non-foaming aerosols and pump/trigger spray products, obtain the test substance by dispensing the product into a sterile vessel for collection. Cap the vessel and use dispensed product within 30 min. ii. For foaming spray formulations, allow the foam to break down for at least 5-10 minutes for the generation of a 1-2 mL liquid sample. Cap the vessel and use dispensed product within 30 min. e. Do not process carriers where the test substance runs off the carrier or does not completely cover the inoculum spot; replace with new carrier(s) and vial(s) if this occurs. f. Treat control carriers last. Each control carrier receives 50 µL of virus CGM equilibrated to 21±3°C, instead of the test substance. Hold the control carriers for the same contact time as used for the test substance. g. Conduct the test at room temperature (21±3°C) for the selected contact time; record temperature on the Test Information Sheet (see section 14). Use a certified timer to ensure that each carrier receives the required contact time.
12.7 Neutralization of test substance and elution of test organism	a. The neutralizer for the control carriers is the same as that for the treated carriers. b. Within ±5 s of the end of the contact period, add 10 mL (maximum of 20 mL) of neutralizer equilibrated to 21±3°C to each vial in the specified order according to the predetermined schedule. Briefly vortex (2-3 s) each vial following the addition of the neutralizer. i. The neutralized vial with carrier is documented as the 10^0 dilution. c. Following the neutralization of the entire set of carriers, vortex each vial

	for 30±5 s at high speed to recover and disaggregate the inoculum; ensure that the liquid and carrier are fully vortexed. Do not remove the carrier from the vial. d. Interaction between the neutralizer and product and its effect on the cell line must be determined prior to testing. Refer to **Appendix 1**.
12.8 Dilution and recovery	a. Initiate dilutions within 30 min after neutralization and vortexing. Initiate inoculation of cell line cultures within 30 min of preparing the dilutions. b. Titrate the samples for virus infectivity using the appropriate cell line using the most probable number (MPN) method. c. Serially dilute using pre-warmed virus CGM and plate samples from the treated and control carriers; process treated carriers first. d. If using virus CGM as the neutralizer, plate 8 wells per dilution if 10 mL neutralizer is used or 16 wells per dilution if 20 mL neutralizer is used (plate at least 80% of the neutralizer if using other sized plates). i. If a virus test system requires an adsorption step, plate as much of the first dilution as practical (e.g., plating 24 wells at 200 µL per well would use 4.8 mL of the neutralizer). Further dilutions can be plated using only 8 wells per dilution on a 24 well plate. e. For further dilutions, make 10-fold dilutions of the eluates with virus CGM as the diluent (e.g., 1 mL + 9 mL virus CGM), equilibrated to the proper temperature. f. Refer to the Virology Supplement for details on enumerating the virus. The elution steps for control carriers are the same as for the test carriers. g. For each test, have at least one well as a negative control (virus CGM alone) and one well as a positive growth control (e.g., one of the dilutions from a control carrier). h. If cytotoxicity was observed in pre-neutralization testing (see **Appendix 1**, Section 1), remove the virus CGM from all wells in the affected dilutions (treated and control) at the appropriate time (one hour minimum) and wash them with pre-warmed DPBS, then replace the DPBS with fresh virus CGM. The DPBS wash step may not be required if the cytotoxicity is mild. i. Incubate test and control plates appropriately. Refer to the Virology

	Supplement for details about a specific test system.
12.9 Recording results	a. Record results as positive or negative in each well after the required incubation period. A positive well may exhibit cytopathic effect (CPE), positive fluorescent staining, or some other effect (refer to the Virology Supplement) and a negative well will not.
13. Data Analysis/ Calculations	1. All observations are recorded and used in calculations to estimate the log reduction based on the MPN. 2. Use values with at least three significant figures when performing calculations (e.g., log density, mean log density). Report the final mean log reduction value with two significant figures (e.g., round up or down to the nearest tenth). 3. The MPN/carrier is calculated using the MLB Virucidal Activity Spreadsheet (Section 14). Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier). 4. Calculate the mean $\log_{10}$ density across treated carriers. 5. Calculate the mean $\log_{10}$ density across control carriers. 6. Calculate the $\log_{10}$ reduction (LR) for treated carriers: $\log_{10}$ reduction = the mean $\log_{10}$ density for control carriers minus the mean $\log_{10}$ density for treated carriers. 7. For a set of 3 treated carriers: when the 10^0 dilution (the contents of the vial with the carrier) is plated either by itself or in addition to other dilutions and the data for each carrier result in zeros for each dilution plated, report the LR as greater than or equal to the mean $\log_{10}$ density for the control carriers.
14. Forms and Data Sheets	1. Appendix 1: Testing for Cytotoxicity**,** Interference with Virus Infectivity, Influence of Soil Load on Host Cells, and Neutralization Confirmation 2. Appendix 2: Carrier Specifications 3. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record Form — MB-29-02_F1.docx Quantitative Method for Virucidal Activity: Organism Culture Tracking Form — MB-29-02_F2.docx Quantitative Method for Virucidal Activity: Virology Calculation Spreadsheet — MB-29-02_F3.xls

	Quantitative Method for Virucidal Activity: Test Information Sheet	MB-29-02_F4.docx
	Quantitative Method for Virucidal Activity: Test Processing Sheet	MB-29-02_F5.docx
	Quantitative Method for Virucidal Activity: Timing Sheet	MB-29-02_F6.docx
	Virology Results Sheet	MB-29-02_F7.docx
	Quantitative Method for Virucidal Activity: Neutralizer or 3-Part Soil Effect on Cell Line	MB-29-02_F8.docx
	Quantitative Method for Virucidal Activity: Cytotoxicity Determination	MB-29-02_F9.docx
	Quantitative Method for Virucidal Activity: Neutralization Test Processing Sheet	MB-29-02_F11.docx
	Quantitative Method for Virucidal Activity: Neutralization Timing Sheet	MB-29-02_F12.docx
15. References	None	

Appendix 1

Testing for Cytotoxicity, Interference with Virus Infectivity, Influence of Soil Load on Host Cells, and Neutralization Confirmation.

(Note: Refer to the Virology Supplement for specific information on a particular test system)

1. **Cytotoxicity Determination.** Prior to performing the neutralization assay, ensure the proposed neutralizer, neutralizer and test chemical, and the soil used do not impact the quality of the cell line by performing the following.

    a. **Neutralizer Effect on Cell Line (for neutralizers other than virus CGM).**

        i. Add 0.5 mL of the proposed neutralizer to 4.5 mL of pre-warmed virus CGM (this is the 10^{-1} dilution). It is suggested to do further dilutions out to 10^{-2} or 10^{-3} depending on the expected cytotoxicity of the neutralizer.

        ii. Remove the cell line CGM from the wells of a 24 well plate with an appropriate cell line confluency and add 1 the neutralizer plus virus CGM solution. Plate at least 4 wells per dilution. Have at least one well as a negative control (e.g., virus CGM with alone).

        iii. Incubate plate as appropriate and observe closely for cytotoxicity.

        iv. If cytotoxicity is observed after one hour, remove the media in a single well of the affected dilution, rinse once with pre-warmed DPBS (the DPBS wash step may be omitted if the cytotoxicity is mild), and replace media.

        v. If cell death occurs in under one hour, the neutralizer cannot be tested.

        vi. The effect of the media change in the single well can be compared to the other wells in the dilution and the negative control.

    b. **Neutralizer Plus Test Chemical Effect on Cell Line.**

        i. Add 50 µL of test chemical and 10 mL (maximum of 20 mL) of neutralizer equilibrated to 21±3°C and vortex 2-3 seconds. Let this solution sit at room temperature for 10 minutes.

        ii. Add 1.0 mL of this solution to 9 mL of virus pre-warmed virus CGM (this is the 10^{-1} dilution). It is suggested to do further dilutions out to 10^{-2} depending on the expected cytotoxicity.

iii. Refer to the Virology Supplement for details on enumerating the virus. Plate at least 8 wells for the 10^0 dilution, 6 wells for the 10^{-1} dilution, and 4 wells is for the 10^{-2} dilution. Extra wells will be needed to observe the effect of no media changes or for further media changes as needed.

iv. For highly toxic test chemicals, washing the cells with pre-warmed DPBS before the addition of the virus CGM will help remove cytotoxicity.

v. Have at least one well on each plate as a negative control (e.g., virus CGM alone).

vi. It is suggested to change the media in the wells as outlined below. Change the media at the lower time interval if they look more toxic. Other media changes can be made at other times if necessary.

- For the 10^0 dilution: On the day of the test, change 2 wells 1-2 hours (1-hour minimum) after the neutralizer/test chemical mixture was added to the cells. Change 2 more wells 3-5 hours after the neutralizer/test chemical mixture was added to the cells. The next day, change 1 each of the 1-2 hour and 3-5 hour wells, as well as another, previously unchanged well.
- For the 10^{-1} dilution: On the day of the test, change 2 wells 3-5 hours after the neutralizer/test chemical mixture was added to the cells. The next day, change 1 of these wells as well as another, previously unchanged well.
- For the 10^{-2} dilution: On the day after the test, change 1 well.

vii. Incubate the plate as appropriate (refer to the Virology Supplement) and observe the cells for cytotoxicity. The test cells should be compared to the negative control cells to determine toxicity.

viii. Score the cells as toxic or non-toxic in each in each test conditions.

ix. Identify the test condition that removed the cytoxicity and use that condition for further neutralization and efficacy testing. Use the test condition that allows the media to stay on the cells for as long as possible.

Example: In the 10^0 dilution, if the unchanged wells are toxic but both the 1 hour and 4 hour media changes are non-toxic, change the media

in the 10^0 dilutions after 4 hours in all future testing.

x. If cell death occurs in under one hour, that test condition cannot be used.

xi. Cytotoxicity past the 10^{-1} dilution is unacceptable for testing. Increased neutralizer volume (up to 20 mL), alternative neutralizers, or media changes may be utilized to reduce cytotoxicity.

c. **3-Part Soil Effect on Cell Line.**

i. Make the 3-part soil (See Section 12.4b but withhold the virus).

ii. Add 10 µL of the soil to 10 mL of pre-warmed virus CGM.

iii. Refer to the Virology Supplement for details on enumerating the virus. Have at least one well as a negative control (e.g., virus CGM alone).

iv. Incubate plate as appropriate (refer to the Virology Supplement) and observe daily for cytotoxicity. No cytotoxicity should be observed.

2. **Neutralization assay:**

a. The test substance is first mixed with a candidate neutralizer. The diluted test virus is then added to the reaction mixture as a suspension.

b. 10 mL of virus CGM is the default neutralizer, equilibrated to 21±3°C. A minimum of 10 mL and up to 20 mL neutralizer may be used if necessary. Other neutralizers may be used if the CGM is found to be ineffective. If a neutralizer other than virus CGM is proposed, an additional Neutralizer Toxicity Control (Treatment 3, below) must be added to determine any toxicity or interference from the proposed neutralizer.

c. Refer to Sections 12.3 and 12.4 for preparation of the final test suspension with soil load.

d. Prepare *Test Suspension A*: Dilute the virus stock suspension in virus CGM to achieve an average recovered concentration of approximately 2.0-3.0 logs (i.e., 100-1000 virus particles) per vial for the Titer Control sample. To achieve this, dilute the virus stock suspension through 10^{-4} (or as necessary); refer to the Neutralization Test Processing Sheet.

e. Prepare *Test Suspension B*: Prepare the soil load: vortex each component and combine 25 µL bovine serum albumin (BSA), 35 µL yeast extract, 100 µL of mucin, and 340 µL of *Test Suspension A* (0.5 mL total volume) and mix well. *Test Suspension B* should be used within 30 minutes of preparation.

Note: Two separate serial dilutions of *Test Suspension A* may be used to prepare two different concentrations of *Final Test Suspension B* to ensure at least one dilution yields an average recovered concentration of 2.0-3.0 logs per vial.

f. **Required Treatments** (Refer to **Figure 1** below):

i. ***Treatment 1: Titer Control.*** Add 10 mL neutralizer to each of three test tubes. At timed intervals, add 10 µL of *Test Suspension B* to each tube. Use a new tip for each tube. Vortex tube for 3-5 s. Proceed with step g.

ii. ***Treatment 2: Neutralizer Effectiveness.*** Add 50 µL of the test substance to each of three test tubes. At timed intervals, add 10 mL neutralizer to each tube and briefly swirl. After 10±2 s, add 10 µL of *Test Suspension B* to each tube. Use a new tip for each tube. Vortex each tube for 3-5 s. Proceed with step g.

iii. ***Treatment 3: Neutralizer Toxicity Control (for neutralizers other than CGM).*** Add 10 mL proposed neutralizer to each of three test tubes. At timed intervals, add 10 µL of *Test Suspension B* to each tube. Use a new tip for each tube. Vortex tube for 3-5 s. Proceed with step g.

Note: Steps should be conducted at timed intervals (e.g., 30 s) to ensure consistent time of contact for each carrier.

g. Hold the mixtures for 10±1 min at room temperature (21±3°C).

h. At the conclusion of the holding period, vortex each tube for 3-5 s. Serially dilute the sample as needed (e.g., remove 1 mL of sample and dilute in 9 mL µL of pre-warmed virus CGM).

Note: Initiate dilution and plating as soon as possible (e.g., within 5 minutes). Two analysts are recommended to perform vortexing and dilution steps to reduce holding time after vortexing.

i. Titrate the samples for virus infectivity using the appropriate cell line using 8 wells per dilution. This is done using the most probable number (MPN) method.

Note: If any 10^0 (vial) dilution is used that does not contain virus CGM (e.g., Treatment 3 with proposed neutralizer), allow it to adsorb on the cells for 1 hr, then remove and replace with fresh virus CGM.

j. Check the cells in all dilutions from Treatment 3 often for cytotoxicity. If cytotoxicity is observed, replace with fresh virus CGM. If the virus CGM is changed for certain dilutions, also wash and replace the virus CGM in the same dilutions for the control treatments. Otherwise, do not replace the virus CGM for the duration of the

incubation.

k. For the neutralizer to be considered effective:

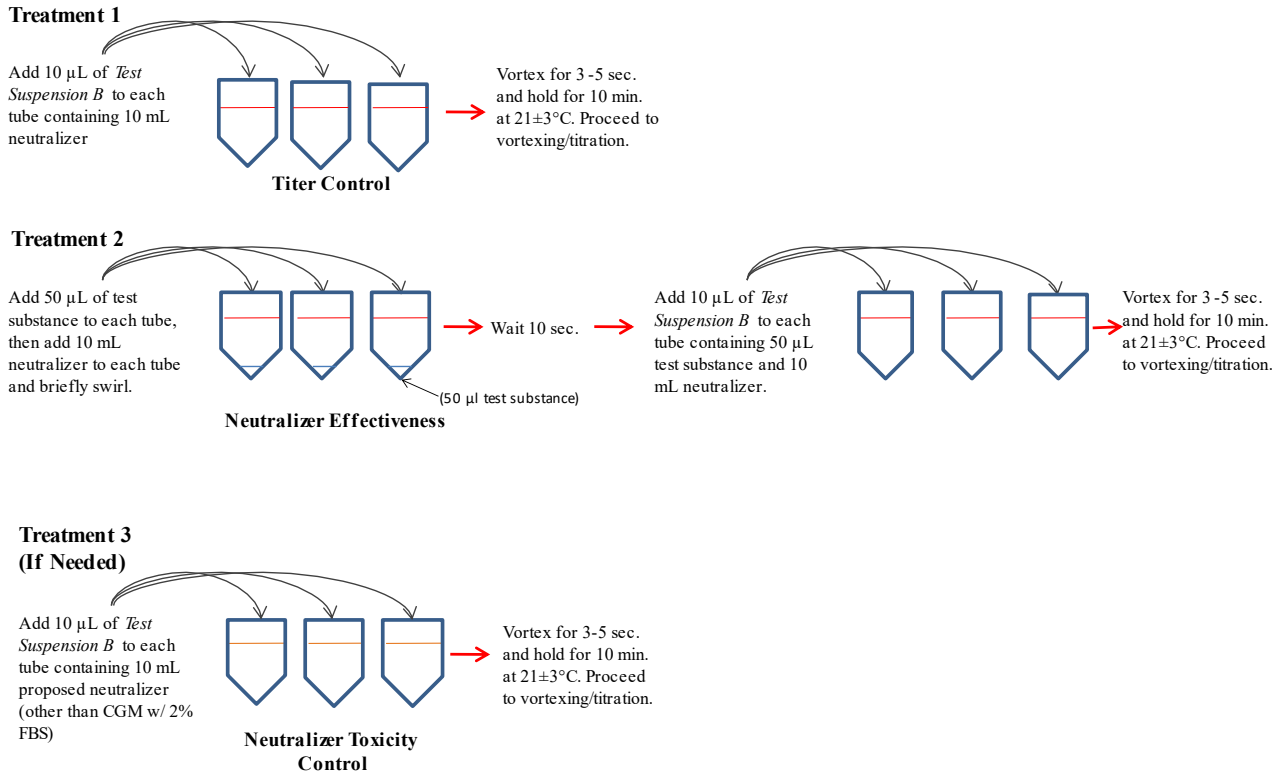
   i. Ensure that the recovered virus (MPN) in the **Titer Control** using *Final Test Suspension B* is between approximately 2.0-3.0 logs per vial.

   ii. The recovered virus (MPN) in the **Neutralizer Effectiveness** treatment is within 0.5 logs of the **Titer Control**; this verifies effective neutralization. A log reduction greater than 0.5 logs indicates that the neutralizer was not effective. Note: an MPN value higher than the **Titer Control** is also deemed valid.

   iii. The recovered virus (MPN) in the **Neutralizer Toxicity Control** (if performed) is within 0.5 logs of the **Titer Control**. A log reduction greater than 0.5 logs indicates that the neutralizer is harmful to the test system. Note: an MPN value higher than the **Titer Control** is also deemed valid.

l. All criteria must be met. If the criteria are not met, another neutralizer or mixture of neutralizers must be identified and verified.

Figure 1: Quantitative Method Neutralization Assay Flow Chart



Appendix 2

Carrier Specifications

(AISI Type 304 Stainless Steel Carriers)

General Description: 1 cm magnetic disc made of AISI Type 304 Stainless Steel (SS) with 150 grit unidirectional brushed finish on one side.

Material: AISI Type 304 Austensic stainless steel consisting of 18% to 20% Chromium, 8% to 10.5% Nickel, and a maximum of 0.8% Carbon.

- European Specification X5CrNi18-10 Number 1.4301
- Japanese Specification: JIS 4303 SUS 304

Carrier Dimensions:

- Diameter: 1cm (±0.5 mm)
- Stainless Steel Sheet Thickness: 22 gauge; carrier manufacturer will provide thickness of the original stainless steel sheet (in mm).
- Flatness: Carrier height not to exceed 110% of the thickness of the uncut sheet of stainless steel from which the carriers are manufactured.

Finish: A ground unidirectional finish obtained with 150 grit abrasive (AISI) on the top side of the stainless steel sheet.

Burr Removal: Remove burrs from the edges of the discs on the bottom side of the carrier using a manual process.

Passivation: Parts are passivated by the carrier manufacturer according to ASTM A967 in a citric acid solution and prepared as follows:

- Degrease with citrus-based degreaser by soaking in the degrease solution for 1 hour
- Rinse with de-ionized water
- Passivate by soaking carriers:
    - 7% citric acid solution
    - 20-30 min at 35±5°C.
- Rinse with de-ionized water
- Air dry

Examples of Physically Screened Carriers[1]

Figure 1: Examples of typical acceptable carriers.

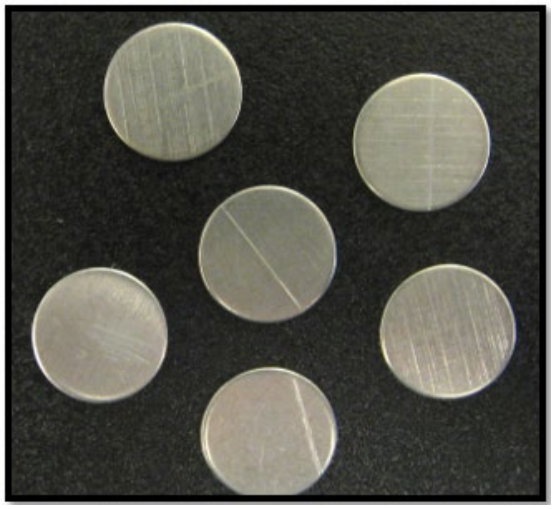


Figure 2: Examples of typical unacceptable carriers.

A B

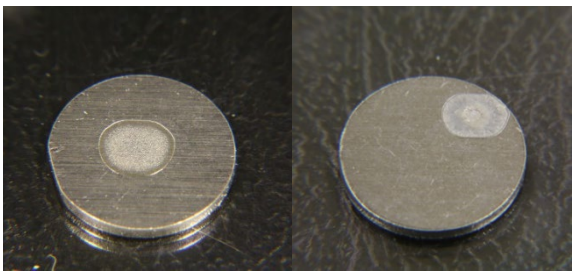


Figure 3: Example of an acceptable (A) and unacceptable inoculated carrier (B).

[1]Carriers are screened without magnification