Quantitative Method for Evaluating the Efficacy of Antimicrobial Test Substances on Soft Surface Textiles against Viruses

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Scope

The Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) recommends that applicants utilize this method to support efficacy criteria for the registration of products bearing claims for use on soft surface textiles. The method provides a quantitative assessment of the performance of antimicrobial substances against viruses on soft surface textiles.

This method provides log reduction (LR) based on viral inactivation as the quantitative measure of efficacy for disinfectants against viruses on a soft surface textile.

Method Overview

In brief, the method uses 1 cm diameter discs or 1 cm^2 squares (carriers) of a set of representative soft surface textiles. Each carrier receives 10 µL of microbial inoculum (with a three-part soil load) deposited in the center of each carrier. The inoculum is allowed to dry and is then exposed to 50 µL of the antimicrobial treatment; control carriers receive an equivalent volume of an innocuous fluid (e.g., growth media). The exposure time is allowed to elapse; a liquid neutralizer is then added to the vial to halt the antimicrobial action. Each vial with the carrier is vortexed, serially diluted, and plated onto cells to recover viable virus particles. The presence of viable virus particles is determined as applicable to the test system (e.g., cytopathic effect (CPE), direct fluorescent antibody (DFA) stain, hemagglutination, etc.). Based on the difference between the mean $\log_{10}$ density values of the untreated control and treated carriers, a mean $\log_{10}$ reduction (LR) in viable virus particles is calculated. The LR value is used as the measure of product effectiveness.

Appropriate safety procedures should always be used when working with laboratory test systems which include human pathogenic microorganisms. Laboratory safety is discussed in the current edition of “Biosafety in Microbiological and Biomedical Laboratories (BMBL)” 6th edition, from the subject matter experts within the U.S. Department of Health and Human Services (HHS), including experts from the Centers for Disease Control and Prevention (CDC) and National Institutes of Health (NIH).

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1) **Special Apparatus and Materials**

a. Test Virus, use virus to be claimed on the label.

b. Cell Line, appropriate for the virus tested.

c. Media and reagents:

i. *Complete Growth Media (CGM).* Consisting of Minimum Essential Media and FBS or other medium specified for the virus being tested. Used for cell line propagation, viral propagation, and serial dilution. Antibiotics and/or antifungals may be added to reduce potential contamination.

1. *Growth Media (GM).* Liquid or powder form (e.g., Eagle's or Dulbecco's Minimal Essential Medium). Used to prepare complete growth media. Prepare per manufacturer's guidelines.

2. *Heat Inactivated Fetal Bovine Serum (FBS).* Compatible for use with cell lines. Often used to prepare complete growth media.

ii. *Neutralizer.* A liquid reagent used to inactivate and/or dilute the antimicrobial treatment to end the contact time.

1. Note: The recommended neutralizer for the test system is the same medium used to grow the virus (e.g., CGM), however, other neutralizers may be used.

iii. *Dulbecco's Phosphate buffered saline (DPBS).* Or other equivalent buffer (e.g., PBS, Earle's Balanced Salt Solution). Prepare per manufacturer's guidelines.

iv. *Tryptic Soy Broth (TSB)*. Used to assess sterility of carriers. Purchase broth from a reputable source or prepare according to manufacturer's instructions.

v. *Antibiotic/antifungal.* 100x Amphotericin B/Penicillin/Streptomycin solution or other equivalent antibiotic/antimycotic solution. May be used to prevent contamination of cell culture.

vi. *Soil load, 3-part.* Use as the soiling agent.

1. BSA: Add 0.5 g bovine serum albumin (BSA, radio immunoassay (RIA) grade or equivalent, CAS# 9048-46-8) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter (polyethersulfone) membrane filter, aliquot (e.g., a minimum of 50 µL), and store at -20±2°C.

2. Yeast Extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter (polyethersulfone) membrane filter, aliquot (e.g., a minimum of 70 µL), and store at -20±2°C.

3. Mucin: Add 0.04 g mucin (from bovine submaxillary gland, CAS # 84195-52-8) to 10 mL of PBS, stir or vortex-mix until thoroughly dissolved, and pass through a 0.2 µm pore diameter (polyethersulfone) membrane filter, aliquot, and store at -20±2°C.

4. The three stock solutions of the soil load are single use only. Do not refreeze; store up to one year at -20±2°C.

vii. *Antimicrobial Test Substance.* Ready-to-use, activated, or concentrated antimicrobial. If the antimicrobial test substance is prepared by diluting a concentrate, adequately mix antimicrobial test substance with the appropriate diluent (e.g., hard water), then use prepared test substance within 3 hours of preparation or as otherwise instructed by the manufacturer. Measuring error increases as delivery volume decreases. To minimize variability due to measuring error, a minimum of 1.0 mL or 1.0 g of concentrated antimicrobial test substance should be used when preparing use-dilutions for testing. Use v/v dilutions for liquid antimicrobial test substances and w/v dilutions for solid antimicrobial test substances. The use of a positive displacement pipette is recommended for viscous liquids.

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

d. Apparatus

i. *Carriers*: Discs (1 cm in diameter) or 1 cm^2 squares cut from soft surface textile. Carriers are single use only. See section 2 for carrier specifications.

ii. *Hole punch:* If necessary, for use in the preparation of 1 cm disc from material. Model number: SKU# HP-MEI448R or equivalent.

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

iv. *Filter paper*. Whatman No. 2, to line glass Petri plates.

v. *Calibrated micropipettes* (e.g., 200 µL, 1 mL) with appropriate corresponding tips, for deposition of test substance on carriers and preparing dilutions.

vi. *Forceps,* straight or curved, non-magnetic, disposable with smooth flat tips to pick up the carriers for placement in vials.

vii. *Vials with lids (plastic or comparable)*. Sterile, flat-bottomed, wide-mouthed (at least 25 mm diameter), approximately 20 mL capacity, for holding inoculated carriers to be exposed to the test chemical and for accommodating neutralizer (e.g., Thermofisher #2116-0015).

1. Transparent vials are more desirable to facilitate application of 50 µL test substance or control substance to inoculated carrier.

viii. *Certified timer.* Readable in minutes and seconds, for tracking of timed events and intervals.

ix. *Desiccation unit* (with gauge to measure vacuum level) with fresh desiccant (e.g., anhydrous $CaCl_2$). For drying inoculated carriers.

x. *Vacuum source.* In-house line or suitable vacuum pump capable of achieving 0.068 to 0.085 MPa, for drying inoculated carriers in desiccation unit.

xi. *Titration kit* (e.g., Hach digital titrator). For measuring water hardness.

xii. *Vortex-style mixer.* For vortex-mixing of various solutions.

xiii. *Conical centrifuge tubes (e.g., 15 mL).* For serial dilutions.

xiv. *Water bath.* To maintain cell culture media at 37±1°C.

xv. *Tissue/cell culture flasks (tissue culture treated).* Flasks for cell propagation.

xvi. *Cell plates* (e.g., 24-well plates) used to assay virus from control and treated carriers.

xvii. *Centrifuge (with swinging bucket rotor).* For preparing frozen virus stock.

xviii. *Ultracentrifuge (capable of spinning 100,000 x g)*. For concentrating virus stock if needed.

xix. *Inverted microscope.* For viewing cells.

xx. *Incubator with/without* CO_2. For incubation of virus/cell line test system.

2) <u>Carriers</u>

a. Carrier Materials (see Figure 1)[1]

i. Privacy Curtain Fabric (PCF-03): 56% Polyester, 44% Fire Resistant (FR) Polyester, 12.93 oz./lin. yd. ± 1.0 oz. CF Stinson, LLC. Mambo MAM34.

ii. Non-PVC Fabric (NVF-01): Polyurethane Face made with Polycarbonate and Polyether Resins, Polyester Backing, 20.3 oz./lin. yd. ± 1.0 oz. CF Stinson, LLC. KID17.

iii. Vinyl Seating Fabric (VF-01): Non-Phthalate Vinyl Surface with Polyester Backing, 30.0 oz./lin. yd. ± 1.0 oz. CF Stinson, LLC. Hopsack HOP24.

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[1]EPA and its employees do not endorse the products, services, or enterprises of nonfederal entities. These carriers were chosen because they demonstrated consistent recovery and repeatability but may not be the only soft surface textiles capable of achieving these results. Use of these carriers does not constitute an endorsement by EPA or its employees.

Figure 1: Examples of carrier materials cut into 1 cm discs; materials 2.a.i, 2.a.ii, and 2.a.iii (from left to right)

Privacy Curtain (PCF-03) | Non-PVC (NVF-01) | Vinyl (VF-01)

b. Carrier Preparation

i. Punch or obtain approximately 1 cm round carriers or use comparable cutting procedure from fabric. Alternatively, 1 cm^2 square carriers can be utilized if punching is unavailable or too difficult.

1. Note: punch can be sharpened by punching aluminum foil.

ii. Visually screen carriers to ensure consistent surface characteristics; trim any jagged edges or loose fabric.

1. Carriers that demonstrate excessive fraying, ripping, backing separation, discoloration, etc. should not be used.

iii. No pre-cleaning of carriers is necessary. Sterilize carriers using a gravity cycle at 121°C for 20 minutes; ensure carriers are dry following sterilization. Test sterility of carriers prior to or concurrently with efficacy testing.

1. Carriers may not be entirely flat after autoclaving; however, minor distortion, such as cupping or doming, of carriers is acceptable for testing if the test substance and inoculum are not affected when applied to the surface of the carrier.
2. Prior to use in testing, document the condition of the screened and sterile carriers (e.g., digital photographs or documentation on the paperwork).
3. To test sterility, place a carrier in tryptic soy broth (TSB) and incubate at 36±1°C for 3-10 days.
4. Use sterilized carriers for up to 6 months. After 6 months, re-sterilize any remaining carriers.

c. Carrier Cytotoxicity Check

i. Each carrier type and lot should be tested for any cytotoxic effects on the cell line once per cell line, prior to or concurrently with testing. Place a carrier into 10 mL of the proposed neutralizer and let soak for ten minutes. Add 1 mL per well (a minimum of 2 wells) of this undiluted solution to a 24 well plate with a confluent monolayer of cells. Incubate plate at the required conditions and time. No cytotoxicity should be observed.

1. Note: it is recommended to monitor cytotoxicity daily.

3) Carrier Inoculation

a. Propagate the virus on the appropriate cell line.
   i. Note: Concentration of the virus stock (e.g., ~100,000 x g for 4 hours at 4ºC) may be necessary to achieve adequate control counts.
b. Defrost a cryovial rapidly to avoid loss in the viability of the preserved virus (e.g., place in a 37ºC water bath and use within 15 min after thawing).
   i. Note: Frozen virus stock can be centrifuged at high speeds (i.e., 100,000 x g) and washed to remove soil used in storage (e.g., FBS) prior to testing.
c. Dilute the virus stock with GM to achieve control counts in the range of 4.0 to 5.5 logs virus particles/carrier.
d. Use the diluted virus to prepare the final test suspension with the addition of the soil load.
e. 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 (smaller 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 microbial test suspension.
   i. 340 µL virus test suspension
f. Briefly vortex the final test suspension with 3-part soil load (at room temperature, 21±3°C) and use to inoculate carriers within 30 min of preparation.
g. It is advisable to briefly rescreen each sterilized carrier for abnormalities prior to inoculation. Place carriers screened side up inside an empty, sterile Petri dish (no more than 20 carriers/dish).
   i. Privacy curtain carriers have no backing material and may be inoculated on either side.
   ii. Non-PVC and vinyl carriers are layered materials comprised of a smooth, colored top surface and a white fabric bottom; only inoculate the top surface.
h. Vortex-mix the final test suspension for 10 s following the addition of the soil load and immediately prior to use.
   i. If a smaller volume test suspension is prepared, pipetting to mix may be used.
i. Inoculate the number of carriers required for the evaluation of the test substance (3 controls and 5 treated) along with a few extra carriers.

j. 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 (screened and sterile), keeping the pipette perpendicular to the carrier during deposition of the final 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-mix the final test suspension 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.
    iv. Discard any inoculated carriers that flip over.
    v. Note: If inoculum soaks/absorbs into the carrier and is no longer visible, carriers are still acceptable to use. Refer to Appendix 3 for examples of inoculated carriers and carriers with 50 µL of test substance added.

k. Transfer the Petri dish(es) with the inoculated carriers into a desiccation unit (with desiccant) and completely remove the lid of the Petri dish(es). Close the desiccation unit door (or lid) and seal the unit. Apply vacuum to evacuate the desiccation unit.
    i. Pressurize the desiccator slowly to lessen the potential for carriers to move or flip.
    ii. Note: do not exceed 40 inoculated carriers per desiccator to ensure carriers dry within the prescribed time.

l. 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) by leaving the vacuum on during the drying period with the desiccator stopcock opened or turning the vacuum off with the stopcock closed.

m. Hold the inoculated carriers in the evacuated desiccation unit 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. Do not use carriers that are visibly wet for testing.
    i. Record the time for all timed events.
    ii. Depressurize the desiccator slowly to lessen the potential for carriers to move or flip.
    iii. Carriers that become stuck or flip during desiccation should be discarded.

n. Use dried inoculated carriers for testing within 30 min following removal from desiccation unit; hold carriers in closed Petri dish(es) at room temperature (21±3°C) until use.

4) **Performance Assessment – Efficacy**

a. Evaluate 3 control carriers and 5 treated carriers for each test substance tested (one virus and contact time/carrier type combination) unless specified otherwise.

   i. One set of control carriers per carrier type may be used for evaluating multiple test substances against the same virus on one test day (assuming the carrier material, neutralizer, and soil load are 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.

   i. Note: An anti-static gun may be aseptically used to reduce the carriers clinging to the side of the vial.

c. Prepare the antimicrobial test substance. Use antimicrobial test substance within 3 hours of preparation or as specified by the manufacturer.

d. In a timed fashion with appropriate intervals, sequentially deposit 50 µL of the test substance (equilibrated to 21±3°C) with a calibrated micropipette over the dried inoculum on each test carrier, ensuring complete coverage.

   i. Note: Gently apply the antimicrobial test substance at a perpendicular angle to the inoculated carrier; do not forcefully deposit the disinfectant.

e. 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.

f. For NFV-01 and VF-01 carriers, 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. When test substance is applied to PCF-03 carriers it is anticipated to soak into and through the carrier (e.g., the test substance may be observed to pool around the carrier).

   i. If the inoculum spot is no longer observable, place the test substance in the area where the inoculum spot was placed (e.g., the center of the carrier).

g. Conduct the test at room temperature (21±3°C) for the selected contact time. Use a certified timer to ensure that each carrier receives the required contact time.

h. Process control carriers last. Each control carrier receives 50 µL 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.

i. Within ±5 s of the end of the contact period, add 10 mL neutralizer equilibrated to 21±3°C to each vial in the specified order according to the predetermined schedule. Briefly vortex-mix (2-3 s) each vial following the addition of the neutralizer.

   i. For calculation purposes, the solution in the neutralized vial with carrier is considered to be the 10^0 dilution.

   ii. The neutralizer for the control carriers is the same as that for the treated carriers.

   iii. If deemed necessary and substantiated by the neutralization assay, a neutralizer volume other than 10 mL (5-20 mL) may be used. Use the same volume of neutralizer for all carriers (treated and controls).

   iv. Ensure that carrier's treated surface comes into contact with the neutralizer by swirling the vial to submerge the carrier prior to briefly vortex-mixing.

      1. Note: The carrier may float back to the surface after vortex-mixing.

j. Following the neutralization of the entire set of carriers, vortex-mix vials at high speed for 30±5 s then proceed as follows:

   i. Allow carriers to sit undisturbed in the vials for approximately 5 minutes.

   ii. Vortex-mix vials at high speed for 30 s (vortex-mix #2).

      1. Ensure that the carriers are submerged in the neutralizer during this step.
      2. Ensure that the liquid and carrier are both spinning in the vial during the vortex-mix.

   iii. Allow carriers to sit undisturbed in the vials for approximately 5 minutes.

   iv. Vortex-mix vials at high speed for 30 s (vortex-mix #3).

      1. Ensure that the carriers are submerged in the neutralizer during this step.
      2. Ensure that the liquid and carrier are both spinning in the vial during the vortex-mix.

k. Initiate dilutions within 30 min after completion of the vortex-mixing. Initiate inoculation of cell line within 30 min of preparing the dilutions.

l. Titrate the samples for virus infectivity using the appropriate cell line.

m. Plate a minimum of 80% of the volume (8 mL for 10 mL neutralizer, 16 mL for 20 mL, etc.) of the 10^0 vial and of each dilution tube.

   i. Remove the growth medium from each well of the plate with a confluent monolayer of cells and replace with the maximum volume of the dilution tube (i.e., add 1 mL per well for a 24 well plate) working from most dilute to least dilute.

n. The elution steps for control carriers are the same as for the test carriers; use 10-fold dilutions to achieve 4.0-5.5 logs viable virus particles/carrier.

o. If cytotoxicity was observed in pre-neutralization testing and/or on the cytotoxicity control, remove CGM from all wells in the affected dilutions at the appropriate time (one hour minimum) and wash them with pre-warmed DPBS, then replace the DPBS with fresh CGM.

    i. All test and control parameters of the same dilution should be changed.

p. Incubate test and control plates as appropriate for the test system.

5) Data Requirements

a. Record all observations (presence/absence of viable virus particles) and use in calculations to estimate the log reduction based on the $TCID_{50}$ or MPN (most probable number) technique.

b. 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 to the nearest tenth).

c. Calculate the $TCID_{50}$/carrier or MPN/carrier using the following equations. The calculated values of $TCID_{50}$/carrier and MPN/carrier represent the log density/carrier.

$$-Log\ TCID_{50}/assayed\ volume = -\log(initial\ plated\ dilution) - \left\{\left[\left(\frac{\sum \%\ mortality\ at\ each\ dilution}{100}\right) - 0.5 \times (1)\right]\right\}$$

$$TCID_{50}/carrier = \left(\frac{anti\log TCID_{50}}{assayed\ volume}\right)\left(\frac{total\ neutralizer\ volume}{assayed\ volume}\right)$$

    i. The volume of virus and test product are insignificant to the calculation and not used in the calculation.

d. Calculate the mean $\log_{10}$ density across treated carriers.

e. Calculate the mean $\log_{10}$ density across control carriers.

f. Calculate the mean $\log_{10}$ reduction (LR) for treated carriers:

    i. Mean $\log_{10}$ reduction = the mean $\log_{10}$ density for control carriers minus the mean $\log_{10}$ density for treated carriers.

Appendix 1

Neutralization Assay

The purpose of this section is to assess the effectiveness of the neutralization processes associated with this method. Perform the neutralization assay prior to or concurrently with testing to demonstrate the neutralizer's ability to inactivate the chemical and determine if there is interference from the carrier itself. Differences in performance (quality) between batches of media may lead to misleading neutralization results.

Select a neutralizing medium that is not inhibitory to the virus and is not cytotoxic to the cells. The acceptance criteria for acceptable neutralization are a 0.5 log difference between the neutralization effectiveness, neutralization toxicity control, titer control, carrier control, and test interference control. Interaction between the neutralizer and product and its effect on the cell line must be determined prior to testing.

1) Prepare *Test Suspension A*: Dilute the virus stock suspension in CGM to achieve an average recovered concentration of approximately 2-3 logs (i.e., 100-1000 virus particles) per vessel for the Titer Control sample. To achieve this, dilute the virus stock suspension through 10^{-4} (or as necessary).
2) Prepare *Test Suspension B*: Prepare the soil load: using a vortex, mix each component and combine 25 µL BSA, 35 µL yeast extract, 100 µL of mucin; then vortex-mix the solution. Combine 340 µL of *Test Suspension A* and the 160 µL of the soil load (SL) and vortex mix for 10 seconds. Use *Test Suspension B* within 30 minutes of preparation.
3) Neutralization Treatments (**Figure 2**)
    a. ***Treatment 1: Neutralizer Effectiveness.*** Add 50 µL of the test substance to each of three reaction vessels. At timed intervals, add 10 mL neutralizer to each vessel and briefly swirl (by hand). After 10±2 s, gently add 10 µL of *Test Suspension B* using a micropipette to each vessel. Use a new tip for each tube. Vortex each tube for 3-5 s. Proceed with step 4.
        i. If deemed necessary, a neutralizer volume other than 10 mL (5-20 mL) may be used. Use the same volume for Treatments 1-5.
    b. ***Treatment 2: Neutralizer Toxicity Control.*** Add 10 mL neutralizer to each of three reaction vessels. At timed intervals, add 10 µL of *Test Suspension B* using a micropipette to each vessel and briefly vortex. Use a new tip for each tube. Proceed with step 4.
        i. Treatment 2 is only required when using a neutralizer other than CGM.
    c. ***Treatment 3: Titer Control.*** Add 10 mL CGM to each of three reaction vessels. At timed intervals, add 10 µL of *Test Suspension B* using a micropipette to each vessel and briefly vortex. Use a new tip for each tube. Proceed with step 4.
    d. ***Treatment 4: Carrier Interference Control***. Add one carrier to each of three reaction vessels. At timed intervals, add 10 mL neutralizer to each vessel and briefly swirl (by hand). After 10 s gently add 10 µL of *Test Suspension B* using a

micropipette to each vessel and briefly vortex. Use a new tip for each tube. Assess one set of three carriers for each carrier type. Proceed with step 4.

e. ***Treatment 5***: ***Test Interference Control***. Add one carrier to each of three reaction vessels. At timed intervals, add 50 µL of test substance and 10 mL neutralizer to each vessel and briefly swirl (by hand). After 10 s gently add 10 µL of *Test Suspension B* using a micropipette to each vessel and briefly vortex. Use a new tip for each tube. Proceed with section 4.

f. Note: Conduct steps at timed intervals (e.g., 30 s) to ensure consistent time of contact for each treatment.

4) Hold the neutralization treatments for 10±1 minutes at room temperature (21±3°C).

5) 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 of CGM).

   a. 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.

   b. Titrate the samples for virus infectivity in the same manner as in the test, using the appropriate cell line – plate a minimum of 80% of the reaction vessel and dilutions.

      i. For each well plated, add the maximum volume of the well (i.e., add 1 mL per well for a 24 well plate).

      ii. Note: If any reaction vessel does not contain CGM (e.g., Treatment 2 with proposed neutralizer), then the first usable dilution will be 10^{-1}.

   c. If cytotoxicity was observed in pre-neutralization testing, remove CGM from all wells in the affected dilutions at the appropriate time (one hour minimum) and wash them with pre-warmed DPBS, then replace the DPBS with fresh CGM. All treatments of the same dilution should be changed.

   d. Incubate test and control plates as appropriate for the test system.

   e. For the neutralizer to be considered effective:

      i. Ensure that the recovered virus in the **Titer Control** using *Test Suspension B* is between approximately 2-3 logs per vessel.

      ii. The recovered virus 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: a value higher than the **Titer Control** is also deemed valid.

      iii. The recovered virus in the **Neutralizer Toxicity Control** 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: a value higher than the **Titer Control** is also deemed valid.

iv. The recovered virus in the **Carrier Interference Control** is within 0.5 logs of the **Titer Control**. A log reduction greater than 0.5 logs indicates that the carrier is harmful to the test system. Note: a value higher than the **Titer Control** is also deemed valid.

v. The recovered virus in the **Test Interference Control** is within 0.5 logs of the **Titer Control**. A log reduction greater than 0.5 logs indicates that the carrier is harmful to the test system. Note: a value higher than the **Titer Control** is also deemed valid.

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

Appendix 2

Cytotoxicity Determination

Prior to performing the neutralization assay, ensure the proposed neutralizer, neutralizer and test chemical, and cytotoxicity determinations do not impact the quality of the cell line by performing the following.

1) Neutralizer Effect on Cell Line (for neutralizers other than CGM).
   a) Add 0.5 mL of the proposed neutralizer to 4.5 mL CGM, equilibrated to 37±1°C (this is the 10^0 dilution). It is suggested to do further dilutions out to 10^{-2} or 10^{-3} depending on the expected cytotoxicity of the neutralizer.
   b) Remove the CGM from the wells of a 24 well plate with cells at the appropriate confluency and add 1 mL per well of the neutralizer plus CGM solution. Plate at least 4 wells per dilution. Have at least one well as a negative control (e.g., CGM with 2% FBS alone).
   c) Incubate plate as appropriate and observe closely for cytotoxicity.
   d) 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.
   e) If cell death occurs in under one hour, the neutralizer cannot be tested.
   f) The effect of the media change in the single well can be compared to the other wells in the dilution and the negative control. If cytotoxicity cannot be overcome with washing and replacing of media, column filtration (e.g., Sephadex) may be used in future testing.
2) Neutralizer Plus Test Chemical Effect on Cell Line.
   a) Add 50 µL of test chemical and the appropriate volume of neutralizer, equilibrated to 21±3°C, and vortex 2-3 seconds. Let this solution sit at room temperature for 10 minutes.
   b) Add 1.0 mL of this solution to 9 mL CGM, equilibrated to 37±1°C (this is the 10^0 dilution). It is suggested to do further dilutions out to 10^{-2} depending on the expected cytotoxicity.
   c) Remove the CGM from the wells of a 24 well plate with cells at the appropriate confluency and add 1 mL per well of the neutralizer plus test chemical solution and dilutions. Plate at least 8 wells for the 10^0 dilution, 6 wells for the 10^0 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.
   d) Have at least one well on each plate as a negative control (e.g., CGM with 2% (v/v) FBS alone).
   e) If necessary, change the media in the wells, an example is 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.

i) 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.

ii) For the 10^0 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.

iii) For the 10^{-2} dilution: On the day after the test, change 1 well.

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

g) Incubate the plate as appropriate and observe the cells for cytotoxicity. The test cells should be compared to the negative control cells to determine toxicity.

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

i) Identify the test condition that removed the cytotoxicity 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.

   i) **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.

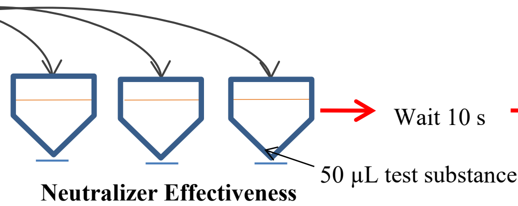
j) If cell death occurs in under one hour, that test condition cannot be used.

k) Cytotoxicity past the 10^0 dilution is unacceptable for testing.

Figure 2: Neutralization Schematic

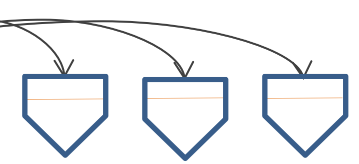
Treatment 1

Add 50 µL of test substance to each vessel. At timed intervals add appropriate amount of neutralizer and swirl by hand.



Wait 10 s

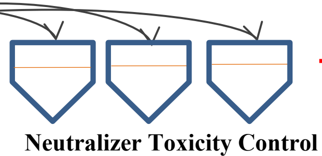
Add 10 µL of *Test Suspension B* to each vessel containing 50 µL test substance and appropriate amount of neutralizer.



Vortex and hold for 10 min at 21±3°C. Proceed to vortexing/filtering.

Treatment 2

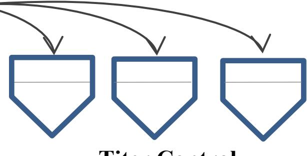
Add 10 µL of *Test Suspension B* to each vessel containing appropriate amount of neutralizer.



Vortex and hold for 10 min at 21±3°C. Proceed to vortexing/filtering.

Treatment 3

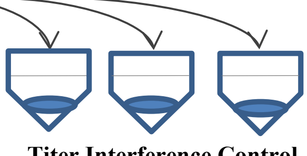
Add 10 µL of *Test Suspension B* to each vessel containing appropriate amount of CGM.



Vortex and hold for 10 min at 21±3°C. Proceed to vortexing/filtering.

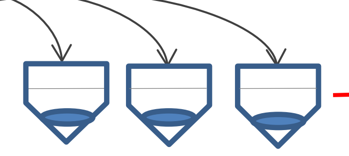
Treatment 4

Add 1 sterile carrier to each vessel. At timed intervals, add appropriate amount of neutralizer and swirl by hand.



Wait 10 s

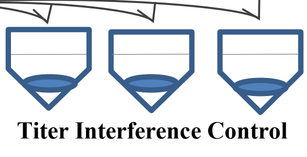
Add 10 µL of *Test Suspension B* to each vessel containing the carrier and appropriate amount of neutralizer.



Vortex and hold for 10 min at 21±3°C. Proceed to vortexing/filtering.

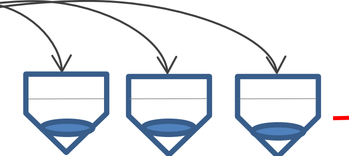
Treatment 5

Add 1 sterile carrier to each vessel. At timed intervals, add 50 µL of test substance and appropriate amount of neutralizer and swirl by hand.



Wait 10 s

Add 10 µL of *Test Suspension B* to each vessel containing the carrier and appropriate amount of neutralizer.



Vortex and hold for 10 min at 21±3°C. Proceed to vortexing/filtering.

Appendix 3

Carrier Examples

Figure 3: Examples of Inoculated Carriers

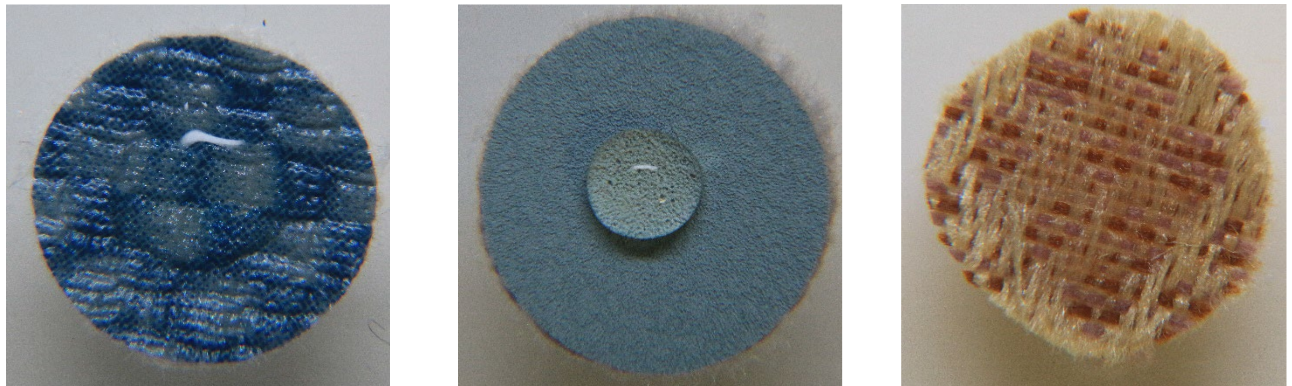


Figure 4: Examples of Inoculated Carriers with 50 µL of Test Substance Added