Interim Quantitative Method for Evaluating the Efficacy of Antimicrobial Test Substances on Porous Surfaces against Viruses

(12/12/2022)

Scope

The Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) recommends that applicants utilize this interim method to support the registration of disinfectant products with claims for efficacy against viruses when used on soft-porous surfaces.

This method is quantitative and provides log reduction (virus inactivation) as the quantitative measure of efficacy for disinfectants against viruses on soft-porous surfaces.

Method Overview

In brief, the method uses 1 cm diameter discs (carriers) of a set of materials to represent soft-porous surfaces. Each disc receives 10 µL of microbial inoculum (with a three-part organic and inorganic 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. 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 $\log_{10}$ reduction (LR) in viable virus is calculated. The LR value is used as the measure of product effectiveness.

Appropriate biosafety 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 appropriate 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. *Minimum Essential Media (MEM).* Liquid or powder form (e.g. Eagle's or Dulbecco's). 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.* 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). If the neutralization confirmation assay demonstrates that CGM is ineffective, 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. *Antibiotic/antifungal.* 100x Amphotericin B/Penicillin/Streptomycin solution or other equivalent antibiotic/antimycotic solution. May be used to prevent contamination of cell culture.

v. *Soil load, 3-part.* Use as the soiling agent. Add to the test suspension in the following manner:

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.

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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.

vi. *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.

vii. *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) cut from porous material. Carriers are single use only. See section 2 for carrier specifications.

ii. *Hole punch:* If necessary, for use in the preparation of 1 cm discs 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.

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) with fresh desiccant (e.g., anhydrous $CaCl_2$). For drying inoculated carriers.

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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 and to perform membrane filtration.

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

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

xiii. *15 mL conical centrifuge tubes*. 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.* 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 5% CO_2*. For incubation of virus/cell line test system

2) Carriers

a. Carrier Materials (see Figure 1)

i. Privacy Curtain Fabric (PCF-03): 54% Polyester, 46% Fire Resistant (FR) Polyester. CF Stinson, LLC. Mambo MAM34 Nights.

ii. Non-PVC Fabric (NVF-01): Polyurethane Face made with Polycarbonate and Polyether Resins, Polyester Backing. CF Stinson, LLC. Kid BlueSky KID17.

iii. Vinyl Seating Fabric (VF-01): Vinyl Face, Polyester Backing. CF Stinson, LLC. Hopsack - HOP24 Fjord.

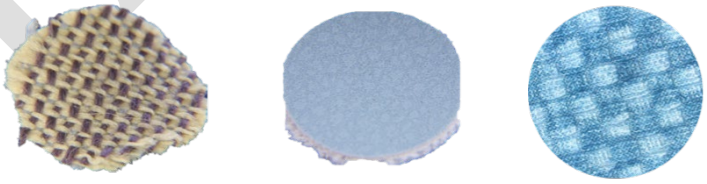

Figure 1: Examples of carrier materials cut into 1 cm discs; materials 2.a.i, 2.a.ii, and 2.a.iii (from left to right)

b. Carrier Preparation

i. Punch 1 cm round carriers or use comparable cutting procedure from fabric.

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

iii. No pre-cleaning of carriers is necessary. To sterilize carriers, sterilize using a gravity cycle, 121°C for 20 minutes; ensure carriers are dry following sterilization. Test sterility of carriers prior to testing.

1. Carriers may not be entirely flat after autoclaving; however, minor distortion of carriers is acceptable for testing.

2. Prior to use in testing, document the condition of the screened and sterile carriers (e.g., digital photographs).

c. Carrier Cytotoxicity Check

i. Each carrier type should be tested for any cytotoxic effects on the cell line. Place a carrier into 10 mL of the proposed neutralizer and let soak for ten minutes. Add 1 mL per well of this solution to a 24 well plate with a confluent monolayer of cells. Incubate plate at the required conditions and time; observe daily for cytotoxicity. No cytotoxicity should be observed.

3) <u>Carrier Inoculation</u>

a. Propagate the virus on the appropriate cell line.

Note: Concentration of the virus stock (~100,000 x g for 4 hours at 4º C) 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).

c. Dilute the virus stock with CGM 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. It is advisable to briefly rescreen each sterilized carrier for abnormalities prior to inoculation. Place carriers screened side up inside an empty, sterile plastic Petri dish (no more than 20 carriers/dish).

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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 the top surface will be inoculated.

g. Vortex-mix the final test suspension for 10 s following the addition of the virus test suspension and immediately prior to use. Inoculate carriers within 30 min of preparation.

i. If a smaller test suspension is prepared, pipetting to mix may be used.

ii. Inoculate the number of carriers required for the evaluation of the test substance (3 controls and 5 treated) along with a few extra carriers.

h. 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); 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.

i. 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.

j. 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) throughout the carrier drying process.

k. Hold the inoculated carriers in the evacuated desiccation unit at 21±3°C for 45 to 60 min. Inspect inoculated carriers to verify that they are not visibly wet 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 avoid the potential for carriers to move or flip.

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

4) <u>Performance Assessment – Efficacy</u>

a. Evaluate 3 untreated control carriers and 5 treated carriers per test substance.

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i. One set of untreated control carriers may be used for evaluating multiple test substances against the same virus on the same 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.

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. 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.

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 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. The vial containing the neutralized solution with carrier is considered to be the 10^0 dilution.

ii. Use the same neutralizer for the control carriers as that for the treated carriers.

iii. If deemed necessary, the final volume of neutralizer may be increased to 20 mL (for all carriers).

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j. Following the neutralization of the entire set of carriers, vortex-mix each vial for 30±5 s at high speed to recover and disaggregate the inoculum. Do not remove the carrier from the vial.

k. Initiate dilutions within 30 min after neutralization and 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 using 8 wells per dilution.

m. Plate a minimum of 80% of the volume (8mL for 10 mL neutralizer, 16 mL for 20 mL) 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 virus particles/carrier.

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

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 probably 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. Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier).

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

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

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

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Attachment 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 testing to demonstrate the neutralizer's ability to inactivate the chemical and determine if there is interference from the carrier itself.

Select a neutralizing medium that is not inhibitory to the virus and is not cytotoxic to the cells. The acceptance criteria for acceptable neutralization is 0.5 log difference between the neutralization effectiveness, neutralization toxicity control, titer control, and carrier 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: 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. 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 test tubes. At timed intervals, add 10 mL (up to 20 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 3.
    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. Use a new tip for each tube. Vortex each tube for 3-5 s. Proceed with step 3.
    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. Use a new tip for each tube. Vortex each tube for 3-5 s. Proceed with step 3.
    d. ***Treatment 4: Carrier Interference Control.*** Add 10 mL CGM and one sterile carrier to each of three reaction vessels. At timed intervals, 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 3.
    e. Note: Steps should be conducted at timed intervals (e.g., 30 s) to ensure consistent time of contact for each carrier.
4) Hold the neutralization treatments for 10±1 minutes at room temperature (21±3°C).

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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 using the appropriate cell – plate a minimum of 80% of the 10^0 vial and all 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 10^0 (vessel) dilution is used that does not contain CGM (e.g. Treatment 2 with proposed neutralizer), allow it to adsorb on the cells for 1 hr, then remove and replace with fresh CGM.

   c. If cytotoxicity was observed in pre-neutralization testing CGM may be removed from all wells in the affected dilutions at the appropriate time (one hour minimum) and wash them with pre-warmed PBS, then replace the PBS with fresh CGM. Also wash with pre-warmed PBS and replace the CGM in the same dilutions for the control carriers.

   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.

   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.

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Attachment 2

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.

1) Neutralizer Effect on Cell Line (for neutralizers other than CGM with 2% CGM).
    a) Add 0.5 mL of the proposed neutralizer to 4.5 mL CGM with 2% (v/v) FBS, equilibrated to 37±1°C (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.
    b) Remove the CGM from the wells of a 24 well plate with an 80-95% confluent monolayer of cells 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 20 mL 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 with 2% (v/v) FBS, equilibrated to 37±1°C (this is the 10^{-1} 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 an 80-95% confluent monolayer of cells and add 1 mL per well of the neutralizer plus test chemical solution and dilutions. Plate at least 8 wells for the 100 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.
    d) For highly toxic test chemicals, washing the cells with pre-warmed DPBS before the addition of CGM with 2% FBS will help remove cytotoxicity.
    e) Have at least one well on each plate as a negative control (e.g., CGM with 2% (v/v) FBS alone).
    f) At a minimum, 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.

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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^{-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.

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

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 100 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^{-1} dilution is unacceptable for testing. Alternative neutralizers or column filtration (e.g., Sephadex) may be used to mitigate cytotoxicity. See ASTM Method E1482-12. Standard Practice for Use of Gel Filtration Columns for Cytotoxicity Reduction and Neutralization (Reapproved 2017) for further information on column filtration.

3) 3-Part Soil Effect on Cell Line.

a) Make the 3-part soil (see section 14e but withhold the virus).

b) Add 10 µL of the soil to 20 mL of CGM, equilibrated to 37±1°C.

c) Remove the CGM from the cells and add 1 mL of this solution to 4 wells on a 24 well plate with an 80-95% confluent monolayer of cells. Have at least one well as a negative control (e.g., CGM alone).

d) Incubate plate as appropriate and observe daily for cytotoxicity. No cytotoxicity should be observed.

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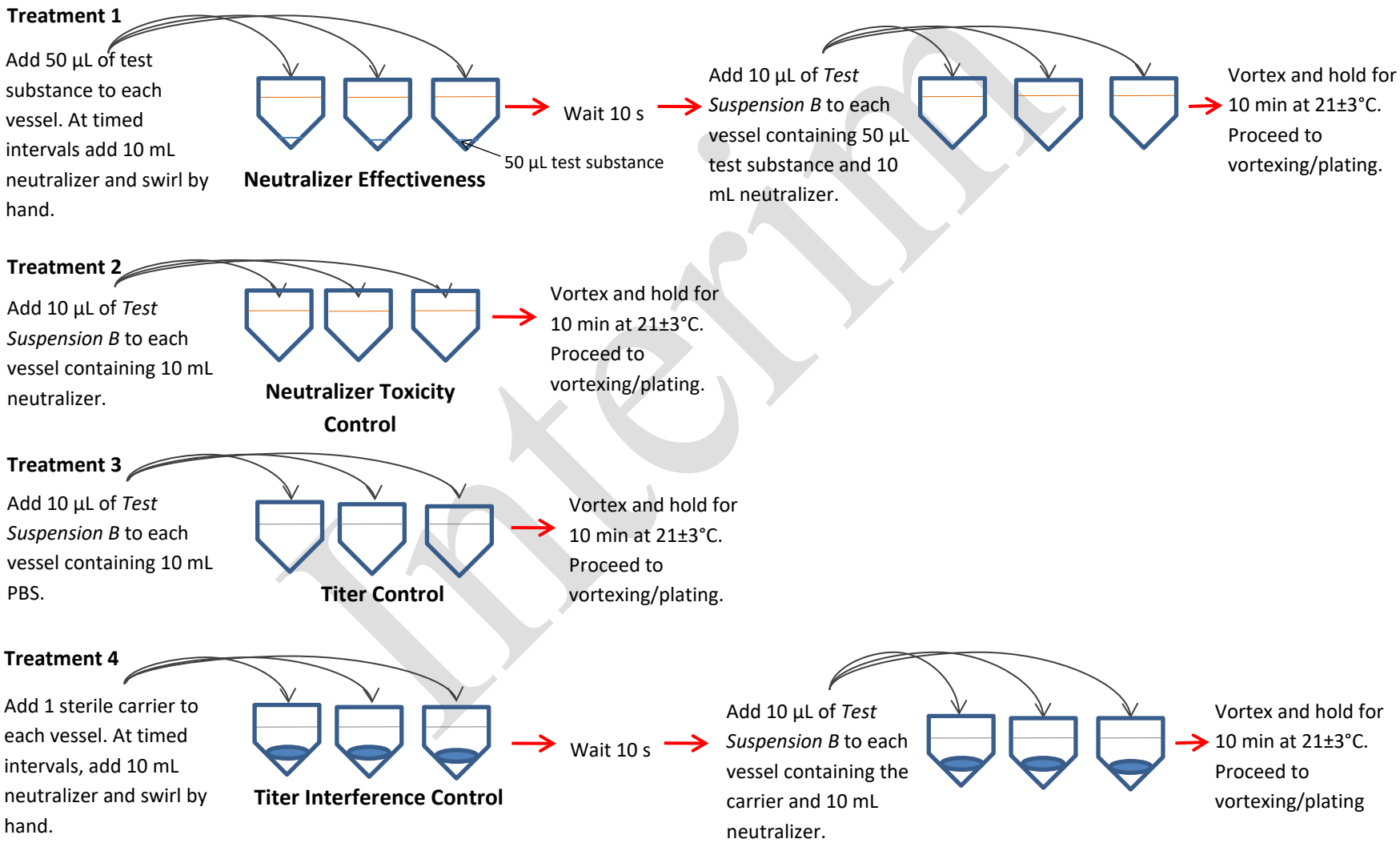
Attachment 3

Figure 2: Neutralization Schematic



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