US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
Standard Practice to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces Using Human Coronavirus

SOP Number: MB-39-01

Date Revised: 08-10-20

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Revisions Made	• N/A, new SOP.

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Scope	The method is used to evaluate the virucidal efficacy of liquid, aerosol, or trigger-spray microbicides intended for use on inanimate, nonporous environmental surfaces. This method is based on ASTM E1053-20. The method will be modified for the product formulation as deemed necessary.
Application	This method provides log reduction (LR) as the quantitative measure of efficacy for liquid disinfectants against the test microbe on a hard, non-porous surface. If cytotoxicity is present, the virus control titer should be increased as necessary to demonstrate a ≥ 3 $\log_{10}$ reduction in viral titer beyond the cytotoxic or neutralization failure level.

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Data SOP issued:	08-10-2020
Controlled copy number:	0
Date SOP withdrawn:	

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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 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. MOI = multiplicity of infection. The ratio of virus to cells. 7. CoV = coronavirus 8. 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) for details on method and frequency of calibration.
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, document the required information 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 **Attachment 5**. Any dilution that does not exhibit successful neutralization cannot be used in the virucidal assay. 2. The test substance or the neutralizer may interfere or be cytotoxic to the cell line. This should be experimentally determined prior to testing. See

	Attachment 5. 3. Inadequate distribution of the applied product over the inoculated carrier may lead to erroneous data. 4. Avoid excess alkalinity of the EMEM and CGM. Place medium in a CO_2 incubator with a loose cap to allow it to reach its normal pH if needed. 5. Presence of atypical CPE (non-CoV) may be indicative of a viral or mycoplasma contaminate. 6. Cytotoxicity of the cell line may compromise the test system. 7. 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 plate recovery control of $10^{4.8}$-$10^{6.3}$ (6.3×10^4 to 2.0×10^6) infective units/control carrier. 2. There must be documented evidence that cytotoxicity mitigation and neutralization verification have been achieved. The virus control titer must demonstrate a $\geq$3 $\log_{10}$ reduction in viral titer beyond the cytotoxic or neutralization failure level. 3. 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 fetal bovine serum (FBS) concentrations can produce inaccurate results. 2. Ensure that the cell line is free from *Mycoplasma* contamination. Test cells regularly with a *Mycoplasma* detection kit (e.g. MycoAlert Plus). 3. Freezing the virus stock in aliquots of less than 1 mL will negatively affect the long-term viability of the virus.
11. Special Apparatus and Materials	1. Test Virus: Human coronavirus 229E (ATCC # VR-740). Biosafety level 2. Other CoV test systems may be used as deemed necessary. 2. Cell Line: MRC-5 (ATCC # CCL-171). Human lung fibroblast cells. 3. Media and Reagents: a. *Neutralizer.* The default neutralizer for this test system is CGM with 2% (v/v) FBS. If the neutralization control (see **Attachment 5**) demonstrates that the neutralizer is ineffective, other neutralizers may be used. b. *Diluent (serial dilutions).* CGM with 2% (v/v) FBS.

	c. *Dulbecco's phosphate buffered saline (DPBS) or other equivalent buffer (e.g. PBS, Earle's Balanced Salt Solution).* Prepare per manufacturer's guidelines. d. *Eagle's Modified Eagle Medium (EMEM).* May be bought in liquid or powder form (e.g. Corning Cellgro 50-011-PC). Prepare per manufacture's guidelines. e. *Heat Inactivated Fetal Bovine Serum (FBS).* Compatible for use with cell lines. Used to grow and propagate the cell line. f. *Complete Growth Media (CGM).* Consisting of EMEM and 10% fetal bovine serum (for cell line propagation) or 2% fetal bovine serum (for viral propagation). Antibiotics and/or antifungals may be added to reduce potential contamination. g. *0.25% Trypsin solution containing 0.1% EDTA*. Used to dissociate adherent cells from the vessel in which they are being cultured. h. *100x Amphotericin B/Penicillin/Streptomycin solution or other equivalent antibiotic/antimycotic solution.* May be used to prevent contamination of cell culture. i. *Sephadex LH-20 powder.* Used to make gel filtration media. See **Attachment 6**. j. *1% bovine serum albumin solution.* Used to suspend Sephadex in solution and to wash gel filtration columns. k. *Cryoprotective Medium.* CGM (with 10% (v/v) FBS) containing 5% dimethyl sulfoxide (DMSO). l. *Soil load.* A 5% soil load (e.g. FBS) is incorporated in the test suspension. Additional soil loads may be used per the Agency's guidance or research protocol. i. See section 12.3 for addition of soil load to inoculum. m. *Test substance.* Antimicrobial test solution or product. If dilution is required, see section 11.3n for diluent. n. *Test substance diluent.* Used for the preparation of dilutable products. The test substance diluent is AOAC hard water; refer to MB-30, Preparation of hard water and other diluents for preparation of antimicrobial products. i. Additional diluents and levels of water hardness may be used per the Agency's guidance or research protocol.

	o. *Water.* De-ionized (DI), distilled water or water with equivalent quality for making reagent solutions and culture media. 4. Apparatus a. *Petri plates (carriers)*: glass, 100 mm diameter, 10-14 mm deep. b. *Calibrated micropipettes* (e.g., 1 mL) with 100-1000 µL tips, for preparing dilutions. c. *Water bath* to maintain cell culture media at 37±1°C. d. *Vacuum*. In-house line or suitable vacuum pump to remove media from plates or flasks. e. *Tissue culture flasks and plates.* Appropriately sized flasks for propagation of the cell line and 24 well plates for MPN determination. f. *10 mL syringe*. Used to make gel filtration columns. g. *Glass wool*. Used to hold gel filtration media inside syringe. h. *50 mL conical centrifuge tubes with ¾ inch hole drilled in the cap (column cap).* Used to hold the gel filtration column. i. *Liquid nitrogen dewar* or comparable freezer capable of long-term storage of cell lines. j. *Certified timer*. Readable in minutes and seconds, for tracking of timed events and intervals. k. *Centrifuge (with swinging bucket rotor and aerosol tight lids).* For preparing frozen virus stock. l. *Vortex-style mixer*. For vortex mixing of various solutions. m. *Inverted microscope* n. *Incubator* with 5% CO_2. o. *Hach digital titrator kit*. For measuring total chlorine and water hardness.
12. Procedure and Analysis	
12.1 Preparation of carriers	a. Visually screen glass Petri plates; discard those with chips, scrapes, or any visible damage. Discard any plates that have large ridges or depressions on the inside bottom of the plate. b. Thoroughly clean glass carriers with non-ionic detergent by hand or use a dishwasher cycle.

	i. Glassware should be determined to be free of detergent residue. c. After cleaning, rinse once with 95% ethanol. d. Rinse with DI water three times. Dry plates prior to sterilization. e. Sterilize appropriately by steam or heat sterilization. f. Record cleaning and sterilization procedural steps on the appropriate preparation sheet. g. 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 line	a. Refer to **Attachments 2-3** for information on preparing 24 well plates for testing. A minimum of three 24 well plates will be needed for a two-lot efficacy test.
12.3 Preparation of test virus	a. Refer to **Attachment 4** for preparation of the frozen virus stock suspension for CoV. b. Thaw a cryovial rapidly to avoid loss in the viability of the preserved virus (e.g., place in a 37°C water bath). Each cryovial is single use only. c. Use an appropriate dilution scheme to achieve control counts in the range of $10^{4.8}$-$10^{6.3}$ (6.3×10^4 to 2.0×10^6) infective units/carrier. Dilute the virus stock (e.g., 1:10 in CGM with 2% FBS) prior to preparing the virus with the soil load. i. The virus may be concentrated by ultracentrifugation (100,000 x g for 4 hours at 4º C) if necessary. d. Use the diluted virus 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 diluted virus for 10-30 s. b. To obtain 1 mL of the final test suspension with 5% soil load, add 50µl of FBS to 950 µl of the diluted virus stock. c. Additional soil loads (e.g., 10% (v/v) FBS) may be used per the Agency's guidance or research protocol. d. Use final test suspension with soil load (at room temperature, 22±2°C) to inoculate carriers within 30 min of preparation.
12.5 Preparation of virus films	a. Vortex the final test suspension for 10 s following the addition of the soil load and immediately prior to use. b. Prepare virus films by adding 200 µL of final test suspension to the inside bottom of the carrier. To help avoid the inoculum from spreading to the side of the carrier, add the 200 µl in approximately 10-12 small

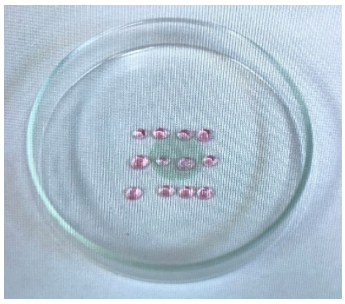
drops in the middle of the carrier. Use a sterile cell scraper to spread the inoculum evenly without touching the sides of the carrier. Any carrier where the inoculum touches the side of the carrier cannot be used. Use 1 carrier for virucidal activity test per lot of disinfectant and 1 carrier for the plate recovery control plus extras. See **Figure 1** below.

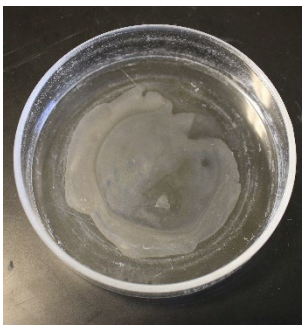
i. Note: the volume of virus inoculum per carrier may be increased depending on the titer of the virus. This volume must be consistent on all carriers. An increased volume will prolong the drying of the inoculum and may lead to increased losses in virus infectivity.

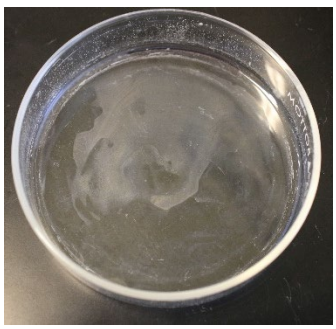
c. Dry the virus films with the lids ajar in the biological safety cabinet (BSC) at 22±2°C until visibly dry (15-60 minutes). Ensure all carriers are dry before beginning the test.

d. Use dried inoculated carriers for testing within 2 hours following drying; hold carriers at room temperature (22±2°C) until use.

Figure 1. Inoculation spot pattern (top). Satisfactory (bottom left) and unsatisfactory (bottom right) dried carriers. The inoculum has run to the side of the unsatisfactory carrier.







e. Take results of cytotoxicity and neutralization into account when determining if the virus culture should be standardized by dilution to

	target a virus endpoint titer of $10^{4.8}$-$10^{6.3}$ (6.3×10^4 to 2.0×10^6) infective units/carrier.
12.6 Test for virucidal activity	a. For each lot of the test substance, treat a dried carrier with 2.0 mL of the use-dilution of a liquid product or the amount of product released during recommended use of the aerosol or trigger spray. Rock the carrier gently to evenly distribute the test substance over the entire surface of the carrier. If the test substance does not cover the carrier within 20 seconds discard that carrier and repeat the assay using a new dried carrier. i. If the product to be tested is an antimicrobial towelette, refer to the study protocol for application instructions. b. The contact time will begin when the carrier has been completely covered by the test substance. Cover the carrier with the top of the Petri dish. Hold the treated carrier for the required contact time. c. Upon completion of the contact time, use one of the following neutralization procedures. (Other neutralization methods may be used per the study protocol). Regardless of the neutralization procedure used, the resulting suspension is the 10^{-1} dilution of the virus. i. <u>Addition of neutralizer</u>. Within ±5 s of the end of the contact time add 2.0 mL neutralizer to the carrier and rock the carrier gently to mix well. Scrape the film with a sterile cell scraper to resuspend the virus/test substance/neutralizer mixture. ii. <u>Gel filtration column only.</u> Scrap the carrier with a sterile cell scraper just prior to the end of the contact time. Add the scraped suspension to a previously prepared gel filtration column. Within ±5 s of the end of the contact time push the solution through the gel filtration column utilizing the syringe plunger. iii. <u>Neutralizer + gel filtration column</u>. Within ±5 s of the end of the contact time add 2.0 mL neutralizer to the carrier and rock the carrier gently to mix well. Add the scraped suspension to a previously prepared gel filtration column and push the solution through the gel filtration column utilizing the syringe plunger. d. Prepare serial 10-fold dilutions (0.5 mL + 4.5 mL CGM with 2% FBS) within 30 minutes of neutralization. e. 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 to at least four replicate cell monolayers per dilution. Process from most dilute to least dilute. Start the plating of the dilutions within 30 minutes of them being made.

	f. If cytotoxicity was observed in pre-neutralization testing (see **Attachment 5**), remove the CGM from all wells in the affected dilutions (treated and control) at the appropriate time (after one hour minimum) and replace with fresh CGM with 2% FBS. g. Incubate plates at 35 ± 1°C in 5% CO_2 for 6-9 days.
12.7 Plate recovery control (control plates)	a. Assay 1 carrier as the carrier recovery control after the virucidal activity test carrier has been processed. b. After drying, overlay the dried control film with 2.0 mL of neutralizer or another buffered solution harmless to the virus and its host cells. Rock the carrier gently to evenly distribute the liquid over the entire surface of the carrier. If the control solution does not cover the carrier within 20 seconds discard that carrier and repeat the assay using a new dried carrier. c. The contact time will begin when the carrier has been completely covered by the control solution. Cover the carrier with the top of the Petri dish. Hold the plate recovery control carrier for the same required contact time used for the treated carrier. d. Upon completion of the contact time, use the same neutralization procedure used for the treated carrier. Regardless of the neutralization procedure used, the resulting suspension is the 10^{-1} dilution of the virus. e. Prepare serial 10-fold dilutions (0.5 mL + 4.5 mL CGM with 2% FBS) within 30 minutes of neutralization. f. Inoculate 1 mL per well to at least four replicate cell monolayers/dilution in a 24 well plate starting from the first ten-fold dilution of the post-neutralized sample within 30 minutes of the dilutions being made. g. Incubate plates at 35 ± 1°C in 5% CO_2 for 6-9 days.
12.8 Cell culture control	a. <u>CGM Control:</u> To ensure the CGM with 2% FBS is not contaminated, remove the growth media on at least one well and replace it with the CGM with 2% FBS used in the test. b. Use an aliquot of the stock virus (i.e. the 10^{-1} dilution tube of the plate recovery control) and inoculate onto the cells to confirm that the cells are susceptible to the virus. A lack of typical virus-induced cytopathic effects invalidates the test. c. Any obvious contamination or degeneration in such monolayers invalidates the test.
12.9 Virus stock titer control	a. If desired, serially dilute an aliquot of the stock virus to confirm that the titer of the stock virus is appropriate for use in the test.

(Optional)	
12.10 Recording results	a. Record results as positive or negative for the presence of CPE in each well after 6-9 total days of incubation.
13. Data Analysis/ Calculations	1. Record all observations (presence/absence of CPE) and use in calculations to estimate the log reduction based on $TCID_{50}$. 2. Record the last cytotoxic dilution (see **Attachment 5**). 3. Record the first dilution that is successfully neutralized (see **Attachment 5**). 4. For calculation purposes, the total volume of liquid on the carrier (test substance + neutralizer) is based on the control carrier. 5. 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). 6. Calculate the $TCID_{50}$/carrier using the Virucidal Activity Spreadsheet (section 14). Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier). 7. Calculate the mean $\log_{10}$ density of the treated plate (section 12.6). 8. Calculate the mean $\log_{10}$ density of the control plate (section 12.5). 9. Calculate the $\log_{10}$ reduction (LR) for treated plate: $\log_{10}$ reduction = the $\log_{10}$ density for the control plate minus the mean $\log_{10}$ density for the treated plate. 10. There can be no virus recovered for 3 logs past the cytotoxic dilution.
14. Forms and Data Sheets	1. Attachment 1: Storage of Cell Line Stocks by Freezing in Liquid Nitrogen 2. Attachment 2: Reviving Cell Line from Liquid Nitrogen Storage 3. Attachment 3: Sub-culturing Cell Line for Work with Viruses 4. Attachment 4: CoV Propagation, Harvest and Titration 5. Attachment 5: Testing for Cytotoxicity, Test Substance Neutralization Control, Influence of Soil Load 6. Attachment 6: Sephadex Column Preparation and Use 7. Test Sheets. Test sheets are stored separately from the SOP under the following file names: ASTM Method for Virucidal Activity: Virucidal Activity Spreadsheet — MB-39-01_F1.xlsx ASTM Method for Virucidal Activity: Test Information Sheet — MB-39-01_F2.docx

	ASTM Method for Virucidal Activity: Test Processing Sheet MB-39-01_F3.docx ASTM Method for Virucidal Activity: Results Sheet MB-39-01_F4.docx
15. References	1. Standard Practice to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces, ASTM E1053-20. 2. Standard Practice for Use of Gel Filtration Columns for Cytotoxicity Reduction and Neutralization, ASTM E1482-12.

Attachment 1

Storage of MRC-5 Cell Line Stocks by Freezing in Liquid Nitrogen

The procedure describes how to freeze aliquots of the cell line. Unless indicated otherwise, the volumes given below are for flasks with a capacity of 75 cm^2. Adjustments will be required if cell culture vessels of other sizes are being used.

Test organisms
Clearly document the source (e.g., ATCC), scientific name, and reference number of the cell line. In addition, maintain records including dates the cell lines were received, sub-cultured, and frozen as initial stock. The complete passage history should be documented—ensuring traceability to the initially frozen vials.

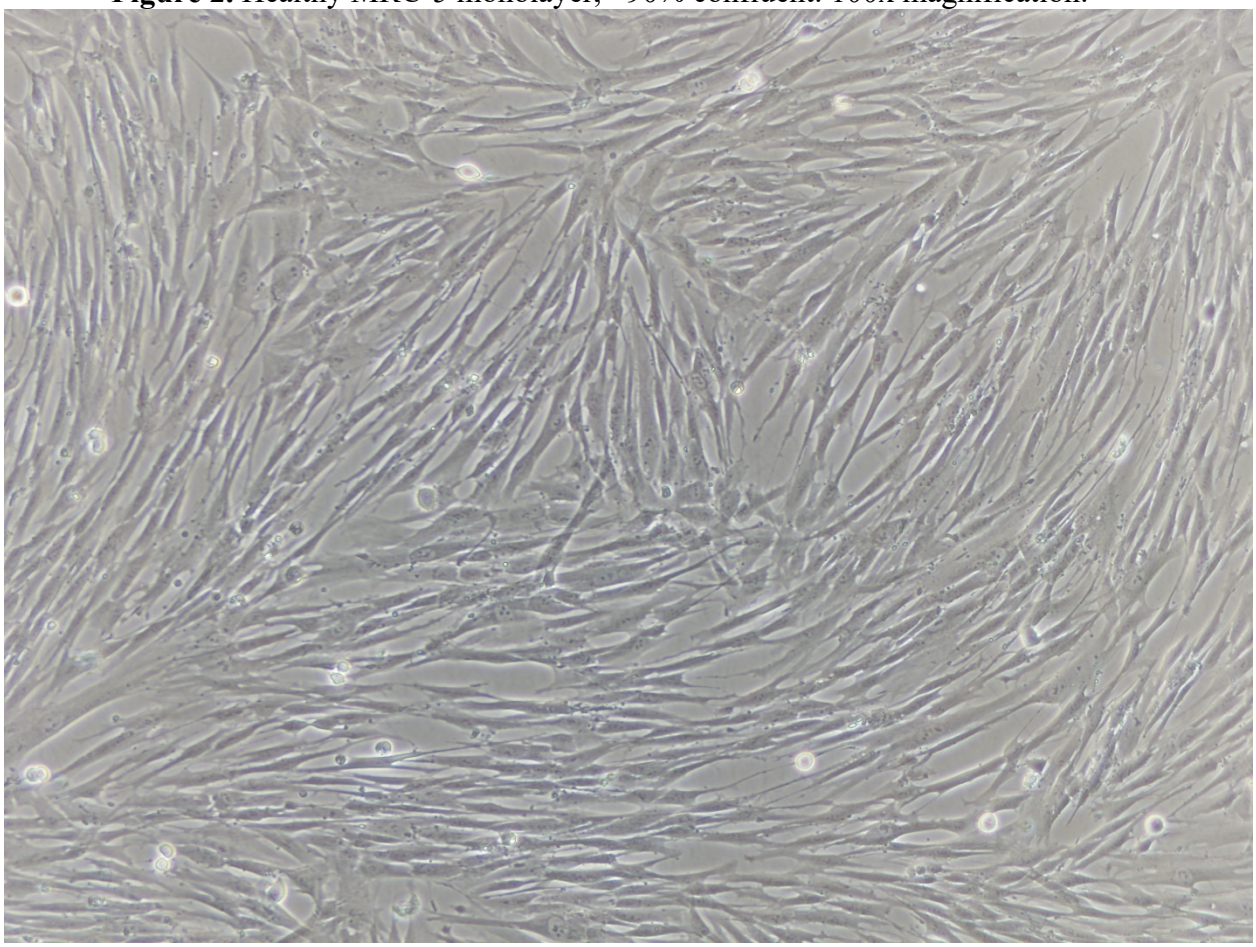
Cell culture media and reagents
Commercially prepared cell culture media and any ingredients purchased to make such media in-house are obtained from commercial sources. Chemicals/reagents are of analytical grade or appropriate for virology purposes. All work must be done with strict adherence to aseptic technique.

Method

1. Place containers of FBS, DPBS and trypsin in water bath or incubator at 37±1°C. Remove EMEM from the refrigerator and inspect it. Place the bottle with the cap loosened in a CO_2 incubator to adjust pH if needed.
2. Make the required amount of CGM with 10% FBS and warm it to 37±1°C (approx. 30 min. for 50 mL or ≥1 hr for 200+ mL).
3. For the cryoprotective medium, make a 5% v/v DMSO solution in CGM with 10% FBS (e.g. add 5 mL of DMSO to 95 mL CGM).
4. Remove the needed number of flasks that exhibit an 80-95% confluent, healthy monolayer from the incubator and check that the cell morphology is normal. See **Figure 2**.
5. Following temperature equilibration of all media, remove spent medium from flasks.
6. Dispense about 10 mL of DPBS into each flask and wash monolayer 2-3 times. Discard the DPBS wash.
7. Dispense 2-3 mL of trypsin into each flask and distribute it evenly over the entire monolayer. Incubate flask at 37±1°C and observe frequently for cell detachment.
8. Just before cells are fully detached from flask (generally 1-3 minutes), gently tap the flask to fully detach cells.
9. Dispense 6-9 mL of cryoprotective medium (three times the amount of trypsin used) and pipet the cell suspension up and down 2-3 times to break up any clumps.
10. Dispense 1 mL aliquots of the cell solution into a sterile cryovials.

11. Put the cryovials on ice for approximately 30 minutes, then move them to -20±2°C freezer for 24 hours. Alternatively, use a controlled freezer or an alcohol bath device (i.e. Mr Frosty) to bring the cells to temperature in a controlled manner.
12. After the initial freeze, load the cryovials into metal canes and immerse them into liquid nitrogen (-196°C) or a comparable mechanical freezer.

Figure 2. Healthy MRC-5 monolayer, ~90% confluent. 100x magnification.



Attachment 2

Reviving MRC-5 Cell Line from Liquid Nitrogen Storage

Purpose
This procedure is for reviving cells frozen in liquid nitrogen.

Method

1. Place containers of FBS in water bath or incubator at 37±1°C. Remove EMEM from the refrigerator and inspect it. Place the bottle with the cap loosened in a CO_2 incubator to adjust the pH if needed.
2. Make the required amount of CGM and warm it to 37±1°C (approx. 30 min. for 50 mL or ≥1 hr for 200+ mL). An antifungal/antibiotic may be added to the media if desired.
3. Label a T-25 cm^2 flask with the identification number, cell line name, and date.
4. Remove vial(s) of cells from liquid nitrogen.
5. Immediately place the vial in a water bath (but do not let the cap get wet) at 37±1°C to thaw the cells rapidly and avoid damage from ice crystal formation. Leave vial in water bath no longer than 5 minutes.
6. Remove the thawed cells with a pipette and place them in a T-25 cm^2 flask. Slowly, over 2 minutes, add 9 mL pre-warmed CGM to the cells.
7. Incubate at 37±1°C and 5% CO_2 and replace the media after 24 hours with 5 mL CGM. An antifungal/antibiotic may be added to the media if desired. Continue to incubate for 2-3 more days and observe daily for cell adherence and growth. Pass cells once they exhibit an 80-95% confluent monolayer (see **Attachment 3**).

Attachment 3

Sub-culturing MRC-5 Cell Line for Work with Viruses

Purpose

This procedure describes how to split (pass) confluent cell monolayers for subculture in a T-75 cm^2 tissue culture flask (working volume of 15 mL). Proportionally reduce or increase the volume for flasks of other sizes. The subcultivation ratio is 1:2 to 1:5. Medium Renewal: 1 to 2 times per week.

Method

1. Place containers of FBS, DPBS and trypsin in water bath or incubator at 37±1°C. Remove EMEM from the refrigerator and inspect it. Place the bottle with the cap loosened in a CO_2 incubator to adjust the pH if needed.
2. Make the required amount of CGM and warm it to 37±1°C (approx. 30 min. for 50 mL or ≥1 hr for 200+ mL). It is recommended to add an antifungal/antibiotic to the media in one backup flask in case the media in the other flasks becomes contaminated.
3. Remove the needed number of flasks that exhibit an 80-95% confluent, healthy monolayer from the incubator and check that the cell morphology is normal (See **Figure 2**). Label new flasks with passage #, cell line name and date.
4. Following equilibration of temperature of all media, remove spent medium from flasks.
5. Dispense about 10 mL of DPBS into each flask and wash monolayer 2-3 times. Discard the DPBS wash.
6. Dispense 2-3 mL of trypsin into each flask and distribute it evenly over the entire monolayer. Place flask on rotator in incubator at 37±1°C and observe frequently for cell detachment.
7. The cells will fully detach from flask in about 2-4 minutes. Avoid tapping the flask as it may lead to excess clumping of the cells.
8. Dispense 6-9 mL of CGM (three times the amount of trypsin used) and pipet the cell suspension up and down 2-3 times to break up any clumps.
9. Transfer the required volume of cell suspension for the desired split ratio, then add CGM up to the working volume of the flask. For example, for a minimum subcultivation ratio of 1:2 to 1:5 (a 1:4 ratio would be 1 T-75 cm^2 flask passed to 4 T-75 cm^2 flasks), add 3 mL cell suspension to each of two new flasks and then add 12 mL fresh CGM. For 24 well plates, add 200-300 µL of the cell suspension to 800-700 µL (respectively) of CGM.
10. Place the flasks into the incubator at 37±1°C and 5% CO_2 and do not disturb then for 2-3 hours to allow for cell adherence to the flask surface.
11. Monolayers prepared this way can be used for making virus pools, for making plates for virus infectivity assays, or for another passage for subsequent experiments.
12. A maximum of around 40 passes can be made before fresh cells should be started. Do not use the cells if they have undergone any morphological change.

Attachment 4

CoV Propagation, Harvest and Titration

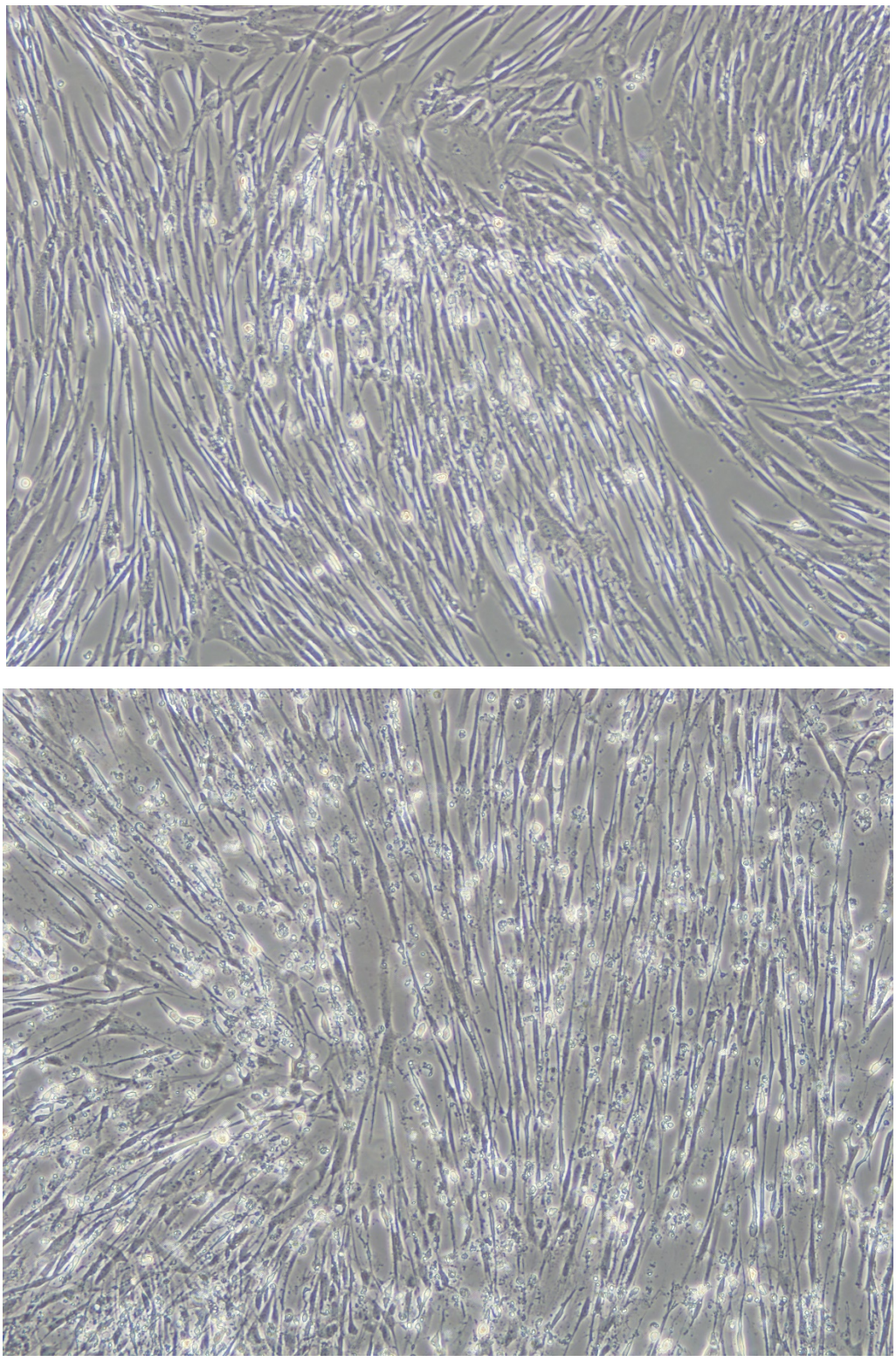
Purpose

This procedure describes how to propagate, harvest, enumerate by titration, and store virus. This procedure is to be used with a T-75 cm^2 tissue culture flask (working volume of 15 mL). Proportionally reduce or increase the volume for flasks of other sizes.

Initiation and Propagation of Frozen CoV as Received from ATCC:

1. Make a T-75 (or larger if desired) flask of MRC-5 cells (ATCC CCL-171) with an 80-90% (not 100% confluent) confluent monolayer. This flask should be incubated at 37±1º C in 5% CO_2 for 24-48 hours.
2. See the ATCC product specification sheet to determine the titer of the frozen CoV as received from ATCC. This value will be used to calculate the correct MOI.
3. Thaw the vial (approximately 1 mL) rapidly in a 37±1°C water bath, being careful not to let the cap get wet. Allow vial to thaw for no longer than 2-3 minutes.
4. When ready to infect the cell line, remove the CGM with 10% FBS from the flask and wash the cells three times with pre-warmed DPBS. Replace with 15 mL of CGM with 2% FBS.
5. Prepare dilution of virus in CGM at a minimum volume (e.g. 1 mL per 25 cm^2), optimized for propagation at an MOI of 0.01-0.1. Add the virus dilution to the flask. Retain the remaining volume of thawed CoV and store in an ultra-freezer at -80 (or -70) ±5º C.
6. Incubate flask at 35±1º C in 5% CO_2 for 2-7 days or until at least 80% of the monolayer shows virus-induced cytopathic effects (CPE). The CPE associated with CoV is visually evident by the presence of rounded cells and sloughing. See **Figure 3**. At the end of the incubation with the desired level of CPE, proceed to virus harvest below.
7. The virus stock solution concentration should be around 7 logs MPN.

Figure 3: CoV CPE on MRC-5 cell line (Top: ~48 hr. Bottom: ~3 days. 100x magnification)



Harvesting and storage of the virus stock suspension:

1. Take the flask containing the virus to be harvested and place it in a freezer (either -20±1º C or -80 (or -70) ±5º C) until frozen. Remove the flask from the freezer and thaw. Do this at least three times to release virus from the infected cells. Transfer contents of the flask into a sterile plastic centrifuge tube. Centrifuge at 1500 × g for 10 minutes to remove cell debris.
2. Collect the virus containing supernatant and aliquot 1 mL of the virus suspension per cryovial and store at -80 (or -70) ±5º C with the vials clearly labeled with name of virus, vial number, and date of preparation. (Note: freezing in aliquots of less than 1 mL will negatively affect the long-term viability of the virus stock).
3. The virus stock suspension does not have an expiration date; however, the virus stock suspension must be tested occasionally using the test system control and return the desired results for it to continue to be stored. Each cryovial is for single use only.

Virus titration:

1. Prepare 24-well tissue culture plates with an 80-90% confluent monolayer (not 100% confluent) of MRC-5 cells in all wells (see **Figure 1**). These plates should be incubated at 37±1°C in 5% CO_2 for 1-2 days.
2. To determine the titer of CoV, prepare a serial ten-fold dilution of CoV stock or carrier eluate from 10^{-1} to 10^{-9} (or as required) in pre-warmed CGM (e.g., 0.5 mL virus + 4.5 mL CGM with 2% FBS).
3. Remove the old CGM from each well needed on the 24 well plate. This process must be performed quickly to minimize cell death due to lack of CGM on the monolayer.
4. Add 1 mL of CGM to a negative control well first.
5. Next, add 1 mL from each dilution to each of eight wells. When done, cover that column with the tissue culture plate cover to mitigate contamination. Process from most dilute to least dilute.
6. The positive control is done last. While ensuring that all other wells are covered by the tissue culture plate cover, add 1 mL of the most concentrated CoV dilution to a positive control well.
7. Place plates back into the incubator and incubate the 24-well tissue culture plate 6-9 days at 35±1º C in 5% CO_2.
8. At the end of the incubation period, score and record the infectious CoV microscopically by observing virus-specific CPE produced in each well of each dilution as either positive for CPE (see **Figure 3**) or the absence of CPE (see **Figure 1**).
9. The positive control wells should demonstrate CPE while the negative control wells should not exhibit any virus-induced CPE. Results will be scored in comparison with the negative control.

Attachment 5

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

Cytotoxicity and Neutralization Determination. Prior to performing the neutralization assay, assess the cytotoxicity of the test substance to the cells and ensure the proposed neutralizer is effective. These controls can also be performed concurrently with the efficacy evaluation if desired.

1. **Cytotoxicity Control.**

   a. Neutralize the disinfectant per the study protocol. Examples of neutralization methods include 1) column filtration (single or multiple) alone, 2) adding 2 mL of disinfectant and 2 mL of a chemical neutralizer, or 3) by adding 2 mL of disinfectant and 2 mL of a chemical neutralizer and then using column filtration. Equilibrate chemical neutralizers to 22±2°C. Vortex and let this solution sit at room temperature (22±2°C) for 10 minutes.

      i. The first suspension following neutralization will be considered the 10^{-1} dilution.

   b. Serially dilute in CGM with 2% FBS, equilibrated to 37±1°C, out to at least the 10^{-4} dilution 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 4 wells for all dilutions. Extra wells may be needed to observe the effect of no media changes or for further media changes as needed.

   d. Use at least one well on each plate as a negative control (e.g. CGM with 2% FBS alone).

   e. Change the media in single wells that are displaying cytotoxicity after a minimum of one hour. Compare this well to the other wells in that dilution to determine if the media change reduced cytotoxicity. If cell death occurs in under one hour, that dilution cannot be used.

   f. Incubate the plate at 35±1°C in 5% CO_2 for 6-9 days and observe the cells for cytotoxicity. The test cells should be compared to the negative control cells to determine toxicity.

   g. Score the cells as toxic or non-toxic in each test conditions. Any dilution that exhibits cytotoxicity cannot be used in the virucidal assay.

2. **Test Substance Neutralization Control:**

   a. To validate neutralization, two treatments are needed: the neutralized test substance and an equal volume of DPBS as a control.

   b. Neutralize the disinfectant per the study protocol. Examples of neutralization methods include 1) column filtration (single or multiple) alone, 2) adding 2 mL of disinfectant

and 2 mL of a chemical neutralizer, or 3) by adding 2 mL of disinfectant and 2 mL of a chemical neutralizer and then using column filtration. Equilibrate chemical neutralizers to 22±2°C. The DPBS control is treated the same as the disinfectant. Collect the column eluate or the disinfectant/neutralizer solution in a sterile test tube and vortex briefly.

i. This suspension will be considered the 10^{-1} dilution.

c. Serially dilute in CGM with 2% FBS, equilibrated to 37±1°C, out to the expected dilution to be used in the efficacy test. For the control only dilute to the 10^{-2} dilution.

d. To each dilution tube, add a low number (1000 to 5000) of virus particles and hold the tubes for at least the contact time at the contact temperature.

e. Titrate the dilutions for infectious virus by inoculating 1 mL per well to at least four replicate cell monolayers/dilution in a 24 well plate starting from the first ten-fold dilution of the sample.

f. Incubate plates for 6-9 days at 35±1º C in 5% CO_2.

g. Comparable levels of infective virus must be recovered from the control and the neutralized treatments for the neutralization to be successful.

h. In case of incomplete neutralization, either another neutralizer or gel filtration columns may be needed. Any dilution that does not exhibit successful neutralization cannot be used in the virucidal assay.

Forms and Data Sheets:

1. Test Sheets. Test sheets are stored separately from the SOP under the following file names:

ASTM Method for Virucidal Activity: Cytotoxicity and Neutralization Verification Sheet	MB-39-01_F5.docx

Attachment 6

Sephadex Column Preparation and Use

Preparation of sterile syringes:

1. Remove the plunger from a 10 mL syringe and place 0.07 ± 0.01 g of glass wool into the barrel of the syringe.
2. Gently push fiber to bottom of syringe and then push in the plunger until it just touches the glass wool.
3. Autoclave on gravity cycle for 20 minutes to sterilize.

Preparing column caps:

1. Using a 3/4" spade bit, drill a hole in the middle of a 50 mL conical lid.
2. Autoclave on gravity cycle for 20 minutes to sterilize.

Making Sephadex columns:

1. Replace 50 mL conical tube lid with sterile column cap.
2. Place sterilized syringe with glass wool into column cap and remove the plunger. Keep plunger sterile.
3. Gently thoroughly mix room-temperature Sephadex solution and add approximately 10 mL into each syringe. Allow the solution to drip by gravity into the 50 mL conical tube for approximately 10 minutes. See **Figure 4** below.
4. Decant eluate from 50 mL conical tube and centrifuge at 600 x g for 3 minutes.
5. Once centrifuged, remove column and column cap and place into a new sterile 50 mL conical tube (save the conical cap, this will be used later).
6. Add solution to be neutralized to the column, insert the syringe plunger, and press the plunger until all liquid has been expressed. Do not press too hard or Sephadex media may contaminate the eluate.
7. The eluate is now ready for dilution and plating or further processing.

Figure 4. Sephadex column (left) and column in 50 mL conical tube (right)