Evaluation of Antiseptic Antiviral Activity of Chemical Agents

Chloé Geller,¹ Chantal Finance,¹ and Raphaël Emmanuel Duval¹

¹Nancy-University, CNRS, Nancy Cedex, France

UNIT 26.10

ABSTRACT

Antiviral antiseptics and disinfection are crucial for preventing the environmental spread of viral infections. Emerging viruses and associated diseases, as well as nosocomial viral infections, have become a real issue in medical fields, and there are very few efficient and specific treatments available to fight most of these infections. Another issue is the potential environmental resistance and spread of viral particles. Therefore, it is essential to properly evaluate the efficacy of antiseptics-disinfectants (ATS-D) on viruses. ATS-D antiviral activity is evaluated by (1) combining viruses and test product for an appropriately defined and precise contact time, (2) neutralizing product activity, and (3) estimating the loss of viral infectivity. A germicide can be considered to have an efficient ATS-D antiviral activity if it induces a >3 or $>4 \log_{10}$ reduction (American and European regulatory agency requirements, respectively) in viral titers in a defined contact time. This unit describes a global methodology for evaluating chemical ATS-D antiviral activity. *Curr. Protoc. Cell Biol.* 51:26.10.1-26.10.22. © 2011 by John Wiley & Sons, Inc.

Keywords: antiseptic • antiviral • chemical agents • neutralization • gel-filtration

INTRODUCTION

Emerging viruses and associated diseases, as well as nosocomial viral infections, have become a real issue in medical fields, along with the potential environmental resistance of viral particles and the possibility of their transfer between contaminated hosts, mainly from the hands to environmental surfaces, and the converse. Antiviral antiseptics and disinfection are crucial for preventing the environmental spread of viral infections. Indeed, very few efficient and specific treatments are available to fight most of these infections.

Proper evaluation of the efficacy of antiseptics-disinfectants (ATS-D) on viruses is very important. Essentially, ATS-D antiviral activity is evaluated by combining viruses and the product to be tested for an appropriately defined and precise contact time, neutralizing product activity, and estimating the loss of viral infectivity due to the product activity. Neutralization of the ATS-D plays a key role in the test procedure; it ensures a precise contact time, the elimination of the residual activity and cytotoxicity of the tested product, and the successful recovery of viruses not killed by the product. These tests require appropriate controls, especially to check the absence of interference due to the test itself on viral infectivity, efficiency of neutralization, removal of cytotoxicity, and reproducible and well defined test conditions (e.g., contact time and environmental temperature). A germicide can be considered to have an efficient ATS-D antiviral activity if it induces a $\log_{10}$ reduction in viral titers, in a defined contact time, higher than 3 or 4 $\log_{10}$, depending on American and European regulatory agencies, respectively (ASTM, 2004; AFNOR, 2007).

This unit describes a global methodology for evaluating ATS-D activity of chemicals on viruses. The viral model used to validate this method is the human coronavirus, strain 229E (HCoV 229E), grown on L-132 cells. Cultivation parameters for the cells and the viruses are described in Support Protocols 1 and 2, respectively. In the assay procedure

Figure 26.10.1 General principle of exclusion-diffusion in ATS-D neutralization by gel-filtration.

described in the Basic Protocol, the neutralization step is based on a gel-filtration method using Sephadex columns prepared in-house (Geller et al., 2009). By the process of exclusion-diffusion, the column retains the small molecules of the tested germicide and releases the larger viral particles (Fig. 26.10.1), which can then be titered. An important step upstream of the virucidal assay is to ensure that viral particles are not retained by the columns and that the methodology itself does not have any influence on viral infectivity (see Support Protocol 1). It is also necessary to ensure the efficiency of the neutralization step. To verify that the tested germicide is retained by the column and thus neutralized, two approaches have been taken, a biological approach checking the absence of cytotoxicity of the filtrates (see Support Protocol 3) and a physicochemical approach, determining the retention rate of the germicide using UV-visible spectrophotometry (see Support Protocol 4). Supported by these preliminary results, the ATS-D virucidal assay can be performed as described in the Basic Protocol. To plan for carrying out the Basic Protocol, review the sequence described in Figure 26.10.2.

BASIC PROTOCOL

QUANTITATIVE SUSPENSION TEST FOR ANTIVIRAL ATS-D ACTIVITY EVALUATION USING SEPHADEX GEL-FILTRATION NEUTRALIZATION

This assay is based on combining viruses and test product during a defined contact time, followed by neutralization of the test product on Sephadex columns. The filtrates, containing viruses, are then placed on confluent cell monolayers, seeded 48 hr earlier (see Support Protocol 1). After the incubation time required to obtain the viral cytopathogenic effect (CPE), viral titers are estimated by the end point dilution method (see Support Protocol 3). Virucidal activity is then evaluated for each tested contact time and each tested concentration, by comparing viral titers with and without addition of the test product. This activity is expressed as the $\log_{10}$ difference between the viral titers.

Figure 26.10.2 Sequence of events in evaluating ATS-D antiviral activity.

NOTE: All steps are carried out using aseptic technique and a level 2 biological safety cabinet (see *UNIT 1.3*).

NOTE: For sterilization by autoclaving and waste decontamination, see *UNIT 1.4*.

Materials

Combed cotton (e.g., Dutscher Scientific cat. no. 030232)
 Test product (cytotoxicity determined in preliminary assays using Support Protocol 3)
 G-10 or G-25 Sephadex suspension (see recipe)
 Deionized, sterile water, pH 7.0 (see important note below)
 7.5% (w/v) sodium bicarbonate (if required)
 1-ml frozen viral suspension (Support Protocol 2)
 Nine 96-wells plates with confluent monolayer of L-132 cells: seeded 48 hr before performing the assay (Support Protocol 1, plates for viral titration)
 Bleach solution with 9.6% (w/v) active chlorine: 250 ml bleach diluted in 2 liters H₂O
 MEM-2: minimum essential medium with Glutamax and Earle's salts (Invitrogen cat. no. 41090-093), supplemented with 2% (v/v) fetal bovine serum (FBS, Invitrogen cat. no. 10270098)

- 1 ml-syringes
 - 1.5-ml microcentrifuge tubes
 - Stainless steel scissors, sterile
 - Stainless steel dissecting forceps, sterile
 - Container for sterilizing column components (e.g., jar with lid)
 - 50-ml conical centrifuge tubes with tops, sterile
 - 0.22- μ m syringe-driven filter unit
 - 200 $\times$ inverted microscope
 - 96-well cell culture plates
 - 8-channel multichannel pipettor and reagent reservoirs
 - Humidified, 37°C, 5% CO₂ cell culture incubator
- Additional reagents and equipment for evaluating viral titers with using the endpoint dilution method (Support Protocol 2)

IMPORTANT NOTE: The quality of water (e.g., hardness due to the presence of ions like chloride, magnesium, or calcium) used in making the test solutions is very important because it can influence product activity. Therefore, deionized water is typically used. In addition, as autoclaving can acidify the water, the pH must be checked after autoclaving and the water neutralized, if necessary, by the addition of 7.5% (w/v) sodium bicarbonate (NaHCO₃). Hard water can be used, but if it is, the hardness should be precisely measured and specified. Indeed, hard water is recommended by some standards because it is more representative of conditions in the actual use of the product. However, deionized water allows easier assay standardization. Furthermore, autoclaving can induce an acidification of the water, so the pH must be checked. Indeed, pH-induced variability in virus infectivity has been demonstrated with coronaviruses (Sturman et al., 1990).

Prepare and sterilize Sephadex column components

1. Stuff 1 ml-syringes with combed cotton, as shown in Figure 26.10.3A.
To facilitate this step, push the cotton with the plunger of the syringe and/or the stainless steel dissecting forceps.
2. Prepare drilled 1.5-ml microcentrifuge tubes, using sterile stainless steel scissors and forceps to make a hole in the center of the cap. Ensure that this hole is large enough to accommodate the tip of the syringe. See Figure 26.10.3A
3. Place these items in a container, and sterilize them by autoclaving.
4. Store them at room temperature in a closed cabinet.

Figure 26.10.3 Steps for Sephadex column fabrication. **(A)** The top of a microcentrifuge tube is drilled, and a syringe is stuffed with combed cotton, followed by sterilization. **(B)** The components from panel A are assembled, and the syringe body is filled with 1 ml of either G-25 or G-10 gel. **(C)** After a centrifugation step (1 min at 4500 $\times$ g) to pack the gel, the column is ready to use.

Prepare Sephadex columns

5. On the day of the experiment, fill the body of the sterilized syringe with 1 ml sterile Sephadex gel (Fig. 26.10.3B).

Eight columns are necessary for each experiment.

The type of Sephadex that should be used depends on the molecular mass of the test product (see the Commentary, Critical Parameters).

6. Place the syringe on the drilled microcentrifuge tube, and then place this entire system in a 50-ml conical centrifuge tube to conserve sterility.
7. Centrifuge 1 min at $4500 \times g$ (mean acceleration), room temperature (20° to 22°C).

The in-house Sephadex column is now ready to use (Fig. 26.10.3C).

The use of the 50-ml conical tube is essential for transport of the columns if the centrifuge is located outside the sterile area, often the case.

Prepare test solutions and thaw virus suspension

8. Prepare ~ 5 ml of the appropriate dilution of test product in sterile, deionized water, and sterilize it by passing it through a $0.22\text{-}\mu\text{m}$ syringe-driven filter unit.

This dilution must take into account the later dilution involved by the addition of viral suspension or water, in the case of controls, to ensure an appropriate final concentration.

9. Thaw 1 ml of viral suspension at room temperature.

Assay antiviral activity

Each assay is repeated three different times and consists of the evaluation of ATS-D activity of a concentration x of the test product and two different contact times (t_1 and t_2).

10. Prepare solutions that will be used for all nine plates as described in Table 26.10.1, in nine 1.5-ml microcentrifuge tubes.

The viruses will be added last to ensure the most accurate contact times.

11. Thirty seconds before the end of the 1st contact time, deposit 500 μl of each solution onto the Sephadex columns, and place each of these columns into sterile 50 ml-centrifuge tubes to maintain sterility.

Thirty seconds is the time needed in our laboratory to deposit the solution and to go to the centrifuge without lengthening the contact time. This timing depends on the organization of the equipment in the laboratory and should be adjusted accordingly.

12. Centrifuge 1 min at $405 \times g$, room temperature (20° to 22°C).
13. Remove four seeded, incubated 96-well plates from the incubator, check cell integrity under an inverted microscope at $200\times$ magnification, and spill the medium into a bleach-containing vessel.

These four plates will be inoculated with filtrates from the t_1 filtration control, neutralization control, and t_1 assays 1 and 2.

The total number of seeded plates for each assay should include enough for each concentration of test product and two contact times, as well as filtration, cytotoxicity, and neutralization controls (see Table 26.10.1).

14. Fill each well with 180 μl MEM-2.

MEM is used for cultivation of L-132 cells, but different media can be used for other cell lines.

The 2% FBS in the medium is necessary to maintain cells in a survival state without interfering too much with virus infectivity.

Table 26.10.1 Outline of the Experiment^a

Solution for:	Volume required	Composition of solution	Contact time
Plate 1: virus control (no filtration) ^b	200 µl	20 µl viral suspension + 180 µl sterile, deionized water	Not filtered ^c
Plate 2: virus control after filtration (t_1) ^d	500 µl	100 µl viral suspension + 900 µl sterile, deionized water	1 st contact time (t_1)
Plate 3: virus control after filtration (t_2) ^d	500 µl	100 µl viral suspension + 900 µl sterile, deionized water	2 nd contact time (t_2)
Plate 4: neutralization control ^e	500 µl	900 µl of test product at concentration x + 100 µl sterile, deionized water	1 st contact time (t_1) ^f
Plate 5: cytotoxicity controls ^g	500 µl	900 µl of test product at concentration x + 100 µl deionized, sterile water	2 nd contact time (t_2) ^g
Plates 6 and 7: assay t_1	2 × 500 µl	900 µl of test product at concentration x + 100 µl viral suspension	1 st contact time (t_1)
Plates 8 and 9: assay t_2	2 × 500 µl	900 µl of test product at concentration x + 100 µl viral suspension	2 nd contact time (t_2)

^aEach assay comprises two contact times in order to save time and materials. In this way, the neutralization and cytotoxicity controls are done only once for both contact times. However, adding more parameters (concentrations and/or contact times) for additional savings would make the experiment too complicated to carry out effectively.

^bThe virus positive control corresponds to the viral titer without filtration and is the reference viral titer.

^cThere is no filtration in the positive control so the contact time is irrelevant.

^dFiltration controls: Plates for t_1 and t_2 ensure that the protocol itself (dilution in sterile water and filtration on Sephadex columns) does not influence viral titers. Thus, if the difference in viral titers between plates 1 and 2, and plates 1 and 3 is $< 0.5 \log_{10}$, the reference viral titer for the evaluation of the virucidal activity for each contact time, will be the corresponding one obtained after the filtration. In this way, the $\log_{10}$ difference will reliably and exclusively evaluate the virucidal activity of the tested product.

^eNeutralization control: cells inoculated with 180 µl of the filtered product plus 20 µl of the viral suspension. This ensures that the product after filtration does not interfere with virus infectivity or produce any virucidal effect.

^fContact times for neutralization and cytotoxicity controls have been assigned arbitrarily to equilibrate the tubes in the centrifuge.

^gCytotoxicity control: cells without virus. This ensures the absence of cell morphological changes due to the presence of filtrates of the test product at a concentration x .

15. Using an 8-channel multichannel pipettor, deposit 20 µl of filtrate into each well of the 3rd column of the appropriate plate, and mix gently with the multichannel pipettor. Transfer 20 µl of the solutions in each well of the 3rd column into the wells of the 4th column, mix gently by pipetting, and repeat this operation until the last column, discarding the 20-µl excess volume from the last wells.

The 1st column is set aside for the negative control (medium only), and the 2nd column is for the positive control (cells with medium).

16. For the neutralization control, mix 180 µl of the test product filtrate with 20 µl of viral suspension and inoculate this mixture on the cell monolayer as described in step 15.
17. Repeat steps 11 to 15 for the 2nd contact time and the cytotoxicity control (solutions 3, 5, 8 and 9).
18. For the virus positive control without filtration, inoculate the wells in column 3 of plate 1 with 20 µl of a 1/10 dilution of unfiltered virus:sterile, deionized water. Carry out a serial dilution as described in step 15.

The 1/10 dilution is adapted for HCoV 229E titration.

Virus suspension is diluted in sterile deionized water to reproduce conditions of the viruses before filtration.

19. Incubate all plates at 33°C (the optimal temperature for HCoV 229E growth) for the time required to see the CPE (6 days for the HCoV 229E).

The temperature and time will vary according to the viral model.

20. Evaluate viral titers using the endpoint dilution method (see Support Protocol 2).

Determine ATS-D virucidal activity of the test product

21. Calculate viral titers as described in Figure 26.10.5
22. Check the controls to determine that the difference in viral titers between the positive control (viral suspension without filtration) and the filtration and neutralization controls is less than 0.5 log₁₀, which ensures their validation.
23. Check the cytotoxicity control under an inverted microscope to be sure that there are no visible signs of cytotoxicity (e.g., morphological changes with round cells, syncytia formation, and destruction of the cell monolayer).
24. Estimate the loss of virus infectivity by determining the difference in viral titers between:

Plate 2 (positive control after filtration, t_1) and plates 6 and 7 (concentration x and contact time t_1).

Plate 3 (positive control after filtration, t_2) and plates 8 and 9 (concentration x and contact time t_2).

L-132 CELL CULTURE

The methods described in this unit were developed using the L-132/human coronavirus strain 229E (HCoV 229E) system, but it can be adapted, with validation, to other cell/virus systems. L-132 is a continuous, adherent cell line, which theoretically allows an infinite number of passages. In the interests of reproducibility, the number of passages is limited to 30 in our laboratory.

Materials

L-132 cells (ATCC #CCL-5)

MEM-5: with Glutamax and Earle's salts (Invitrogen cat. no. 41090-093), supplemented with 5% (v/v) fetal bovine serum (FBS, Invitrogen cat. no. 10270098)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Trypsin/EDTA (Invitrogen cat.no.25300-059)

FBS

Dimethyl sulfoxide (DMSO)

Isopropanol

Liquid nitrogen

MEM-10: MEM with Glutamax and Earle's salts (Invitrogen cat. no. 41090-093), supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen cat. no. 10270098)

MEM: MEM with Glutamax and Earle's salts (Invitrogen cat. no. 41090-093), without serum

4% (v/v) trypan blue

SUPPORT PROTOCOL 1

Viruses

26.10.7

25-cm² and 75-cm² tissue culture flasks
Humidified, 37°C, 5% CO₂ cell culture incubator
200× inverted microscope
2-ml cryotubes (Sarstedt cat.no.72.699.406)
Freezing container (e.g., by Nalgene, Thermo Scientific cat. no. 5100-0001)
Hemocytometer
96-well plates (Sarstedt cat.no.83.1835.300)
50-ml test to be, sterile

Maintain cell lines

1. Grow L-132 cells in 75 cm²-culture flasks in 20 ml MEM-5 in a humidified, 37°C, 5% CO₂ cell culture incubator.

See UNIT 1.1 for general information about culturing mammalian cells.

2. Split the cells when monolayer becomes confluent, two to three times a week, as described in steps 3 to 6.
3. Wash the cell monolayer three times with 8 ml PBS, and add 0.5 ml trypsin-EDTA.

It is better to perform three washings with 8 ml of PBS than two washings with 10 ml of PBS, is to obtain a better cell dissociation. It also better eliminates the excess FBS, which can interfere with trypsin-EDTA activity.

4. Incubate the flasks for a few minutes at 37°C.
5. Add 4.5 ml MEM-5, and dissociate the cells by gently pipetting up and down. Check for dissociation under a 200× inverted microscope.
6. Leave 1 ml of cell suspension in the flask and add 19 ml MEM-5 to maintain the flask culture.

The remaining 4 ml of cell suspension can be discarded or used to make more culture flasks. They can also be used to freeze stock cells, or seed 96-well plates for cytotoxicity assays or viral titration, as described in the following steps.

Prepare and revive frozen cells stocks

7. Dispense into 2-ml cryotubes the following:

800 µl cell suspension (80% v/v)
100 µl FBS (10% v/v)
100 µl DMSO (10% v/v).

8. Place the cryotubes in an isopropanol-containing freezing container. Place this at –80°C for 24 hrs, allowing a progressive fall in temperature, and then in liquid nitrogen. Store up to several years in liquid nitrogen (–196°C).
9. To revive the cells, deposit 1 ml of thawed cell suspension (thawed at room temperature) into a 25-cm² flask and add 9 ml MEM-10.

The 10% FBS in MEM-10 is used to protect cells from DMSO, which is cryoprotectant, but is also cytotoxic.

10. Replace the medium the next day with fresh MEM-5 to eliminate the DMSO, after ensuring adhesion of cells to the flask.
11. Split the culture in the 25-cm² flask 1:2 when the confluence is reached.

Enumerate cells

12. After dissociating and passaging a 75 cm²-flask of confluent cells (step 6), mix 50 μ l cell suspension with 50 μ l trypan blue and 400 μ l serum-free MEM, and enumerate using the trypan blue exclusion method.
13. Fill the hemacytometer, with special care for complete adherence of the cover glass, count dead (blue) and living (colorless) cells under a 200 $\times$ inverted microscope, and convert the results to live cells/ml.

The chamber of a Malassez type hemacytometer corresponds to a volume of 1 μ l. For more information on the use of a hemacytometer see UNIT 1.1.

Seed plates for cytotoxicity assays (Support Protocol 3)

- 14a. Calculate the volume of cell suspension required for an inoculum of 2×10^3 cells/well of a 96-well plate. Determine the volume of medium that must be added to the cell suspension to make 50 μ l. Multiply these volumes by the total number of wells to be inoculated (plus a few extra), and combine the resulting volumes of cell suspension and medium in a 50-ml test tube.

For example, for four 96-well plates, we prepare the volume necessary for 400 wells. If 39 μ l of a cell suspension is required to inoculate each well, $0.39 \times 400 = 156 \mu$ l of cell suspension is diluted in $(50 - 0.39) \times 400 = 19.844$ ml MEM-5.

- 15a. Mix the adjusted cell suspension and deposit 50 μ l of the suspension into the each well in columns 2 to 11.
- 16a. Add 200 μ l MEM-5 to each well in columns 1 and 12 (negative controls), and add 150 μ l MEM-5 to each well in columns 2 to 11.
- 17a. Incubate the plates 48 hr at 37°C, 5% CO₂, to reach 40% confluence.

Inocula have been optimized for L-132 cells in order to have the same experimental conditions for both the MTT and NR cytotoxicity assays (Support Protocol 3) and to obtain a confluence of ~40%.

Seed plates for viral titration (Basic Protocol and Support Protocol 2)

- 14b. Calculate the volume of cell suspension required for an inoculum of 10^4 cells/well of a 96-well plate. Determine the volume of medium that must be added to the cell suspension to make 50 μ l. Multiply these volumes by the total number of wells to be inoculated (plus a few extra), and combine the resulting volumes of cell suspension and medium in a 50-ml test tube.
- 15b. Mix the adjusted cell suspension, and deposit 50 μ l of the suspension into the each well in columns 2 to 11.
- 16b. Add 200 μ l MEM-5 to each well in column 1 (negative control), and add 150 μ l MEM-5 to each well in columns 2 to 12.
- 17b. Incubate the plates 48 hr at 37°C, 5%CO₂, to reach confluence.

VIRAL CULTURE AND TITRATION BY THE END POINT DILUTION METHOD

This protocol provides methods for the culture, freezing, and titration of the HCoV 229E, cultured on L-132 cells. This assay is used to titrate the virus inoculum and the filtered virus control, to ensure the nonretention of viruses by the Sephadex columns used in the Basic Protocol. The difference between viral titers with and without filtration should not exceed 0.5 log₁₀. While the HCoV 229E/L-132 system is used in this unit, the general parameters may be optimized for other virus/cell systems.

SUPPORT PROTOCOL 2

Viruses

26.10.9

Materials

75 cm²-flasks with confluent monolayers of L-132 cells (two flasks; 48 hr incubation; Support Protocol 1)
Phosphate-buffered saline (PBS; APPENDIX 2A)
1 ml frozen virus suspension (HCoV 229E, ATCC #VR 740), ~10⁶ infectious particles/ml
MEM: minimum essential medium with Glutamax and Earle's salts (Invitrogen cat. no. 41090-093), serum-free
MEM-2: MEM, supplemented with 2% (v/v) fetal bovine serum (FBS, Invitrogen cat. no. 10270098)
96-well plates of confluent L-132 cell monolayers seeded for viral titration (Support Protocol 1)
Bleach solution with 9.6% (w/v) active chlorine: 250 ml bleach diluted in 2 liters H₂O
May-Grünwald solution (Merck cat. no.101424)
Giemsa solution (Merck cat. no.109204)
33°C, 5% CO₂ cell culture incubator
2-ml cryovials
8-channel multichannel pipettor

Create virus stock suspensions

1. Remove the medium from two 75 cm²-flasks with confluent monolayers of L-132 cells and wash the monolayers three times with 8 ml PBS to eliminate any trace of FBS, which can interfere with virus infectivity.

2. Thaw 1 ml of virus suspension, and dilute 1/5 in serum-free MEM.

The initial viral titer of the frozen suspension should be $\geq 10^6$ infectious particles/ml for virucidal assays.

3. Add the viral suspension to one flask, and swirl to distribute it. Mock infect the second flask with 1 ml serum-free MEM.

The mock-infected control flask ensures the absence of any contamination.

4. Incubate the flasks 1 hr at 33°C.

This incubation allows virus adsorption.

The optimal temperature for HCoV 229E growth has been shown to be 33°C (Bradburne, 1972).

5. Add 11 ml MEM-2, and incubate the flasks ~48 h at 33°C (before the CPE kills all cells), to recover the maximum number of infectious viral particles.

6. Freeze and thaw (at room temperature) three times to lyse cells.

7. Transfer the cell suspension from the flasks to 15-ml centrifuge tubes, and centrifuge 5 min at 2000 × g, room temperature, to eliminate cellular fragments.

8. Delicately recover the virus-containing supernatant, being careful not to disturb the pellet, and dispense 1-ml aliquots into it 2-ml cryovials.

This volume is appropriate for the antisepsis assays (1 ml for each assay). In this way, samples do not need to be refrozen, which can alter their quality.

9. Store stock viruses suspensions up to at least 4 years at –80°C.

Figure 26.10.4 L-132 cells: (A) uninfected monolayer and (B) cellular lysis and monolayer destruction after 6 days of infection by the HCoV 229E. L-132 cells were fixed and colored by May-Grünwald-Giemsa coloration (see Geller et al., 2009).

Carry out viral titration

10. Remove the medium in 96-well plates of confluent L-132 monolayers seeded for viral titration by discarding it in a container with bleach solution. Add 180 μ l fresh MEM-2 to all of the wells.
- 11a. *To titrate a viral suspension:* Thaw a cryovial of viral suspension (step 9) and dilute it 1/10 in MEM (without serum).
- 11b. *To titrate a viral filtrate:* Filter the viral suspension through a Sephadex column as described in the Basic Protocol, steps 5 to 7 (for preparing the column) and 11 and 12 (for depositing 500 μ l of the 1/10 viral suspension and centrifuging the column at 1 min at $405 \times g$).
12. Add 20 μ l of the diluted virus or the undiluted filtrate to each well in the 3rd column, perform a serial 10-fold dilution through the last column (using an 8-channel multichannel pipettor), and discard the excess 20 μ l from the last column.
13. Incubate the plates 6 to 7 days at 33°C, until the appearance of the viral CPE, which in the case of the HCoV 229E, is cellular lysis (Fig. 26.10.4).
14. At the end of this incubation period, discard the medium in a container with bleach solution.
15. Add 50 μ l/well of May-Grünwald solution and incubate 5 min at room temperature to fix the cells.
16. Discard the May-Grünwald solution, and rinse the plates gently under tap water.
17. Add 50 μ l/well of Giemsa solution (diluted 1/10 in PBS) and let stand for 15 min at room temperature. Rinse the plates again under tap water.
18. Evaluate viral titers following the Reed and Muench method (Reed and Muench, 1938, Fig. 26.10.5) by counting infected wells under a 200 $\times$ inverted microscope, calculating the cumulated percentage and the so-called 50% cell culture infective dose (CCID₅₀), which can also be expressed as the number of infectious particles/ml (see Fig. 26.10.5B).

B

Inoculated viral dilutions	Number of wells		Cumulative sum of wells		% of infected wells
	Infected	Not infected	Infected (a)	Not infected (b)	$[(a/(a+b))] \times 100$
10 ⁻¹ (column 3)	8	0	27	0	100
10 ⁻² (column 4)	8	0	19	0	100
10 ⁻³ (column 5)	6	2	11	2	84.62
10 ⁻⁴ (column 6)	3	5	5	7	41.67
10 ⁻⁵ (column 7)	2	6	2	13	13.33
10 ⁻⁶ (column 8)	0	8	0	21	0
10 ⁻⁷ (column 9)	0	8	0	29	0
10 ⁻⁸ (column 10)	0	8	0	37	0
10 ⁻⁹ (column 11)	0	8	0	45	0
10 ⁻¹⁰ (column 12)	0	8	0	53	0

Figure 26.10.5 Viral titration by the Reed and Muench method; 96-well plate setup for viral titration, whatever the assay (viral titration alone, viral titration after filtration on Sephadex column, neutralization control, ATS-D assay). **(A)** The 96-well plate setup for controls, viral dilutions, and infection. Column 1 is the negative control (medium only) and column 2 is the positive control (medium plus cells). Column 3 is inoculated with viral suspension and serially diluted 10-fold in columns 4 through 12. **(B)** The proportional distance between the two dilutions surrounding the 50% of infected wells is calculated as follows: $(84.62 - 50)/(84.62 - 41.67) = 0.81$. The CCID₅₀ (50% cell culture infectious dose) then corresponds to the dilution of 10^{-3.81} of the initial inoculum or $1/10^{-3.81} = 6.5 \times 10^3$ for an initial volume of 20 μ l. Thus the CCID₅₀ of the viral suspension inoculum is 3.2×10^5 infectious particles/ml.

SUPPORT PROTOCOL 3

Evaluation of Antiseptic Antiviral Activity of Chemical Agents

26.10.12

CYTOTOXICITY ASSAYS

Cytotoxicity assays are essential preliminary steps, carried out before performing the ATS-D assay in the Basic Protocol to help determine the concentration of test product that can be neutralized when carrying out the ATS-D assay. They determine the cytotoxicity threshold of the product and the efficiency of filtration on the in-house Sephadex columns in removing this potential cytotoxicity. Two complementary tests are used: (1) the MTT assay, which estimates the cellular viability, and (2) the NR assay, which allows differentiation of dead cells from living cells. Each test begins by the seeding and the incubation of four 96-well plates with L-132 cells (see Support Protocol 1) for each solution to be tested: for three contact times (24, 48, and 168 hr), plus an extra plate in case of a problem (e.g., contamination of a plate). These three contact times have been chosen to observe cellular behavior during an extended period of contact with the drug and because of the length of the HCoV 229E CPE period (~6 days). Note that

the cytotoxicity control in the Basic Protocol is based only on microscopic observation of the cells. These preliminary tests provide more information about the cytotoxicity of the product and its kinetics.

Materials

Test product

Sterile, deionized water, pH 7.0

96-wells plates (four for each test) with 40% confluent L-132 cells: seeded 48 hr before performing the assay (Support Protocol 1, plates for cytotoxicity testing)

MEM-5: minimum essential medium with Glutamax and Earle's salts (Invitrogen cat. no. 41090-093), supplemented with 5% (v/v) fetal bovine serum (FBS, Invitrogen cat. no. 10270098)

Bleach solution with 9.6% (w/v) active chlorine

Phosphate-buffered saline (PBS: *APPENDIX 2A*)

MTT (methyl thiazol tetrazolium) solution (see recipe)

SDS (sodium dodecyl sulfate) solution (see recipe)

NR solution (see recipe)

Solution A (see recipe)

Solution B (see recipe)

0.22- μ m filter

8-channel multichannel pipettor

Scanning multiwell spectrophotometer

Additional reagents and equipment for preparing and using in-house Sephadex columns (Basic Protocol)

Prepare solutions for cytotoxicity testing

- 1a. *To test cytotoxicity of a test solution:* Prepare the solutions of the test product in sterile, deionized water, and sterilize by passing through a 0.22- μ m filter.
- 1b. *To test cytotoxicity of a filtrate:* Filter test solutions from step 1 through in-house Sephadex columns as described in the Basic Protocol, steps 5 to 7 (for preparing the column) and 11 and 12 (for depositing 500 μ l of the test solution and centrifuging the column at 1 min at $405 \times g$).

Perform cytotoxicity testing

2. Remove from the incubator four 96-well plates containing 40% confluent cells for each solution to be tested for each test, and replace the medium with 180 μ l of fresh MEM-5 per well.
3. Using a multichannel pipettor, add 20 μ l of the test solution or filtrate to each well of the 3rd column of each plate and carry out a serial 10-fold dilution through the 12th column, discarding the extra 20 μ l at the end.

The 1st column is the negative control (medium only), the 2nd column is the positive control (medium + cells), and the 12th is also a negative control (medium + drug). See Figure 26.10.6.
4. Incubate the plates for the appropriate contact times at 37°C, 5% CO₂.

Carry out MTT assay (modified from Mosmann, 1983)

- 5a. At the end of each contact time (24, 48, and 168 hr), discard the medium in a container with bleach solution.
- 6a. Add 100 μ l/well PBS and 10 μ l/well MTT solution.

- 7a. Incubate the plates 4 hr at 37°C.
- 8a. Add 100 µl/well SDS solution to dissolve the formazan dark blue crystals produced by reduction of MTT by succinate mitochondrial dehydrogenate.
- 9a. Incubate the plates 4 hr at 37°C.
- 10a. Measure absorbance at 540 nm (test wavelength) and 690 nm (reference wavelength for nonspecific absorbance) with a scanning multiwell spectrophotometer.
- The absorbance at 540 nm is proportional to the number of viable cells.*
- 11a. Calculate the % viability using the following equation,

$$\% \text{ viability} = \left(\frac{A_{\text{sample}} - A_{\text{control Z}}}{A_{\text{control Y}} - A_{\text{control X}}} \right) \times 100$$

Equation 26.10.1

where

A corresponds to the (average absorbance in a column at 540 nm) – (average absorbance in a column at 690 nm)

Control X is medium only (plate column 1)

Control Y is medium plus cells (plate column 2)

Control Z is medium plus drug (plate column 12).

See Figure 20.10.6.

Figure 26.10.6 Plate diagram for cytotoxicity assays.

12a. Graphically determine the IC₅₀ value (50% inhibitory concentration).

See, e.g., Figure 26.10.8 in the Anticipated Results section of the Commentary.

Carry out NR assay (modified from Borenfreund and Puerner, 1985)

- 5b. At the end of each contact time, discard the medium in a container with bleach solution. Rinse cell monolayers with 200 µl PBS/well and discard.
- 6b. Add 200 µl/well NR solution. Incubate the plates 3 hr at 37°C to allow the uptake of NR by living cells.
- 7b. Remove the dye-containing medium. Rinse each well with 200 µl of solution A, and discard.
- 8b. Add 200 µl/well of solution B, which causes living cell lysis and the release of incorporated dye.
- 9b. Incubate 20 min at 37°C.
- 10b. Obtain spectrophotometric measurements at 540 nm.
- 11b. Calculate the % cytotoxicity according to the following formula,

$$\% \text{ cytotoxicity} = 100 - \left[\left(\frac{A_{\text{sample}} - A_{\text{control Z}}}{A_{\text{control Y}} - A_{\text{control X}}} \right) \times 100 \right]$$

Equation 26.10.2

where

A is average absorbance at 540 nm in a column

Control X is medium only (plate column 1)

Control Y is medium plus cells (plate column 2)

Control Z is medium plus drug (plate column 12).

See Figure 26.10.6.

12b. Graphically determine the 50% cytotoxic concentration (CC₅₀).

EVALUATION OF RETENTION RATES BY SEPHADEX COLUMNS AND UV-VISIBLE SPECTROPHOTOMETRY

To confirm and determine precisely the retention rates of test solution by the Sephadex columns molecules, spectrophotometric measurements must be made. Moreover, in a hypothetical case of a bad retention, it is preferable to perform these assays before the cytotoxicity assays to save time and money.

Materials

Test product

Sterile, deionized water, pH 7.0

UV-visible spectrophotometer

Additional reagents and equipment for preparing and using in-house Sephadex columns (Basic Protocol)

**SUPPORT
PROTOCOL 4**

Viruses

26.10.15

Establish spectrophotometric parameters

1. Prepare a series of 1:10 dilutions of the test product in deionized water.
2. On a UV-visible spectrophotometer, establish a calibration curve, where absorbance is a function of concentration, or $A = f(c)$, to determine the following specific parameters for the molecule being tested:

Maximum absorption wavelength ($\lambda_{\max}$)

Molar absorption coefficient (ϵ)

Detection limits.

Determine retention rates

3. Prepare dilutions of the test product (the same dilutions that will be used to evaluate the ATS-D antiviral activity) in deionized water.
4. Prepare in-house Sephadex columns (Basic Protocol, steps 5 to 7).
5. Filter 500 μ l of the test product dilutions on the appropriate type of Sephadex columns (see Basic Protocol, steps 11 and 12 for filtering the product).
6. Determine the residual concentration by UV-visible spectrophotometry.
7. Calculate the retention rate according to equation 26.10.3,

$$RR = \left[\frac{(C_i - C_f)}{C_f} \right] \times 100$$

Equation 26.10.3

where

RR is the retention rate

C_i is the initial concentration (before filtration)

C_f is final concentration (after filtration).

See the Anticipated Results section in the Commentary for an example and discussion of retention rates.

Retention rates should be >90% for the tested concentration for that concentration to be considered acceptable to use in assays.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

MTT solution, 5 mg/ml

50 mg MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
10 ml phosphate-buffered saline (PBS; APPENDIX 2A)

Remove the PBS from the refrigerator $\sim 1/2$ hr before making the solution, to better facilitate MTT dissolution (at room temperature). Store at 4° C until the presence of contamination (indicated by cloudiness or change in color) makes it necessary to make a new solution.

Whether contamination occurs will depend on how quickly the solution is used up, as well as general laboratory practices.

NR solution

To make 4 mg/ml NR stock solution:

Dissolve 20 mg of neutral red (NR; Sigma cat. no. 861251) in 5 ml deionized water, and stir well.

Centrifuge 10 min at $405 \times g$, room temperature, to eliminate aggregates. Remove the supernatant and sterilize it by passing through a 0.22- μ l syringe-driven filter. Store up to 15 days at 4°C.

To make 50 μ g/ml working NR solution:

Dilute 625 μ l of NR stock solution in 49.375 ml of DMEM without glutamine and without phenol red (Invitrogen cat. no. 51200-046)

Filter this solution through a 0.45- μ m syringe-driven filter to remove the fine precipitate that can form when NR is mixed with medium. Use immediately.

SDS solution, 10% (w/v)

Dissolve 50 g of sodium dodecyl sulfate (SDS) in 500 ml of phosphate-buffered saline (PBS; *APPENDIX 2A*).

Add 445 μ l of 0.01 M hydrochloric acid.

Mix with a magnetic stirrer, with a light heating, until the mixture becomes clear. Store up to several months at room temperature.

Sephadex G-25 and G-10 suspensions

Prepare a suspension of G-10 or G-25 Sephadex beads by placing 7.50 g of Sephadex in a 250-ml glass bottle containing a magnetic stirrer and adding 250 ml of phosphate-buffered saline (PBS; *APPENDIX 2A*) for a final concentration of 30 g/liter.

An excess of PBS is necessary to reach a neutral pH.

Stir this mix for at least 3 hr at room temperature to allow the gel to swell and the pH to equilibrate to neutral. Sterilize this suspension by autoclaving.

Pipet off excess PBS to obtain a suspension with a ratio of Sephadex/PBS of 1:1 v/v (~75 ml of Sephadex gel suspension). Store this sterile gel suspension up to ~4 weeks, or longer in the absence of contamination or breakdown, at 4°C.

Sephadex media are a range of cross-linked dextran gels of variable porosity, according to the degree of cross-linking. They are manufactured in a bead form and need to be suspended in a buffer.

It is better not to prepare more than 250 ml since the Sephadex columns are made with only 1 ml of the gel suspension. This volume avoids contamination risks due to frequent handling.

Solution A

54.8 ml of aqueous 36.5% (v/v) formaldehyde solution

5.0 g CaCl_2

445.2 ml deionized water

Store up to several months at room temperature.

Solution A is (4% (v/v) aqueous formaldehyde, 1% (w/v) CaCl_2 , and 95% (v/v) deionized water.

Solution B

5.0 ml acetic acid
250.0 ml absolute ethanol
245.0 ml deionized water
Store up to several month at room temperature.

Solution B is 1% (v/v) acetic acid, 50% (v/v) absolute ethanol, and 49% (v/v) deionized water.

COMMENTARY

Background Information

The evaluation of ATS-D antiviral activity still needs some standardization, e.g., the viral model used, contact times, and presence and nature of interfering substances (e.g., FBS) to mimic organic material in which viruses are naturally embedded and protected from the action of an ATS-D. One major concern is the neutralization step. Neutralization can be achieved by different methods. The most common and simplest method proposed in most of the standards is dilution. However, this presents two major disadvantages: (1) the toxicity of many chemicals cannot be neutralized by a 1/10 or even a 1/100 dilution, and (2) only a few viruses can be grown at enough high titers to support such dilutions. Thus, other neutralization methods must be considered. Chemical neutralization offers the advantage of stopping the activity of the tested germicide at a precise time point, ensuring a consistent contact time. Unfortunately, it is restricted to specific chemicals, e.g., neutralization of aldehyde functions by glycine. Another solution, the one developed in this unit, is the gel-filtration method. Many variations can be envisaged, e.g., the type of the gel chosen. European and American standards propose, as well, different substitutive methods (AFNOR, 2007; ASTM, 2004). This last technique offers the possibility of neutralizing a large range of molecules, independently of their functional groups and without extra dilution. However, some drawbacks persist, e.g., difficulty in retaining the smallest molecules such as alcohols, which are widely used as ATS-D.

Critical Parameters

Choice of viral model choice

Several considerations are necessary in the choice of a viral model for evaluating ATS-D activity:

The chosen virus must be known to be responsible for human diseases and for epidemics within institutional structures.

Starting titers must allow the estimation of the performance criteria of 3 to 4 log₁₀ reduction, i.e., virus titers must be at least 10⁶ infectious particles/ml. Moreover, viruses should be titered with a method that estimates infectious viral particles (end point dilution method or plaque assay). Molecular biological methods such as PCR are not amenable to this type of assay. Indeed, the correlation between number of infectious particles and nucleic acids quantity is difficult, or even impossible.

The chosen virus must be known to survive under different conditions, i.e., on environmental surfaces, on hands, in different conditions of temperature and relative humidity, or in fluids, including biological fluids.

Viruses must be carefully selected for their safety to laboratory workers and the environment (level 2 containment).

Choice of Sephadex type

The type of Sephadex is chosen according to the molecular mass of the test product. Two types have been selected, covering a large range of ATS-D: Sephadex G-10, which can retain molecules from 100 to 1000 g/mol, and Sephadex G-25, which can retain molecules from 1000 to 5000 g/mol.

Choice of contact times

Contact times should be representative of ATS-D use under field conditions, and are generally short. Contact times of 5, 15, 30, and 60 min can be applied. Contact times depend on the use of the tested germicide (e.g., hand washing, or surface or instrument disinfection).

Anticipated Results

First of all, retention rates, determined by UV-visible spectrophotometry should be evaluated, and they should be higher than 90% to be acceptable. As shown in the example given in Figure 26.10.7, the specific absorption peaks of chlorhexidine (CHX) are not detectable any more after filtration of solutions at

Figure 26.10.7 UV-visible spectra of chlorhexidine (CHX) alone (heavy blue line) and after filtration (six spectral scans) on Sephadex G-25 columns. Sephadex gel filtrates of CHX solutions of (A) 10^{-2} M, (B) 10^{-3} M, and (C) 10^{-4} M and their unfiltered counterparts are represented. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb2610>.

10^{-3} and 10^{-4} M on Sephadex G-25 columns (Fig. 26.10.7B, C). However, this is not the case for solutions of 10^{-2} M (Fig. 26.10.7A), where an overload of the columns seems to occur (Geller et al., 2009). The 200-nm peak in Figure 26.10.7B, C corresponds to nonspecific absorbance.

Cytotoxicity assays must demonstrate the disappearance of the product's cytotoxicity after filtration on Sephadex columns, as shown in Figure 26.10.8. The results presented here are consistent with the ones obtained with UV-visible spectrophotometry. Indeed, CHX without filtration has an IC_{50} value of $\sim 5 \cdot 10^{-6}$ M after 24 hr; yet, this cytotoxicity disappears after filtration of solutions of 10^{-3} M and at 10^{-4} M. However, after filtration of 10^{-2} M solutions, toxicity recurs with an IC_{50} value of

$\sim 2.5 \times 10^{-5}$ M, which indicates incomplete retention.

After validating these parameters, a virucidal assay can be performed. Final results for CHX activity toward HCoV 229E are presented in Figure 26.10.9. Although not shown here, the difference in viral titers between the positive control (viral suspension without filtration) and the different filtration, neutralization, and cytotoxicity controls was less than 0.5 $\log_{10}$, and there were no morphological change due to cytotoxicity. Results of ATS-D antiviral activity are expressed in $\log_{10}$ differences for each tested concentration and contact time. Depending on the reference used (American or European), CHX either has, or has not, an efficient ATS-D activity toward HCoV 229E (Geller et al., 2009).

Figure 26.10.8 L-132 cell viability determined by the MTT assay for unfiltered and Sephadex G-25 column-filtered chlorhexidine (CHX) solutions. MTT assays were performed using L-132 cells incubated with serial dilutions of unfiltered 10^{-2} M and Sephadex G-25 column-filtered 10^{-2} M, 10^{-3} M, and 10^{-4} M CHX solutions for (A) 24 hr, (B) 48 hr, and (C) 168 hr. The circles in panel A point out the loss of cytotoxicity due to the filtration, which moves the IC_{50} value to the left. The arrow for increasing cytotoxicity indicates that the greater the dilution required to obtain an IC_{50} (moving the IC_{50} to the right), the greater the toxicity of the test product. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb2610>.

Figure 26.10.9 Evaluation of ATS-D activity of chlorhexidine (CHX) on human coronavirus (HCoV 229E) according to European and American standards (Geller et al., 2009).

Until requirements are standardized, it is absolutely necessary to precisely define the test condition and the regulatory document referenced, when evaluating ATS-D antiviral activity of a germicide.

Time Considerations

Preliminary steps should be performed before any virucidal assay. Determination of retention rates of a germicide on Sephadex columns determined by UV-visible spectrophotometry is the simplest, fastest, and least expensive method and should be done first. This procedure can take several hours to establish spectra of the unfiltered product and its spectrophotometric parameters and then the spectra of the same product after filtration on Sephadex columns, which have also must prepared.

If the potential germicide is retained by Sephadex columns, cytotoxicity assays are then performed, determining cytotoxicity of the product and its elimination through gel-filtration. Each assay is repeated three times and takes 9 days (2 days for cell monolayer growth and 7 days of contact time with the drug). This period can be shortened according to the length of the CPE for different viral models.

Finally, the virucidal assay is performed. The time of the experiment itself depends on chosen contact times, but 3 hr can be considered as a mean time for carrying out the assay. The incubation time necessary for the appearance of the viral CPE depends on the nature of the viral model. The HCoV 229E CPE is quite long, ~6 days, but the poliovirus (still recommended in numerous standards) CPE is much shorter and lasts ~3 days.

Literature Cited

- AFNOR (Agence Française de NORmalisation). 2007. Essai virucide quantitatif de suspension pour les antiseptiques et désinfectants chimiques utilisés en médecine humaine - Méthode d'essai et prescriptions (phase 2, étape 1). NF EN 14476+A1. <http://www.afnor.org>.
- ASTM (American Society for Testing and Materials). 2004. Standard Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations. E1482-04.
- Borenfreund, E. and Puerner, J.A. 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol. Lett.* 24:119-124.
- Bradburne, A.F. 1972. An investigation of the replication of coronaviruses in suspension cultures of L132 cells. *Arch. Gesamte Virusforsch.* 37:297-307.

- Geller, C., Fontanay, S., Finance, C., and Duval, R.E. 2009. A new Sephadex-based method for removing microbiocidal and cytotoxic residues when testing antiseptics against viruses: Experiments with a human coronavirus as a model. *J. Virol. Methods* 159:217-226.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55-63.
- Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* 27:493-497.
- Sturman, L.S., Ricard, C.S., and Holmes, K.V. 1990. Conformational change of the coronavirus peplomer glycoprotein at pH 8.0 and 37°C correlates with virus aggregation and virus-induced cell fusion. *J. Virol.* 64:3042-3050.