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bio-protocol www.bio-protocol.org/e2855 Vol 8, Iss 10, May 20, 2018 DOI:10.21769/BioProtoc.2855

Virucidal and Neutralizing Activity Tests for Antiviral Substances and Antibodies

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[Abstract] In a narrow definition, virucidal activity represents the activity by which to interact with and physically disrupt viral particles. In a broad definition, it includes the activity by which to functionally inhibit (neutralize) viral infectivity without apparent morphological alterations of the viral particles. The viral infectivity can be measured in cell culture system by means of plaque assay, infectious focus assay, 50% tissue culture infectious dose ($TCID_{50}$) assay, *etc*. Morphologically, disruption of viral particles can be demonstrated by negative staining electron microscopic analysis of viral particles. In this article, we describe methods to assess virucidal activity in a broad definition.

Keywords: Virucidal activity, Neutralizing activity, Viral particle, Antiviral substance, Antibody, Viral infectivity assay, Negative staining electron microscopic analysis

[Background] Viruses are small intracellular parasites that hijack host cell machinery to replicate their own genome. At the initial step of the viral life cycle, infectious viral particles attach (bind) to particular host proteins, called viral receptors, on the surface of the target cells, followed by viral penetration (internalization and/or fusion) into intracellular compartments of the host cells, where the subsequent steps of the viral life cycle proceed to produce progeny virions (Scheel and Rice, 2013).

Virucidal activity in a narrow definition represents the activity by which to interact with and physically disrupt viral particles. In a broad definition, it includes the activity by which to interact with and functionally inhibit (neutralize) viral infectivity without apparent morphological alterations of viral particles, as in the case of antibody-mediated neutralization.

We have recently reported that an isoform of secreted phospholipase A_2 obtained from snake venom (Chen *et al.,* 2017) and a peptide from scorpion venom (El-Bitar *et al*., 2015) possess strong virucidal activity against viruses that belong to the family *Flaviviridae* by targeting the lipid bilayer of the viral envelope, which is acquired from the endoplasmic reticulum membrane of the host cells. It was also reported that one of the host defense peptides from the skin of the South Indian frog has a strong virucidal activity against H1 hemagglutinin-bearing human influenza virus by targeting the conserved stalk of H1 hemagglutinin (Holthausen *et al*., 2017). In this article, we describe a number of useful methods by which to measure virucidal activity in a broad definition, such as plaque assay, infectious focus assay, 50% tissue culture infectious dose ($TCID_{50}$) assay and negative staining electron

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microscopic analysis.

Materials and Reagents

1. Disposable tips
   a. 10 µl capacity (Thermo Fisher Scientific, Molecular BioProducts, catalog number: 3510-05)
   b. 200 µl capacity (Thermo Fisher Scientific, Molecular BioProducts, catalog number: 3900)
   c. 1 ml capacity (FUKAEKASEI and WATSON, catalog number: 110-502C)
2. 100 mm culture dish (Corning, Falcon®, catalog number: 353003)
3. 6-well culture plate (Corning, Falcon®, catalog number: 353046)
4. 12-well culture plate (Corning, Falcon®, catalog number: 353043)
5. 24-well culture plate (Corning, Falcon®, catalog number: 353047)
6. 96-well culture plate (Corning, Falcon®, catalog number: 353072)
7. 1.5 ml microcentrifuge tube (FUKAEKASEI and WATSON, catalog number: 131-715C)
8. 15 ml tube (Corning, Falcon®, catalog number: 352196)
9. Cover slip (13 x 13 mm; Matsunami Glass, catalog number: C013001)
10. Microscope slide (Matsunami Glass, catalog number: S2215)
11. Disposable serological pipette
    a. 1 ml capacity (Corning, Falcon®, catalog number: 356521)
    b. 5 ml capacity (IWAKI, catalog number: 7153-005)
    c. 10 ml capacity (IWAKI, catalog number: 7154-010)
12. Filter paper (ATTO, catalog number: CB-06A-20A)
13. Viruses (Chen *et al.,* 2017):
    a. Hepatitis C virus (HCV, J6/JFH-1 strain)
    b. Dengue virus (DENV, Trinidad 1751 strain)
    c. Japanese encephalitis virus (JEV, Nakayama strain)
    d. Influenza A virus (FLUAV, A/Udorn/307/72[H3N2])
    e. Sendai virus (SeV, Fushimi strain)
    f. Herpes simplex virus type 1 (HSV-1, CHR3 strain)
    g. Coxsackievirus B3 (CV-B3, Nancy strain)
    h. Vesicular stomatitis New Jersey virus (VSNJV)
    i. Sindbis virus (SINV)
    j. Encephalomyocarditis virus (EMCV, DK-27 strain)
14. Huh7it-1 cells (Apriyanto *et al.,* 2016)
    Note: Huh7it-1 cells are susceptible to all viruses described above (HCV, DENV, JEV, FLUAV, SeV, HSV-1, CV-B3, VSNJV, SINV and EMCV).
15. Vero cells (ATCC, catalog number: CCL-81)
    Note: Vero cells are susceptible to all viruses described above (HCV, DENV, JEV, FLUAV, SeV,

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HSV-1, CV-B3, VSNJV, SINV and EMCV).

16. Antibodies
    a. Rabbit polyclonal antibody against DENV PrM (Gene Tex, catalog number: GTX128093)
    b. Mouse monoclonal antibody against DENV type 2 (3H5; Hotta *et al*., 1984)
    c. UV-inactivated anti-HCV human serum (Bungyoku *et al.*, 2009)
    d. Anti-HCV E2 neutralizing antibody #55 (Shimizu *et al*., 2013)
    e. Rabbit antiserum against CV-B3 (DENKA SEIKEN, catalog number: 300638)
    f. Rabbit antiserum against FLUAV (Shimizu *et al.,* 1985)
    g. Rabbit antiserum against SeV (Hayashi *et al.,* 1991)
    h. Rabbit antiserum against HSV-1 (Hayashi *et al.,* 1986)
    i. FITC-conjugated goat anti-human IgG (MEDICAL & BIOLOGICAL LABORATORIES, catalog number: 104AG)
    j. Alexa Flour488-conjugated goat anti-mouse IgG (Thermo Fisher Science, catalog number: A-11001)
    k. Alexa Flour488-conjugated goat anti-rabbit IgG (Thermo Fisher Science, catalog number: A-11008)
17. High glucose Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, catalog number: 044-29765)
18. Phospholipase A_2 from *Naja mossambica* snake venom (Sigma-Aldrich, catalog number: P7778) (Chen *et al.,* 2017)
19. Trypsin-EDTA solution (Wako Pure Chemical Industries, catalog number: 209-16941)
20. Crystal violet (Wako Pure Chemical Industries, catalog number: 038-04862)
21. MEM with non-essential amino acids (Thermo Fisher Science, Gibco™, catalog number: 10370021)
22. Fetal bovine serum (FBS; Biowest, catalog number: S1820)
23. Penicillin-Streptomycin solution (Wako Pure Chemical Industries, catalog number: 168-23191)
24. Methyl Cellulose 4000 (Wako Pure Chemical Industries, catalog number: 136-02155)
25. 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries, catalog number: 163-20145)
26. Formaldehyde solution (AppliChem, catalog number: A3592,0500)
27. Gram Hacker's Stain Solution I (MUTO PURE CHEMICALS, catalog number: 41162)
28. Triton X-100 (Wako Pure Chemical Industries, catalog number: 169-21105)
29. Bovine serum albumin (BSA; Wako Pure Chemical Industries, catalog number: 015-21274)
30. Hoechst 33342 solution (Thermo Fisher Scientific, Molecular Probes, catalog number: H3570)
31. Vectashield mounting solution (Vector Laboratories, catalog number: H-1000)
32. Formvar-coated nickel grid (Electron Microscopy Sciences, catalog number: FF200-Ni)
33. 2% phosphotungstic acid (Wako Pure Chemical Industries, catalog number: 582-66852)
34. Sodium chloride (NaCl; Wako Pure Chemical Industries, catalog number: 191-01665)
35. Potassium chloride (KCl; Wako Pure Chemical Industries, catalog number: 163-03545)

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36. Disodium Hydrogen Phosphate ($Na_2HPO_4 \cdot 12H_2O$, NACALAI TESQUE, catalog number: 31722-45)
37. Potassium phosphate monobasic (KH_2PO_4; Wako Pure Chemical Industries, catalog number: 169-04245)
38. Complete medium for cell culture (see Recipes)
39. 10x phosphate-buffered saline (PBS[-]) (see Recipes)
40. Overlay medium (see Recipes)

Equipment

1. Micropipette (Gilson, P20, P200, P1000)
   a. P20 (Gilson, catalog number: F123600)
   b. P200 (Gilson, catalog number: F123601)
   c. P1000 (Gilson, catalog number: F123602)
2. Multichannel micropipette (10-100 µl) (Eppendorf, catalog number: 3125000036)
3. Hemocytometer chamber (*e.g.*, Erma, catalog number: 03-303-1)
4. Biosafety cabinet (*e.g.*, PHC, model: MHE-S1301A2)
5. CO_2 incubator (*e.g.*, PHC, model: MCO-20AIC)
6. Autoclave (*e.g.,* TOMY DIGITAL BIOLOGY, model: SX-500)
7. Refrigerated tabletop centrifuge (*e.g.*, Eppendorf, model: Centrifuge 5424)
8. Vortex (*e.g.*, Scientific Industries, model: Vortex-Genie 2)
9. -80 °C freezer (*e.g.,* PHC, model: MDF-384)
10. Inverted microscope (*e.g.*, Olympus, model: CKX53)
11. Fluorescent microscope (*e.g.*, ZEISS, model: Axio Vert. A1)
12. Multilabel Plate Counter (PerkinElmer, model: 1420 ALBOSX)
13. Transmission electron microscope (Hitachi, model: HT7700 TEM)

Procedure

Part I: Virucidal and neutralization reactions (Figure 1)

1. Prepare serial dilutions of PLA_2 (or anti-HCV E2 neutralizing antibody) in DMEM in 1.5 ml tubes.
   Note: In our study, the PLA_2 concentrations in each tube are 2, 20, 200 and 2,000 ng/ml and the anti-HCV E2 antibody concentrations are 0.2, 2, 20, 200 and 2,000 µg/ml.
2. Dilute HCV stock in DMEM to a concentration of 2×10^4 focus-forming unit (FFU) per 200 µl in a 15 ml tube.
3. Mix 100 µl of each dilution of PLA_2 (or anti-HCV E2 antibody) with 100 µl of the HCV solution in a 1.5 ml tube and gently vortex.

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Note: After being mixed with the HCV solution, the final concentration of PLA_2 (or anti-HCV E2 antibody) in each tube is 1, 10, 100 and 1,000 ng/ml (or anti-HCV E2 antibody: 0.1, 1, 10, 100 and 1,000 μg/ml), respectively.

4. Incubate for 1 h at 37 °C.
5. Determine remaining viral infectivity by fluorescent antibody (FA) method, plaque assay or $TCID_{50}$ assay. See Part II for detailed procedure.

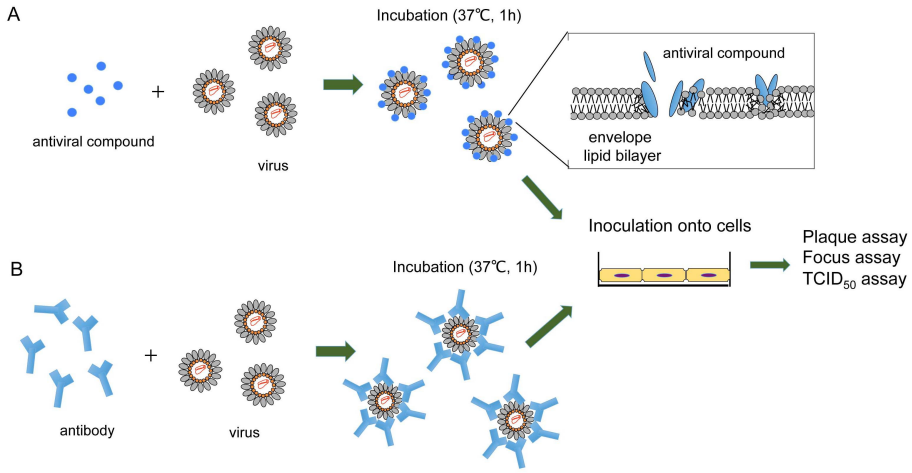

Figure 1. Illustration of the flowchart of virucidal and neutralizing activity tests. A fixed amount of virus is incubated with serial dilutions of an antiviral compound (*e.g.*, PLA_2) (A. virucidal activity test) or an antibody (B. neutralizing activity test) at 37 °C for 1 h before inoculation to cultured cells. Antiviral activities of the test samples are assessed by appropriate procedures, such as plaque assay, focus assay and $TCID_{50}$ assay.

Part II: Determination of viral infectivity

Virus titers are expressed as plaque-forming unit (PFU)/ml, focus-forming unit (FFU)/ml, cell-infectious unit (CIU)/ml and 50% tissue culture infectious dose ($TCID_{50}$)/ml.

A. Plaque assay (for SINV, VSNJV and EMCV)

Plaque assay is one of the standard methods to determine infectious titers of viruses that cause strong cytopathic effect (CPE). A confluent monolayer of cells are infected with virus at various dilutions and cultured in a solid or semisolid overlay medium containing agarose or methylcellulose. This minimizes subsequent viral spread only to neighboring cells in the monolayer. Virus-infected cells undergo cell death by CPE and an area where a group of dead cells have detached is called a plaque (Figure 2). The infectivity titer is expressed as PFU/ml.

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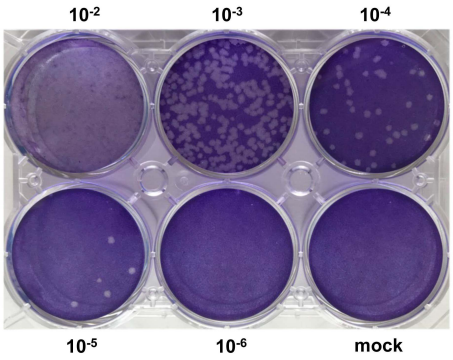

Figure 2. Representative image of a plaque assay plate of VSNJV. Plaques are visualized by staining with Gram Hacker's Stain Solution I. Cell monolayers infected with serial 10-fold dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of VSNJV and mock-infected are shown.

1. Seed Huh7it-1 cells (5 x 10^5 cells) in a final volume of 3 ml of complete medium in each well of a 6-well plate.
2. Incubate for 20-24 h at 37 °C in a 5% CO_2 incubator. (Cells should be 90-100% confluent.)
3. To prepare virus solution, dilute 100 µl of virus-PLA_2 (or neutralizing antibody) mixture from Part I in 900 µl culture medium (10^{-1} dilution) and make subsequent 10-fold serial dilutions of the virus (10^{-2} to 10^{-6} dilution).
4. Remove culture medium from each well.
5. Inoculate virus solution (10^{-2} to 10^{-6} dilution) to the cells (300 µl/well).
6. Incubate for 1 h at 37 °C in a 5% CO_2 incubator.
7. Remove the inoculum and rinse with medium (3 ml/well).
8. Gently add overlay medium containing 1% methylcellulose (3 ml/well).
   Note: Overlay medium contains 2% FBS.
9. Incubate at 37 °C in a 5% CO_2 incubator for 2 to 4 days – VSNJV and EMCV(2 days), SINV (3 to 4 days).
10. Aspirate the overlay medium.
    Note: As overlay medium is viscous, aspirate it slowly.
11. Fix the cells with 10% formaldehyde solution in PBS(-) (1 ml/well) for 20 min at room temperature.
12. Add Gram Hacker's Stain Solution I (1 ml/well).
13. Incubate for 30 min.
14. Discard the staining solution, rinse with PBS (3 ml/well) and dry the plate.
15. Count the number of plaques (Figure 2).

B. Focus forming assay by FA method (for HCV, DENV, JEV, FLUAV, SeV, HSV-1, CV-B3)

Focus forming assay is particularly useful to measure infectivity of viruses that do not cause strong CPE. Virus-infected cells are incubated for a duration of a single round of the viral life cycle (one-step growth). Virus-infected cells are detected by an FA method using antibodies specific to

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the respective viral antigens (Figure 3). The infectivity titer is expressed as FFU/ml or CIU/ml.

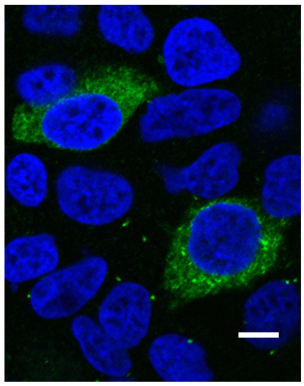

Figure 3. Visualization of virus-infected cells by FA method. Virus-infected cells are stained in green. Nuclei are stained in blue. Scale bar = 10 μm.

1. Seed Huh7it-1 cells (1 x 10^5 cells per 1ml) in each well of a 24-well plate containing a sterile glass slip.
2. Incubate for 20-24 h at 37 °C in a 5% CO_2 incubator.
3. To prepare virus solution, dilute 100 μl of virus-PLA_2 (or neutralizing antibody) mixture from Part I in 900 μl culture medium (10^{-1} dilution) and make subsequent 10-fold serial dilutions of the virus (10^{-2} to 10^{-6} dilution).
4. Remove culture medium from each well.
5. Inoculate virus solution to the cells (200 μl/well).
6. Incubate for 1 h at 37 °C in a 5% CO_2 incubator.
7. Remove the inoculum and rinse with medium (1 ml/well).
8. Add complete medium (500 μl/well).
9. Incubate for 24 h at 37°C in a 5% CO_2 incubator.
10. Remove culture medium and rinse twice with PBS (200 μl/well).
11. Fix the cells with 200 μl of 4% paraformaldehyde solution.
12. Incubate for 20 min at room temperature.
13. Rinse cells three times with PBS (200 μl/well).
14. Add 200 μl of 0.1% Triton X-100 in PBS.
15. Incubate for 20 min at room temperature.
16. Add 200 μl of 1% BSA in PBS.
17. Incubate for 1 h at room temperature.
18. Prepare primary antibodies in PBS.

    Note: We use UV-inactivated anti-HCV human serum (1:500 dilution), rabbit polyclonal antibody against DENV PrM (1:500 dilution), mouse monoclonal antibody against DENV type 2 (1:500 dilution), rabbit antiserum against CV-B3 (1:100 dilution), rabbit antiserum against FLUAV (1:1,000 dilution), rabbit antiserum against SeV (1:1,000 dilution) and rabbit antiserum against HSV-1 (1:1,000 dilution).

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19. Remove 1% BSA solution.
20. Add primary antibodies against the respective viruses (200 µl/well).
21. Incubate for 1 h at room temperature.
22. Prepare FITC-conjugated secondary antibodies in PBS (1:800 dilution, 200 µl/well).
23. Rinse cells three times with PBS.
24. Add secondary antibodies (200 µl/well).
25. Incubate for 1 h at room temperature in a dark box.
26. Rinse cells three times with PBS (500 µl/well).
27. Add Hoechst 33342 solution (1 mg/ml) in PBS (200 µl/well).
28. Incubate for 15 min at room temperature.
29. Rinse cells three times with PBS (500 µl/well).
30. Mount the glass coverslip on a microscope slide using Vectashield mounting solution.
31. Observe under a fluorescence microscope and count the number of virus-infected (stained in green) cells (Figure 3).

C. $TCID_{50}$ assay (for DENV, JEV, HSV-1, SINV, VSNJV and EMCV)

$TCID_{50}$ assay can be used to measure infectivity of viruses that cause strong CPE. $TCID_{50}$ represents a dilution of virus that makes 50% of the test wells show cell detachment (Figure 4). The infectivity titer is expressed as $TCID_{50}$/ml.

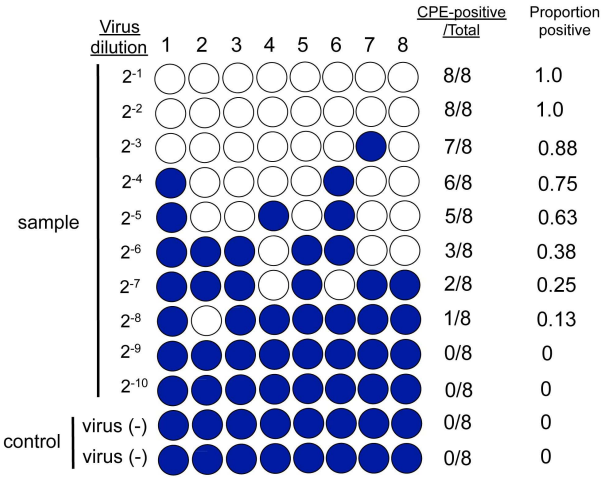

Figure 4. Schematic presentation of a result of $TCID_{50}$ assay. White circles indicate the wells in which the cells have undergone cell death to detach from the plastic plate due to strong CPE. Blue circles indicate the wells in which the cells remain intact to be stained with crystal violet.

1. Seed Vero cells (2 x 10^4 cells in 100 µl) in complete medium in each well of a 96-well plate.
2. Incubate for 20-24 h at 37 °C in a 5% CO_2 incubator.
3. To prepare virus solution, dilute 100 µl of virus-PLA_2 (or neutralizing antibody) mixture from

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Part I in 100 µl culture medium (2^{-1} dilution) and make subsequent 2-fold serial dilutions of the virus (2^{-2} to 2^{-10} dilution).

4. Remove culture medium from each well.
5. Inoculate the virus solution to the cells (100 µl/well)
   Note: Aliquots of the same sample should be inoculated to 4 to 8 wells.
6. Incubate for 2 h at 37 °C in a 5% CO_2 incubator.
7. Remove the inoculum and add overlay medium.
   Note: Overlay medium contains 2% FBS.
8. Incubate at 37 °C in a 5% CO_2 incubator.
   Note: DENV (5 to 8 days), JEV (5 to 6 days), HSV-1 and SINV (4 days), VSNJV and EMCV (2 days).
9. Count the number of wells with or without CPE under an inverted microscope.
10. Remove culture medium and rinse with PBS (100 µl/well).
11. Fix the cells with 10% formaldehyde solution in PBS(-) (100 µl/well) for 20 min at room temperature.
12. Rinse the cells with PBS (100 µl/well).
13. Add crystal violet solution to each well (100 µl /well).
14. Incubate for 10 min at room temperature.
15. Discard the crystal violet solution, rinse with PBS (100 µl/well), and dry the plate.
16. Count the number of wells with or without CPE by the naked eye (Figure 4).

D. Negative staining electron microscopic analysis (HCV, DENV, JEV, FLUAV, SeV, HSV-1, CV-B3, SINV, VSNJV and EMCV)

   Negative-staining electron microscopy of viruses requires adequate concentrations of virus particle (> 10^8/ml).

   1. Add 5 to 10 µl of a purified virus solution onto a Formvar-coated nickel grid.
   2. Wait for 5 min at room temperature so that the viral particles are adsorbed to the grid.
   3. Remove the solution using the tip of a strip of filter paper.
   4. Add 5 to 10 µl of 2% phosphotungstic acid in distilled water onto the grid and incubate for 2 min.
   5. Remove the solution using a piece of filter paper.
   6. Add 10 µl of PBS to wash the grid. (Repeat the Steps D5 and D6 three times.)
   7. Air-dry the grid.
   8. Observe under a transmission electron microscope.

Data analysis

Determination of 50% inhibitory concentration (IC_{50}): IC_{50} of an antiviral substance against a given virus can be obtained based on the percent inhibition of viral infectivity mediated by serial dilutions

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of the antiviral substance. This model can be used for typical dose-response curves and receptor-ligand binding assays in pharmacological studies.

1. Plot data using ImageJ software and draw a sigmoid curve (Figure 5).
2. Four parameter (A, B, C, D) logistic equation is obtained.
   y = D + (A - D)/(1 + (x/C)^B)
   A = minimum asymptote; B = slope factor; C = concentration corresponding to the response midway between A and D; and D = maximum asymptote.
3. Parameter C is calculated as the estimate of IC_{50}.

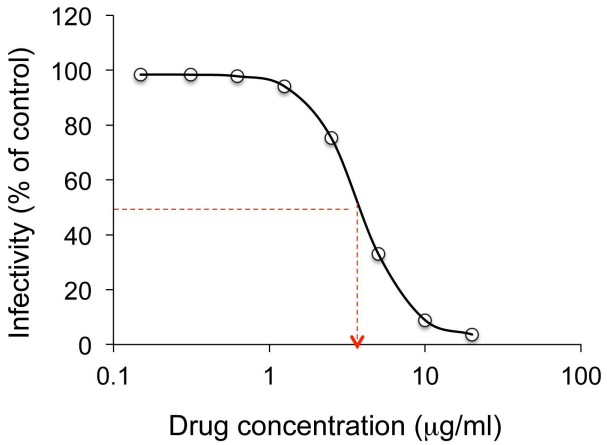

Figure 5. A typical sigmoid curve showing inhibition of viral infectivity by serial dilutions of an antiviral drug. The red line shows the IC_{50} value of the drug.

Notes

Manipulation of infectious viruses requires special biosafety laboratories. The degree of biocontainment is revised in accord with laws in the country/region where research will be conducted.

Recipes

1. Complete Dulbecco's modified Eagle's medium (complete medium)
   1x non-essential amino acids
   100 U/ml penicillin and streptomycin
   10% fetal bovine serum (FBS; heat-inactivated at 56 °C for 30 min)
2. 10x phosphate buffered saline (PBS)
   a. Dissolve NaCl (80 g), KCl (2 g), $Na_2HPO_4 \cdot 12H_2O$ (28.8 g) and KH_2PO_4 (2.4 g) in 800 ml H_2O
   b. Adjust volume to 1 L with dH_2O
   c. Autoclave at 121 °C for 20 min. Dilute to 1x with distilled water

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3. Overlay medium
   a. Heat DMEM (500 ml) at 100 °C in an autoclavable glass bottle
   b. Put a magnet bar in the bottle
   c. Add methylcellulose (5 g) to the hot DMEM (500 ml) and stir well
      Note: Methylcellulose is not yet dissolved (turbid).
   d. Autoclave at 121 °C for 20 min
   e. When the temperature of the 1% methylcellulose-containing DMEM drops to about 60 °C (the solution is still turbid), rapidly cool the bottle in ice bath while stirring the content with a magnetic stirrer. Methylcellulose starts to dissolve and the medium will become translucent
   f. Add FBS and other necessary reagents for cell culture

Acknowledgments

This work was supported in part by grants-in-aid for Research on Viral Hepatitis from the Ministry of Health, Labour and Welfare, and from the Japan Agency for Medical Research and Development (AMED). This work was also supported in part by a grant-in-aid for Special Research on Dengue Vaccine Development from Tokyo Metropolitan Government. This protocol was adapted from procedures published in Chen *et al.* (2017). The authors do not have any conflicts of interest or competing interests to declare.

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