Dengue Virus: Isolation, Propagation, Quantification, and Storage

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ABSTRACT

Dengue is a disease caused by infection with one of the four dengue virus serotypes (DENV-1, -2, -3, and -4). The virus is transmitted to humans by *Aedes* sp. mosquitoes. This enveloped virus contains a positive single-stranded RNA genome. Clinical manifestations of dengue can have a wide range of outcomes varying from a mild febrile illness to a life-threatening condition. New techniques have largely replaced the use of DENV isolation in disease diagnosis. However, virus isolation still serves as the gold standard for detection and serotyping of DENV and is common practice in research and reference laboratories where clinical isolates of the virus are characterized and sequenced, or used for a variety of research experiments. Isolation of DENV from clinical samples can be achieved in mammalian and mosquito cells or by inoculation of mosquitoes. The experimental methods presented here describe the most common procedures used for the isolation, serotyping, propagation, and quantification of DENV. *Curr. Protoc. Microbiol.* 27:15D.2.1-15D.2.24. © 2012 by John Wiley & Sons, Inc.

Keywords: dengue • infection • titration • plaque assay • flow cytometry • mosquito inoculation • immunofluorescence

INTRODUCTION

Dengue virus (DENV), a flaviviridae, comprises four related but antigenically distinct DENV serotypes (DENV-1, -2, -3, and -4) (Westaway and Blok, 1997). The positive single-stranded RNA genome (~10 kb) of DENV encodes three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Mukhopadhyay et al., 2005). DENV is transmitted to humans through the bite of infected *Aedes* sp. mosquitoes (Gubler, 1998). Individuals infected with DENV can be asymptomatic or exhibit a wide range of illnesses going from a mild non-specific febrile syndrome to severe dengue (World Health Organization, 2009). Laboratory diagnosis of dengue is best made during the acute phase of the disease (first 5 days with symptoms), when the virus is present in the blood and when detecting the virus, or virus components such as the viral RNA or antigen, is feasible (Vorndam and Kuno, 1997; World-Health-Organization, 2009). IgM antibodies for the virus rise at approximately day 4 of illness. Infection by DENV confers long-term immunity to the infecting serotype but not to the others. Therefore, people in dengue-endemic countries are likely infected multiple times over their lifetime. Secondary infection with another serotype often results in higher viremia and risk for developing severe illness (Halstead, 1970, 1988; Libraty et al., 2002; Vaughn et al., 2000; Wang et al., 2003).

This unit describes the methods utilized for isolation (Basic Protocols 1 and 2), immunofluorescence detection (Basic Protocol 3), titration (Basic Protocol 4 and Alternate Protocol 1), propagation (Basic Protocol 5), and purification/concentration (Basic Protocol 6) of DENV-1 to -4. Procedures for the maintenance and storage of C6/36 and Vero cells are also provided (Supporting Protocols 1 to 4). DENV does not reach titers

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as high as those of other viruses or as high as desired for their use in biological assays. Although DENV grows in many different cell lines derived from both vertebrate and invertebrate cells, the most common cell lines used for virus isolation are *Aedes albopictus* mosquito C6/36 cells; titration and propagation of the virus are usually achieved also in C6/36 or in *Cercopithecus aethiops* (African green monkey) kidney epithelial cells (Vero cells). Confirmation of virus isolation and DENV serotype can be achieved through immunofluorescence assays. Detection of DENV in samples with low viremia or viruses that do not grow well in culture can be achieved through confirmation of virus infectivity in mosquitoes. DENV detection and quantitation/titration of DENV can be achieved through direct visualization of plaques in agarose overlays of infected cells in culture or with the use of flow cytometry of infected cells after immunostaining.

CAUTION: According to the Centers for Disease Control manual *Biosafety in Microbiological and Biomedical Laboratories* (*http://www.cdc.gov/biosafety/publications/bmbl5/index.htm*), DENV is classified as a Biosafety Level 2 (BL-2) virus. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Handling and use of DENV should include the use of a class II biological safety cabinet (BSC). Infectious DENV may be decontaminated by applying a 10% bleach solution. The virus-inactivated liquids treated with this solution may be discarded in a laboratory sink. Other DENV-contaminated materials (e.g., plasticware) should be placed in an autoclavable bag and autoclaved before discarding as regular trash. The surfaces of benches, BSC cabinets, and glassware used in experiments with infectious DENV should be decontaminated by applying a 10% bleach solution for 10 min and then cleaning the area with a 70% ethanol solution.

BASIC PROTOCOL 1

DENGUE VIRUS ISOLATION FROM DIAGNOSTIC SAMPLES IN C6/36 CELLS

DENV can be derived from blood, serum, or plasma samples obtained from viremic people by incubating the virus in *Aedes albopictus* cells (C6/36) in culture. Virus titers in these diagnostic samples are usually unknown; hence, a precise amount of inoculum for viral infection cannot be determined. To overcome this limitation, and to avoid diluting the virus excessively, virus isolation is first carried out at a low scale, using a relatively small amount of the original infected human sample to inoculate C6/36 cells previously grown in 16-mm tissue culture tubes. All procedures should be carried out using sterile techniques and in a biosafety cabinet.

Materials

C6/36 cells (see Support Protocol 1)
Phosphate-buffered saline (PBS) without calcium or magnesium (*APPENDIX 2A*)
0.05% trypsin-EDTA: 0.05% (w/v) trypsin in 1 mM EDTA
DMEM-5 and DMEM-2 media (see recipes)
7.5% (w/v) sodium bicarbonate (Gibco, cat. no. 25080-094)
Fetal bovine serum (FBS)

Incubator (33°C)
16 × 125-mm screw-capped tissue culture tubes
Slanted tube racks, 5° and 20° angles
0.02-µm × 13-mm filter (optional)
Pillow
Transfer pipet

Cell preparation

One 75-cm^2 flask, with a fully confluent monolayer of C6/36 cells, seeds approximately forty 16 × 125-mm tissue culture tubes. (Estimated concentration is 5×10^5 cells per ml).

1. Discard old growth medium from one 75-cm^2 flask of C6/36 cells and wash flask twice with PBS.
2. Gently knock flask to detach cell monolayer.
3. Add 1.5 ml of 0.05% trypsin-EDTA and incubate at 33°C for 2 min.
4. Resuspend cells in 10 ml of DMEM-5 medium, place in a 16 × 125-mm screw-capped tissue culture tube, and centrifuge suspension 5 min at 500 × *g*, room temperature.
5. Discard medium and resuspend cell pellet in 120 ml of DMEM-5.
6. Dispense 3 ml of the cell suspension into a screw-capped 16 × 125-mm tissue culture tube (40 tubes total).
7. Incubate in a slanted rack (20° angle) at 33°C for 24 hr.

   Tubes should have 90% cell confluence; if confluence is not achieved after 24 hr, extend the incubation period.

Cell infection

8. Discard growth medium from culture tube and add 1 ml of fresh DMEM-2 medium warmed to 33°C.
9. Add 50 µl of the serum sample to the culture tube and incubate at a small slant angle (5°) for 1 hr in a 33°C incubator.
10. Add 2 ml of DMEM-2 medium to the culture tube and incubate at a 20° slant angle for 5 days in a 33°C incubator.

    For serum samples that are not sterile, virus isolation is still possible if the serum is first filtered using a 0.2-µm, 13-mm diameter filter.

    pH adjustment is usually necessary around the third day of incubation. Add small amounts of 7.5% sodium bicarbonate stock solution (approx. 20 µl) to the culture tube until the medium regains its original color.

Virus harvest

11. Readjust the pH level using 7.5% sodium bicarbonate and add 690 µl (23% v/v) FBS to the culture tube.
12. Knock down cell monolayer by tapping culture tube on a pillow.
13. Centrifuge culture tube 5 min at 500 × *g*, 4°C.
14. Extract supernatant from tube using a transfer pipet without disturbing the cell pellet.
15. Transfer supernatant to a labeled vial or prepare aliquots of the virus harvest.
16. Store harvested virus at −70°C.

    Cell pellet can be discarded or used for an immunofluorescence assay (Basic Protocol 3).

DENGUE VIRUS INOCULATION OF MOSQUITOES IN VIVO

BASIC PROTOCOL 2

The mosquito inoculation technique is a highly sensitive and affordable method for isolating DENV. The technique requires skillful labor and the existence of facilities to grow and maintain *Aedes aegypti* or *Toxorhynchites amboinensis* mosquitoes. The latter is not a natural vector of dengue, nor is it able to transmit the virus, and it is considerably

larger than *Aedes* sp., providing a safe and efficient in vivo system for DENV rescue. It is ideal for rescuing viruses from samples of patients with low viremia, viral isolates with a very low titer, or viruses with low infectivity in cell cultures. Compared to other isolation techniques such as cell culture, mosquito inoculation requires a small volume of sample. Therefore, this technique is a viable option on occasions where there is only a small quantity of sample from which the virus is being isolated. This procedure is based on previously published studies (Gubler et al., 1984; Rosen and Gubler, 1974; Rosen et al., 1985).

Materials

Adult *Aedes aegypti* or *Toxorhynchites amboinensis* mosquitoes (15 per sample plus 20 for controls)
DENV-containing sample (e.g., serum, plasma, or cell culture supernatant from infected cells)
10% (w/v) sucrose in dH_2O
Acetone, chilled to –20°C
BA-1 diluent (see recipe)

Borosilicate glass tubing (internal diameter 0.4 mm; outside diameter 0.7 to 1.0 mm; 30 in. length)
Bunsen burner
Jeweler's forceps
Rubber stamp with lines marked 1 mm apart
Injection apparatus [see Rosen and Gubler (1974) for assembly details]
  Metal holder for glass needle
  Plastic tubing [internal diameter 3/16 in., wall thickness 1/16 in., outside diameter 5/16 in. (7.9 mm)]
  50-ml syringe
  3-way stopcock
Dissecting (stereo) microscope
28°C incubator
Cotton balls
Scalpel
Poly-lysine–treated glass slides
2.0 ml microcentrifuge tubes, snap-cap
4.5-mm premium-grade copper or stainless steel BBs
TissueLyser mixer mill or vortex

Inoculation

1. Select 15 adult *Aedes aegypti* or *Toxorhynchites amboinensis* mosquitoes for each sample to be tested and 10 mosquitoes each for negative and positive controls.

2. Prepare a capillary needle by drawing the borosilicate glass tubing to a point after flaming with a Bunsen burner and break the tip with forceps to form a needle (Figure 15D.2.1A-D). Mark the capillary needle with divisions using a rubber stamp (Figure 15D.2.1E).

   If the indicated size of borosilicate glass tubing is used, 1 mm is equivalent to 0.17 μl.

3. Tranquilize the mosquitoes by putting them in a box of ice (~0°C) for 10 min.

4. Attach the borosilicate glass needle to a metal holder, attach the metal holder to plastic tubing, and attach the other end of the plastic tubing to a syringe with a 3-way stopcock (Figure 15D.2.1F). Fill the needle with inoculum.

5. While looking through a stereomicroscope, inoculate the mosquitoes intrathoracically with 0.17 μl of the sample by applying gentle pressure on the syringe.

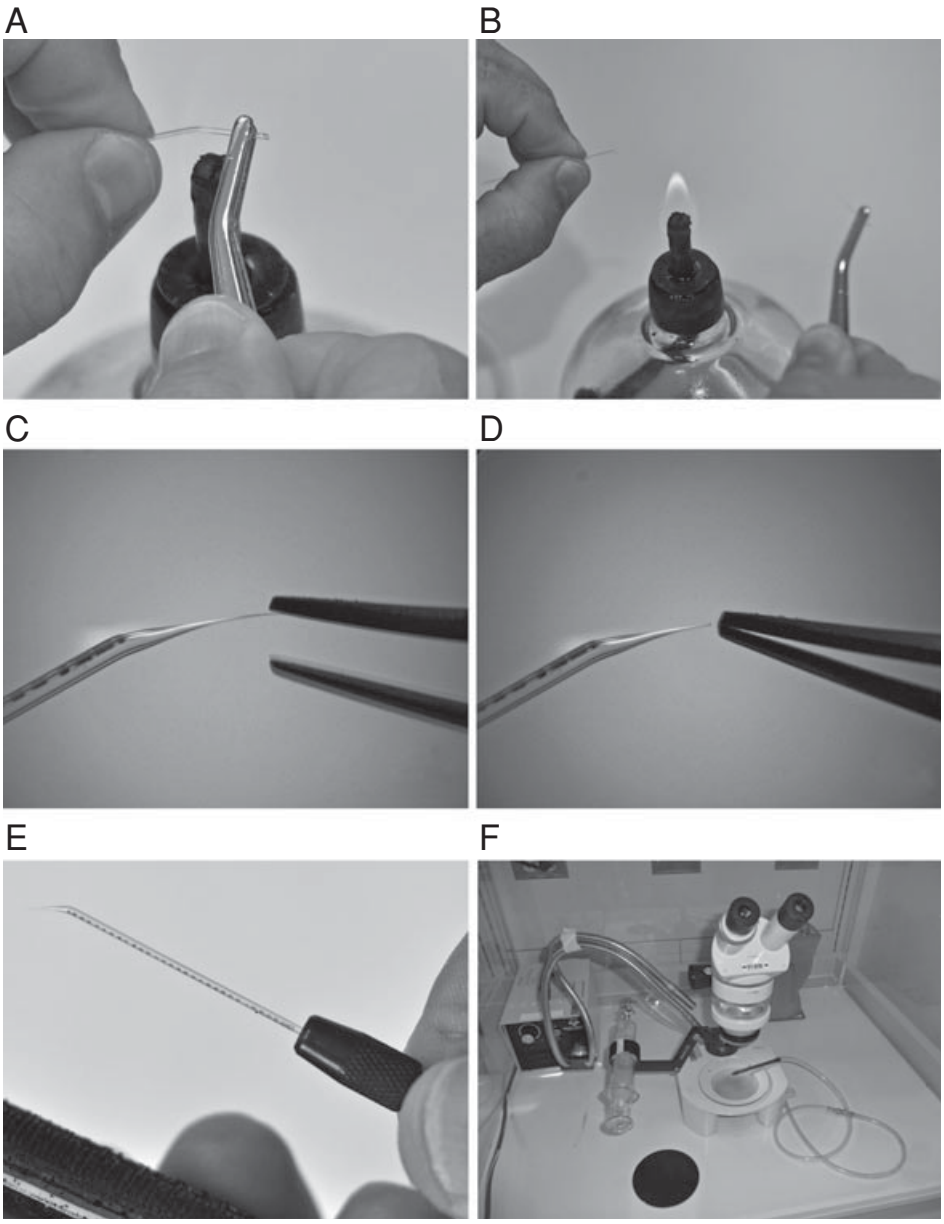

Figure 15D.2.1 Photograph sequence demonstrating how to construct a mosquito inoculation needle out of a fine borosilicate tube. The tube is placed in the flame; as soon as it becomes malleable, it is pulled apart (**A** and **B**). Needle tip refinement is achieved under a stereomicroscope by using forceps to break the closed end of the tube (**C** and **D**). The needle is then stamped with the measuring guides (**E**) and attached to the syringe system in the stereomicroscope (**F**).

Toxorhynchites should be inoculated with 0.34 μl of inoculum through the soft cuticle between the sclerites (Figure 15D.2.2).

Aedes aegypti can be inoculated at the membranous area anterior to the mesepisternum and below the spiracle (females) or through the neck membrane (males and females) (Figure 15D.2.3).

6. Maintain the mosquitoes at 28°C for 14 days, feeding them daily with cotton balls soaked in 10% sucrose in dH_2O.

7. Check mosquitoes daily and remove any dead insects.

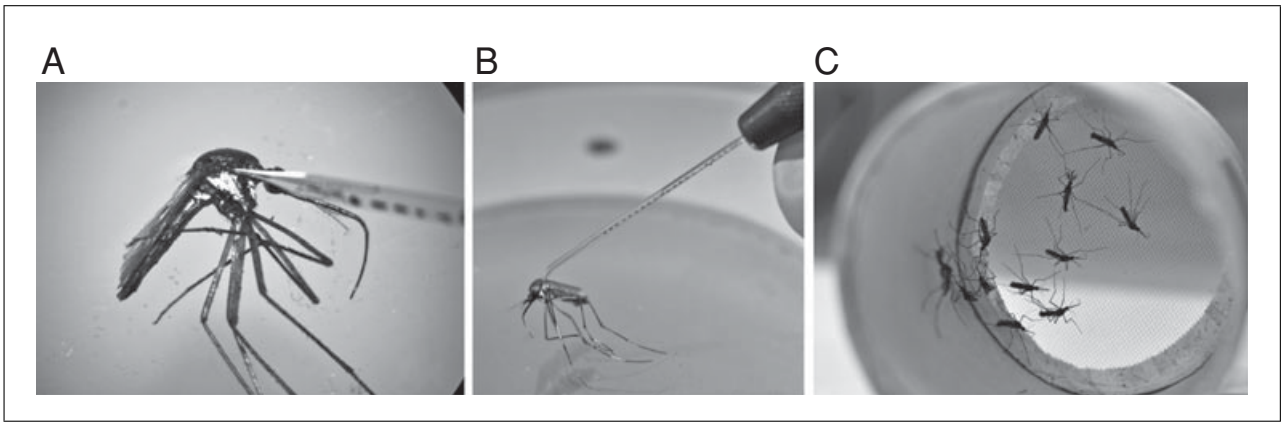

Figure 15D.2.2 Photograph sequence of an intrathoracic inoculation of a *Toxorhynchites amboinensis* mosquito. A virus inoculum of 0.34 μl is injected through the soft cuticle between the sclerites of the mosquito (**A**). The inoculated mosquito is removed from the microscope and placed in a container for incubation using the needle's grasp (**B**). Inoculated mosquitoes are stored at 28°C for 14 days to obtain optimal virus replication (**C**).

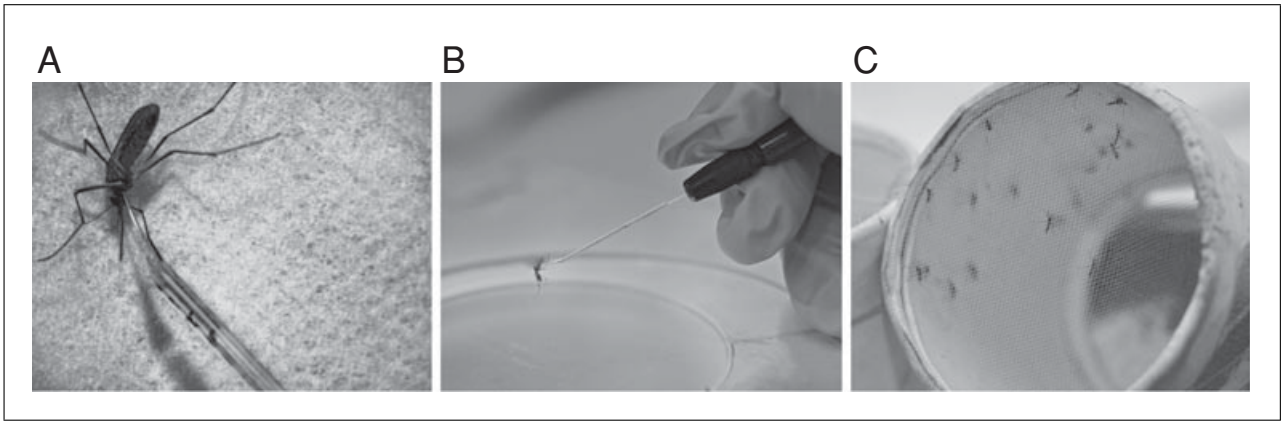

Figure 15D.2.3 Photograph sequence of an intrathoracic inoculation of an *Aedes aegypti* mosquito. A virus inoculum of 0.17 μl is injected into the membrane anterior to the mesepisternum of the mosquito (**A**). The inoculated mosquito is removed from the microscope and placed in a container for incubation using the needle's grasp (**B**). Inoculated mosquitoes are stored at 28°C for 14 days to obtain optimal virus replication (**C**).

8. Kill mosquitoes by freezing them (−20°C) for 20 min.
9. Remove the heads with a scalpel and macerate them onto a poly-lysine–treated glass slide.
10. Air dry the slides.
11. Fix with chilled acetone (−20°C) for 15 min and air dry.
12. Perform direct or indirect immunofluorescence assay on the heads (Basic Protocol 3).
13. Macerate the bodies of virus-positive mosquitoes in BA-1 diluent for RT-PCR testing, virus isolation, or −70°C storage, as described in steps 14 to 19.

Mosquito grinding using a TissueLyser mixer mill or vortex

14. Place 2-3 BBs and up to 50 mosquitoes in a labeled 2.0-ml microcentrifuge tube with 0.75 ml of BA-1 diluent.
15. Load the sealed tubes (make sure they are capped tightly) onto the mixer mill racks (24 per rack) and place about $\frac{1}{2}$ sheet of a large paper towel, folded, on top of the tubes, then put the rack top in place and place racks into the mixer mill according to the manual.

16. Run the machine at 25 cycles/sec for 4 min.

    If a TissueLyser Mixer Mill machine is not available, the mosquitoes can be ground by vortexing each individual tube for 5 min.

17. Remove racks from mill, and centrifuge the tubes 2 min at 5200 × *g* (7000 rpm), 4°C.

18. Carefully pipet the supernatant into clean 2.0-ml microcentrifuge tubes.

19. Freeze tubes in a −70°C freezer for storage.

    The supernatant can be used for PCR testing or inoculating tissue culture cells.

IMMUNOFLUORESCENCE ASSAYS

BASIC PROTOCOL 3

The immunofluorescence technique allows the visualization of the DENV in infected cells. Since the DENV infection can be empirically corroborated, the immunofluorescence assay is considered the gold standard for DENV diagnostic testing.

The two methods of immune staining used to determine DENV infectivity in cells are direct fluorescence and indirect fluorescence. In direct fluorescence staining, a polyclonal primary antibody is chemically conjugated with a fluorescent dye (usually FITC). When the conjugated antibody binds to the virus epitope, and excess antibody is washed away, the fluorescent stain reveals the presence of the virus in infected cells. DENV infection can be confirmed by this method; however, since the primary antibody is polyclonal, the virus serotype cannot be determined. In the indirect fluorescence method, a primary DENV serotype–specific monoclonal antibody attaches to viral epitopes and then a secondary antibody labeled with a fluorescent dye is used to recognize the primary antibody. DENV serotype can be determined with this technique; however, it requires a separate staining procedure for each of the four DENV serotypes. This assay is based on published methods (Igarashi, 1978; Gubler et al., 1984; Kuno et al., 1985; Kuno and Oliver, 1989).

Materials

0.005% (w/v) poly-lysine
C6/36 DENV-infected cells (Basic Protocol 1 or 5)
Acetone
Primary FITC-conjugated polyclonal antibody
Phosphate-buffered saline (PBS) without calcium or magnesium (*APPENDIX 2A*)
90% (v/v) glycerol in PBS
Primary monoclonal antibodies (mAbs) for all four DENV serotypes (for use in indirect method)
  DENV-1: 15F3-1 (purified from hybridoma ATCC #HB-47)
  DENV-2: 3H5-1 (purified from hybridoma ATCC #HB-46)
  DENV-3: 5D4-11 (purified from hybridoma ATCC #HB-49)
  DENV-4: 1H10-6 (purified from hybridoma ATCC #HB-48)
Human sera (for use in indirect method)
Secondary FITC-conjugated anti-mouse antibody (for use in indirect method)

Teflon-masked 12-well slides
Pillow
Transfer pipet
Coplin jar
37°C incubator
Humidified chamber (e.g., plastic box containing moistened paper towels)
Slide coverslip
Fluorescence microscope

Slide preparation

1. Prepare Teflon-masked slides (12-well) by dipping the slides in a solution of 0.005% poly-lysine for 20 min. Wash the slides once in water and allow to air dry. Store prepared slides at −20°C for up to 1 year.

Seeding cells

2. Knock loose infected cells from tube or flask by firmly tapping the container against a pillow.
3. Spot one drop (approx. 20 µl) of the cell suspension with a transfer pipet on a well of the slide.

   Always make duplicates of each sample.

4. Allow the slide to air dry.
5. Fix the cells by placing the slide in a Coplin jar containing chilled acetone (−20°C) for 15 min.
6. Allow to air dry.
7. Store at −20°C for up to 1 year until fluorescence assay is performed.

Immunofluorescence assay (direct or indirect)

Direct immunofluorescence assay (DFA):

8a. Spot 10 µl of previously titered, diluted primary FITC-conjugated polyclonal antibody onto each well of the slide.

   A primary FITC-conjugated polyclonal antibody is used here, but this protocol can also be followed with the FITC-conjugated monoclonal antibodies listed in the Materials list. However, the immunofluorescence would then be serotype specific. Alternatively, the monoclonal antibodies 2H2 and 4GH2 listed in Alternate Protocol 1 can be used for pan-dengue detection.

9a. Incubate in a humidified chamber for 30 min at 37°C.

10a. Tap slide against a paper towel to remove excess conjugate.

11a. Wash slide by immersing it in a Coplin jar containing PBS for 10 min at room temperature.

12a. Fill wells with a glycerol solution (90% glycerol, 10% PBS) and cover with a slide coverslip.

13a. Look for the presence of positive cells under fluorescent light with a microscope using 200× total magnifying power.

   Positive wells should contain cells with a green fluorescent color. Negative cells lack the green color.

   Wells that are not at least 30% covered with cells should be repeated.

Indirect fluorescence antibody (IFA) method:

8b. Add 10 µl of previously titered, monoclonal antibodies (mAbs) to the corresponding well.

   There are specific mAbs to each DENV serotype in the Materials list that can be used. Alternatively, other suitable DENV mAbs can be obtained from commercial sources. Use a primary FITC-conjugated polyclonal antibody for the positive control and normal sera for the negative control and follow the procedure for the direct immunofluorescence assay (steps 8a to 13a).

9b. Incubate in a humidified chamber for 30 min at 37°C.

10b. Remove slide from incubator and tap on paper towel to remove excess mAbs.

11b. Wash slide by immersing it in a Coplin jar containing PBS for 5 min at room temperature and air dry.

12b. Add 10 µl of diluted FITC-conjugated anti-mouse antibody onto each well of the slide.

13b. Incubate in a humidified chamber for 30 min at 37°C.

14b. Tap slides against a paper towel to remove excess conjugate.

15b. Wash slide by immersing it in a Coplin jar containing PBS for 5 min at room temperature.

16b. Fill wells with a glycerol solution (90% glycerol, 10% PBS) and cover with a slide coverslip.

17b. Look for the presence of positive cells under fluorescent light with a microscope using 200× total magnifying power.

Positive wells should contain cells with a green fluorescent color. Negative cells lack the green color.

Wells that are not at least 30% covered with cells should be repeated.

TITRATION OF DENGUE VIRUS BY PLAQUE ASSAY

BASIC PROTOCOL 4

A plaque assay is performed to establish the viral concentration of a predetermined DENV stock. The assay consists of inoculating Vero cell monolayers with DENV at multiplicities of infection (MOIs) low enough that the plaques of destroyed cells can be directly visualized. Viral plaques are visible clear, often rounded or irregular structures formed within an otherwise confluent cell culture. In order to observe the plaques, it is frequently necessary to cover the cells with an overlay medium followed by staining of the cells. This assay is based on a previously published method with modifications (Sukhavachana et al., 1966).

Materials

Vero cells (Support Protocol 2)
M199-5 medium (see recipe)
10 mg/ml gentamicin stock solution
7.5% (w/v) sodium bicarbonate stock solution (Gibco, cat. no. 25080-094)
SeaKem ME agarose
2× Ye-Lah overlay medium (see recipe)
30% (v/v) FBS in PBS (see *APPENDIX 2A* for PBS recipe)
3.2% (w/v) neutral red in PBS (see *APPENDIX 2A* for PBS recipe)

Hemacytometer
6-well plates
Light microscope
37°C incubator with 10% CO_2
45°C water bath

Prepare plates (6-well plates)

1. Knock flask to detach Vero cells.
2. Determine the concentration of cells in the suspension by using a hemacytometer.

For plates to be confluent in 24 hr, a cell concentration of 6×10^5 cells/well is needed.

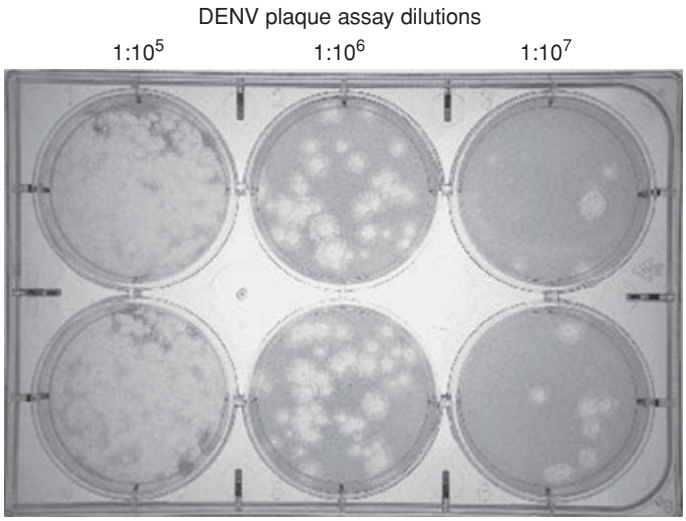

Figure 15D.2.4 Example of DENV plaque assay. Vero cells were infected with serial 1:10 dilutions of dengue virus strain 16681 in duplicate. Plaques were stained with 3.2% neutral red solution 5 days after infection, and visualized 24 hr later for counting.

3. Once the needed cell suspension volume is established, add that volume to each well of a 6-well plate. Bring the final volume of each well to 3 ml with M199-5 medium supplemented with 1% (v/v) of the gentamicin stock solution and 3% (v/v) of the sodium bicarbonate stock solution.

4. After placing the cell mix in the wells, shake plates in a cross pattern, let rest, and repeat in 15 min. Examine plates under the microscope to verify that cells are spreading evenly throughout the well.

5. Incubate plates at 37°C with 10% CO_2 for 24 hr.

Overlay medium

6. Make a 1% agarose solution with dH_2O as diluent. The volume needed is 2 ml per well.

7. Autoclave agarose solution in a cycle for liquids (15 min at 121°C)

8. Keep agarose in a 45°C water bath until use, to prevent solidification.

9. Warm the 2× Ye-Lah overlay medium to 45°C. The volume needed is 2 ml per well.

10. Combine the agarose solution and the 2× Ye-Lah overlay medium (1:1) and supplement it with 3% (v/v) of the sodium bicarbonate stock solution. Keep final overlay solution in a 45°C water bath until use, to prevent solidification.

Plaque assay

11. Dilute the virus in tenfold dilutions from 10^{-1} through 10^{-9}. Use PBS supplemented with 30% FBS as the solvent.

12. Remove the culture medium from the plates.

13. Add a 150-µl inoculum of each dilution into duplicate wells.

14. Incubate at room temperature for 1 hr. Rock plates every 15 min to prevent cells from drying.

15. Remove inoculum and add 4 ml of the overlay solution to each well.

16. Incubate at 37°C with 10% CO_2 for 5 days.

17. Stain plates with 3.2% neutral red in PBS (1 ml of stain solution per well).
18. Incubate at 37°C with 10% CO_2 for 24 hr.
19. Remove excess stain solution from plate and count the plaques in each well (Figure 15D.2.4).

Virus titer

20. Convert the results of the plaque assay into plaque-forming units per milliliter (pfu/ml). The conversion formula is as follows:

    FD × FC × plaque avg. = pfu/ml

    The dilution factor (FD) is the dilution value for the end-point dilution. For a 6 well plate the end-point dilution has a plaque count between 30 and 35 plaques.

    The conversion factor (FC) equals one milliliter over the volume of the inoculum (1 ml/150 μl).

    The average number of plaques per well (plaque avg.) refers to the average of plaques in the two wells of the end-point dilution.

TITRATION OF DENGUE VIRUS BY FLOW CYTOMETRY

ALTERNATE PROTOCOL 1

This assay is based on published protocols (Lambeth et al., 2005) with modifications. Infectivity of DENV is measured in Vero cells at 24 hr post infection (hpi), by enumerating cells that are positive for intracellular expression of dengue E protein (by using the 4G2 monoclonal antibody) or prM/M protein (by using the 2H2 mAb by flow cytometry). This method is best employed for quantification of viruses with high concentrations ($>10^4$ pfu/ml). The 4G2 mAb is cross-reactive with all flaviviruses and binds to E protein domain II. We have observed that the 2H2 mAb has a better correlation with results obtained from plaque assays. A BSL-2 biological cabinet should be used for steps involving live virus. Work can proceed outside of the biological cabinet once the virus is inactivated by paraformaldehyde (Cytofix/Cytoperm fixation).

Materials

Vero cells (Support Protocol 2)
10^6 pfu/ml DENV stock (Basic Protocols 1, 2, 5, or 6)
M199-0 and -5 (see recipes)
Phosphate-buffered saline (PBS) without calcium or magnesium (see *APPENDIX 2A*)
1× Hanks balanced salt solution (HBSS) (optional) (see *APPENDIX 2A*)
0.05% trypsin-EDTA: 0.05% (w/v) trypsin in 1 mM EDTA
10% (v/v) FBS in PBS
BD Cytofix/Cytoperm (BD Biosciences, cat. no. 554722)
BD Cytoperm/Cytowash (BD Biosciences, cat. no. 554723)
Pan-DENV fluorophore-conjugated mAb
  4G2 (purified from hybridoma ATCC #HB-112) or
  2H2 (purified from hybridoma ATCC #HB-114)
24-well plates
37°C incubator
Aspirator
Light microscope
96-well V-bottom plates
Plate adaptors for centrifuge
Multichannel pipettor
Parafilm (optional)
Microtiter tubes
Flow cytometer and analysis software

Day 1: Seeding Vero cells into 24-well plates

1. Seed Vero cells grown in M199-5 medium in 24-well plates by either (a) seeding at a density of 1.25×10^5 cells per well the day before the infections, or (b) seeding at a density of 2.5×10^4 cells per well on a Friday for a Monday infection. The monolayer should be ~95% confluent at the time of infection.

   This protocol can be adapted for 96-well plates by dividing the infection and serial dilution volumes by 4; however, the volumes for staining for flow cytometry would remain the same. In this protocol we recommend using 24-well plates for infecting cells and then transferring to 96-well plates for staining. Processing samples in the flow cytometer is much faster when it is done this way. When using a 96-well plate for infecting cells, you have the advantage of transferring the infected cells quickly from a flat-bottom to a V-bottom plate using a multi-channel micropipet.

Day 2: Infection of Vero cell monolayers with serial dilutions of DENV

2. Starting with a 1:2 dilution of DENV stock in M199-0 medium, make 4-fold or 10-fold-serial dilutions of the DENV stock in medium (M199-0). It is recommended to test at least 3 to 4 dilutions. Three to four 4-fold dilutions are adequate for viral titers that are approximately in the 10^6 range. If higher viral titers are expected, perform 10-fold dilutions.

3. Make 2-fold dilutions by transferring 225 µl of viral stock into tubes containing 225 µl of medium. Start successive 4-fold dilutions by taking 150 µl of the initial virus dilution and adding it to 450 µl of medium.

4. Remove the medium from the Vero cells and add 120 µl of each virus dilution into duplicate wells. Incubate for 1 hr at 37°C. Rock every 15 min.

5. Remove the inocula completely. Add 1.0 ml of M199-5 to each well and incubate at 37°C for 24 hr.

6. Make sure that each experiment includes 2 mock-infected wells and 2 virus-containing positive-control wells.

Day 3. Harvesting and fixing infected cells

7. Remove medium from the plates. This can be done fast by decanting the medium into a tall waste tray containing paper towels, followed by patting the plate dry on a stack of paper towels, or by using an aspirator.

8. Wash 2× with 0.5 ml 1× PBS or 1× HBSS per well. Decant each wash.

9. Add 0.1 ml of 0.05% trypsin-EDTA per well. Incubate ~5 min at 37°C. Check under the microscope to make sure the monolayer is detached.

10. Add 0.1 ml per well of PBS with 10% FBS. Place plates on ice. Gently pipet cells up and down to make a single-cell suspension. Keep plates on ice while pipetting.

11. Transfer the content of each well to a 96-well V-bottom plate and keep on ice. The rest of the fixing and staining will be done in this 96-well plate.

12. Place plates in centrifuge plate adaptors and spin 5 min at $500 \times g$, 4°C.

13. Aspirate the supernatant with care to avoid losing the pelleted cells. *Alternative:* Remove the supernatant by gently dumping into a waste tray containing paper towels, and without inverting the plate, gently drying the plate on paper towels. Be careful not to lose the pelleted cells.

14. Wash cells with 0.2 ml of cold PBS. Add 200 µl/well to each row with a multichannel pipettor and pipet up and down gently to resuspend the cells. Change tips and add 200 µl/well to the next row, resuspend, and repeat until all rows are completed.

15. Spin plate 5 min at 500 × *g*, 4°C.
16. Remove the supernatant as in step 7.
17. Add 100 µl/well of cold Cytofix/Cytoperm and resuspend cells by gently pipetting up and down with a multichannel pipettor.
18. Incubate in the dark on ice for 20 min.
19. Add 100 µl/well of cold Cytoperm/Cytowash.
20. Spin 5 min at 750 × *g*, 4°C.
21. Remove the supernatant as in step 7. Be careful as the pellet is now looser.
22. Add 0.2 ml of cold Cytoperm/Cytowash. Resuspend gently.
23. Spin 5 min at 750 × *g*, 4°C.
24. Repeat steps 22 and 23.
25. Spin 5 min at 750 × *g*, 4°C.
26. Remove the supernatant as in step 7.
27. Add 0.2 ml of cold Cytoperm/Cytowash. Resuspend gently. If time permits, continue to protocol steps for Day 4.
28. (Optional) Wrap the plate in Parafilm and store at 4°C in the dark until staining (no more than a day or two).

Day 4. Staining and analyzing by flow cytometry

29. Prepare diluted antibody. We use monoclonal antibody 2H2 conjugated to Alexa 488, which recognizes a conserved epitope on M in all four DENV serotypes. The anti-DENV monoclonal antibody 4G2 conjugated to an Alexa dye can also be used for titration.

    For each batch of conjugated antibody, determine in a prior experiment the optimal dilution for it to be in excess. Recommended dilution for 2H2-Alexa 488 ranges from 1:400 to 1:2000, and for 2H2-Alexa 647 ranges from 1:12,500 to 1:20,000. Recommended dilution for 4G2-Alexa 488 ranges from 1:400 to 1:1000.

30. Spin 5 min at 750 × *g*, 4°C.
31. Aspirate the supernatant with care to avoid losing the pelleted cells. *Alternative:* Remove supernatant by gently dumping the supernatant in a single movement and padding once in absorbent towel for 5 sec. Immediately turn the plate back upright.
32. Add 100 µl of diluted 4G2 or 2H2 Alexa-conjugated antibody to each well. Use a multichannel pipettor and pipet up and down gently to resuspend the cells. Change tips, add 100 µl antibody/well to the next row, resuspend, and repeat until all rows are completed.
33. Incubate on ice for 1 hr in the dark.
34. Add 100 µl/well of cold Cytoperm/Cytowash. Resuspend gently.
35. Spin cells 5 min at 750 × *g*, 4°C.
36. Remove supernatant as in step 31.
37. Add 0.2 ml of cold Cytoperm/Cytowash. Resuspend gently.
38. Spin 5 min at 750 × *g*, 4°C.

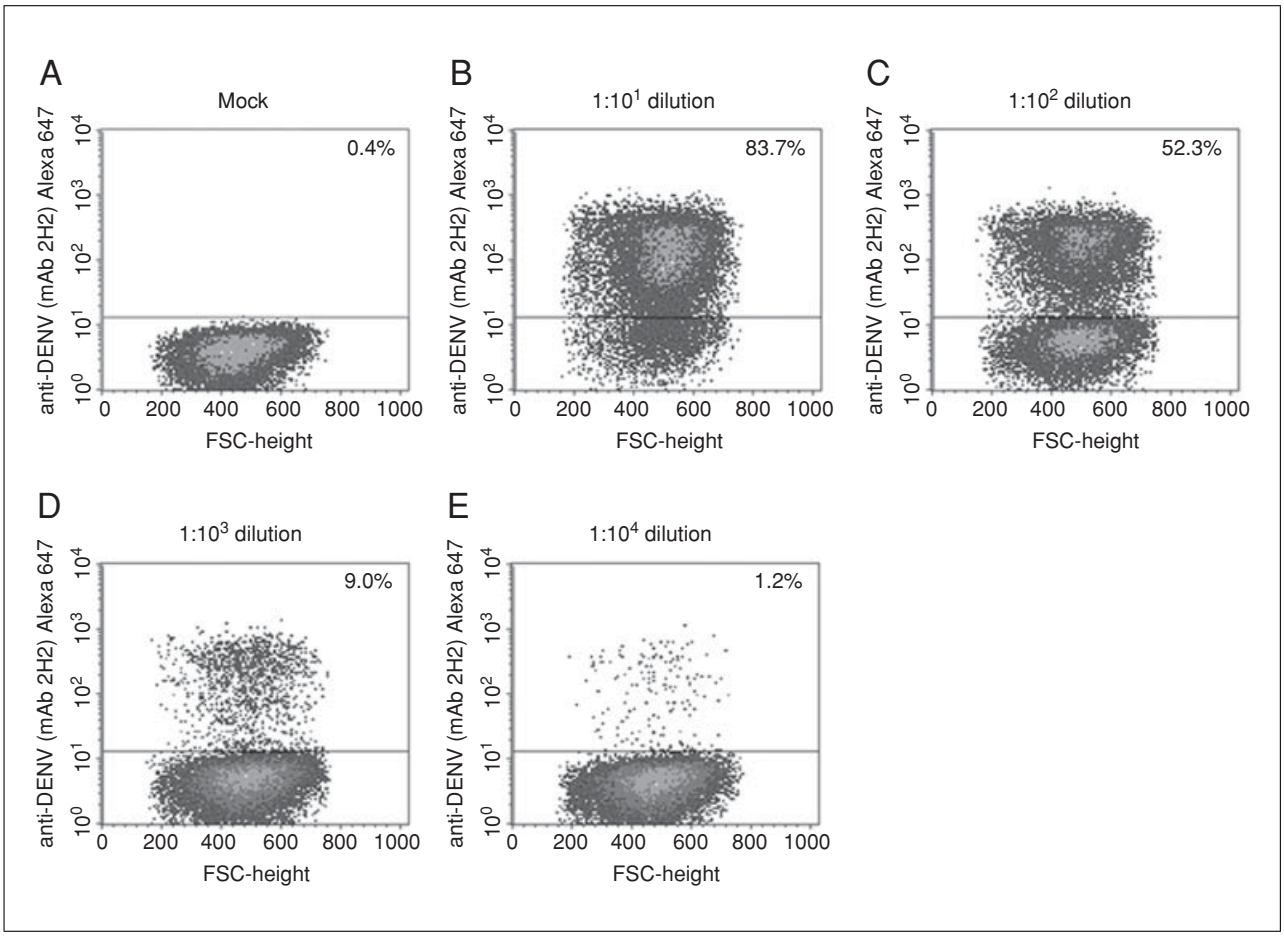

Figure 15D.2.5 Titration of dengue virus by flow cytometry. Vero cells were infected with serial 1:10 dilutions of dengue virus strain 16681 for 24 hr. Cell fluorescence was measured on a BD FACS Calibur and data analysis was conducted using BD Cell Quest software to determine the percent of dengue-positive cells (see upper-right-hand corner of each graph).

39. Repeat steps 37 and 38.
40. Resuspend cells in 200 µl of Cytoperm/Cytowash.
41. Transfer to microtiter tubes containing 150 µl of Cytoperm/Cytowash.
42. Keep cells on ice in the dark if analysis is to be performed the same day. Keep at 4°C in the dark if analysis is to be performed within a couple of days.
43. Analyze by flow cytometry, preferably on the same day but definitely within a couple of days. Count at least 20,000 events per sample.

Calculations for obtaining virus titers in infectious units (IU)/ml

44. Calculate the virus titer as follows:

[(avg. % positive DENV infected cells – avg. % positive mock-infected cells) × (total number of cells in well) × (dilution factor)]/ml of inoculum added to cells = titer (FACS infectious units/ml)

Example for calculating FACS IU using data from Figure 15D.2.5D:

$[(0.09 - 0.004) \times (1.25 \times 10^5$ cells$) \times (1000)/0.12$ ml $= 8.96 \times 10^7$ FACS IU/ml

The linear area of the curve or effective range is when 0.2% to 25% of the cells are infected.

DENGUE VIRUS PROPAGATION

Reference DENV strains and clinical isolates can be further propagated in order to increase the virus titer and the volume of the virus stock. Usually, an MOI of 0.01 and an incubation period of 3 days is recommended for harvesting low-passage isolates at an optimum titer. However, depending on the virus strain the MOI could fluctuate between 0.0001 and 0.1. The incubation period (time between inoculation and harvest of virus) may take up to 5 days. The success of this procedure will depend on the initial titer of the virus and its MOI, its ability to replicate in C6/36 cells, and the cytotoxic effect of the virus on the cells.

Materials

C6/36 cells (Support Protocol 1)
Phosphate-buffered saline (PBS) without calcium or magnesium
DMEM-0, -2 (see recipe)
FBS

75-cm^2 seal-cap tissue culture flasks
33°C incubator
Light microscope
15-ml sterile conical polypropylene centrifuge tubes
Millipore Centricon Plus-20 (UFC2BHK08) or Centricon Plus-70 (UFC710008) (optional)

Infect cells and harvest supernatant

1. Remove old culture medium from flask of C6/36 cells.

   Flasks of 75-cm^2 with 95% C6/36 cell confluence are used for virus propagation.

2. Rinse cells with PBS and remove remaining PBS with a pipet.

3. Add calculated volume of inoculum to flask.

   Inoculum formula: (cells per flask)(MOI)/(virus titer in pfu) × (1000 μl) = virus inoculum (μl)

   Example: $(1.2 \times 10^7$ cells)(0.01)/$(5.5 \times 10^6$ pfu) × (1000 μl) = 22 μl

4. Bring flask volume to 3 ml with DMEM-0.

5. Incubate flask at 33°C for an hour. Gently rock the flask every 15 min to prevent the monolayer from drying and to spread the virus evenly.

6. Add 7 ml of DMEM-2 to the flask for a final volume of 10 ml.

7. Incubate at 33°C for 3 to 4 days. Check cells every day under the microscope to verify cell mortality.

   Only some cytopathogenicity should be observed near the time of virus harvest. Titers will be low if all of the cells are dead.

8. Harvest virus by transferring all the flask supernatant to a 15-ml centrifuge tube. If no concentration step is planned, add 23% FBS; if concentrating the sample (optional step 11), do not add FBS.

9. Centrifuge 10 min at 4,000 × *g*, 4°C.

10. Place the clean supernatant in a new conical tube and discard the tube with cell debris.

11. (Optional) To increase the virus titer, concentrate collected supernatant by using a Centricon Plus-20 (up to 15 ml) or Centricon Plus-70 (up to 60 ml) centrifugal

concentrator or equivalent. Add the supernatant to the filtration device and centrifuge 30 min (or until the desired volume of the virus concentrate solution is reached) at 4000 × *g*, 4°C.

12. Store supernatant aliquots in a −70°C freezer for future experiments.

    The virus titer may be measured from a sample thawed on ice by using the methods described in Basic Protocol 4 and Alternate Protocol 1.

BASIC PROTOCOL 6

PURIFICATION OF DENGUE VIRUS BY SUCROSE GRADIENTS

Sucrose gradients are an effective way to concentrate virus stocks. Gradients increase virus titer by eliminating all non-viral elements of the stock. The formed virus pellet is usually resuspended in a much smaller volume than the original stock. Although the new virus stock is at a higher titer, its smaller volume might be restrictive. Purifying DENV through sucrose gradients increases its titer by 100-fold on average.

NOTE: The purification protocol requires an environment as sterile as possible. It is recommended to autoclave or sterilize all of the materials that are going to be used during the procedure.

Materials

20% (w/v) sucrose in dH_2O
Virus supernatant (Basic Protocol 5)
DMEM-30 (see recipe)
Disposable bottle-top 0.2-μm SFCA membrane filter unit

Polyallomer 25 × 89-mm ultracentrifuge tubes (Beckman)
Ultracentrifuge with SW-28 Beckman rotor

Sucrose gradient

1. Prepare a solution of 20% sucrose in dH_2O and filter it with a 0.2-μm bottle-top filter unit.
2. Add 24 ml of virus supernatant to an ultracentrifuge tube.
3. Very slowly add 7 ml of the sucrose solution to the bottom of the ultracentrifuge tube.
4. Balance the tubes in their respective ultracentrifuge buckets.
5. Centrifuge tubes 3.5 hr at 100,715 × *g*, 4°C.
6. Decant and discard supernatant and leave the virus pellet drying upside-down inside the biosafety cabinet at room temperature for 20 min.
7. Add 200 μl of DMEM-30 to each pellet and wait 30 min for pellets to dissolve.
8. Combine all the virus solutions into one.
9. Make aliquots of desired volume (e.g., 50 μl).

SUPPORT PROTOCOL 1

GROWTH AND SPLITTING OF C6/36 CELLS

Aedes albopictus (Asian tiger mosquito) larva cells of clone C6/36 were originally adapted to grow at 28°C in Eagle minimum essential medium and shown to efficiently replicate flaviviruses (including DENV) to high titers (Igarashi, 1978). C6/36 are loosely adherent, non-tumorigenic cells that maintain a diploid chromosome number. DENV has mammalian hosts (mainly humans) whose body temperatures are higher than the ambient temperature in which poikilothermic organisms live. The upper thermal limit of C6/36

cells was determined to be 36°C (Kuno and Oliver, 1989). The protocol described here uses C6/36 cells adapted to grow at 33°C. The replication rate of DENV is increased under these conditions, resulting in higher viral titers with decreased incubation periods for harvest (Kuno and Oliver, 1989). The method used is a modification based on previously published protocols (Igarashi, 1978).

Materials

C6/36 cells (ATCC #CRL-1660)
DMEM-5 (see recipe), 33°C
Phosphate-buffered saline (PBS) without calcium or magnesium (see *APPENDIX 2A*), 33°C
0.05% trypsin-EDTA: 0.05% (w/v) trypsin in 1 mM EDTA

33°C incubator
15-ml conical centrifuge tubes
75-cm^2 seal-cap tissue culture flasks

Cell propagation

1. Maintain C6/36 cells at 33°C in 75-cm^2 seal-cap tissue culture flasks containing 30 ml of DMEM-5.

   Subculturing can be done by mechanically knocking off the cell monolayer since the cells do not attach firmly to the substrate. However, a protocol of trypsinization (described below) is preferred for a higher yield of healthy cells in suspension.

Cell splitting

2. Discard old growth medium from flask.
3. Rinse twice gently with warmed (33°C) PBS.
4. Add 1.5 ml of 0.05% trypsin-EDTA and incubate at 33°C for 2 min.
5. Gently knock flask to detach cell monolayer.
6. Resuspend cells in 10 ml of DMEM-5. Transfer cell suspension to a sterile 15-ml conical tube and centrifuge 5 min at 500 × *g*, room temperature.
7. Discard medium and resuspend cell pellet in 10 ml of DMEM-5.
8. Subculture cells in a new tissue culture flask at a ratio of 1:4 to 1:10 with 30 ml of fresh DMEM-5.
9. Incubate at 33°C until cell confluence is reached.

   Perform cell maintenance at least twice weekly, depending on the subcultivation ratio utilized.

GROWTH AND SPLITTING OF VERO CELLS

SUPPORT PROTOCOL 2

Vero cells are mammalian cells derived from the kidney of an adult *Cercopithecus aethiops* (African green monkey). These cells have an epithelial morphology and have adherent growth properties. The Vero cell line is suitable for performing plaque assays as well as plaque reduction neutralization tests (PRNT) for DENV.

Materials

Vero cells (ATCC #CCL-81)
M199-5 (see recipe)
Phosphate-buffered saline (PBS) without calcium or magnesium (see *APPENDIX 2A*)
0.05% trypsin-EDTA: 0.05% (w/v) trypsin in 1 mM EDTA

150-cm^2 seal-cap tissue culture flasks
37°C incubator

Cell propagation

1. Grow Vero cells at 37°C in 150 cm^2 seal-cap tissue culture flasks containing 30 ml of M199-5 tissue culture medium.

Cell splitting

2. Discard old growth medium.
3. Rinse gently twice with warmed (37°C) PBS.
4. Add 2 ml of 0.05% trypsin-EDTA and incubate at 37°C for 2 min.
5. Gently knock flask to detach cell monolayer.
6. Resuspend cells in 10 ml of M199-5 medium. Transfer cell suspension to a sterile 15 ml conical tube and centrifuge 5 min at 500 × *g*, room temperature.
7. Discard medium and resuspend cell pellet in 10 ml of M199-5 medium.
8. Transfer cells to a new tissue culture flask at a ratio of 1:5 with 30 ml of fresh M199-5 medium.
9. Incubate at 37°C until cell confluence is reached.

   If using a 1:5 dilution, perform cell maintenance twice weekly. If working with a different dilution, perform split when the monolayer confluence reaches 100%.

SUPPORT PROTOCOL 3

FREEZING C6/36 AND VERO CELLS

C6/36 and Vero cells can be stored long-term in liquid nitrogen, as described below.

Materials

C6/36 cells (Support Protocol 1) or Vero cells (Support Protocol 2)
DMEM-5 medium (for C6/36 cells) or M199-5 medium (for Vero cells) with 5% (v/v) DMSO

Hemacytometer
Cryovials
Freezing container (Mr. Frosty)

1. Harvest cells:
   a. For C6/36 cells, follow the cell-splitting protocol (Support Protocol 1) up through step 7.
   b. For Vero cells, follow the cell-splitting protocol (Support Protocol 2) up through step 7.
2. Determine the cell count by using a hemacytometer.
3. Centrifuge 5 min at 500 × *g*, room temperature, and then resuspend cells in cold (4°C) medium with 5% DMSO at a concentration of 10^7 cells/ml.
4. Make 1-ml aliquots in cryovials and label with cell information.
5. Place aliquots in a freezing container.
6. Place freezing container in a −70°C freezer overnight.
7. Transfer aliquots to liquid nitrogen and log the storage information.

THAWING FROZEN C6/36 AND VERO CELLS

The viability of cultures grown from a frozen stock depends on the careful thawing and handling of the frozen cell aliquot.

Materials

Cryovial of C6/36 or Vero cells frozen at −70°C and stored in liquid nitrogen (Support Protocol 3)
DMEM-5 medium (for C6/36 cells) or M199-5 medium (for Vero cells) with 5% DMSO

15-ml conical test tube
25-cm^2 tissue culture flask
Incubator (33°C for C6/36 cells or 37°C for Vero cells)

1. Remove cells from liquid nitrogen and place in a 37°C water bath to thaw quickly.
2. Transfer the thawed cell suspension to a 15-ml conical tube containing 10 ml of the appropriate medium for the cell line.
3. Pipet gently and centrifuge cells 5 min at 500 × *g*, room temperature.
4. Discard supernatant and resuspend cells in 10 ml of fresh medium.

   This step removes the DMSO left from the medium in which the cells were frozen.

5. Transfer cell suspension to a 25-cm^2 tissue culture flask.
6. Incubate at appropriate temperature for cell line.

REAGENTS AND SOLUTIONS

Use tissue-culture-grade water in all recipes and protocols. Media recipes are calculated for 1 liter of product but can be modified for smaller or higher quantities.

BA-1 diluent

Combine the following, then filter:
100 ml 10× M199 with Hanks' salts, without L-glutamine (Sigma, cat. no. M9163)
200 ml 5% (w/v) bovine serum albumin
50 ml 1 M Tris·Cl pH 7.5 (see *APPENDIX 2A*)
10 ml L-glutamine (Gibco, cat. no. 2503-081)
4.5 ml 7.5% (w/v) sodium bicarbonate (Gibco, cat. no. 25080-094)
10 ml 100× pen-strep (Gibco, cat. no. 15140-122)
1 ml 1000× Fungizone (amphotericin) (Sigma, cat. no. A9528)
624.5 ml dH_2O
Store at 4°C for up to 6 to 8 weeks

DMEM-0, -2, -5, -30 (DMEM containing 0%, 2%, 5%, or 30% FBS)

Combine the following, then filter:
13.37 g powdered Dulbecco's Modified Eagle Medium (DMEM)
1% (10 ml) sodium pyruvate (Gibco, cat. no. 11360-70)
1% (10 ml) nonessential amino acids (Gibco, cat. no. 11140-050)
1% (10 ml) sodium bicarbonate (Gibco, cat. no. 25080-094)
1% (10 ml) MEM vitamins (Gibco, cat. no. 11120-052)
0%, 2%, 5%, or 30% (0 ml, 20 ml, 50 ml, or 300 ml) FBS, to prepare DMEM-0, -2, -5, and -30, respectively.
dH_2O to 1 liter final volume
Store at 4°C for up to 4 to 6 weeks

10× Earle's balanced salts solution (BSS) without phenol red

Core BSS:
68 g NaCl (final concentration in 10× Earle's BSS is 1.164 M)
4.0 g KCl (final concentration 0.054 M)
1.25 g $NaH_2PO_4 \cdot H_2O$ (final concentration 0.0091 M)
10.0 g dextrose (final concentration 0.0555 M)
700 ml dH_2O
Autoclave and let cool

100× $CaCl_2$:
26.49 g $CaCl_2 \cdot 2H_2O$ (final concentration 0.018 M)
1000 ml dH_2O
Autoclave and let cool

100× $MgSO_4$:
20.46 g $MgSO_4 \cdot 7H_2O$ (final concentration 0.00831 M)
1000 ml dH_2O
Autoclave and let cool

To prepare 10× Earle's BSS:
700 ml Core BSS
100 ml 100× $CaCl_2$
100 ml 100× $MgSO_4$
100 ml dH_2O
Store all solutions at 4°C for up to 12 months

M199-0, -2, -5 (M199 medium containing 0%, 2%, or 5% FBS)

Combine the following, then filter:
10% (100 ml) 10× M199 medium (Sigma, cat. no. M9163)
1% (10 ml) HEPES (Gibco, cat. no. 15630-080)
1% (10 ml) L-glutamine (Gibco, cat. no. 25030-081)
1% (10 ml) sodium bicarbonate (Gibco, cat. no. 25080-094)
0%, 2%, or 5% (0 ml, 20 ml, or 50 ml) FBS, to prepare M199-0, -2, and -5, respectively
dH_2O to 1 liter
Store at 4°C for up to 4 to 6 weeks

Ye-Lah medium

Dissolve 20 g yeast extract in 1000 ml dH_2O.
Dissolve 100 g lactalbumin hydrolysate in 1000 ml dH_2O.
Combine yeast extract solution and lactalbumin hydrolysate solution 1:1, autoclave 15 min, aliquot, and label.
Store at 4°C for up to 3 to 4 months

2× Ye-Lah overlay medium

Combine the following, then filter:
196 ml 10× Earle's BSS without phenol red (see above)
66 ml Ye-Lah medium (see above)
40 ml FBS
2 ml 1000× Fungizone (amphotericin) (Sigma, cat. no. A9528)
2 ml 1000× gentamicin (Gibco, cat. no. 15710-064)
694 ml dH_2O
Store at 4°C for up to 4 to 6 weeks

COMMENTARY

Background Information

Dengue is a human illness caused by infection with any one of four related DENV serotypes (DENV-1, -2, -3, and -4). An estimated 50 million people in approximately 100 countries present with mild or severe dengue annually (World Health Organization, 2010). The increased incidence and emergence of dengue epidemics in previously unaffected areas have made it the most important arthropod-borne viral disease of humans. A large proportion of the human population remains at risk of infection with more than 2.5 billion people living in dengue-endemic areas (World Health Organization, 2009). Dengue is a significant cause of morbidity and mortality in many endemic countries. The disease caused by DENV has been known as dengue fever and dengue hemorrhagic fever. Individuals infected with DENV exhibit varied clinical manifestations that result in either non-severe or severe clinical outcomes (Gubler, 1998; Guzman and Kouri, 2002). In 2009, the WHO revised their patient classification guidelines to determine disease severity based on a set of clinical and laboratory parameters (World Health Organization, 2009). Patients are now classified as having non-severe or severe dengue. Patients with non-severe dengue are further subclassified as having or not having warning signs. Patients with probable dengue must live or have traveled to a dengue-endemic area and have fever (typically 2 to 7 days) with two of the following symptoms: nausea and/or vomiting, rash, muscle and bone aches, tourniquet test positive, leucopenia, any warning sign. Warning signs include abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleed, lethargy, restlessness, liver enlargement >2 cm, and laboratory increase in hematocrit concurrent with rapid decrease in platelet count. As fever subsides, more severe hemorrhagic manifestations may ensue due to increased capillary permeability; severe cases can display severe plasma leakage, severe hemorrhage, and severe organ impairment. Intervention for severe cases consists of intravenous rehydration therapy. Failure to recognize or adequately manage severe cases may lead to shock and sometimes death. Appropriate treatment can reduce the case fatality rate to less than 1%. Approximately 75% of DENV infections are asymptomatic. Persons with symptomatic DENV infection usually present with symptoms when they are viremic (acute phase); this period may last 2 to 7 days.

The dramatic global spread of dengue has driven the divergence of distinct genetic variant populations of each DENV serotype (termed genotypes and sublineages) that are often associated with specific geographical regions (Holmes and Twiddy, 2003; Rico-Hesse, 2003; McElroy et al., 2011). Extensive study of the evolution of the viral genome scrutinized at the population level has identified significant correlations between genotypes, increased epidemic potential, and the emergence of severe disease in particular regions of the world (Rico-Hesse et al., 1997; OhAinle et al., 2011). These associations demand further monitoring of each relevant genotype and sublineage relative to the epidemiology of the disease. In order to perform effective dengue surveillance, virus isolation from infected patients is required; therefore, standardized protocols are necessary to isolate, identify, quantify, handle, and store viral strains for further investigation.

Probable dengue infections are confirmed through laboratory tests that identify viral nucleic acid or antigens, detect DENV-specific antibodies, or enable isolation of the virus in cell culture. Virus isolation can be time-consuming and slow; however, this method is still considered the gold standard of dengue diagnosis. The use of isolated DENV strains can greatly enhance virologic studies that involve serologic, biologic, genomic, and epidemiological components. Growing the virus from blood samples (usually serum) of infected individuals during their acute phase (usually less than 7 days after the onset of symptoms) can be achieved in a number of tissue culture systems. *Aedes albopictus* C6/36 cells are the most commonly used cell type for the rescue of DENV. Studies show that higher virus isolation rates are obtained early in illness and in primary DENV infections. This is likely due to antibody complex formation and circulating antibodies neutralizing free virus (Gubler et al., 1981). C6/36 cells are also preferred over Vero cells for isolations, as they are more easily infected than mammalian cells, the viruses may be isolated as early as in 2 days (depending on virus strain), and they acquire fewer mutations per passage than mammalian cells. One of the disadvantages of using C6/36 cells is that not all clinical isolates grow well in the first passage and it is not as sensitive as mosquito inoculation. In addition, C6/36 cells have high susceptibility to serum toxicity, which limits the amount of blood specimen that can be inoculated in a given volume of

medium. Confirmation of virus isolation and DENV serotype can be achieved through immunofluorescence. Although the isolation of DENV directly from serum is efficient, the more recently developed RT-PCR assays can be very sensitive and fast. Titration of DENV is performed by the classic method of plaque assays; however, some DENV strains are difficult to quantify through this method due to the size and/or form of the plaques. An alternative method utilized for DENV titration is flow cytometry. This method is a non-biased, highly-quantitative approach that eliminates the difficulty of discerning plaques.

Critical Parameters and Troubleshooting

DENV is quite stable for years when stored at −80°C in medium containing 25% FBS. Relatively higher temperatures will progressively reduce the virus concentration. At 4°C, the virus will survive for several days, but its titer will start to decrease significantly if kept at that temperature. At room temperature, virus degradation is even more accelerated, so it is recommended when working with any aliquot of the virus to maintain the virus in a bucket with ice. The virus can be rapidly inactivated by placing it in a 56°C water bath for 30 min. Inactivated virus can still be detected through procedures such as RT-PCR; however, the virus loses its ability to replicate, so it cannot be measured with procedures that rely on the infectivity of the virus, such as immunofluorescence or plaque assays.

Considering the high rate of mutation of DENV, it is advisable to keep the virus stocks at the lowest possible number of passages. It is recommended that viruses with a high passage history be sequenced to ensure that no significant mutations have altered the virus. Both the C6/36 cell lines and the Vero cell lines are allowed to grow only up to 20 passages. This is a precautionary procedure to prevent cell mutations that can affect virus replication.

Culturing of virus-infected cells for an extended period of time will result in cell destruction due to cytotoxic effects. Therefore, it is critical to harvest the virus at the pinnacle of virus replication, before the critical period of cell toxicity is reached.

The quality of patient serum samples can be compromised depending on the conditions under which the sample was taken or the handling and transportation of the samples. Since isolating these viruses from serum samples tends to result in contamination, it is a standard practice to filter the serum samples when inoculating the cell monolayer.

Cell culture isolation of dengue is the favored routine method of virus recovery. Despite the large virus inoculum that can be utilized in mosquito or mammalian cell culture isolation of DENV, the use of mosquito inoculation to recover virus isolates remains the gold standard for sensitivity. This technique is utilized when the patient's viremia or inoculating virus titer is low or if there is only a small sample volume remaining.

Toxorhynchites amboinensis mosquitoes are the preferred choice of host for the isolation and expansion of DENV viruses. The susceptibility of *Aedes albopictus* and *Toxorhynchites amboinensis* mosquitoes to all DENV serotypes is very similar in both sexes (Rosen, 1981). One of the major advantages of using *Toxorhynchites amboinensis* is that the adults do not feed on blood and do not pose a risk for human infection. When necessary, the use of *Aedes albopictus* and *Aedes aegypti* for virus amplification should be limited to areas in which they are already present. The large size of *Toxorhynchites* facilitates its use in the preparation of mosquito triturates and detection of DENV by immunofluorescence in mosquito head squashes. The disadvantage of using *Toxorhynchites* lies in the difficulty of rearing this species.

Inoculating mosquitoes requires precise injection in the correct anatomical section of the insect. A deep puncture or an excessive volume of inoculum is certain to result in the death of the mosquito.

During the staining of infected cells with the conjugated antibody in the immunofluorescence assay, avoid the use of excessive light as it might affect the fluorescence quality of the fluorophore. Also, if the FITC-conjugated antibody does not contain an anti-fade reagent, then specimen reading under the microscope should be performed immediately after exposing the cells to the green light frequency because the fluorescence decays quickly. Positive and negative controls are always necessary to establish the fluorescence parameters and therefore eliminate any background.

Anticipated Results

DENV titers used in the laboratory typically range between 10^4 and 10^7 pfu/ml. However, these titers can be increased by several orders of magnitude by purifying the virus with a sucrose gradient (Basic Protocol 6) or concentrating the virus with a centrifugal filter device (Basic Protocol 5).

Immunofluorescence assays are not as sensitive as other DENV detection protocols such as the Centers for Disease Control and Prevention (CDC) real-time RT-PCR assay for DENV. Hence, it is common that samples with a very low level of the virus give a false-negative result. To put things in perspective, it is uncommon to successfully isolate a virus from a sample with an RT-PCR threshold cycle (CT) value over 25; however, samples are considered DENV-positive for CT values as high as 37.

There is a wide range of plaque size among virus strains. Virus strains that create a small number of large plaques might affect a higher percentage of cells than a virus strain with smaller but more abundant plaques, thus creating a bias when reading and analyzing the plaque assay (Basic Protocol 4). Other protocols such as titration through flow cytometry (Alternate Protocol 1) eliminate this problem because cells are analyzed individually.

Time Considerations

The appropriate time point to harvest DENV from the C6/36 cell line will vary depending on the strain used. Typically when inoculating at a low MOI (0.001 to 0.1), the optimal time frame to harvest is on day 3 post-infection. Some strains require an additional day for best results. Longer exposure of the cells to the virus-rich supernatant tends to result in the destruction of the cell monolayer due to cytotoxicity. If working with diagnostic samples of unknown viral concentration, the cells should be infected with a small amount of inoculum and can be left to replicate the virus up to day 5 post-infection.

When inoculating mosquitoes, 1-day-old adult hatchlings are ideal to survive the 14-day incubation period that it takes the virus to replicate inside the mosquito vector.

Keep in mind when setting up experiments that precise cell confluence for both the C6/36 and the Vero cell line can take up to 3 days, depending on the dilution ratio used for cell splitting.

DENV can be titered by flow cytometry in as little as 3 days if the number of viruses is small. It is recommended to split the procedure into 4 days, as described in Alternate Protocol 1, when dealing with samples containing a large number of viruses.

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