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Preparation of Cell Cultures and Vaccinia Virus Stocks

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Abstract

The culturing of cell lines used with vaccinia virus, both as monolayer and in suspension, is described. The preparation of chick embryo fibroblasts (CEF) is presented for use in the production of the highly attenuated and host range-restricted modified vaccinia virus Ankara (MVA) strain of vaccinia virus. Protocols for the titration and trypsinization of vaccinia virus stocks, as well as viral DNA preparation and virus purification methods are also included.

Keywords

cell culture; vaccinia virus; modified vaccinia virus Ankara

This unit describes the maintenance of cell lines used with vaccinia virus, both in monolayer cultures (see Basic Protocol 1) and in suspension (see Basic Protocol 2). The suspended cell culture is then used in the preparation of vaccinia virus stocks (see Basic Protocol 3). The preparation of chick embryo fibroblasts (CEF) is also presented (see Basic Protocol 4), for use in the production of the highly attenuated and host range–restricted modified vaccinia virus Ankara (MVA) strain of vaccinia virus (see Basic Protocol 5). For preparation of viral DNA, to eliminate infected-cell debris, or to obtain a very high titer stock, virus purification methods are described (see Basic Protocol 6). Additionally, support protocols are presented for the titration of standard and MVA vaccinia virus stocks (see Support Protocols 1 and 3, respectively) and for trypsinization of virus stocks (see Support Protocol 2).

Because standard vaccinia virus strains have a broad host range, there is considerable latitude in the selection of cell lines; those described below (see Basic Protocols 1 and 2) have been found to give good results. BS-C-1 cells exhibit strong cytopathic effects and therefore give the best results for a plaque assay, whereas HeLa cells are preferred for preparation of virus stocks. Human thymidine kinase–negative (TK^-) 143B cells are used when TK selection is employed (*unit 14A.4*), but they can be used for transfection as well as for a plaque assay. Either CEF or BHK-21 cells are used to propagate MVA and prepare recombinant MVA. Table 14A.3.1 presents a summary of the uses for specific cell lines.

CAUTION: Proceed carefully and follow biosafety level 2 (BL-2) practices when working with standard vaccinia virus.

NOTE: Carry out all procedures in this unit using sterile technique, preferably in a biosafety cabinet.

BASIC PROTOCOL 1

CULTURE OF MONOLAYER CELLS

Frozen cells are thawed and grown in appropriate complete medium containing twice the maintenance amount of serum (see below). When the cells are confluent, they are treated with trypsin/EDTA, diluted, and maintained in appropriate complete medium containing 8% FBS (see Table 14A.3.2).

Materials

Frozen vial of cells (Table 16.16.2): BS-C-1 (ATCC CCL26), HuTK$^-$ 143B (ATCC CRL8303), *or* BHK-21 (ATCC CCL10)

70% ethanol

Start-up medium (Table 16.16.2): complete MEM-16, complete DMEM-16, *or* complete MEM-16/BrdU (see recipes), 37°C

Maintenance medium (Table 16.16.2): complete MEM-8, complete DMEM-8, *or* complete MEM-8/BrdU (see recipes), 37°C

PBS (optional; *APPENDIX 2A*)

Trypsin/EDTA: 0.25% (w/v) trypsin/0.02% (w/v) EDTA, 37°C

50 ml centrifuge tube

75-cm^2 and 162-cm^2 tissue culture flasks

Humidified, 37°C, 5% CO_2 incubator

Begin the culture

1 Quickly thaw a frozen vial of cells in a 37°C water bath. Remove from 37°C immediately after thawing.

2 Sterilize the outside of the vial with 70% ethanol and transfer the cells with a pipet into a 50-ml centrifuge tube containing 30 ml of warm start-up medium. Centrifuge 5 min in a Sorvall H-6000A at 1200 rpm (420 × *g*) at room temperature, and discard supernatant. Resuspend pellet in 1 ml of start-up medium then transfer to a 75-cm^2 tissue culture flask containing 30 ml of start-up medium. Rotate the flask to evenly distribute the cells and place overnight in a humidified, 5% CO_2 incubator at 37°C.

3 Aspirate the start-up medium and replace with appropriate maintenance medium. Return cells to the CO_2 incubator at 37°C and check daily for confluency.

Cells should be passaged when they become confluent.

Maintain the culture

4 When the cells are a confluent monolayer, aspirate medium.

5 Wash cells once with PBS or trypsin/EDTA to remove remaining serum from the cells by covering cells with the solution and pipetting it off.

6 Overlay cells with 37°C trypsin/EDTA using a volume that is just enough to cover the monolayer (e.g., 1 ml for a 75-cm^2 flask, 2ml for a 162-cm^2 flask). Incubate up to 5 min (cells should become detached) and shake the flask to completely detach cells.

7 Add 4 or 8 ml of appropriate maintenance medium. Pipet the cell suspension up and down several times to disrupt clumps.

8 Remove 0.5 ml of cell suspension and add it to a new 162-cm^2 tissue culture flask containing 30 ml of maintenance medium. Rotate the flask to evenly distribute the cells and place in a CO_2 incubator at 37°C until the cells are confluent (~1 week). Maintain the cells by splitting ~1:20 in maintenance medium at approximately weekly intervals.

Cells can be maintained in smaller flasks if desired. If so, volumes should be adjusted proportionately.

BASIC PROTOCOL 2

CULTURE OF CELLS IN SUSPENSION

HeLa S3 cells are maintained in complete spinner medium-5.

Materials

Frozen vial of HeLa S3 cells (ATCC CCL2.2)

70% ethanol

Complete MEM-8 (see recipe), 37°C

Trypsin/EDTA: 0.25% (w/v) trypsin/0.02% (w/v) EDTA, 37°C

Complete spinner medium-5 (see recipe), 37°C

75-cm^2 tissue culture flask

Humidified, 37°C, 5% CO_2 incubator

Sorvall H-6000A rotor (or equivalent)

50-ml centrifuge tube

100- or 200-ml vented spinner bottles and caps with filters (Bellco)

Additional reagents and equipment for counting cells with a cell counter or hemacytometer (*APPENDIX 4A*)

The authors use a Nexcelom Cellometer

Begin the culture

1 Thaw a frozen vial of HeLa S3 cells in a 37°C water bath.

2 Sterilize the outside of the vial with 70% ethanol, and transfer the cells with a pipet into a 50-ml centrifuge tube containing 30 ml of warm start-up medium. Centrifuge 5 min in a Sorvall H-6000A at 1200 rpm (420 × *g*), room temperature, and discard supernatant. Resuspend pellet in 1 ml of start-up medium then transfer to a 75-cm^2 tissue culture flask containing 30 ml of complete MEM-8. Rotate the flask to evenly distribute the cells and place overnight in a humidified, 5% CO_2 incubator at 37°C.

3 Aspirate medium. Overlay cells with 1 ml of 37°C trypsin/EDTA and incubate for up to 2 min.

Since these cells do not attach firmly to the flask, they should not be washed prior to trypsinization.

4 Add 10 ml of complete spinner medium-5 and transfer cells to a 50-ml centrifuge tube. Centrifuge for 5 min in a Sorvall H-6000A rotor at 1200 rpm (420 × *g*), room temperature, and discard supernatant.

5 Suspend cell pellet in 5 ml of complete spinner medium-5 by pipetting up and down to disrupt clumps.

6 Add 50 ml of complete spinner medium-5 to a 100- or 200-ml vented spinner bottle and transfer the cell suspension to this bottle.

7 Remove 1 ml of cell suspension and count the cells using a cell counter or hemacytometer (*APPENDIX 3f*). Add complete spinner medium-5 to adjust the cell density to 3–4 × 10^5 cells/ml. Place cells in a 37°C incubator without CO_2 and stir continuously.

The initial high density is used because some cells are not viable.

8 Grow cells for two successive days, counting cells daily and adding complete spinner medium-5 as necessary to maintain a concentration of 3–4 × 10^5 cells/ml.

Maintain the culture

9 Remove 1 ml of cell suspension and count the cells using a cell counter or hemacytometer.

10 When the density is 4–5 × 10^5 cells/ml, dilute the cells to 1.5 or 2.5 × 10^5 cells/ml with complete spinner medium-5 for alternate day or daily feeding, respectively.

11 Place a 100- or 200-ml vented spinner bottle containing 50 ml or 100 ml cells, respectively, in 37°C incubator without CO_2 and stir continuously. Passage every 1 to 2 days.

HeLa S3 cells are grown and maintained in complete spinner medium-5 in vented spinner bottles at 37°C without CO_2. Cells are diluted with fresh medium at 1- to 2-day intervals to keep the cell density between 1.5 × 10^5 and 5 × 10^5 cells/ml.

BASIC PROTOCOL 3

PREPARATION OF A VACCINIA VIRUS STOCK

To prepare a vaccinia virus stock, HeLa S3 cells from a spinner culture (see Basic Protocol 2) are plated the day before infection and allowed to attach. They are then infected with sonicated virus. After several days, the infected cells are harvested and lysed during repeated freeze-thaw cycles.

The virus stock is then sonicated, aliquoted and stored at ⁻80°C. To titer this stock, see Support Protocol 1. The protocol can be modified for monolayer cultures.

Materials

HeLa S3 cells from suspension culture (see Basic Protocol 2)

Complete MEM-8 and −2.5 (see recipe), 37°C

Vaccinia virus (ATCC VR1354 or equivalent)

Sorvall H-6000A rotor (or equivalent)

162-cm^2 tissue culture flask

Cup sonicator (e.g., Ultrasonic Processor VCX-750 from Sonics and Materials)

Humidified, 37°C, 5% CO_2 incubator

Additional reagents and equipment for counting cells with a cell counter or hemacytometer (*APPENDIX 4A*)

Prepare cells

1 Count HeLa S3 cells from a suspension culture using a cell counter or hemacytometer (*APPENDIX 4A*).

2 Centrifuge 5×10^7 cells 5 min in a Sorvall H-6000A rotor at 2500 rpm (1800 × *g*), room temperature, and discard supernatant.

3 Resuspend cells in 25 ml of 37°C complete MEM-8, dispense in a 162-cm^2 tissue culture flask, and lay flat overnight in a humidified, 5% CO_2 incubator at 37°C. Cells will attach to the flask and grow as a monolayer culture.

Increase the number of HeLa cells proportionately if more than one 162-cm^2 flask is to be infected.

As an alternative to HeLa suspension cells, prepare 162-cm^2 flasks of monolayer cells as described (see Basic Protocol 1) and continue with step 4 below.

Sonicate virus

4 Just prior to use, thaw virus and keep on ice for the remainder of the procedure. Sonicate 30 seconds in ice water, followed by a 30 sec rest on ice, and another 30 sec sonication.

Virus stocks are usually at a titer of ~1 × 10^9 pfu/ml, but may be significantly lower depending on the source.

Vortexing usually breaks up any clumps of cells. However, if there are still visible clumps, chill to 0°C and sonicate an additional 30 sec in ice water. Sonication can be repeated several times but the sample should be allowed to cool on ice between sonications. For larger aliquots (> 1ml) increase sonication time to 1 min.

Infect cells

5 Dilute sonicated virus in complete MEM-2.5 at 2.5–7.5 × 10^7 pfu/ml. Decant or aspirate medium from the 162-cm^2 flask of cells and add 2 ml of diluted, sonicated virus. Place 2 h in a CO_2 incubator at 37°C, rocking flask by hand at 30-min intervals.

The optimal multiplicity of infection (MOI) is 1 to 3 pfu/cell. Multiplicities of 0.1 pfu/cell may be necessary if the titer of the initial virus stock is low.

6 Overlay cells with 25 ml of complete MEM-2.5 and place in a CO_2 incubator at 37°C.

Cytopathic effect should be apparent in the infected cells, i.e. cells will become rounded-up and detach readily from the flask within 3 days.

7 Detach the infected cells from the flask by shaking and pour or pipet into a sterile plastic screw-cap tube. Centrifuge 5 min at 1800 × *g*, 5° to 10°C, and discard supernatant.

8 Resuspend cells in 1 ml complete MEM-2.5 (per initial 162-cm^2 flask) by gently pipetting or vortexing.

Harvest virus stock

9 Lyse the cell suspension by freeze-thaw cycling as follows: freeze in dry ice/ethanol, thaw in a 37°C water bath, and vortex. Carry out the freeze-thaw cycling a total of three times.

10 Keep the virus stock on ice. Sonicate twice for 30 sec in ice water, with a 30 sec rest on ice between, and divide it into 0.5- to 2-ml aliquots. Store the aliquots indefinitely at ⁻80°C.

SUPPORT PROTOCOL 1

TITRATION OF VACCINIA VIRUS STOCKS BY PLAQUE ASSAY

Serial dilutions of the sonicated virus stock (see Basic Protocol 3) are used to infect the appropriate cell line. Trypsinization may be used as an alternative to sonication (see Support Protocol 2). After several days growth, the medium is removed and the cells are stained with

crystal violet. Plaques appear as 1- to 2-mm-diameter areas of diminished staining due to the retraction, rounding, and detachment of infected cells.

Additional Materials (also see Basic Protocols 1 and 3)

BS-C-1 cells from confluent monolayer culture (see Basic Protocol 1)

Virus stock (see Basic Protocol 3)

0.1% (w/v) crystal violet (Sigma) in 20% ethanol (store indefinitely at room temperature)

Complete MEM-2.5 containing 0.5% methylcellulose

6-well, 35-mm^2 tissue culture dishes

Additional reagents and equipment for counting cells with a cell counter or hemacytometer (*APPENDIX 4A*)

Prepare cell and virus stocks

1. Trypsinize confluent monolayer of BS-C-1 cells as described (see Basic Protocol 1, steps 4 to 7).
2. Count the cells using a cell counter or hemacytometer (*APPENDIX 4A*).
3. Seed wells of a 6-well, 35-mm^2 tissue culture dish with 5×10^5 cells per well BS-C-1 cells in 2 ml complete MEM-10. Place overnight in a humidified, 5% CO_2 incubator at 37°C to reach confluency.
4. Sonicate virus stock (see Basic Protocol 3, step 4).
5. Make nine 10-fold serial dilutions of the sonicated virus in complete MEM-2.5, using a fresh pipet for each dilution.

Perform assay

6. Remove medium from BS-C-1 cells and infect cells in duplicate wells with 1 ml of the 10^{-6}, 10^{-7}, and 10^{-8} sonicated virus dilutions. Place 1 to 2 hr in a CO_2 incubator at 37°C.
7. Aspirate virus inoculum and overlay cells in each well with 2 ml of complete MEM-2.5 with methylcellulose and place 2 days in a CO_2 incubator at 37°C.
8. Remove medium and add 0.5 ml of 0.1% crystal violet to each well. Incubate 20 min at room temperature.
9. Aspirate crystal violet, rinse plates with water, and allow wells to dry.
10. Determine the titer by counting plaques within the wells and multiplying by the dilution factor.

    Most accurate results are obtained from wells with 20 to 80 plaques.

SUPPORT PROTOCOL 2

TRYPSINIZATION OF VACCINIA VIRUS STOCKS

Trypsinization digests cell debris and increases virus titer approximately 3-fold and may be used instead of sonication. Trypsinization is done just prior to use, and trypsinized virus must be diluted at least ten-fold to reduce trypsin activity before applying to cells. Only trypsinize the amount of virus to be used in a given experiment as storage of trypsinized virus may result in a reduction in infectivity. To accurately determine the multiplicity of infection used in experiments, the titer of virus stock that has been trypsinized should be calculated by performing a plaque assay. When determining titer, remember to take into account the 1:1 dilution of the virus stock with trypsin.

Materials

Virus stock (see Basic Protocol 3)

0.25 mg/ml trypsin (2× crystallized and salt-free; Worthington; filter sterilize and store at ⁻20°C)

Trypsinize virus—Just prior to use, mix an equal volume of vaccinia virus stock and 0.25 mg/ml trypsin, and vortex vigorously. Incubate 30 min in a 37°C water bath, vortexing at 5- to 10-min intervals throughout the incubation.

BASIC PROTOCOL 4

PREPARATION OF CHICKEN EMBRYO FIBROBLASTS

The attenuated, replication-deficient modified vaccinia virus Ankara (MVA) can be used as an alternative to standard strains of vaccinia virus, for use as a vector to express foreign genes. MVA was derived from the vaccinia virus strain Ankara by multiple (>570) passages in chick embryo fibroblasts (CEF). Although MVA expresses recombinant proteins efficiently, the assembly of infectious particles is interrupted in human and most other mammalian cells lines, providing an added degree of safety to laboratory personnel. The NIH Intramural Biosafety Committee has determined that individuals do not need to be vaccinated to work with MVA, and can do so under biosafety level 1 (BL-1) conditions if no other vaccinia virus strains are being manipulated at the same location. Recombinant MVA expressing bacteriophage T7 RNA polymerase, which can be used for high-level transient expression, has also been constructed (Elroy-Stein and Moss, 2001).

CEF or Syrian hamster kidney cells (BHK-21, see Basic Protocol 1) are permissive for growth of MVA. MVA is titered by immunostaining (see Support Protocol 3), because it does not form distinct plaques for accurate quantitation.

In this protocol, ten-day-old chicken embryos are trypsinized and plated in appropriate medium. After the cells form a confluent monolayer, they are transferred directly to a 31°C incubator where they can be held for up to 2 weeks before making secondary CEF for virus infection. With each passage, CEF require longer to reach confluency; therefore use of up to the second passage is recommended.

Materials

Ten 10-day-old embryonated eggs (Specific Pathogen Free Eggs, SPAFAS)

70% ethanol

MEM with no additives, 37°C

FBS

Trypsin/EDTA: 0.25% (w/v) trypsin/0.02% (w/v) EDTA, 37°C

Complete MEM-8 (Table 16.16.2; see recipe), 37°C

Sterile dissecting scissors and forceps

100-cm^2 sterile petri dishes

10-ml syringes

Sterile trypsinization flask with magnetic stir bar

Humidified, 37° and 31°C, 5% CO_2 incubators

500-ml beaker with two layers of gauze taped over top

Sorvall H-6000A rotor and 250-ml centrifuge bottles (or equivalent)

50 ml centrifuge tubes

162-cm^2 tissue culture flasks

Prepare tissue

1 Position ten 10-day-old embryonated eggs with air space (blunt end) up and spray with 70% ethanol.

2 Crack the top of an egg with sterile dissecting scissors, and cut off shell to just above the membrane while keeping the latter intact. Remove membrane with sterile forceps. Repeat with remaining eggs.

NOTE: *Healthy eggs have well-formed blood vessels.*

3 Remove the embryo from each egg and combine in a 100-cm^2 sterile petri dish.

4 Remove head, wings and feet from each embryo and place the rest of the body in a 100-cm^2 sterile petri dish containing 10 ml MEM with no additives. Mince embryos by squeezing through 6-cc syringes (~5 embryos/syringe) into a sterile trypsinization flask.

Dissociate cells

5 Add 50 ml of 37°C trypsin/EDTA and incubate for 10 min in a humidified, 5% CO_2 incubator at 37°C, with stirring.

6 Decant fluid from the trypsinization flask into a 500-ml beaker covered with gauze, and add 10 ml of FBS to reduce trypsin activity.

7. Add 50 ml of fresh trypsin/EDTA to the remaining tissue in the trypsinization flask, and incubate 5 min at 37°C.
8. Pour digest into the same 500-ml beaker covered with gauze. Transfer filtrate to the 250-ml centrifuge bottle and bring volume up to 150 ml with MEM-8.
9. Centrifuge for 15 min at 1200 × *g*, 4°C.
10. Aspirate and discard supernatant from pellet, add 10 ml complete MEM-8, resuspend by pipetting ~10 to 15 times, and adjust volume to 45 ml.
11. Transfer to 50-ml centrifuge tube and centrifuge for 15 min at 1200 × *g*, 4°C.
12. Resuspend pellet in 10 ml of complete MEM-8 and adjust volume to 40 ml.

Prepare confluent culture

13. Add 1 ml of cell suspension to each of thirty 162-cm^2 tissue culture flasks containing 30 ml of complete MEM-8.
14. Incubate at 37°C for several days until cells are confluent, and move flasks to a humidified, 31°C, 5% CO_2 incubator for storage.

    The CEF cell cultures can be held for up to 2 weeks at 31°C without further attention. Primary CEF can also be used directly for virus growth, or the trypsinized stock of CEF can be frozen in liquid nitrogen for future use.

BASIC PROTOCOL 5

PREPARATION OF AN MVA STOCK

To prepare an MVA stock, CEF (see Basic Protocol 4) or BHK-21 (see Basic Protocol 1) cells are plated one to several days before infection and allowed to approach confluency. They are then infected with MVA. After several days, the infected cells are harvested, lysed by repeated freezing and thawing, sonicated, dispensed in small aliquots, and stored at ⁻80°C.

NOTE: BHK-21 cells should be acquired from ATCC, as cells from alternative sources may support lower levels of MVA replication. BHK-21 should be used at low passage (<20) as virus replication efficiency diminishes with higher passage cells.

Materials

162-cm^2 tissue culture flasks of nearly confluent CEF (see Basic Protocol 4) or BHK-21 cells (see Basic Protocol 1)

Complete MEM-8 and -2.5 (see recipe), 37°C

Modified vaccinia virus Ankara (MVA; ATCC VR-1508)

162-cm^2 tissue culture flasks

Humidified, 37°C, 5% CO_2 incubator

Sorvall H-6000A rotor and sterile 250-ml centrifuge bottles (or equivalent)

Additional reagents and equipment for trypsinizing cells (see Basic Protocol 1)

Prepare cells

1 Trypsinize seven 162-cm^2 tissue culture flasks of nearly confluent CEF *or* two of BHK-21 cells as described (see Basic Protocol 1, steps 4 to 7), using 2 ml trypsin/EDTA and 8 ml maintenance medium for the larger flask. Distribute to twenty 162-cm^2 flasks. Incubate at 37°C in a humidified, 5% CO_2 incubator until nearly confluent monolayers have formed (usually 2 days).

CEF and BHK-21 cells are split 1:3 and 1:10, respectively. It is advisable to use monolayers of BHK-21 cells at 90% confluency, as completely confluent cultures degrade within 48 hr.

2 Remove medium and add 30 ml complete MEM-2.5 to each 162-cm^2 flask.

Sonicate virus

3 Thaw MVA virus and sonicate 30 sec in ice water to break up clumps.

Trypsinizaton of virus is avoided with MVA because it may lower the titer.

Infect cells

4 Add 1 to 3 infectious units of virus/cell to the medium and place cells in a CO_2 incubator for 3 days at 37°C.

A monolayer of CEF or BHK-21 cells in a 162 cm^2 flask contains ~1–2 × 10^7 cells. If there is insufficient virus, then use fewer or smaller flasks of confluent cells.

Harvest virus stock

5 Detach the cells with a cell scraper or, if possible, by shaking. Transfer cells and medium to a sterile 250-ml centrifuge bottle and centrifuge for 10 min at 1200 × *g*, 4°C. Discard supernatant.

6 Resuspend cells in 1 ml of complete MEM-2.5 (per 162-cm^2 flask) by pipetting or vortexing.

7 Lyse cells by freeze-thaw cycling: freeze in dry ice/ethanol, thaw in a 37°C water bath, and vortex. Carry out the freeze-thaw cycling a total of three times.

8 Sonicate stock in ice water for 1 min, pause for 1 min, and repeat sonication. Dispense in 0.5-ml aliquots and store indefinitely at ⁻80°C.

SUPPORT PROTOCOL 3

TITRATION OF MVA STOCKS BY IMMUNOSTAINING

The titer of MVA is routinely determined by immunostaining because MVA does not form distinct plaques in CEF or BHK-21 cells. To determine the MVA titer, serial dilutions of virus stock are prepared and used to infect CEF or BHK-21 cells. At 24 hr after infection, the cells are fixed with acetone/methanol and immunostained using a polyclonal vaccinia virus antiserum and a secondary peroxidase-conjugated antibody. A focus of infected cells appears as a 0.3- to 0.4-mm reddish brown–stained area. If the cells are left for two days before immunostaining, secondary foci may form by virus spread giving inaccurate titers.

Materials

CEF (see Basic Protocol 4) or BHK-21 cells (see Basic Protocol 1) in a 162-cm^2 tissue culture flask

Complete MEM-8 and -2.5 (see recipe), 37°C

MVA stock (see Basic Protocol 5)

1:1 (v/v) acetone/methanol

PBS (*APPENDIX 2A*), with and without 3% FBS

Rabbit anti-vaccinia antibody (e.g., Pierce via ThermoScientific or see Linscott, 1998)

Horseradish peroxidase–conjugated whole anti–rabbit Ig antibody (HRP-anti-rabbit; Pierce via ThermoScientific)

Dianisidine, or premade peroxidase substrate kit (Vector Laboratories)

PBS/H_2O_2 (add 10 µl 30% H_2O_2 to 10 ml PBS immediately before use)

6-well, 35-mm^2 tissue culture dishes

Humidified, 37°C, 5% CO_2 incubator

Additional reagents and equipment for trypsinizing cells (see Basic Protocol 1)

Infect and fix cells

1 Trypsinize a 162-cm^2 tissue culture flask of CEF or BHK-21 cells as described (see Basic Protocol 1, steps 4 to 6), using 2 ml of trypsin/EDTA for the larger flask. Resuspend cells by repeated pipetting in 8 ml complete MEM-8, and add an additional 50 ml of complete MEM-8.

2 Add 2 ml of cell suspension to each well of a 6-well, 35-mm^2 tissue culture dish. Incubate overnight in a humidified, 5% CO_2 incubator at 37°C to reach near confluency.

3 Remove medium and replace with 1 ml of complete MEM-2.5.

4 Thaw MVA stock and sonicate for 30 sec in ice water.

5 Make eight 10-fold serial dilutions of the virus in complete MEM-2.5, using a fresh pipet for each dilution.

6 Add 1 ml of each of the 10^{-7}, 10^{-8}, and 10^{-9} dilutions to cells in duplicate wells. Swirl gently to mix, and incubate 24 hr in a CO_2 incubator at 37°C.

7 Remove fluid and fix cells 2 min with 1 ml of 1:1 acetone/methanol. Remove fixative and add 2 ml PBS to each well.

At this point, the plates can be immunostained immediately or stored at 4°C for several weeks.

Perform immunostaining

8 Dilute rabbit anti-vaccinia antibody in PBS containing 3% FBS and add 1 ml/well. Incubate for 1 hr at room temperature, with gentle rocking.

The optimal dilution for each antibody must be determined empirically, but a 1:500 or 1:1000 dilution is a good place to start.

9 Wash twice with 2 ml of PBS.

10 Dilute HRP-anti-rabbit secondary antibody in PBS containing 3% FBS and add 1 ml/well. Incubate for 30 to 45 min at room temperature, with gentle rocking.

The optimal dilution for each antibody must be determined, but a 1:500 or 1:1000 dilution is a good place to start. If another anti-vaccinia virus antibody is used in step 8, the appropriate HRP-conjugated anti-species secondary antibody should be substituted here.

11 Wash twice with 2 ml PBS.

12 Make a saturated solution of dianisidine in 0.5 ml ethanol, vortex, incubate for 5 min at 37°C, and clarify by microcentrifugation at maximum speed for 1 min. Add 0.2 ml dianisidine solution to 10 ml PBS/H_2O_2. Alternatively, prepare substrate from a kit according to manufacturer's instructions.

CAUTION: *Dianisidine is carcinogenic and the powder should be manipulated with gloves in a fume hood. 30% H_2O_2 is caustic to skin and should be handled with gloves.*

13 Add 0.5 ml of dianisidine substrate solution or substrate solution from kit to each well. Rotate dish gently and let stand ~10 min. Check microscopically for foci of infected cells. When development is complete, wash dishes with water and overlay with 1 ml water to preserve stain.

Weaker antibody may take longer to develop.

14 Count the number of stained foci and multiply by the dilution factor to express titer as infectious units/ml.

Most accurate results are obtained from wells with 20 to 100 plaques.

BASIC PROTOCOL 6

PURIFICATION OF VACCINIA VIRUS

Vaccinia virus is usually purified by zonal sucrose gradient centrifugation. Purification is useful in studies in which contaminating infected-cell proteins are undesirable, and to increase virus titer. For large-scale purification (as in this protocol, which is for 1-liter cultures or multiples thereof) it is preferable to use HeLa cell suspensions for infection rather than monolayer cultures. If monolayer cells are used, follow the alternative procedure for monolayers (Basic Protocol 3) then continue with step 14 of this protocol. For many purposes, virus that is partially purified (through step 14 of this protocol) may suffice. For purification of MVA, virus is not trypsinized and either CEF or BHK-21 cells are used instead of HeLa.

Materials

Vaccinia virus stock (see Basic Protocol 3)

HeLa S3 cells growing in suspension culture (see Basic Protocol 2)

Complete spinner medium–5 (see recipe)

10 mM and 1 mM Tris·Cl, pH 9.0 (*APPENDIX 2A*)

36% (w/v) sucrose solution in 10 mM Tris·Cl, pH 9.0

40%, 36%, 32%, 28%, and 24% (w/v) sucrose solutions in 1 mM Tris·Cl, pH 9.0

Gradient Master (BioComp Instruments) (optional)

Requires 25% and 40% sucrose solutions in 1 mM Tris·Cl, pH 9.0

Sorvall centrifuge with H-6000A rotor (or equivalent)

2-liter vented spinner flasks (microcarrier type; Bellco)

15 ml or 40 ml Dounce homogenizer, glass, with tight pestle

Cup sonicator (e.g., Ultrasonic Processor VCX-750 from Sonics and Materials)

Ultracentrifuge with Beckman SW 27 or SW 28 rotor (or equivalent) and sterile centrifuge tubes

Spectrophotometer

ViroCyt virus counter (optional)

Additional reagents and equipment for tissue culture and counting cells (*APPENDIX 4A*), and titering virus (see Support Protocol 1)

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly. Incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Infect the cells

1. Just prior to use, thaw virus and keep on ice for the remainder of the procedure. Sonicate 30 sec in ice water, followed by a 30 sec rest on ice, and another 30 sec sonication.

   Virus stocks are usually at a titer of ~1×10^9 pfu/ml, but may be significantly lower depending on the source.

   Vortexing usually breaks up any clumps of cells. However, if there are still visible clumps, chill to 0°C and sonicate 30 sec in ice water. Sonication can be repeated several times but the sample should be allowed to cool on ice between sonications.

2. Count HeLa S3 cells using a cell counter or hemacytometer (*APPENDIX 4A*).

3. Transfer 5×10^8 HeLa S3 cells into a centrifuge tube, then centrifuge for 10 min at 1800 × *g* (2500 rpm in an H-6000A rotor), room temperature, and discard supernatant.

4. Resuspend cells in complete spinner medium-5 at 2×10^7 cells/ml.

5. Add sonicated virus (from step 1) at an MOI of 5 to 8 pfu/cell and incubate for 30 min with stirring.

6. Transfer cells to a vented spinner flask containing 1 liter complete spinner medium-5 and incubate for 2 to 3 days with stirring.

7. Centrifuge cells 5 min at 1800 × *g*, 5° to 10°C, and discard supernatant.

8. Resuspend cells in 14 ml of 10 mM Tris·Cl, pH 9.0. Keep samples on ice for the remainder of the protocol.

Lyse the cells

9. Homogenize cell suspension with 30 to 40 strokes in a glass Dounce homogenizer with tight pestle. Check for cell breakage by light microscopy.

10. Centrifuge 5 min at 300 × *g* (900 rpm in H-6000A rotor), 5° to 10°C, to remove nuclei. Save the supernatant.

11. Resuspend cell pellet in 3 ml of 10 mM Tris·Cl, pH 9.0. Centrifuge for 5 min at 300 × *g*, 5° to 10°C. Save supernatant and pool with supernatant from step 10.

12. Sonicate pooled supernatants (lysate), keeping the lysate cold the entire time, using a cup sonicator as follows.

    a. Split the sample into 3-ml aliquots to be sonicated separately.

    b. Fill the cup with ice-water (~50% ice). Place the tube containing the lysate in the ice-water and sonicate for 1 min at full power.

    c. Repeat this three to four times, placing the lysate on ice for ≥30 sec between sonications.

Because sonication melts the ice, it is necessary to replenish the ice in the cup.

Obtain purified virus

13 Layer the sonicated lysate onto a cushion of 17 ml of 36% sucrose in a sterile SW 27 (or SW 28) centrifuge tube. Centrifuge 80 min at 32,900 × *g* (13,500 rpm in an SW 27 rotor), 4°C. Aspirate and discard the supernatant.

14 Resuspend the viral pellet in 1 ml of 1 mM Tris·Cl, pH 9.0.

15 Sonicate once for 1 min as in step 12.

16 Prepare a sterile 24% to 40% continuous sucrose gradient in a sterile SW 27 centrifuge tube the day before it is needed by carefully layering 6.8 ml each of 40%, 36%, 32%, 28%, and 24% sucrose. Let sit overnight in refrigerator to allow some diffusion.

If a gradient maker is used, 25% and 40% sucrose solutions are prepared. The 2 solutions are combined in the centrifuge tubes and mixed according to the manufacturer's instructions.

17 Overlay the sucrose gradient with 1 ml of sonicated viral pellet from step 15. Centrifuge for 50 min at 26,000 × *g* (12,000 rpm in an SW 27 rotor), 4°C.

18 Observe the virus as a milky band near the middle of the tube. Aspirate the sucrose above the band and discard. Carefully collect the virus band (~10 ml) with a sterile pipet, place in a sterile tube, and save.

19 Collect aggregated virus from the pellet at the bottom of the sucrose gradient after aspirating the remaining sucrose from the tube. Resuspend the viral pellet by pipetting up and down in 1 ml of 1 mM Tris·Cl, pH 9.0.

20 Sonicate resuspended pellet once for 1 min as in step 12.

21 Reband the virus from the pellet as in steps 16 to 18 and pool band with band from step 18. Add 2 vol of 1 mM Tris·Cl, pH 9.0, and mix. Transfer to sterile SW 27 centrifuge tubes.

The total volume should be ~60 ml, which is enough to fill two SW 27 centrifuge tubes. If less volume is obtained, fill the tubes with 1 mM Tris·Cl, pH 9.0.

22 Centrifuge 60 min at 32,900 × *g*, 4°C, then aspirate and discard supernatant.

23 Resuspend the virus pellets in 1 ml of 1 mM Tris·Cl, pH 9.0. Sonicate as in step 12 and divide into 200- to 250-µl aliquots. Save one aliquot for step 24 and freeze remainder at ⁻80°C.

Quantitate virus

24 On the unfrozen aliquot, estimate the amount of virus spectrophotometrically at 260 nm.

One optical density unit is ~1.2×10^{10} virus particles, which is ~$2.5–5 \times 10^{8}$ pfu. This value is due to light scattering, not absorbance, and therefore may vary with different spectrophotometers.

25 Titer the virus by sonicating 20 to 30 sec in ice water (see step 12), preparing 10-fold serial dilutions down to 10^{-10}, and infecting, in duplicate, confluent BS-C-1 cell monolayers in 6-well tissue culture plates using the 10^{-8}, 10^{-9}, and 10^{-10} dilutions (Support Protocol 1)

26 If a ViroCyt virus counter is available, measure the number of particles according to the manufacturer's instructions. Best results are obtained with 5×10^{6} to 10^{9} particles/ml.

REAGENTS AND SOLUTIONS

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

Complete DMEM-8 or -16

Dulbecco's minimum essential medium (DMEM) containing:

8% or 16% fetal bovine serum (FBS)

0.03% glutamine

100 U/ml penicillin

100 μg/ml streptomycin sulfate

Store up to several months at 4°C

Add supplements from stock solutions prepared in water at the following initial concentrations: 3% glutamine (100×), 20,000 U/ml penicillin (200×), and 20 mg/ml streptomycin (200×). Filter sterilize stock solutions. Store 100× glutamine 4 months at 4°C, and 200× penicillin/streptomycin 4 months at −20°C.

FBS is added at 8% to complete medium for maintenance of cells and at 16% to encourage initial growth upon thawing.

Complete MEM-2.5, -8, or -16

Minimum essential medium (MEM) containing:

2.5%, 8%, or 16% FBS

0.03% glutamine

100 U/ml penicillin

100 μg/ml streptomycin sulfate

Store up to several months at 4°C

Add supplements from stock solutions as described for complete DMEM (see recipe). Also see annotation to DMEM recipe concerning growth versus maintenance levels of serum.

Complete MEM-8/BrdU or -16/BrdU

Prepare as for complete MEM-8 or -16 (see recipe) and add a 5 mg/ml solution of 5-bromodeoxyuridine (BrdU) to 25 μg/ml final. Store up to several months at 4°C.

Prepare the 5 mg/ml BrdU stock solution (200×) in water and filter sterilize. Store in the dark at ⁻20°C. After thawing 5 mg/ml BrdU, vortex to be sure it is in solution before adding to MEM.

Complete MEM-2.5 with Methylcellulose

Final formulation is identical to complete MEM-2.5 (see recipe) but with 0.5% w/v methylcellulose.

Weigh 2.5g methylcellulose and place into a glass bottle containing a stir bar. Autoclave to sterilize. Allow to cool. Add 500 ml MEM and, stir at 4°C until methycellulose is dissolved (this may take several days). A sterile pipette may be used to break up clumps. This should be performed in a biological safety cabinet. Once methycellulse is dissolved, add supplements from stock solutions as described for complete MEM.

Complete spinner medium-5

MEM spinner medium

5% horse serum

Store up to several months at 4°C

Supplements are in MEM spinner medium; no addition is necessary. Horse serum is used because it is cheaper than FBS and may give less cell clumping.

COMMENTARY

Background Information

Vaccinia virus, the prototype species of the poxvirus family, was used as a live vaccine to prevent smallpox and most of what we know about poxvirus biology has come from studies with vaccinia virus. Like all poxviruses, vaccinia virus replicates entirely in the cytoplasm and encodes numerous enzymes and factors for transcription, DNA replication and assembly as well as host defense. Vaccinia virus contains a linear double stranded DNA genome of approximately 200,000 base pairs with covalently linked ends. The 200+ genes are divided into early, intermediate and late classes that are transcribed by a virus-coded multisubunit DNA-dependent RNA polymerase in conjunction with early, intermediate and late transcription factors. The early transcription system is packaged in the infectious virus particle allowing expression immediately after infection, whereas the intermediate and late

genes are transcribed following DNA replication at 4 h. The assembly of infectious particles begins at about 6 h with the formation of crescent structures that enlarge to form spherical immature virions within the DNA replication centers known as virus factories. The immature virions undergo morphological changes to form brick-shaped mature infectious virions. Some of the mature virions become wrapped in a double membrane derived from the trans-Golgi or endosomal membranes and are transported via microtubules to the periphery of the cell where exocytosis occurs. The extracellular virions have two membranes – the original mature virion membrane and an additional wrapping membrane. Most of the extracellular virions remain associated with the plasma membrane, though some are released into the medium depending to some extent on the virus strain. Importantly, most of the infectivity remains cell associated as intracellular mature virions and also as cell associated enveloped virions. More information on the biology of vaccinia virus and other poxviruses may be obtained in a review (Moss, 2013).

HeLa cells, which consistently give high yields of virus, are routinely used for preparation of virus stocks. HeLa S3 cells, as obtained from the ATCC, grow well in monolayer culture but can also be put into suspension culture. After repeated passages in suspension, they do not adhere well to flasks and grow poorly in monolayer cultures. The authors prefer HeLa cells adapted to suspension culture because large numbers can be grown in a single spinner bottle. However, suspension cultures require more maintenance than monolayer cultures and the latter may be more convenient.

Suspension cells are allowed to form a monolayer before infection to increase the chances of cell-to-cell spread if not all cells are initially infected with the viral inoculum. Thus, good yields of virus may be obtained even if the inoculum is <1 pfu/cell. HeLa cells—even those adapted to monolayer culture—are fairly round to begin with and therefore show little visible evidence of infection. BS-C-1 cells, by contrast, are long and spindle-shaped but round up dramatically a few hours after infection. This property accounts for the highly visible plaques obtained with BS-C-1 cells, and their preference for titration by plaque assay.

The Western Reserve (WR) strain of vaccinia virus (ATCC VR1354; NCBI Ref. Seq: NC-006998.1) is widely used for laboratory studies. It gives high yields of cell-associated virus, discrete plaques, and is well adapted to mice and other laboratory animals. Other strains of vaccinia virus are available from the ATCC and private sources.

Horse serum is used for growth of HeLa cells in suspension because it is cheaper than FBS and may give less cell clumping. Horse serum is used in spinner medium for growth of HeLa cells in suspension. All monolayer cells are grown in medium with FBS.

Modified vaccinia virus Ankara (MVA; Mayr et al., 1975) was one of several highly attenuated strains of vaccinia virus that were developed but were not extensively used for smallpox vaccination, in part because of the successful eradication of the disease with the conventional vaccine strains. Restriction enzyme analysis (Meyer et al., 1991) and DNA sequencing (Antoine et al, 1998; GenBank: AY603355.1) demonstrated that MVA had suffered multiple deletions during its long passage history in CEF that may account for its severe host restriction, including an inability to replicate efficiently in human and most other

mammalian cells (Carroll and Moss, 1997). It was somewhat surprising to find that the replication defect occurred at a late stage of virion assembly and consequently that viral or recombinant gene expression was unimpaired (Sutter and Moss, 1992). Therefore, MVA can be viewed as an efficient single-round expression vector with little potential to spread. Although MVA causes less cytopathic effects than standard vaccinia virus in some cell lines, in others the effect is quite severe (Carroll and Moss, 1997).

Because of its host-range restriction, recombinant MVA were initially made and propagated exclusively in primary or secondary CEF. A stable Syrian hamster kidney cell line, BHK-21, is also permissive for MVA growth and could be used for making recombinant viruses (Carroll and Moss, 1997). Although many laboratories may prefer to use a stable cell line, the authors continue to employ CEF because they give higher MVA titers, can be stored in a dormant state for several weeks (whereas BHK-21 cells must be passaged every 5 days), and are better suited to immunostaining because of greater adherence than BHK-21 cells to plastic dishes.

Critical Parameters

During purification of vaccinia virus (see Basic Protocol 6) it is important to check the extract after Dounce homogenization to be sure that cells are broken; if they are not, repeat with more force for ten more strokes and check again. In addition, be sure to perform all steps on ice (this is particularly important when sonicating). Use sterile technique throughout.

Proper maintenance of actively growing cell lines is important in order to achieve efficient infections and high yields of virus. This is especially true for HeLa S3 cell suspension cultures, which should be maintained at $1.5–5 \times 10^5$ cells/ml (requiring passaging every 1 to 2 days). HeLa cells can achieve a density of $8–10 \times 10^5$ cells/ml, but exhibit a lag period upon dilution before optimal growth is resumed; prolonged maintenance at high density will lead to cell death. HeLa S3 cells adapted to grow in suspension cultures do not grow well in monolayer cultures. However, the cells will adhere to flasks, which allow the cells to be infected on the following day (see Basic Protocol 3).

Since most progeny viruses remain cell-associated, infected cells must be disrupted by freeze-thaw cycling and sonication or trypsinization. These procedures are important for releasing virus from the host cells. An entire stock of virus can be subjected to freeze-thaw cycling and sonication, but only the portion to be used should be trypsinized. Sonication is unnecessary if the virus stock has been trypsinized. Unlike standard replication-competent vaccinia protocols, trypsin is not used as a pretreatment to enhance the infectivity of MVA. Pretreatment of MVA with trypsin can decrease virus titer.

Although vaccinia virus is relatively stable, stocks should be kept on ice while in use and should be stored at ⁻80°C. A vaccinia virus stock should have a stable titer for many years when stored at this temperature. Although the stock can be frozen and thawed several times without loss of infectivity, storing in aliquots is recommended.

The preparation of a seed stock of virus that is sufficient for future experiments is strongly recommended. A common mistake is to continually passage the virus.

Anticipated Results

A vaccinia stock should have a titer of ≥1–2 × 10^9 pfu/ml. MVA stocks can approach a titer of 1 × 10^{10} infectious units/ml when prepared as described (see Basic Protocol 5), and recombinant virus can reach 1–3 × 10^9 infectious units/ml. The viral stock should have a stable titer for many years when stored as indicated above (see Critical Parameters).

Time Considerations

During purification of vaccinia virus, the viral amplification takes 2 to 3 days. After harvesting the infected cells, the entire purification can be done in 1 day. However, the protocol can be stopped after resuspension at step 12 or after collecting the band at step 23. The virus can be stored overnight at 4°C or at ⁻70°C for longer periods.

Actively growing cells should be prepared in advance, as it will take ≥1 week to revive frozen cells. For preparation of a vaccinia virus stock, infected cells should be incubated for 3 days. During this period, the BS-C-1 cells for plaque titration can be prepared. For a small volume of stock (i.e., from one 162-cm^2 flask), harvesting and freeze-thaw cycling can be done in <1 hr. Determination of the titer of a virus stock requires 2 days of incubation to allow for development of plaques.

Preparation of CEF cells from embryonated eggs requires ~2 hr. Thereafter, they can be stored for several weeks with no maintenance. Starting BHK-21 cells from frozen cultures can take >1 week and requires passaging every 5 days. For preparation of MVA stocks, 3 days are required for virus growth, 2 hr for harvesting, freeze-thaw cycling, and dispensing, and 1 day of incubation for determination of the virus titer by immunostaining. The immunostaining procedure takes ~3 hr.

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Table 14A.3.1

Cell Lines Used in Specific Vaccinia Protocols

Cell line	Use[a]	Procedure
HeLa S3	Virus stock preparation	*UNIT 14A.3* Basic Protocol 3
	Virus purification	*UNIT 14A.3* Basic Protocol 6
BS-C-1	Plaque assay	*UNIT 14A.3* Support Protocol 1
	Transfection	*UNIT 14A.4* Basic Protocol 1
	Plaque size screening	*UNIT 14A.3* Basic Protocol 2
	Fluorescent protein screening	*UNIT 14A.4* Basic Protocol 2
	XGPRT selection	*UNIT 14A.4* Basic Protocol 2
	Plaque amplification	*UNIT 14A.4* Basic Protocol 3
$HuTK^-$ 143B	TK selection	*UNIT 14A.4* Basic Protocol 2
	Plaque assay (optional)	*UNIT 14A.3* Support Protocol 1
	Transfection (optional)	*UNIT 14A.4* Basic Protocol 2
CEF	MVA procedures	*UNIT 14A.3* Basic Protocol 5, Support Protocol 3; *UNIT 14A.4*
		Basic Protocol 4
BHK-21	MVA procedures	*UNIT 16.16* Basic Protocol 5, Support Protocol 3; *UNIT 16.17*
		Basic Protocol 4

[a]The preferred use(s) for each cell line is listed first; if optional is indicated, the cell line can be used for the indicated procedure, but the results may not be as good as those from the preferred cell line.

Table 14A.3.2

Media Used for Growth and Maintenance of Cell Lines[a]

Cell line	Maintenance medium	Start-up medium[A]
BHK-21	Complete MEM-8	Complete MEM-16
BS-C-1	Complete MEM-8	Complete MEM-16
CEF	Complete MEM-8	Complete MEM-16
HeLa S3	Complete spinner medium-5	Complete MEM-8
$HuTK^-$ 143B	Complete MEM-8/BrdU	Complete MEM-16/BrdU

[a]See Reagents and Solutions for recipes.