Methods for Growing and Titrating African Swine Fever Virus: Field and Laboratory Samples

Angel L. Carrascosa,[1] M. Jose Bustos,[1] and Patricia de Leon[1]

[1]Centro de Biologia Molecular "Severo Ochoa" (CSIC-UAM), Universidad Autonoma de Madrid, Madrid, Spain

ABSTRACT

Growing African swine fever virus (ASFV) isolates obtained mainly from the field, but also engineered in the laboratory, is a critical step for diagnosis, titration, or virus infection studies. This unit describes a set of methods and protocols to produce and titrate any ASFV strain in cell cultures. The procedures include (1) basic techniques to prepare virus-sensitive target cells; (2) strategies for growth, concentration, and purification of virus stocks; and (3) the semi-quantitative (end dilution) and quantitative (plaque) assays for the determination of viral titers, and the use of different ASFV-sensitive cells as targets for virus production and titration. *Curr. Protoc. Cell Biol.* 53:26.14.1-26.14.25.

Keywords: African swine fever virus (ASFV) • susceptibility • production • purification • titration • hemadsorption • cytopathic effect • plaque assay • target cells • Vero • COS-1 • IPAM • WSL • swine macrophages

INTRODUCTION

African swine fever virus (ASFV) is the causative agent of a highly contagious disease of swine mainly affecting many sub-Saharan African countries, but emerging lately in the Caucasus region and Russian Federation in the Euro-Asian continent. The disease courses within a range of conditions from acutely fatal hemorrhagic fever to chronic or unapparent persistent infection (Vinuela, 1985) and can be transmitted by ticks of the Ornithodoros genus. In domestic pigs, the virus infects tissue macrophages, blood monocytes, and, to a lesser extent, specific lineages of reticular, polymorphs, and megakaryocytic cells (Casal et al., 1984; Wilkinson, 1989).

As there is no vaccine available against ASFV, the rapid and accurate laboratory diagnosis of ASFV-positive and carrier animals is critical for the control of virus outbreaks. All of the diagnostic procedures developed thus far, including ELISA, immunoblotting, or PCR (Barderas et al., 2000; Aguero et al., 2003; Zsak et al., 2005) depend on the concentration and quality of the virus samples harvested from the outbreak and may require a preliminary amplification in the laboratory by infecting a suitable virus-sensitive cell culture. Protocols for the preparation of swine peripheral blood monocytes or alveolar macrophages, and the conditions to infect several established cell lines (Vero, COS-1, IPAM, and WSL) that allow for the production of specific ASFV isolates are described in this unit. Methods for growth and purification of ASFV samples at a medium-scale, based in Percoll gradients, are also presented. Assay of infectivity, end-point titrations by hemadsorption or cytopathic effect on swine macrophages, and plaque assay on alveolar macrophages, Vero, or COS-1 cells are also described. All of these protocols can be applied, with minor modifications, to other virus and cell models.

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NOTE: All virus manipulations described in this unit must follow the international recommendations and biosafety guidelines required for this agent (Biosafety Level 3).

BASIC PROTOCOL 1

PREPARATION OF SWINE PERIPHERAL BLOOD MONOCYTES

All of the ASFV isolates obtained from natural outbreaks can be grown in peripheral blood monocytes, the basis for the most traditional method of specifically detecting and titrating ASFV samples. Blood monocytes can also be used for infectivity assays, as hemadsorbing virus strains form erythrocyte rosettes around infected swine blood monocytes. Swine monocytes are one of the in vitro systems of choice for growing virus stock, as wild-type ASFV isolates from natural outbreaks were not found to replicate in conventional established cell cultures. This protocol is an adaptation of Enjuanes et al. (1976).

Materials

Swine (large white, 20 to 30 kg)
70% ethanol
100-ml glass bottles containing anticoagulant and antibiotics (40 IU/ml heparin/100 IU/ml penicillin/0.1 mg/ml streptomycin)
Phosphate-buffered saline (PBS; see recipe)
Heparin
Swine serum (see Support Protocol 1)
Erythrocyte lysis buffer (see recipe)
Dulbecco's modified Eagle medium (DMEM; *APPENDIX 2A*)

Restraining ropes
Atraumatic needles (22- or 23-G; Novartis) connected to a Multifix Mini peristaltic pump with a sterile tygon tube (or to a vacutainer)
37°C incubator
Refrigerated centrifuge

Collect cells from animal

The pigs should be handled calmly and quietly by appropriately trained persons.

1. Place the animal in a dorsal recumbence position and immobilize it by using snout and leg ropes, keeping the head not stretched too far backwards, to allow the air flow through the throat region.
2. Prepare sternum area with 70% ethanol.
3. Insert the 22- or 23-G atraumatic needle near the first rib up the sternum at a 30° angle.
4. Gently advance the needle until blood enters the syringe, then stop the advance and collect the desired sample (50 ml) into a 100-ml glass bottle containing anticoagulant and antibiotics (40 IU/ml of heparin/100 IU/ml penicillin/0.1 mg/ml streptomycin).
5. Mix gently and allow bottle to stand tilted at an ~45° angle for 30 to 60 min at 37°C to sediment most of the red blood cells.
6. Carefully collect the leukocyte-enriched upper phase (about one-half of the original volume) in 100-ml bottles and mix with 3 vol (75 ml) of PBS containing 20 IU of heparin/ml.
7. Centrifuge cells 5 min at 1000 × *g*, room temperature and carefully remove the supernatant.

8. Resuspend the cells in 3 vol (75 ml) of PBS-heparin and repeat wash step two to three additional times.

Lyse erythrocytes

9. Resuspend cell pellet in 0.1 vol (2.5 ml) of ice-cold PBS with 5% swine serum, and add 0.025 vol (0.6 ml) of erythrocyte lysis buffer, then gently pipet up and down while maintaining the sample entirely in ice.

10. After 4 to 5 min, observe lysis of erythrocytes by change in turbidity (easily monitored also in a phase-contrast microscope), and then collect leukocytes by centrifuging 5 min at 1000 × *g*, 4°C.

    It is very important to keep the temperature <4°C while the cells are in lysis buffer.

11. Wash the erythrocyte ghosts two times by resuspending the cells in 5 ml of DMEM with 5% swine serum and 20 IU heparin/ml and centrifuging 5 min at 1000 × *g*, 4°C.

12. Resuspend the last pellet in 6 ml of culture medium (DMEM with 10% swine serum and 20 IU heparin/ml). These cells can be stored using the same conditions as Alternate Protocol 1, steps 12 and 13.

    A typical preparation contains ~20 × 10^6 leukocytes/ml, of which ~6% are monocytes. This concentration of cells can be seed up to ten 60-well Microtest I plates (Falcon).

PREPARATION OF SWINE SERUM

SUPPORT PROTOCOL 1

To prepare the serum to be used in the culture medium for swine monocytes, obtain a sample of blood (without coagulants) from the immobilized pig before collecting heparinized-samples, as indicated in Basic Protocol 1. This is a critical component of many of the protocols in this unit.

Materials

Restrained pig
250-ml glass bottles
37°C incubator
Gauze
50-ml conical centrifuge tubes (Falcon)
Batch filtration device (Sartorius model SM16263/67)
Sterile membranes (Millipore) with pore mean sizes of 1.2, 0.45, and 0.22 μm

1. Collect the desired volume of blood (100 ml) into a 250-ml glass bottle (see Basic Protocol 1, steps 1 to 4).

2. Incubate 2 to 3 hr at 37°C, with occasional agitation to disrupt the blood clot.

   Alternatively, incubate overnight at 4°C.

3. Filter the content of the glass bottle through gauze placed on top of a 50-ml conical centrifuge tube.

4. Centrifuge 10 min at 5000 × *g*, room temperature. Carefully decant the supernatant into a clean tube and discard the sediment.

5. Centrifuge as in step 4 two or three additional times, until no sediment is produced.

6. Sterilize the clean serum by pressure filtration through a batch filtration device containing a cascade of sterile membranes with pore mean sizes of 1.2, 0.45, and 0.22 μm (higher to lower pore size, each with its own pre-filter).

7. Store frozen in 10-ml aliquots up to 10 years at −20°C.

ALTERNATE PROTOCOL 1

PREPARATION OF SWINE ALVEOLAR MACROPHAGES

Swine alveolar macrophages may be used interchangeably with swine blood monocytes, as they share many of the same characteristics, such as ability to grow isolates from natural ASFV outbreaks. They are, therefore, another excellent choice for an in vitro system to grow virus stocks and to mimic natural ASFV infections. In addition, swine alveolar macrophages may be particularly useful for plaque assays. This protocol is adapted from Carrascosa et al. (1982).

***Additional Materials** (also see Basic Protocol 1)*

Swine (large white, 20 to 30 kg)
Ketamine (Ketolar 50; Sigma Chemicals)
Midazolam (Dormicum; Sigma Chemicals)
Atropine (Atropine B; Sigma Chemicals)
Fetal calf serum, heat inactivated, cold
DMSO (dimethyl sulfoxide; Hybri-Max, Sigma, cat. no. D2650)

Mechanical cutters (scalpels, scissors, sternal saw)
Sternal retactor
Hemostatic clamps and forceps
1-liter sterile bottles
2-ml cryotubes (Nunc, cat. no. 375418)
Cryo cooler (Nalgene Cryo 1°C freezing container, cat. no. 5100-00001)
37°C water bath

Euthanize and prepare animal

Pigs are handled and immobilized as indicated in Basic Protocol 1. Usually the extraction of blood sample is performed before the bronchoalveolar lavage to prepare autologous swine serum for the culture medium of swine monocytes and macrophages.

1. Anaesthetize the pig by a combined treatment with ketamine (12 mg/kg Ketolar 50), midazolam (0.5 mg/kg Dormicum), and atropine (0.03 mg/kg Atropine B).
2. Wash the sternum area with 70% ethanol and make an incision using a scalpel to reveal the sternum, then cut the sternum using a medical sternal saw to open up the rib cage.
3. Once opened, retract the thoracic cavity using a sternal retractor to gain access to the upper part of the trachea, which is then dissected and clamped with a hemostat.

Perform bronchoalveolar lavage

4. Remove lungs, heart, and trachea as a single package with the trachea clamped off.
5. Carefully separate the heart, blood vessels, and esophagus residues, and wash externally with PBS (lungs should appear as in Fig. 26.14.1A).
6. Fill the lungs with ~600 ml of PBS containing 10 IU heparin/ml until the lungs are distended (Fig. 26.14.1B), clamp off trachea, and gently hand massage lungs to sponge and distribute the liquid in both lobes of the lung.
7. Decant the lavage fluid into a sterile 1-liter bottle.
8. Repeat lung lavage two additional times to fully recover the free alveolar cell population.

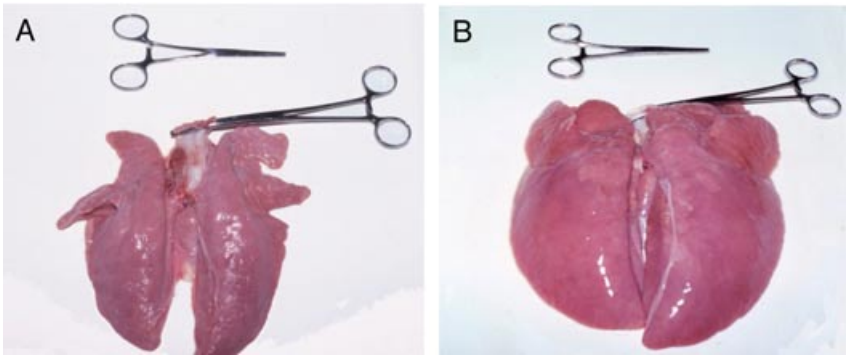

Figure 26.14.1 Porcine lungs as extracted from the thoracic cavity (**A**) and distended by filling with PBS (**B**).

Wash alveolar cells

9. Centrifuge the pooled cell suspensions 10 min at 2000 × *g*, room temperature, decant the supernatant, and resuspend the pellet in 500 ml of PBS-heparin. Repeat wash two additional times.

10. Before decanting the last supernatant, remove, by gently agitation, a white layer ("fluff") on top of the cell pellet.

    Avoid detaching the cell sediment, which is mainly composed of cell debris and platelets. Discard the supernatant.

11. Resuspend the cells in culture medium (DMEM with 10% swine serum and 20 IU heparin/ml) for cell counting.

    Expect to recover ~1.5 to 2.0 × 10^9 alveolar cells, with <10% of erythrocyte contamination (easily recognized as the smaller non-nucleated spherical cells). About 50% of the alveolar cells are macrophages according to different criteria (phagocytosis, adherence, and non-specific esterase staining).

Prepare cells for storage

The alveolar cells are now ready to use for culture (see below) or be stored in liquid nitrogen.

12. Remove culture medium by centrifuging 5 min at 2000 × *g*, room temperature, and resuspend the alveolar cells at a concentration of 15 × 10^6 cells/ml in cold, heat-inactivated fetal calf serum supplemented with 10% dimethyl sulfoxide as cryoprotective agent.

13. Dispense suspension into aliquots of suitable working volume (1 ml) in sterile plastic 2-ml cryotubes, and freeze at a 1°C/min constant cooling rate in a Cryo Cooler before storing in liquid N_2.

    Alveolar cells stored under these conditions remain viable for at least 10 years.

Prepare cell culture from frozen cells

14. Thaw the sample (1-ml tube) with continuous agitation in a 37°C water bath.

15. Transfer cell suspension to a centrifuge tube containing 4 ml of pre-warmed culture medium and gently resuspend the mixture.

16. Centrifuge tube 3 min at 1000 × *g*, room temperature, and decant the supernatant.

17. Resuspend the cell sediment in culture medium, seed, and make use of them as with the original (non-frozen) alveolar cells.

ALTERNATE PROTOCOL 2

PREPARATION OF ESTABLISHED CELL LINES

While alveolar macrophages and blood monocytes are useful, in many cases, it is difficult to obtain these cells in large quantities and reproducibility of results is frequently hindered by variation among different lots of cells. Where a significant quantity of cells is needed, for example, to evaluate virus titers, established cell lines provide the necessary quantity and experimental controls. Several established cell lines, such as Vero, COS-1, IPAM, and WSL are sensitive to ASFV.

All of the established cell lines used here, either from monkey (Vero and COS-1) or from porcine (IPAM and WSL) species, are cultured according to standard protocols in a 37°C, 5% CO_2 humidified incubator.

Established cell lines are grown in plastic tissue culture plates (obtained from several providers as Falcon, Nunc, and Costar) and subcultured by trypsinization according to the following protocol.

Additional Materials *(also see Alternate Protocol 1)*

Trypsin-EDTA solution (see recipe)
Established cell lines from monkey (Vero and COS-1) or from porcine (IPAM and WSL) species and their corresponding cell medium (see recipes for IPAM cell medium, Vero and COS-1 cell medium, and WSL cell medium)

37°C, 5% CO_2 humidified incubator
Tissue culture plates (10-, 5-, or 2-cm diameter dishes or multi-well plates)

1. Prepare trypsin-EDTA solution by mixing 4 vol of 0.02% EDTA and 1 vol of 0.25% trypsin (made up both in PBS).
2. Aspirate the cell medium from the confluent cell culture (60-mm dish).
3. Add 1 ml of trypsin-EDTA solution, spread the liquid onto the entire surface by tilting the plate, and then immediately remove as much liquid as possible.
4. Add 1 ml of trypsin-EDTA, incubate for 30 sec, and remove the liquid.

   There is no need to dry as much as in the previous wash step 3.
5. Place dish for at least 3 min in a 37°C, 5% CO_2 humidified incubator, and observe cell detachment by microscopy and also visually observe monolayer disruption after tapping the base of the plate.

   Sometimes it may take 10 to 15 min, e.g., in the case of IPAM cells, which are more resistant to trypsinization.
6. Add 2 ml of culture medium to the plate and resuspend the cells by pipetting up and down to disaggregate cell clumps.
7. Seed the cells (up to 1/10 dilution) in new plates that are convenient for the purpose of the experiment.

   Cell lines must be subcultured two times a week for maintenance. Usually, cultures are passaged up to 15 times, and then a new sample is thawed from a large frozen stock to keep the characteristics of the cell line used for virus infections consistent.

   To prepare a stock of cells for liquid N_2 storage, a number of cultures must be grown in 10-mm dishes up to a pre-confluent density ($\sim 1.5 \times 10^5$ cells/ cm^2).
8. Remove the culture medium and trypsinize the cell monolayers as described in steps 2 to 6.

9. Centrifuge cells 3 min at 1000 × *g*, room temperature. Resuspend the last cell pellet at a concentration of 10×10^6 cells/ml in cold culture medium supplemented with 10% DMSO.

10. Divide the suspension in 1-ml aliquots into sterile 2.0-ml cryotubes and freeze at a 1°C/min constant cooling rate in a Cryo Cooler before storing in liquid N_2.

Established cell lines stored in these conditions remain viable for up to 10 years.

GROWTH AND PURIFICATION OF SUSCEPTIBLE CELLS (VERO AND COS-1) IN ROLLER BOTTLES

BASIC PROTOCOL 2

When studying ASFV, many assays require a steady and reliable supply of the virus. An initial step in virus production is to produce ASFV-susceptible cells.

For the production of large amounts of virus, susceptible cells (COS-1 or Vero) can be grown and infected in roller bottles. A minimum of one confluent 10-mm dish is needed to seed one glass roller bottle with a surface of 500 cm^2.

Materials

Vero or COS-1 cells (10-mm confluent plates)
Vero/COS-1 cell medium (see recipe), 37°C
ASFV
Saline or PBS (see recipe)

Sterile 1000-ml glass bottles (e.g., borosilicate 3.3; VWR, cat. no. 215-1595)
CO_2 injecting device
Sterile cotton-plugged pipets
Roller apparatus (e.g., Modular Cell Production Model III, Wheaton Instruments, for up to 90 bottles) integrated into an incubator (Hotpack model 1650, with forced-air circulation)
Centrifuge tubes

Additional reagents and equipment for trypsinizing cells (see Alternate Protocol 2)

Prepare cells and seed in roller bottles

1. Release a trypsinized suspension of Vero or COS-1 cells from one 10-mm confluent plate ($10–15 \times 10^6$ cells) and resuspend in 5 ml of Vero/COS-1 cell medium (see Alternate Protocol 2, steps 2 to 6).

2. Dilute up to 50 ml in pre-warmed fresh Vero/COS-1 cell medium.

3. Decant suspension into a sterile 1000-ml glass bottle and equilibrate the inner atmosphere by injecting CO_2 through a sterile cotton-plugged pipet (the same type of CO_2 used in the cell incubator).

The gas flow must be slow and injected for a few seconds to obtain a suitable pH of the medium inside the bottle (this can be optically checked after an equilibration for ~5 min when a bright orange color develops, indicating a neutral pH in the culture medium).

4. Place the bottle in an incubator equipped with a roller bottle apparatus set to a temperature of 37°C and a rotational speed of 1 rpm.

5. Incubate cells 3 to 4 days to reach confluence ($1–2 \times 10^5$ cell/cm^2, $50–100 \times 10^6$ cells per bottle). Monitor cultures daily for pH and temperature.

The pH should be neutral; if the color is neither bright orange nor pink, more CO_2 should be injected, as in step 3.

6. Trypsinize cells in the bottle as in Alternate Protocol 2, steps 2 to 6, increasing the wash volume to 5 to 10 ml.
7. Seed the cells in ten roller bottles and adjust the pH of the culture medium as indicated in step 3. Incubate 3 to 4 days at 37°C, until cultures are ready for subculture or virus infection.
8. Repeat cell seeding to grow up to 100 roller bottles, if required.

Infect with virus

9. Carefully decant the medium of the Vero or COS-1 cell cultures grown in roller bottles to near-confluence ($\sim 10^5$ cells/cm^2).
10. Add 5×10^6 plaque forming units (pfu) of ASFV in 10 ml of fresh culture medium.

    If there are 50×10^6 cells per bottle, the multiplicity of infection (MOI) will be 0.1 pfu per cell.

11. Incubate in the roller 1 hr at 37°C.

    Virus adsorption is best performed in a reduced volume.

12. Bring the final volume to 75 ml by adding fresh medium and extend the incubation until an extensive cytopathic effect is observed under a (non-inverted) microscope (usually at 3 to 4 days after infection).

Harvest infected cells

13. Collect cells and medium by decanting into a 250-ml centrifuge tube and clarify by centrifuging at low-speed 10 min $2000 \times g$, 4°C, to sediment most of the infected cells and cell debris in the culture.

    This sediment can be stored as a source for intracellular virus.

14. Decant the supernatant into a clean centrifuge tube.

    The supernatant contains virus particles released from the infected cells, in addition to cellular contaminants like vesicles, membrane fragments, and subcellular organelles.

Concentrate virus

15. Centrifuge at high-speed 6 hr at 15,000 $\times$ g, 4°C, to concentrate the particulate materials, and remove supernatant.

    A consistent sediment must appear in a base corner and along the lower side of the wall near the pellet.

16. Resuspend the sediment in a small volume (1/300th of the initial volume before centrifugation) of saline (PBS, for further purification) or in culture medium (for concentrated virus to produce virus infections at high MOI).

BASIC PROTOCOL 3

VIRUS PURIFICATION (PERCOLL METHOD)

In this protocol, purification of any ASFV sample, including virulent, attenuated, hemadsorbing (or not), cell-adapted, recombinant or deletion mutant, is described. ASFV samples may be obtained either from natural outbreaks in the field or generated by molecular manipulations in the laboratory. This protocol has been adapted from Carrascosa et al. (1985).

Materials

Percoll (GE Healthcare, cat. no. 17-0891-01)
10× PBS (see recipe)
Extracellular ASFV concentrated in PBS (see Basic Protocol 2)
Tris-sucrose solution (10 mM Tris·Cl (pH 7.5)/0.25 M sucrose)

Sephacryl S-1000 superfine (GE Healthcare, cat. no. 17-0476-01)
10% trichloroacetic acid (TCA), cold
Liquid N_2

26-ml polycarbonate ultracentrifuge bottles (with liquid-tight cap assembly, 25 × 89–mm)
Refrigerated ultracentrifuge
Fixed-angle ultracentrifuge T865 rotor (Sorvall) and Sorvall AH-650 rotor
Pasteur pipets
Vacuum
Chromatographic column (e.g., 1-cm diameter, 20-cm high, 15-ml total volume; Pharmacia Fine Chemicals)
Fraction collector (laboratory-made or commercial; e.g., LKB 2112 Redirack)
Spectrophotometer (e.g., Shimadzu, model no. UV-1201)

Perform Percoll gradients

As an example, the pellet corresponding to 900 ml of culture medium from virus-infected roller bottle cultures should be resuspended after high-speed centrifugation, in a final volume of 3 ml in PBS.

1. Prepare a solution of 45% Percoll in PBS by mixing 2.1 ml of 10× PBS, 10.8 ml of Percoll (from the commercial stock), and 8.1 ml of sterile deionized water.
2. Add the virus sample (3 ml), mix again, fill a 26-ml ultracentrifuge bottle, and close tightly.
3. Ultracentrifuge in the fixed-angle T865 rotor 30 min at 40,000 × *g*, 4°C.

   A density gradient is auto-formed during the centrifugation, and two heavy bands will be found in the tube at the end of the run—one thick and heterogeneous in a lighter position (vesicles, density ∼1.050 g/ml) and a second one (virus) near the lower third of the tube, more homogeneous and at a density ∼1.095 g/ml. Density marker beads can be run in the same tube.

4. Collect the virus band first by carefully removing the material above the virus band with a 5-ml pipet, and then eliminating the liquid below the virus band with a Pasteur pipet.

   As this is being done, one repeatedly crosses the virus band, take care to avoid pumping up or aspirating while passing through the band. The remaining liquid material in the centrifuge tube corresponds to the purified-virus band, although a compact sediment of Percoll is found in a pellet at the bottom of the tube. Collect the virus sample (∼3 ml) after gently moving around the tube (the Percoll sediment will not be disturbed).

5. Prepare a new solution of 45% Percoll in PBS by mixing 2.1 ml of 10× PBS, 9.45 ml Percoll, and 9.45 ml water, in a new centrifuge bottle.
6. Carefully deposit the virus sample (with a Pasteur pipet) into the bottom of the centrifuge bottle.

   The virus sample now has a higher density than the 45% Percoll solution.

7. Ultracentrifuge as in step 3 and collect the clean virus band at higher density as in step 4.

   A very slight (or even no) band will appear in the lighter "vesicle" region.

Remove Percoll by gel filtration

Percoll contamination can be removed from the virus by gel filtration using a Sephacryl S-1000 column.

8. Prepare and sterilize 500 ml of Tris-sucrose buffer.

9. Equilibrate ~50 ml of Sephacryl S-1000 superfine solution (preswollen slurry) in Tris-sucrose buffer.

   Add 50 ml of Tris-sucrose and remove fines after settling most of the matrix particles in the bench and pouring off the supernatant by aspiration. Repeat several times to fully substitute the original buffer with Tris-sucrose.

10. Subject the solution to deaeration under vacuum with frequent agitation (not stirring) for at least 1 hr at room temperature and an additional 1 hr in ice to remove dissolved gases, particularly oxygen, which may produce air bubbles in the column.

11. Pack Sephacryl solution in a chromatographic column (1-cm diameter, 20-cm high, 15-ml total volume) while passing several volumes of Tris-sucrose from an upper reservoir (10 to 20 cm above the inlet of the column).

    Column packing is preferably performed in a cold room because the separation of virions and Percoll particles must be done at 4°C. The column is stored at 4°C in Tris-sucrose in the presence of 0.02% sodium azide, which is removed before use by washing with Tris-sucrose. The sample volume for this column is 1 to 3 ml (up to 20% of the total volume) to obtain a suitable separation of virus and Percoll particles after gel filtration.

12. Remove the buffer over the Sephacryl matrix gel and gently place the virus sample (band collected after the second Percoll gradient) in a volume of 1 to 3 ml on top of the matrix. Open the outlet of the column and allow the sample to enter into the column, while a 3 ml layer of Tris-sucrose buffer is applied on top of the sample.

    The sample and buffer will not mix since the Percoll solution in the sample is denser than the Tris-sucrose solution.

13. Refill the column with buffer and connect it to the buffer in the reservoir.

14. At this point, start (either manually or automatically) collection of fractions. Calibrate by time or drops to collect ~1 ml/fraction, and a total of 25 fractions.

15. Locate the position of the Percoll particles in the effluent of the column by checking the turbidity at 600 nm in an aliquot of each fraction (0.008 ml) precipitated with 1 ml of cold 10% TCA.

    In a typical run, a peak of Percoll will be found in fractions 13 to 25 (Fig. 26.14.2), which must appear clear or slightly colored by dye residues from the culture medium.

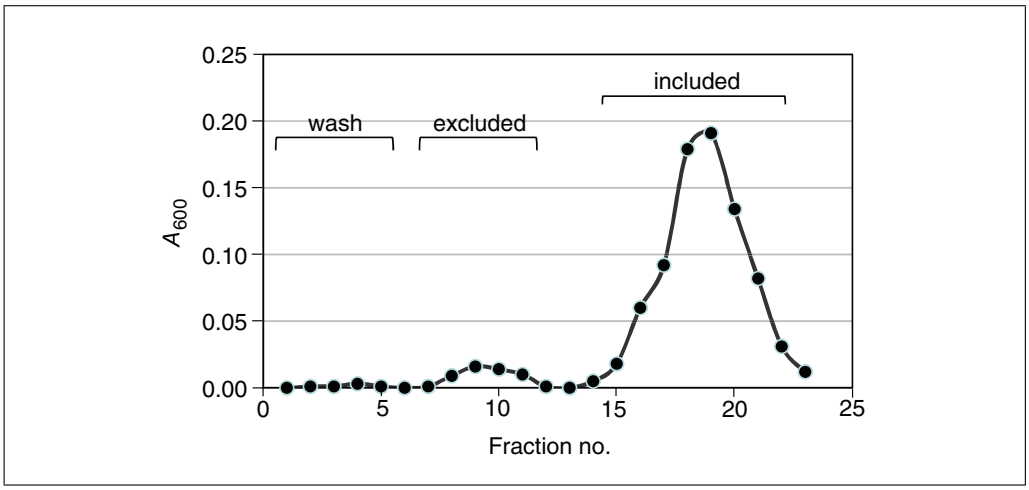

Figure 26.14.2 Purification of ASFV. Gel filtration on Sephacryl S-1000. Extracellular ASFV purified by Percoll sedimentation and subjected to chromatography on Sephacryl S1000 column. Determination of Percoll by precipitation with 10% TCA. Virus protein detected in the excluded fractions and Percoll particles in the included volume.

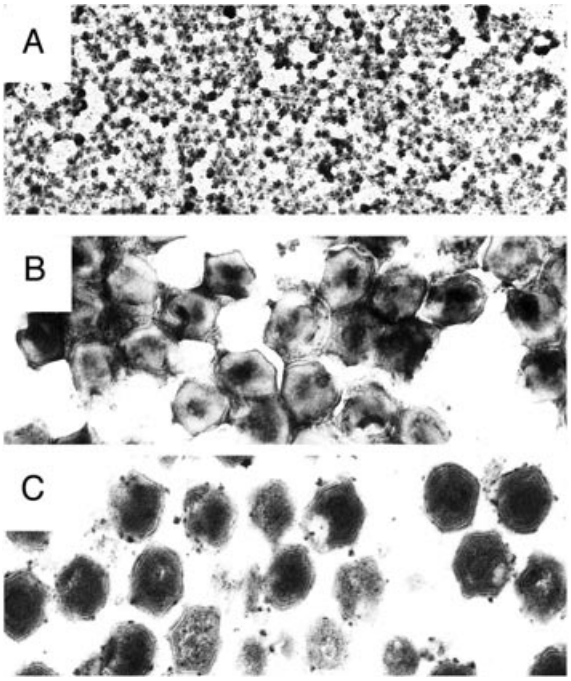

Figure 26.14.3 Micrographs of extracellular ASFV particles purified by Percoll sedimentation. (**A**) Negative staining, low magnification. (**B**) Negative staining, high magnification. (**C**) Ultra-thin section.

16. Select the virus-containing fractions.

    The excluded volume contains particles of higher size, like virions and vesicles, e.g., in fractions from 6 to 12 (with a slightly turbid material). Sometimes a peak of precipitated protein is also detected by TCA precipitation in coincidence with the virus fractions, when a highly-concentrated sample is applied (Fig. 26.14.2). In any case, this peak will be clearly separated from that of the Percoll contaminants present in the included volume. The column can be reused after regeneration by washing with 0.01% Triton X-100 in Tris-sucrose.

17. Pool the virus-containing fractions and store in 100- to 300-µl aliquots indefinitely under liquid N_2, or concentrate the purified virus by high-speed centrifuging, if required, in a Sorvall AH-650 rotor, 1 hr at 145,000 × *g*, 4°C, to sediment the purified virus particles, gently removing the supernatant and resuspending the pellet in the appropriate buffer.

    A sample of purified ASFV analyzed in the electron microscope will provide images full of homogeneous and complete virus particles, free of cell debris, vesicles, and Percoll granules, both in negative staining or in ultra-thin sections (Fig. 26.14.3).

INFECTIVITY ASSAY: HEMADSORPTION ON SWINE MONOCYTES/MACROPHAGES

BASIC PROTOCOL 4

The titration of ASFV stocks can be performed by evaluation of a structural component of the virus particle or a virus product induced during the infection, using techniques like ELISA, PCR, western blotting, or the fluorescent focus assay (Pastor et al., 1989, 1992; Oura et al., 1998; Barderas et al., 2000; Aguero et al., 2003; Zsak et al., 2005). However, the most interesting values come from tests in which the ability to produce infective virus is evaluated, i.e., infectivity assays. One of them is based on the formation of erythrocyte rosettes around infected swine blood monocytes (Enjuanes et al., 1976), a characteristic feature of the ASFV-infected cell, which has been widely used for diagnostic purposes

but it is obviously restricted to hemadsorbing virus strains. Other methods are based on the evaluation of the cytopathic effect produced by endpoint dilutions of virus samples in sensitive cell cultures, yielding, as in the case of hemadsorption, semi-quantitative data. The more simple and quantitative test for virus titration is based on the plaque formation assay, which has been described in Vero (Enjuanes et al., 1976), alveolar swine macrophages (Carrascosa et al., 1982), and in COS-1 cells (Bustos et al., 2002). A detailed description of the corresponding protocols is presented in this unit.

The hemadsorption (HAD) microassay is developed in tissue culture Microtest I plates (Falcon) with 60 wells per plate, which is inoculated with five serial ten-fold dilutions of a virus sample (12 wells per dilution). The indicator cell must be an ASFV-sensitive cell capable of adhering erythrocytes on its surface membrane when infected with the virus, as it is the case of peripheral blood monocytes or alveolar macrophages, prepared from swine as indicated.

Materials

Swine erythrocyte suspension (see Basic Protocol 1) *or* frozen macrophages (see Alternate Protocol 1)
DMEM (*APPENDIX 2A*)
Autologous or homologous (HAD-compatible) swine serum (see Support Protocol 1)
Virus samples (see Basic Protocol 3)
Heparinized swine peripheral blood (see Basic Protocol 1)
PBS (see recipe)

Microtest I plates (60-well, Falcon 3034)
Electronic adjustable pipet
Hamilton microsyringe coupled to an automatic repeating dispenser
37°C, CO_2 incubator
96-well plates (e.g., Falcon, Costar, or Nunc)
Contrast-phase inverted microscope and hemacytometer

Plate cells

1. Resuspend a sediment of swine leukocytes prepared as in Basic Protocol 1 from 5 ml of peripheral blood in 0.6 ml of culture medium (DMEM supplemented with 10% autologous swine serum), and seed 0.01 ml per well in a Microtest I plate using either an electronic adjustable pipet or a Hamilton microsyringe coupled to an automatic repeating dispenser.

   Alternatively, thaw a 1-ml aliquot of frozen alveolar macrophages and wash as indicated in Alternate Protocol 1. Resuspend the pellet in 7.2 ml of culture medium (supplemented with autologous swine serum), and distribute cell suspension into twelve Microtest I plates (0.01 ml per well).

2. Incubate the plate 24 hr (for macrophages) or 48 hr (for leukocytes) at 37°C.

Prepare virus dilutions

3. Prepare ten-fold dilutions (final volume of 0.2 ml) of the virus samples in culture medium. For multiple titrations, prepare dilutions in a 96-well plate, which can hold up to twelve series of eight ten-fold dilutions.

4. Prefill the appropriate number of wells with 0.18 ml of culture medium. Dilute 0.02 ml of the virus sample into the first well, mix, and transfer 0.02 ml to the next well.

5. Repeat as needed to generate the required dilutions to titrate each virus sample.

   Care must be taken to avoid systematic errors and imprecisions (change tips with each transfer, avoid bubbles or drops outside the tips, etc.) in each one of the tips in each step.

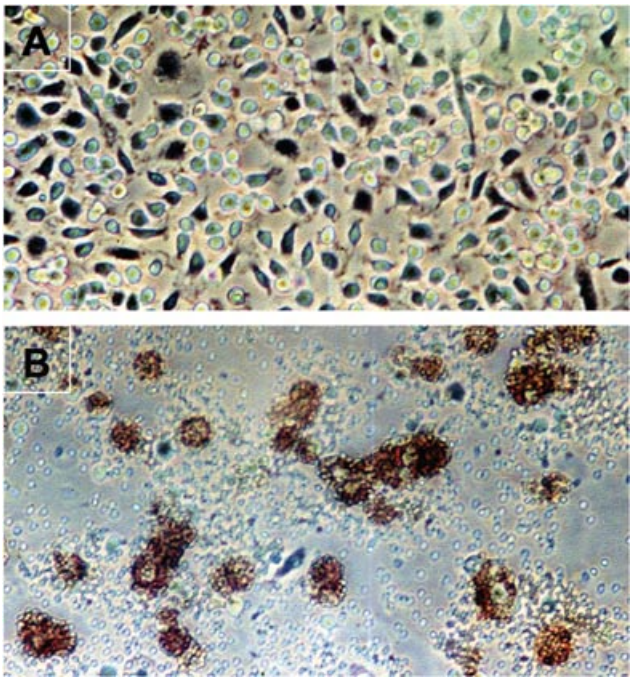

Figure 26.14.4 Hemadsorption in ASFV-infected cells. (**A**) Peripheral blood monocytes incubated for 2 days after removing the non-adherent cells. (**B**) A similar culture after ASFV infection and addition of swine erythrocytes to develop HAD rosettes.

Infect cells

6. Gently aspirate the medium (avoid draining each well) of 12 wells and inoculate them with 0.005 ml per well of the less concentrated dilution of the virus sample to titrate.
7. Repeat with successive dilutions in increasing order in terms of virus concentration (a single tip may be used to titrate each virus sample), to complete the 60-well plate (5 dilutions $\times$ 12 well).
8. Incubate overnight at 37°C.
9. Prepare a fresh suspension of autologous swine erythrocytes by diluting 1 ml of heparinized swine peripheral blood in ~8 ml of PBS and centrifuging for 5 min at 1000 $\times$ *g*, room temperature. Discard the supernatant and repeat washing with PBS until a clear supernatant is obtained (usually three to four times). Resuspend the last sediment in 1 ml PBS and count red blood cells with a hemacytometer ($\sim 10^9$ cells/ml).
10. Prepare a solution with 1.6×10^7 erythrocytes/ml in culture medium (it will contain 80,000 erythrocytes in 0.005 ml).
11. Add 5 µl of this suspension to each well of the Microtest I plate, starting from the less concentrated dilution, with the electronic pipette.

    Change the tip when required (in each loading in the cell suspension or when moving to a new virus sample).

12. Further incubate the plates for 3 to 4 days and examine the development of HAD rosettes in each well (see Fig. 26.14.4) with an inverted microscope at 100-fold magnification (the entire surface of one well will be covered by the field of view of the microscope under these conditions).

Dilution	(+) / total score	(+) cumulative	(-) cumulative	(+) / total cumulative	Percentage of infection
10^{-1}	12 / 12	35	0	35 / 35	100.0
10^{-2}	12 / 12	23	0	23 / 23	100.0
10^{-3}	9 / 12	11	3	11 / 14	78.6
10^{-4}	2 / 12	2	13	2 / 15	13.3
10^{-5}	0 / 12	0	25	0 / 25	0.0

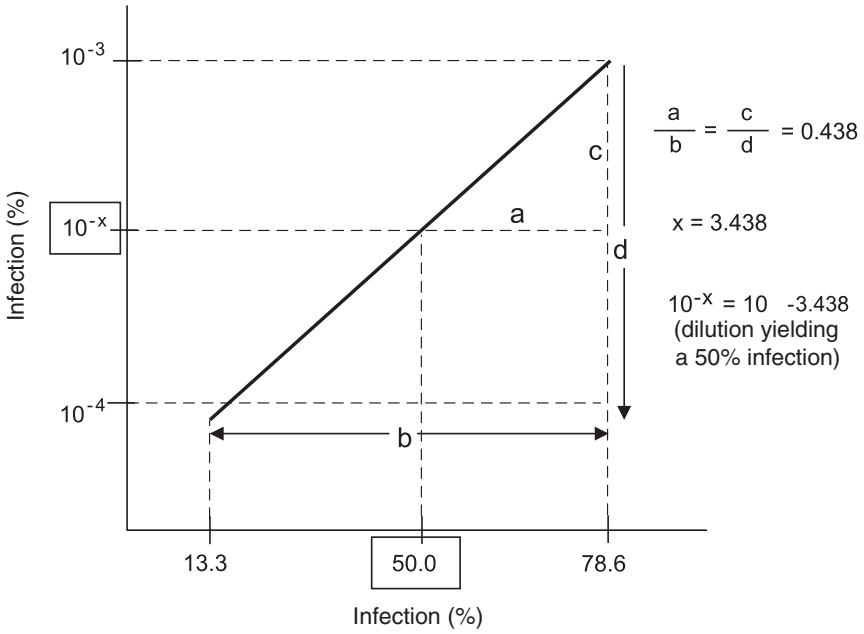

Figure 26.14.5 Calculating $HADU_{50}$: A practical case.

Calculate HAD units

13. Score data for each virus sample and calculate the titer in HAD units yielding a 50% of cumulative infection ($HADU_{50}$) per milliliter (see example in Fig. 26.14.5).

The calculation is based on the percentage of wells positive for HAD throughout a series of successive ten-fold dilutions of the virus sample. It does not take into account the number of HAD rosettes per well, but reflects the percentage of HAD-positive wells (presenting one or more rosettes) in each dilution.

ALTERNATE PROTOCOL 3

CYTOPATHIC EFFECT ON SWINE MACROPHAGES

A semi-quantitative assay of virus infectivity can also be performed by evaluating cytopathic effect rather than HAD. The following protocol is designed for end dilution in swine alveolar macrophages, but it might be applied to any other cell line (Vero, COS-1, etc.) susceptible to a lytic infection of ASFV able to provoke a massive cytolytic result in the cell monolayer.

Additional Materials (also see Basic Protocol 4)

2% crystal violet in 5% formaldehyde

Plate cells

1. Defrost a 1-ml aliquot of frozen alveolar macrophages and wash as indicated in Alternate Protocol 1.
2. Resuspend the pellet in 9.6 ml of culture medium, and distribute the cell suspension in a 96-well plate (100 µl per well).

   Each well must contain 1.5 × 10^5 alveolar cells, about one half of them being macrophages.

3. Incubate plate 24 hr at 37°C.

Infect cells with virus dilutions

4. Prepare ten-fold dilutions (final volume of 0.2 ml) of the virus samples in culture medium. Use a 12-channel mechanical pipettor for multiple titrations, as indicated in Basic Protocol 4.
5. Gently aspirate the medium from five wells (or a higher number of wells to increase the precision of the test) and inoculate them with 20 µl per well of the less concentrated dilution of the virus sample to titrate.
6. Repeat with successive dilutions (e.g., from 10^{-3} to 10^{-7}) to inoculate 30 (out of 96) wells (6 dilutions × 5 well) in the plate.
7. Incubate 2 hr at 37°C and refill each well to 0.1 ml with culture medium.
8. Further incubate at 37°C until extensive cytopathic effect develops in positive cultures (≥5 days).

Calculate TCID

9. Remove the medium (containing the detached cells and cellular debris).

   This can be done by holding the plate (without the lid) and generating a quick 180° flick over a wide waste receptacle.

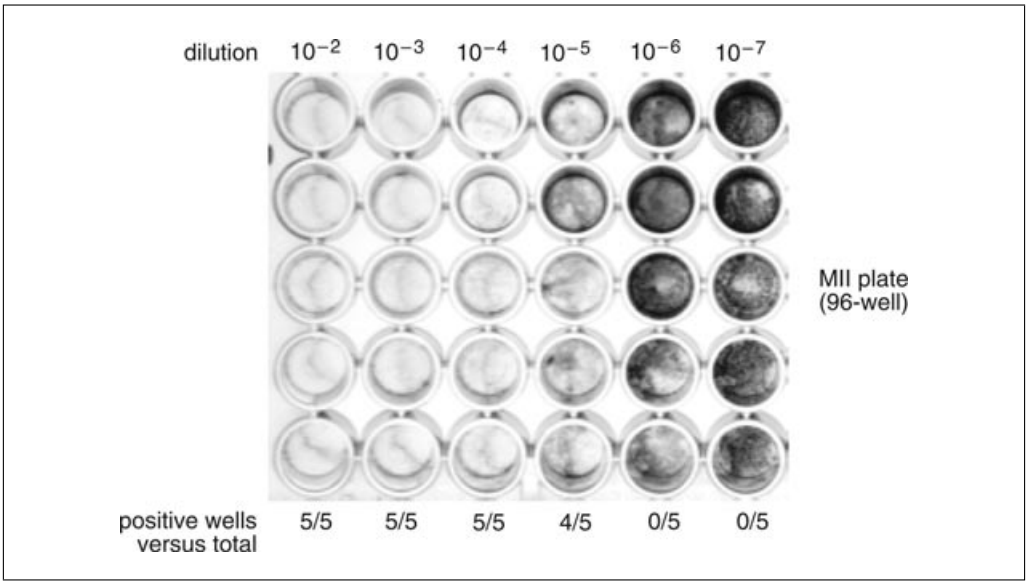

Figure 26.14.6 Titration of an ASFV sample by cytopathic effect assay. Cultures of swine alveolar macrophages in 96-well plate, inoculated with serial ten-fold dilutions of the virus sample and stained with crystal violet 5 days after infection. With the data scored in this case, and considering a volume of 0.02 ml of inoculum per well, the titer of the virus sample is: $1/10^{-5.375} \times 0.02 = 1.18 \times 10^6$ $TCID_{50}$/ml.

10. Stain the cell cultures by adding 20 µl of 2% crystal violet in 5% formaldehyde to each well. Incubate for 30 min at room temperature and remove the liquid under a moderate flow of tap water not directed against the cell monolayer. Remove lids from the plates to dry over filter paper to identify positive wells versus non-infected wells.

    A visual examination of the plates will define the positive wells in which massive cytopathic effect lead to the extinction of the cell monolayer, while the non-infected wells will appear heavily stained in blue (Fig. 26.14.6).

11. Score the data as percentage of positive wells for each virus sample and calculate the titer in tissue culture infective dose yielding 50% of infection ($TCID_{50}$) per milliliter, as indicated in Figure 26.14.5.

    Alveolar macrophages are primary cells and do not develop compact monolayers as compared with established cell lines. For this reason, the blue staining of healthy cultures shown in Figure 26.14.6 is not as intense as that developed by established cell line monolayers like Vero or COS-1 cells (Fig. 26.14.8).

ALTERNATE PROTOCOL 4

PLAQUE ASSAY ON SWINE MACROPHAGES

The more simple and quantitative test for virus titration is based on the plaque formation assay, which has been described in Vero (Enjuanes et al., 1976), alveolar swine macrophages (Carrascosa et al., 1982), and COS-1 cells (Bustos et al., 2002). Swine macrophages are the model of choice when examining strains from a natural outbreak.

***Additional Materials** (also see Basic Protocol 4)*

Swine serum (see Support Protocol 1)
Solid agar-medium solution (see recipe)
2% crystal violet (prepared in 5% formaldehyde)

24-well plates
Filter paper

Plate cells

1. Defrost a 1-ml aliquot of frozen alveolar macrophages and wash as indicated in Alternate Protocol 1.
2. Resuspend the pellet in 10 ml of culture medium (DMEM supplemented with 10% homologous swine serum), and distribute the cell suspension into 20 wells of a 24-well plate (0.5 ml per well). Each well must contain 7.5×10^5 alveolar cells

    The optimal seeding concentration is 390,000 cells per cm^2 (Bustos et al., 2002).

3. Incubate for 1 to 2 days at 37°C.

Perform plaque assay

4. Prepare ten-fold dilutions (final volume of 0.2 ml) of the virus samples in culture medium. Use a 96-well plate and a 12-channel mechanical pipettor for multiple titrations.
5. Gently aspirate the medium from three wells (to test three dilutions per virus sample) and inoculate them with 150 µl/well of the corresponding dilution, starting from the less concentrated.
6. Incubate 1 hr at 37°C, shaking every 10 min both for a better spreading of the virus inoculum and to prevent cultures from drying out.
7. Prepare a solid agar-medium solution.

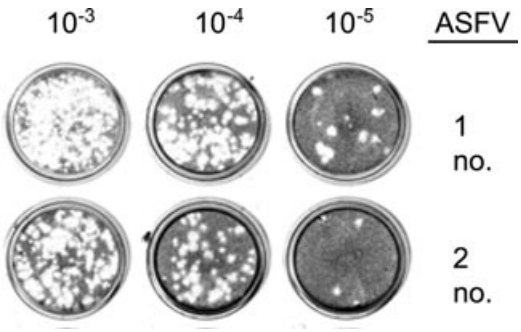

Figure 26.14.7 Plaque assay of ASFV samples on swine macrophages. Swine alveolar cells grown in 24-well plates (390,000 cells/cm^2) were infected with ten-fold dilutions of virus samples, overlaid with agar-medium and incubated 5 days at 37°C, before stained with crystal violet.

8. Add 1.5 ml of solid agar-medium solution to each well, without removing the virus inoculum, and allow the plate to stand quiet at room temperature until the overlay is solidified.
9. Incubate cultures 5 days at 37°C.

Calculate PFU

10. Add 0.5 ml of 2% crystal violet (prepared in 5% formaldehyde) over the solid medium on each well and incubate overnight in an appropriate fume hood.

    Never leave it standing in the CO_2 incubator since the toxic vapors may affect other living cell cultures.

11. Remove the liquid and the agar overlay under a moderate flow of tap water not directed against the cell monolayer.
12. Remove lids from the plates to dry over filter paper and score the number of lysis plaques in a suitable dilution of each sample (Fig. 26.14.7).
13. Calculate the virus titers in pfu/ml. For example, if 50 plaques are counted in a well inoculated with 0.15 ml of a 10^{-5} dilution, the titer of the virus sample is $= (50 \times 10^5)/0.15 = 3.3 \times 10^7$ pfu/ml.

PLAQUE ASSAY ON ESTABLISHED CELL LINES (VERO AND COS-1 CELLS)

BASIC PROTOCOL 5

A more simple and quantitative test for virus titration is based on the plaque formation assay.

With only slight differences, ASFV samples can be titrated by plaque assay on established cell lines (Vero or COS-1 cells) as described for swine macrophages with a few of the following modifications. The list of materials is similar to Alternate Protocol 4, substituting fetal calf serum for swine serum. The culture medium is DMEM supplemented with 5% (v/v) fetal calf serum. A lower number of cells are seeded per well ($\sim 1.2 \times 10^5$ cells/well in a 24-well plate), since these cell lines grow readily during the assay until reaching confluence or being infected by the virus. Ten-fold dilutions must be carefully prepared. The solid agar-medium solution is also composed of DMEM and agar, but supplemented with 10% fetal calf serum instead of 20% swine serum. The plates must be examined to assess the development of lysis plaques from the third day after virus inoculation (some ASFV isolates can produce large plaques in COS cell monolayers (Hurtado et al., 2010) that may grow during the subsequent 3 to 4 days. Stain with crystal

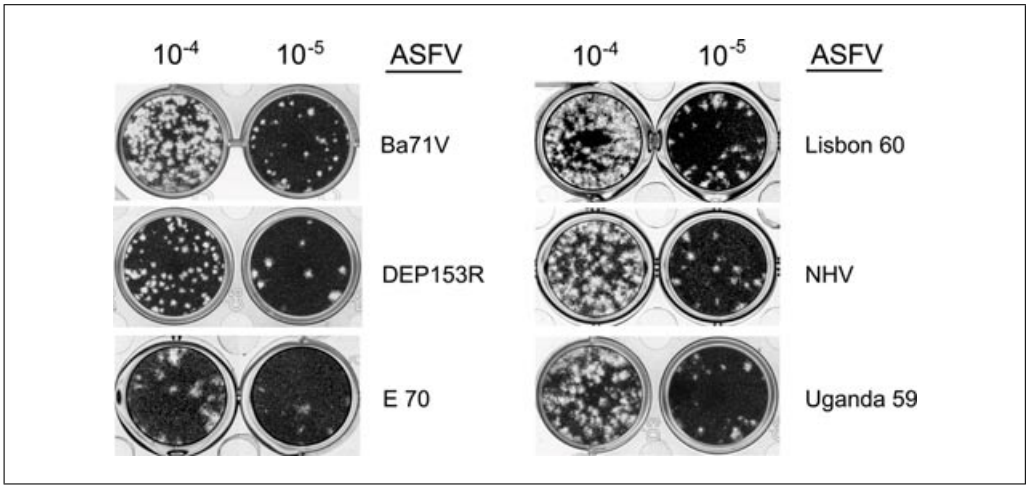

Figure 26.14.8 Plaques developed in COS cell monolayers by different ASFV isolates. COS-1 cells grown in 24-well plates (120,000 cells/well) were infected with ten-fold dilutions of virus samples, overlaid with agar-medium and incubated 5 days at 37°C, before stained with crystal violet.

violet in formaldehyde and calculate the titer as indicated in Alternate Protocol 4. An example of lysis plaques developed in COS cell monolayers by different ASFV isolates is shown in Figure 26.14.8.

BASIC PROTOCOL 6

INFECTION OF SENSITIVE CELL LINES BY DIFFERENT ASFV ISOLATES

All of the ASFV isolates obtained from natural outbreaks can be grown both in peripheral blood monocytes and in alveolar macrophages, and many of them are able to develop the hemadsorption of erythrocytes around the infected cell. This was the basis for the most traditional method to specifically detect and titrate the ASFV samples. However, several virus isolates from natural outbreaks have been shown to be non-hemadsorbing, and cannot be identified nor titrated by this assay. A number of ASFV isolates have been adapted to grow in established cell lines (Vero, CV1/2, etc.), facilitating many biochemical and structural studies of the virus, but restricting the analysis to those adapted virus strains. Hurtado et al. (2010) has described the use of COS-1 cells to grow and titrate many different ASFV isolates, either from the field or generated in the laboratory, allowing the direct infection of these cell cultures with any ASFV sample without selection or adaptation. The use of porcine cell lines derived from macrophages to perform studies of ASFV infection in a more natural context (swine) and target cell (monocyte-macrophage) is recommended—both IPAM and WSL cell lines were derived from pig alveolar cells, and seem to be infected by several ASFV isolates.

A common protocol is described for the infection of different cell lines (COS-1, IPAM, WSL, and swine alveolar macrophages) with several ASFV isolates (Ba71V, ΔEP153R, E70, NHV, and Lisbon 60), with the only difference of the culture medium used to grow and infect the cells (described in the Commentary, Preparation of Target Cells section).

Materials

Cell lines (COS-1, IPAM, WSL, and swine alveolar macrophages) and corresponding cell culture media
ASFV isolates (Ba71V, ΔEP153R, E70, NHV, and Lisbon 60)

24-well plates
37°C incubator
Ultrasonic bath (e.g., Bransonic 12)

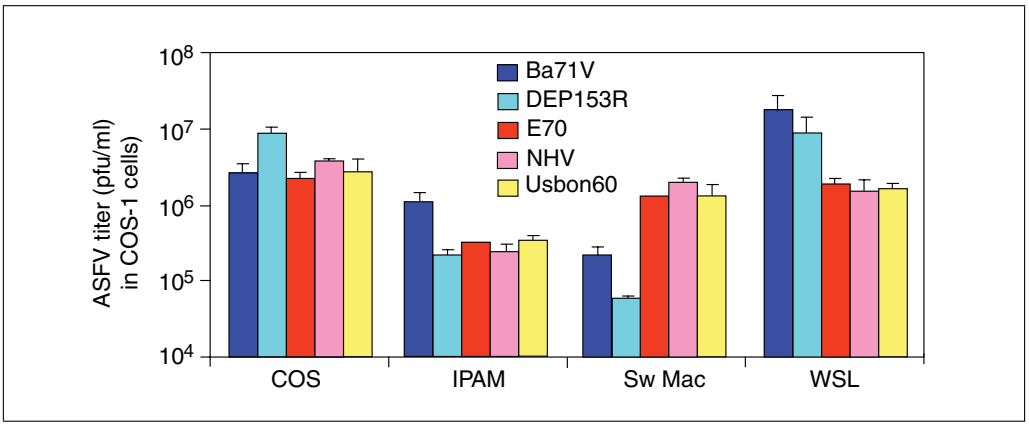

Figure 26.14.9 Production of infective virus in four virus-susceptible cells infected with different ASFV isolates. Cell cultures infected at an MOI of 3 pfu/cell were collected at 3 to 4 days after infection and the total virus titrated in COS-1 cell monolayers.

1. Seed or grow the corresponding cells in 24-well plates at a concentration of 2×10^5 cells/well, and infect them with each one of the ASFV isolates, at an MOI of 3 pfu/cell. Incubate 2 hr at 37°C in a reduced volume (150 µl) with frequent agitation.
2. Remove the virus inoculum and wash two times with 500 µl of culture medium.
3. Refill with 500 µl of culture medium per well and incubate for 3 to 4 days at 37°C.
4. Collect the cells and medium and disrupt the infected cells by a brief sonication in an ultrasonic bath.

   The lysis of the cells can be easily monitored at 60× magnification in a non-inverted microscope providing that the sample is collected in a transparent tube.
5. Titrate the production of total infective virus in duplicate samples by plaque assay in COS-1 cell monolayers as described in Basic Protocol 5.

   An example of the virus production yielded in each cell line is shown in Figure 26.14.9. Both swine alveolar macrophages and IPAM cells produced 10^5 to 10^6 pfu/ml when infected by any one of the five ASFV isolates tested, while COS-1 and WSL cells delivered even higher yields of infective virus (10^6 to 10^7 pfu/ml). Productive infections have also been verified with up to 18 (in COS-1 cells) and 12 (in WSL cells) different ASFV isolates obtained either from the field or generated in the laboratory (data not shown), with no signals of cell resistance to the virus samples tested thus far.

REAGENTS AND SOLUTIONS

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

Erythrocyte lysis buffer

150 mM NH_4Cl
0.1 mM EDTA (*APPENDIX 2A*)
10 mM $KHCO_3$
Adjust pH to 7.4 using NaOH or HCl
Sterilize by filtration using a 0.2-µm filter
Supplement with 20 IU/ml of heparin and 5% (v/v) swine serum
Store indefinitely at 4°C

IPAM cell medium

RPMI-1640 prepared from powder (Gibco, cat. no. 23400) as indicated by the manufacturer
10% (v/v) fetal calf serum
Add gentamycin (100 μg/ml) to the medium

Store up to 6 months at 4°C

PBS (10×)

1.38 M NaCl (*APPENDIX 2A*)
26.8 mM KCl (*APPENDIX 2A*)
81.0 mM Na_2HPO_4 (*APPENDIX 2A*)
14.7 mM KH_2PO_4 (*APPENDIX 2A*)
Adjust pH to 7.3 with HCl
Sterilize by autoclaving

Store up to 1 year at room temperature

PBS, pH 7.3

138 mM NaCl (*APPENDIX 2A*)
2.68 mM KCl (*APPENDIX 2A*)
8.1 mM Na_2HPO_4 (*APPENDIX 2A*)
1.47 mM KH_2PO_4 (*APPENDIX 2A*)
Adjust pH to 7.3 with HCl
Sterilize by autoclaving (120°C, 20 min, 1 atm)

Store up to 1 year at 4°C

Solid agar-medium solution

(1) 2× DMEM: Prepare DMEM from powder (Gibco, cat. no. 52100), according to manufacturer's instructions, and sterilize by filtration. Just before use, supplement with usual products (e.g., glutamine, non-essential amino acids, and antibiotics). Add 160 μg/ml DEAE-dextran (from a pre-sterilized solution at 80 mg/ml in PBS) And 20% (v/v) swine serum (for swine macrophages) or 10% (v/v) fetal calf serum (for Vero or COS-1 cells). Equilibrate to 45°C in a water bath.

(2) Agar solution: Dissolve 1.4% (w/v) agar in deionized water. Weigh desired amount of agar and place into a bottle, which will be filled with the appropriate volume of water. Mix the suspension and boil in a microwave oven for several minutes (better with a 50% power to avoid the liquid spilling when boiling starts) until the solution is completely transparent. Boiling for several minutes is also important for the sterilization of the agar solution. Equilibrate to 45°C in a water bath.

(3) Mix equal volumes of 2× DMEM and 1.4% agar pre-equilibrated to 45°C just before use. The mixture remains liquid for several minutes at room temperature while it is being used to overlay the cultures. In multiple titrations, it is recommended to distribute the mixture into several aliquots to keep at 45°C while the working solution is used in the tissue culture cabinet.

Swine cell medium

Dulbecco's modified Eagle's medium (DMEM) can be either prepared in the laboratory from powder (Gibco, cat. no. 52100) or acquired in a ready-to-use liquid form (Gibco, cat. no. 12800-017), and must be completed after filtration

continued

(and before use) as indicated by the manufacturer (usually with 2 mM glutamine and non-essential amino acids)
10% (v/v) swine serum
Add gentamycin (100 μg/ml)
(Optional) Add 20 IU heparin/ml when required

Store up to 6 months at 4°C

Tris-sucrose buffer

10 mM Tris·Cl, pH 7.5
250 mM sucrose
Store at 4°C

Trypsin-EDTA solution

0.02% EDTA in PBS (see recipe), sterilized by autoclave (110°C, 30 min, and 0.5 atm)
0.25% trypsin (Difco Trypsin250, cat. no. 215240) in PBS (see recipe) supplemented with 1.1 g/liter of glucose, sterilize by filtration
Store at −20°C until used
Mix 4 vol EDTA and 1 vol of trypsin and use immediately

Store up to 6 months at 4°C

Vero/COS-1 cell medium

Dulbecco's modified Eagle's medium (DMEM)
5% (v/v) fetal calf serum
Add gentamycin (100 μg/ml) to the medium
Store up to 6 months at 4°C

WSL cell medium

Mixture (1:1) of DMEM and RPMI-1640
5% (v/v) fetal calf serum
Add gentamycin (100 μg/ml) to the medium

Store up to 6 months at 4°C

COMMENTARY

Background Information

ASFV is an enveloped double-stranded DNA virus that belongs to the family *Asfarviridae* (Dixon et al., 2004) and infect domestic and wild pigs of the *Suidae* family, as well as ticks of the Ornithodoros genus. As wild-type ASFV isolates from natural outbreaks were not found to replicate in conventional established cell cultures, swine monocytes and macrophages were the in vitro system of choice to grow virus stocks and to mimic natural ASFV infections. However, primary cells are difficult to obtain in large amounts and often do not provide reproducible results because of lot-to-lot variations. These problems can be both overcome by using established cell lines, and some ASFV isolates were adapted to grow in different cell lines (mostly derived from African green monkey kidney), allowing many experimental approaches and the development of suitable plaque formation assays to evaluate the virus titers using a more simple, reproducible, and quantitative method (Parker and Plowright, 1968; Enjuanes et al., 1976), but only for those cell culture–adapted ASFV strains. An established cell line, e.g., COS-1, susceptible to all of the ASFV isolates tested thus far (Hurtado et al., 2010) has solved two critical problems in ASFV research by (1) allowing the amplification of any virus sample for diagnosis, detection, and production; and (2) enabling the optimization of plaque assays for natural and laboratory-engineered ASFV strains, to perform infectivity titrations and also the manipulations required for the construction of virus deletion mutants (Garcia

et al., 1995; Galindo et al., 2000; Granja et al., 2006). Additionally, the possibility to infect cell lines like IPAM or WSL, derived from swine alveolar macrophages, facilitates studies in which a more natural environment (swine, macrophage) is required to determine the host cell response and/or to mimic more precisely the course of in vivo ASFV infection.

A set of protocols are described in this unit to allow for the analysis in terms of detection, titration, production, and purification, of any ASFV sample (including virulent, attenuated, hemadsorbing or not, cell-adapted, recombinant or deletion mutant) obtained either from natural outbreaks in the field or generated by molecular manipulations in the laboratory. It is up to the individual investigator to select the more suitable method/assay.

Critical Parameters and Troubleshooting

Preparation of target cells

The most important issue in animal experiments is to ensure that the manipulations are performed by well-trained animal handlers and in the appropriate animal research establishment according to the biosafety rules and ethical requirements. These facilities will also provide specific equipment and training for animal management and successful preparation of both peripheral blood leukocytes and alveolar cells from swine. Once in the laboratory, the primary cells must be handled carefully, following protocol instructions regarding low-speed centrifugation, cell resuspension, and proper temperatures. A critical point regarding the blood leukocyte preparation is to accurately fit the conditions (cell concentration, cold temperature, and gentle cell resuspension) recommended to specifically lyse the erythrocytes without affecting the rest of the blood cells, and particularly the leukocytes.

A critical point to maintain cell viability during cold storage is to ensure a constant cooling rate of 1°C/min when freezing the cell suspension (best performed in commercial devices like CryoCoolers), a quick transfer to the liquid phase of the N_2 for storage, and quick thawing of the cryotubes in a 37°C water bath with continuous agitation. Gently washing and resuspending cell pellets will lead to cell cultures with optimal viability and these recommendations apply not only to primary cells but also to established cell lines.

Trypsinization is another crucial operation in cell culture. Care must be taken to avoid cell damage by over-trypsinization, and conditions (enzyme concentration, temperature, and time of incubation) must be adjusted for each particular cell line, and always perform visual and microscopic monitoring of the whole process to overcome any contingency.

Regarding the culture medium for porcine primary cells (leukocytes and macrophages), the use of fetal bovine serum as a supplement in the culture medium is a common practice in many laboratories. However, the authors have found that it was not convenient for ASFV infection since it reduces 80% to 90% of the production of infective virus particles in swine macrophages infected with several ASFV isolates in the presence of medium supplemented with swine serum (Bustos et al., 2002). As a practical consideration, testing the ability of each particular batch of swine serum to support the production of infective virus is recommended, since the yield could be different depending on the alveolar cell stock (data not shown).

Virus production and purification

To obtain large amounts of ASFV particles purified from cell contaminants, the method of choice is the Percoll gradients. This is by far the most effective system to separate the virions from the membrane fragments, vesicles and cellular rests, while maintaining a reasonable infectivity in the virus sample. To produce the concentrated ASFV suspension required to perform the Percoll centrifugation, it is recommended to grow target cell monolayers (Vero or COS-1 cells) in roller bottles.

This is not particularly complicated but may require previous training to acquire the expertise to produce healthy cell cultures with regular growth and homogeneous distribution in rolling surfaces. Specific issues to consider are (1) keep trypsinization at the less aggressive conditions to avoid cell damage and cluster formation, (2) monitor pH of the culture medium after injecting CO_2 into the bottle (and throughout the cell growth phase) to keep pH near neutral (a basic culture grows more slowly and becomes more resistant to ASFV infection), and (3) take extra care in aseptic manipulations and test for microbial infection in each step (the use of antibiotic-free medium may be helpful to elude hidden bacterial contaminations).

For the purification process, take care of selecting clean and transparent centrifuge bottles (without apparent leaks) to best recognize the cell and virus pellets. As Percoll solutions cannot be sterilized, increase the aseptic policies

both during centrifugation and gel filtration, which must be performed in sterile conditions and at 4°C (and meeting the biosafety requirements associated to this pathogen).

Titration assays

The selection of the infectivity assay to titrate a particular ASFV isolate will depend on the specific characteristics of the virus sample. The HAD-based technique is a semi-quantitative assay suitable only for hemadsorbing isolates, but exhibit a high specificity and sensitivity (still important for detection and identification). It is recommended to use an autologous swine serum for the leukocyte culture to avoid unspecific hemagglutination that may be confused with the HAD rosettes. If it is not available, a compatible homologous swine serum can be tested by preparing a suspension of the swine erythrocytes (1.6×10^7/ml) to be used in the HAD assay in culture medium supplemented with 10% of the corresponding lots of homologous serum to analyze: after an incubation of 2 hr at 4°C, the compatible serum will not exhibit signals of cell agglutination (hemagglutination) and can be used for HAD assays.

The classical assay of infectivity for ASFV field isolates involves swine monocyte-macrophages, either by HAD, cytopathic effect, or plaque assay. However, the latter two techniques depend on the quality of the quasi-monolayer established by a primary (non-growing) cell culture, sometimes difficult to reproduce and subjected to lot-to-lot variations. Another consideration regarding the semi-quantitative methods is that the precision of these tests depends on the number of cultures assayed per dilution, and this can be increased by multiplying the test wells in each dilution, although with increase in costs and complexities.

In the case of a cell-adapted ASFV strain, the titration method will include the corresponding cell line (Vero, CV1/2), and the test can be performed by plaque assay (or cytopathic effect, immunofluorescence, etc. if required and available).

As a general rule, the plaque assay is the method of choice because it is quantitative, simple, less costly, and reproducible than any other infectivity assay, and facilitates the selection and purification of virus isolates or recombinants engineered with selectable marker genes. The recent description of COS-1 as an ASFV-susceptible cell line may introduce a universal plaque assay for any ASFV sample, providing that this observation is extended and corroborated in as many laboratories as possible. A set of alternative protocols is described in this unit for evaluating the infectivity of any ASFV sample.

Anticipated Results

To facilitate planning of an infection experiment, the number of cells expected in the preparation of target cells (peripheral leukocytes, alveolar macrophages, roller bottles) is described in the corresponding protocols. For example, to prepare target cells for virus titrations by the HAD assay, a sample of 500 ml of swine peripheral blood will yield $\sim 1200 \times 10^6$ leukocytes (6% monocytes), enough to seed 100 Microtest-I plates (Enjuanes et al., 1976), while $\sim 2000 \times 10^6$ alveolar cells (50% macrophages) are obtained from the bronchoalveolar lavage of a 20- to 30-kg swine (Carrascosa et al., 1982), allowing for seeding 1600 Microtest-I plates (this is the best option since the cells can be stored in aliquots indefinitely under liquid N_2).

For the preparation of ASFV particles purified by Percoll gradients (sedimentation), the infection of, e.g., 13 roller bottles with Vero cells will result in 1 liter of infected culture medium (with $\sim 10^7$ pfu/ml), yielding a preparation of purified extracellular virus (after Percoll sedimentation and gel filtration) with the following characteristics: 200 μg of virus protein, $1–2 \times 10^7$ pfu/μg of protein, a $15.2 \pm 8.8\%$ recovery of the initial infectious virus, a DNA/protein ratio of 0.18 ± 0.02 and a sediment coefficient of 3500 ± 300 S. These data correspond to a typical purification of the extracellular virus produced in Vero cells after infection with the Ba71V cell–adapted ASFV strain (Carrascosa et al., 1985). Although not explicitly performed, it is expected that the Percoll sedimentation will proceed perfectly well also in the purification of ASFV virions produced in COS-1 cell monolayers cultured in roller bottles, as described for Vero cells.

As discussed above, a number of ten-fold dilutions must be prepared to titrate a virus sample, which will be used to inoculate from one to twelve cultures per dilution to quantify the virus infectivity according to the different titration methods described. The number of dilutions and cultures per dilution is important not solely for the precision of the assay but also to define the limits (upper and lower) of detection of infective units per milliliter of sample. As a rule, the semi-quantitative methods will require a large number of dilutions and cultures per dilution than the plaque assays.

The susceptibility of the porcine cell lines (IPAM and WSL) to ASFV may suggest the possibility of using these cultures for virus titration instead of the monkey-derived cell lines (COS or Vero). However, the alveolar cell lines display only a slight (or even unnoticeable, depending on the virus isolate) cytopathic effect when infected with ASFV, which makes its use unlikely as indicator cells in infectivity assays.

Time Considerations

The preparation of peripheral blood leukocytes or alveolar macrophages for use or to freeze takes 1 working day. Thawing of alveolar cells from frozen stocks will expend just a few minutes. After seeding, cell cultures must be incubated for 1 or 2 days before virus infection.

To calculate the time for virus production, consider the MOI and the duration of the virus infection cycle (from 20 to 30 hr, depending on the target cell); therefore, with an MOI of 2 to 3, most of the cells in the culture (85% to 95%) will become infected in the first round of infection, and total virus should be collected at 48 hr after infection. With an MOI of 0.1 to 0.2, only a small fraction (9% to 18%) of the culture will be infected in the first cycle, and the rest of the cells will keep growing until being infected by the progeny virus produced in the first round, resulting in an increase of ~1 additional day to reach the highest amount of virus production. As indicated above, Vero cell cultures infected in roller bottles by Ba71V isolate at an MOI of 0.1 pfu/cell according to Basic Protocol 2, should need 3 to 4 days to yield the maximum production of extracellular virus.

For virus titrations, the experiment will span to ~1 week. Day 1 for plating the cells, day 2 for virus inoculation, and 3 to 5 additional days until the final data is scored, depending on the titration assay considered.

Virus purification will also require 2 to 3 days: 1 day for collection of culture medium with the infected cells, clarification, and virus concentration (high-speed centrifugation), 1 day for Percoll sedimentation (two centrifugations) and gel filtration, and an additional 1 day may be needed for final virus concentration and further analysis and distribution of the purified virus preparation.

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