The Ferret Model for Influenza

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ABSTRACT

A major challenge in influenza research is the selection of an appropriate animal model that accurately reflects the disease and protective immune response to influenza infection in humans. Ferrets are exquisitely susceptible to infection with human influenza viruses and are widely believed to be the ideal small animal model for influenza research. Mice have also been used for influenza vaccine research for decades. Ferrets are used as an animal model for the study of influenza because they are susceptible to human influenza viruses and develop some of the symptoms of influenza that are seen in humans. Although they are not discussed in detail in this unit, hamsters, guinea pigs, and both cotton rats (*Sigmodon*) and rats (*Rattus*) have also been used for influenza research. *Curr. Protoc. Microbiol.* 13:15G.2.1-15G.2.29. © 2009 by John Wiley & Sons, Inc.

Keywords: influenza • animal models • ferrets • virus titration

INTRODUCTION

Several animal species can be experimentally infected with influenza viruses (Luke and Subbarao, 2008; van der Laan et al., 2008). Ferrets and mice (*UNIT 15G.3*) are the most commonly used models; however, hamsters (Abou-Donia et al., 1980; Heath et al., 1983; Subbarao et al., 1993), guinea pigs (Phair et al., 1979; Lowen et al., 2006), cotton rats (*Sigmodon*; Niewisk and Prince, 2002; Ottolini et al., 2005), and rats (*Rattus*; Teh et al., 1980) have also been used for influenza research. The protocols in this unit describe intranasal administration of influenza virus to lightly anesthetized ferrets, observation of clinical illness where relevant, collection of organs for virologic analysis, methods to quantify virus load in tissues, and processing of tissues for histopathologic examination. Ideally, data should be gathered from use of more than one outcome in a single model (e.g., clinical outcomes and virus titration) or from more than one animal model (ferrets and mice). The protocols in this unit can be applied to study viral pathogenesis and virulence or the efficacy of measures to prevent or treat influenza with antibodies, antiviral drugs, or vaccines. As in any experiment, the use of appropriate controls is critical.

In a typical experiment to evaluate virulence, two outcomes can be evaluated in ferrets: clinical disease and quantitative virology. To assess clinical illness, serial 10-fold dilutions of the virus are administered to groups of lightly anesthetized animals and parameters such as weight loss, activity, temperature, and mortality are recorded. The ability of the virus to replicate in the respiratory tract and extrapulmonary organs provides data that complements but does not replace clinical disease data.

In a typical experiment to evaluate the efficacy of a vaccine, groups of ferrets are immunized with one or two doses of the vaccine at a 4-week interval (Fig. 15G.2.1). Sera are collected before the vaccine is administered (on day 0) and 14 to 28 days after each dose of vaccine to evaluate the antibody response to the vaccine. At a selected time-point (2 to 4 weeks) after the last dose of vaccine, the animals are challenged intranasally with the homologous wild-type virus. The animals are observed for evidence of clinical disease (weight loss or mortality) or are euthanized on the day of peak replication of the

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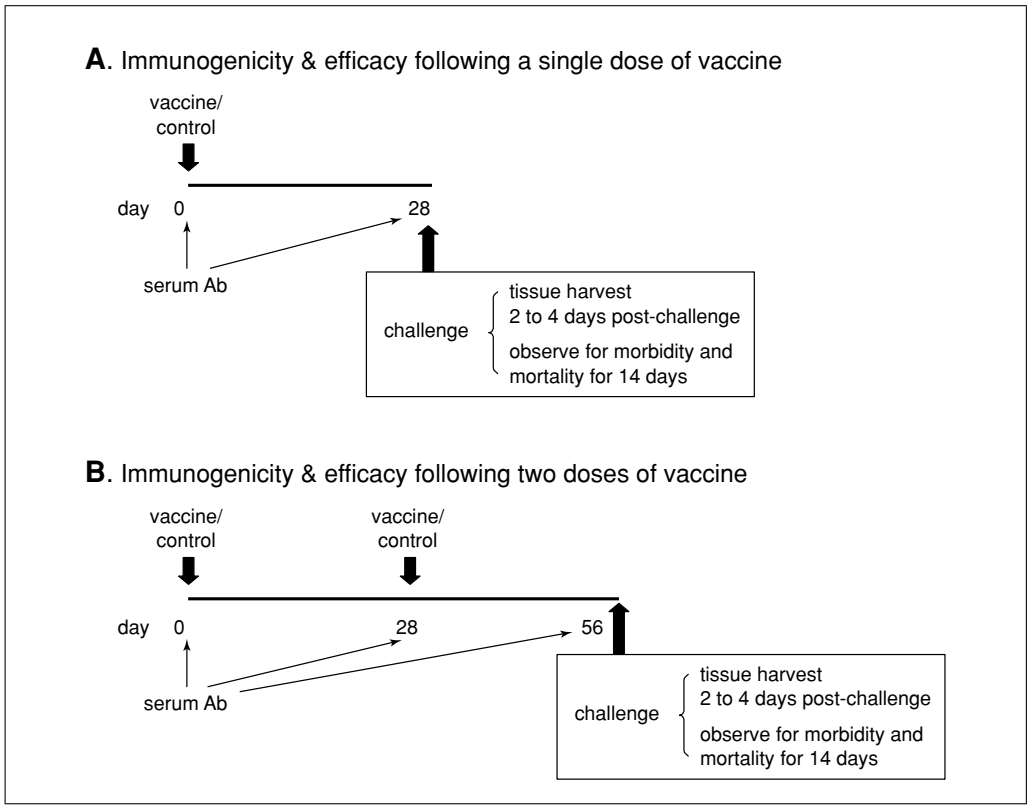

Figure 15G.2.1 A typical study design for a one (**A**) or two dose (**B**) immunization study.

challenge virus and nasal turbinates, lungs, and other organs of interest are harvested for virus titration. The level of replication of the challenge virus is compared with the virus titer in the corresponding organs of mock-immunized animals. The breadth of protection conferred by a vaccine can be assessed by including a heterologous challenge virus in the efficacy study.

Ferrets are used as an animal model for the study of influenza because they are susceptible to human influenza viruses and develop some of the symptoms of influenza that are seen in humans. Ferrets often develop a fever, have respiratory signs such as sneezing and a runny nose, and may become lethargic following intranasal infection with influenza viruses. Ferrets are large enough to monitor clinical parameters such as temperature, pulse, and respiratory rate, and relatively large amounts of sera can be obtained for use in serologic and antigenic characterization of influenza isolates.

Because ferrets are susceptible to human influenza viruses, it is important to screen ferrets for pre-existing antibodies to influenza before using them in experiments. A drawback to using ferrets for influenza research is the lack of immunological reagents. Ferrets are more difficult to handle than mice, are expensive to purchase and maintain, and require additional oversight from Animal Care and Use Committees due to animal welfare issues. Ferrets also require additional animal enrichment due to their intelligent and playful nature.

Younger ferrets tend to develop more severe upper respiratory tract infections than older ferrets (Rosenthal, 2004). We typically use 8- to 10-week-old ferrets to assess the ability of influenza viruses to replicate in the respiratory tract and we use older (>12 week old) ferrets to generate post-infection antisera for antigenic characterization of influenza viruses. We have used ferrets of either sex in our research. In our laboratory, we collect nasal turbinates and lungs for analysis at necropsy; however, if serial sampling of the

ferret is important, nasal wash samples can be obtained from ferrets as described by Zitzow and colleagues (Zitzow et al., 2002). Briefly, PBS is instilled into the nose of the ferret and nasal secretions that are expelled when the ferret sneezes are collected in a petri dish.

CAUTION: The protocols presented in this unit are for use with contemporary human influenza virus subtypes, which must be handled under Biosafety level 2 (BSL-2) conditions. For biosafety levels recommended for noncontemporary or nonhuman influenza viruses, refer to the Influenza Agent Summary Statement in the 5th edition of Biosafety in Microbiological and Biomedical Laboratories manual published by the Centers for Disease Control and Prevention and the National Institutes of Health (*http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm*). All work with infectious influenza virus should be conducted in a Class II biological safety cabinet (BSC). Refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for instructions on safe handling of microorganisms.

CAUTION: The protocols presented in this unit are for use with influenza virus subtypes that can be handled under Biosafety level 2 (BSL-2) conditions. As noted above, for guidance on handling noncontemporary or nonhuman influenza viruses, refer to the Influenza Agent Summary Statement in the 5th edition of Biosafety in Microbiological and Biomedical Laboratories manual published by the Centers for Disease Control and Prevention and the National Institutes of Health (*http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm*). Follow all appropriate guidelines for the use and handling of infected animals. Perform all procedures in a Class II biosafety cabinet using aseptic technique. Spray surfaces of the biosafety cabinet with a disinfectant such as Cidex solution or 70% ethanol and wipe down with paper towels. Place absorbent pads in the work area. Discard waste in appropriate waste containers and clean the biosafety cabinet with disinfectant. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

IMPORTANT NOTE: Highly pathogenic avian influenza viruses have been classified as Select Agents by the United States Government. Refer to the CDC Select Agent Program for more information (*http://www.cdc.gov/od/sap*). Also refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*).

NOTE: Protocols using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee or must conform to governmental regulations regarding the care and use of laboratory animals.

STRATEGIC PLANNING

Safety Considerations

Ferrets will bite. Leather, Kevlar, or metal mesh (chain mail) gloves may be used to handle unfamiliar or aggressive ferrets. Handle ferrets firmly by the scruff of the neck, lifting all four legs off the ground (Fig. 15G.2.2). Most ferrets should relax in this position.

Smaller ferrets are placed into a mouse cage or other clear cage to transport them from their home cage to the biosafety cabinet for manipulation.

Evaluation of Clinical Signs of Disease in Ferrets

Ferrets exhibit clinical signs of influenza infection including elevated temperature, reduced activity, and respiratory signs such as sneezing, coughing, and rhinorrhea. These symptoms can be scored using the method developed by Reuman et al. (1989), which is summarized below (Table 15G.2.1).

Table 15G.2.1 Ferret Clinical Sign Scoring[a]

Score	Nasal symptoms	Activity level (playfulness)
0	No symptoms	Fully playful
1	Nasal rattling or sneezing	Responds to play overtures but does not initiate play activity
2	Nasal discharge on external nares	Alert but not playful
3	Mouth breathing	Not playful, not alert

[a]See Reuman, 1989.

Figure 15G.2.2 Hold ferrets firmly by the scruff of the neck.

Ferrets may develop an acute febrile response to influenza that lasts a few days. Take the ferret's temperature before inoculation as a baseline measurement. Implantation of a microchip capable of measuring the animal's temperature reduces the amount of handling necessary to record daily temperatures, thereby reducing the stress for both the ferret and handler. It is important to record the temperature at approximately the same time each day since the animal's body temperature fluctuates throughout the day. The normal temperature range for ferrets is 37.8° to 40°C, with an average of 38.8°C. (Brown, 2004).

Quantification of Virus by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR technology has been used for influenza diagnostics, surveillance, and research. The primary application of qRT-PCR is to detect any type A influenza virus by amplifying the conserved matrix (*M*) gene. The method is sensitive, specific, and rapid. It is particularly useful for research involving highly pathogenic avian influenza viruses, which requires an enhanced-Biosafety Level 3 (e-BSL3) laboratory for handling live viruses since once RNA is extracted from a specimen (in e-BSL3), the subsequent qRT-PCR and analysis can be performed in a BSL-2 laboratory. The limitation of this method is that it detects viral nucleic acid from viable and nonviable virus particles.

Extraction of RNA from specimen

Different types of RNA extraction kits are commercially available. Some examples that we have used are:

1. QIAamp Viral RNA Mini Kit (Qiagen): Good for respiratory wash and swab specimens and culture supernatants.

2. RNeasy Mini Kit (Qiagen): Good for most tissue samples and for culture supernatants.

3. MagNAPure LC system (Roche): Automated nucleic acid isolation system that is fast and minimizes cross contamination.

qRT-PCR

There are several established protocols based on detection of the *M* gene that are endorsed by the WHO, mainly for human and H5 avian influenza viruses and by the USDA, for influenza virus surveillance in wild birds and poultry markets. The generation of cDNA can be achieved in a combined step of reverse transcription (RT) and PCR or in two separate reactions. The initial steps and reaction conditions such as primer concentration and reaction settings should be optimized for each protocol. See Guidelines on Laboratory Diagnosis of Avian Influenza (see Internet Resources) and Spackman and Suarez (2008) for more information.

Antibody Response of Ferrets to Influenza Virus Infection

The serum antibody response to influenza virus infection can be measured by several methods including ELISA, hemagglutination inhibition (HI; *UNIT 15G.1*) assay, or neutralizing antibody assay. ELISA measures binding antibodies while neutralization assays measure antibodies that are able to neutralize virus infectivity. HI antibodies are commonly accepted as a surrogate for neutralization. By convention, a four-fold rise in antibody titer is considered significant and indicative of infection.

Standard assay protocols for HI and microneutralization assays are available at *http://www.who.org* and the WHO Manual on Animal Influenza Diagnosis and Surveillance: *http://www.wpro.who.int/NR/rdonlyres/EFD2B9A7-2265-4AD0-BC98-97937B4FA83C/0/manualonanimalaidiagnosisandsurveillance.pdf.*

ISOFLURANE ANESTHESIA OF FERRETS

BASIC PROTOCOL 1

Ferrets can be lightly anesthetized with isoflurane for quick procedures such as intranasal inoculation or for subcutaneous insertion of transponder microchips. It is important to have all of the materials for the subsequent procedure assembled prior to the administration of the anesthetic since the animals will only be anesthetized for a short time. Large induction chambers normally used for rats or hamsters may be used for juvenile ferrets. Even larger induction chambers are available by special order for larger ferrets.

Materials

Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether)
8- to 10-week-old ferrets

Isoflurane induction chamber setup (Viking Medical; see Fig. 15G.2.3)
Chain mail or leather gloves to handle the ferrets (chain mail gloves are easier to disinfect)

1. Carefully pour isoflurane into the vaporizer up to the fill line.
2. Place the induction chamber inside the Class II biosafety cabinet.

Figure 15G.2.3 Induction chamber in the BSC is connected to an isoflurane vaporizer through tubing.

3. Adjust the oxygen flow rate to 1.5 liter/min.
4. Adjust the isoflurane rate on the vaporizer to 5%.
5. Place the ferret in the induction chamber, making sure to reseat the lid. Hold the lid down with your hand if the ferret is especially active and pushes on the lid.
6. Wait until the ferret loses its righting response.

   The ferret will be lightly anesthetized for a few minutes. This is enough time for intranasal inoculation or transponder microchip insertion.

BASIC PROTOCOL 2

INJECTABLE ANESTHESIA OF FERRETS

Injectable anesthesia is commonly used when bleeding animals and prior to euthanasia since it produces a deeper anesthesia. Injectable anesthesia can be used for intranasal inoculation when isoflurane anesthesia is not available.

Materials

Anesthetics (see Table 15G.2.2) including:
  Ketamine
  Xylazine
  Atropine
8- to 10-week-old ferrets
70% ethanol spray/squirt bottle

Transport cages
Scale
1-ml syringes
25-G needles
Microcentrifuge tubes (e.g., Eppendorf)
Microcentrifuge tube rack

NOTE: Ketamine is a controlled substance that must be obtained in accordance with federal regulations. These drugs need to be obtained through the facility veterinarian.

Table 15G.2.2 Drug Doses[a,b]

Drug	Dosage (mg drug/kg animal body weight)
Ketamine	25 mg/kg
Xylazine	2 mg/kg
Atropine	0.05 mg/kg

[a]The three drugs will be mixed in a microcentrifuge tube and then injected into the ferret.
[b]Drug concentrations can vary; check the bottle each time.

Figure 15G.2.4 A mouse cage can be used to hold small ferrets for weighing.

Weigh the ferret

1. Weigh the empty ferret transport cage and tare the scale.
2. Place a ferret in the transport cage.
3. Weigh the ferret in the transport cage (Fig. 15G.2.4).
4. Record the weight of the ferret.

Prepare anesthesia drugs for injection

5. Calculate the amount of each injectable anesthetic solution to give the ferret based on its weight using the following formula and the information in Table 15G.2.2:

   Ferret dosage (ml) = [weight of ferret (kg) × drug dose (mg/kg)] ÷ drug concentration (mg/ml)

   IMPORTANT NOTE: *Drug concentrations can vary; check the bottle each time before preparing doses.*

6. Calculate the volume of ketamine needed.

   For example, if a ferret weighs 400 g = 0.4 kg and the concentration of ketamine provided = 100 mg/ml, then the volume of ketamine needed = (0.4 kg × 25 mg/kg) ÷ 100 mg/ml = 0.1 ml.

7. Use a 1-ml syringe equipped with a 25-G needle to draw up the appropriate ketamine dose and transfer into a sterile microcentrifuge tube.

   The same syringe may be used to draw up additional doses of ketamine, as long as the syringe remains sterile and it is not used to draw up any other drug.

8. Calculate the volume of xylazine needed.

   For example, if a ferret weighs 400 g = 0.4 kg and the concentration of xylazine is 20 mg/ml, the volume of xylazine needed = (0.4 kg × 2 mg/kg) ÷ 20 mg/ml = 0.04 ml.

9. Use a new 1-ml syringe equipped with a 25-G needle to draw up the appropriate xylazine dose and transfer into the microcentrifuge tube containing the ketamine.

10. Calculate the volume of atropine needed.

    For example, if a ferret weighs 400 g = 0.4 kg and the concentration of atropine is 0.4 mg/ml, the volume of atropine needed = (0.4 kg × 0.05 mg/kg) ÷ 0.4 mg/ml = 0.05 ml.

11. Use a new 1-ml syringe equipped with a 25-G needle to draw up the appropriate atropine dose and dispense it into the microcentrifuge tube containing the ketamine and xylazine.

12. Use a new 1-ml syringe equipped with a 25-G needle to draw up the mixture of three drugs for administration to the ferret.

Inject the ferret

13. While holding the ferret securely by the scruff of the neck with one hand, clean the inner thigh of the ferret (injection area) with 70% ethanol.

    If needed, one person can restrain the ferret and another can clean the injection site and inject the anesthetic.

14. Inject the anesthetic solution into fatty tissue of the inner thigh.

15. Wait for 2 or 3 min; squeeze a foot to check the ferret for a response—if it does not move, the animal is anesthetized.

    Animal should be under anesthesia for ~10 min.

    Larger animals (>1 kg) may be under anesthesia for 20 min because of the larger drug dosage.

    It is not advisable to anesthetize more than three animals at a time because they may wake up before you complete the planned procedures.

BASIC PROTOCOL 3

INTRANASAL INOCULATION OF FERRETS WITH INFLUENZA VIRUS

A respiratory tract infection is achieved by administering the virus inoculum by the intranasal route to lightly anesthetized ferrets. Light anesthesia is important for the inoculum to be inhaled fully; awake ferrets often sneeze and expel the inoculum. The administration of light anesthesia also reduces stress for both the ferret and the investigator.

Materials

Virus inoculum at appropriate dilution
96-well plate of Madin Darby canine kidney (MDCK) cells (optional for immediate titration)
Ice packs or wet ice
8- to 10-week-old ferrets

Tubes to hold inoculum
Rack for tubes

200-μl pipettor
200-μl pipet tips

Additional reagents and equipment for anesthetizing the ferret with inhaled isoflurane (Basic Protocol 1) and injectable drugs (Basic Protocol 2)

Prepare inoculum

1. Calculate the volume of inoculum needed for the number of ferrets to be inoculated and include an aliquot to save for back-titration of the inoculum. We prepare at least two extra doses to ensure that we have enough.

   Ferrets are typically given 10^7 $TCID_{50}$ of virus in 200 μl of L15 medium.

   For virus stocks grown in eggs, try to dilute the stock at least 1:2 to dilute egg proteins that might induce an inflammatory reaction.

2. Keep the virus inoculum on ice packs or on wet ice.

3. Save an aliquot to back titer (~100 μl) on a 96-well plate of MDCK cells to confirm the dose administered.

   Have a 96-well plate of MDCK cells ready if you plan to titrate the inoculum immediately or freeze the aliquot at −70°C to titrate later.

Anesthetize the ferret

4. Anesthetize the ferret either by isoflurane (see Basic Protocol 1) or injectable drug (see Basic Protocol 2).

Inoculate the ferret

5. Keep the virus inoculum on wet ice/ice packs in biosafety cabinet (see Critical Parameters).

6. Draw up 200 μl of inoculum with a 200-μl pipettor.

7. Once the ferret is anesthetized, use one hand to hold the ferret by the scruff of the neck with the nose pointing upward.

8. With the 200-μl pipettor in the other hand, dispense the inoculum into both nares of the ferret.

9. Hold the ferret somewhat upright to allow the virus to be inhaled thoroughly.

10. Return the ferret to its home cage.

11. Change gloves, clean pipettor, and clean out transport cage(s) between different viruses.

MICROCHIPPING OF FERRETS

BASIC PROTOCOL 4

It is easier to insert a microchip into an anesthetized (isoflurane or injectable) ferret though it can also be done while the ferret is awake. If you are using injectable anesthetics, the microchip can be inserted and a blood sample can be collected at the same time.

Materials

8- to 10-week-old ferrets
70% ethanol

Transponders IPTT-300 (BioMedic Data Systems; *http://www.bmds.com*)
Wand reader system (Fig. 15G.2.5; BioMedic Data Systems; *http://www.bmds.com*)

Additional reagents and equipment for anesthetizing the ferret with inhaled isoflurane (Basic Protocol 1) and injectable drugs (Basic Protocol 2)

Figure 15G.2.5 Implantable temperature and ID transponder and wand reader system.

Prepare the transponder

1. Program the transponder with the ID number for the ferret (consult the software manual).
2. Use the wand to confirm that the transponder ID number is correct before inserting it into the ferret.
3. Write the transponder number on the plunger of the injector to keep track of the ID.

Insert the microchip

4. Anesthetize the ferret with inhaled isoflurane (see Basic Protocol 1) or injectable drugs (see Basic Protocol 2).
5. Place the ferret in ventral recumbency.
6. Clean the area between the shoulder blades with 70% ethanol.
7. Tent the skin between the shoulder blades with one hand.
8. Insert the transponder needle subcutaneously up to the base of the needle.
9. Push the plunger to insert the transponder and remove the needle.
10. Gently rub the injection site to ensure that the transponder does not come out.

BASIC PROTOCOL 5

PERFORMING TEMPERATURE READING

Consult the software manual for instructions on how to record the temperature from an implanted transponder. The temperature can be recorded without connecting the wand to a computer or touching the ferret with the wand. To monitor the temperature, slowly wave the wand over the scruff of the neck. Record the temperature from the transponder readout. Try to take temperatures around the same time each day (±1 hr) for at least 5 days, beginning before the inoculation, to establish the normal baseline temperature for each ferret.

BLEEDING FERRETS VIA ANTERIOR VENA CAVA

Relatively large amounts of blood can be collected from anesthetized ferrets using this approach. Consult your facility veterinarian to determine sample volume limits; typically, no more than 10% of the blood volume of the animal can be safely withdrawn at one time in a normal ferret.

Materials

8- to 10-week-old ferrets
70% ethanol

Electric clippers
1-ml syringe
23-G needles
Serum separator tubes (Sarstedt, cat. no. 41.1378.005)
Microcentrifuge
Microcentrifuge tubes (e.g., Eppendorf)

Additional reagents and equipment for anesthetizing the ferrets with injectable anesthetics (Basic Protocol 2)

Prepare the ferrets

1. Anesthetize the ferret with injectable anesthetics (see Basic Protocol 2)
2. Position the ferret in dorsal recumbency with the head towards you.
3. Using electric clippers, shave the chest area of the ferret from the base of neck along the sternum.
4. Clean the shaved area with 70% ethanol.

Collect blood

5. Locate the sternum by palpation.
6. In order to access the vena cava, insert the needle into the chest to the right of the sternum under the first rib, aiming towards the left hind foot keeping the needle bevel side up and at a 30° angle (Fig. 15G.2.6).
7. Apply slight suction on the syringe while slowly withdrawing the needle until blood begins to fill the syringe.
8. When blood is seen in the syringe, hold it steady and collect the required amount of blood (typically 1 ml).
9. Once the syringe is full, withdraw the needle and apply slight pressure to the injection site to stop any bleeding.
10. Return the ferret to its cage and monitor recovery.

Separate serum

11. Carefully transfer the blood into serum separator tube(s).

    Do not dispense blood quickly or red blood cells will lyse.

12. Allow the blood to clot in the tubes for at least 30 min.
13. Centrifuge the serum separator tubes in a microcentrifuge 5 min at 10,000 × *g*, room temperature.
14. Transfer sera to appropriately labeled microcentrifuge tube(s).
15. Store the sera at 4°C or −20°C.

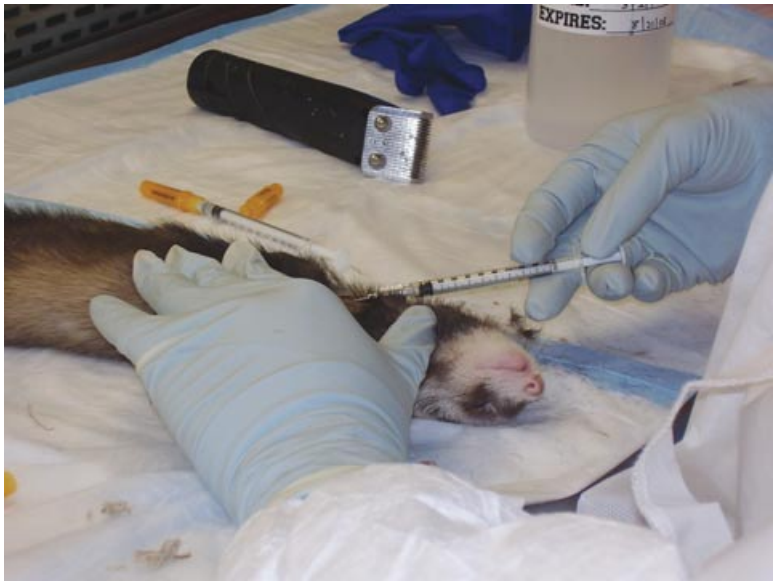

Figure 15G.2.6 Needle is inserted under the sternum into the vena cava.

BASIC PROTOCOL 7

EUTHANASIA OF FERRETS

Animals must be euthanized humanely. Check with your Institutional Animal Care and Use Committee for specific guidelines. In this protocol, ferrets are anesthetized with injectable anesthetics and then given an injection of Euthanasia-III solution via cardiac puncture. Large amounts of blood can be collected via cardiac puncture after anesthesia but prior to euthanasia.

Materials

8- to 10-week-old ferrets
Euthanasia-III (Med-Pharmex, ANADA cat. no. 200-280)

1-ml syringes
23-G needles

Additional reagents and equipment for anesthetizing the ferrets with injectable anesthetics (Basic Protocol 2)

Prepare the ferrets

1. Anesthetize the ferret with injectable anesthetics (see Basic Protocol 2)
2. Position the ferret in dorsal recumbency with the head away from you.
3. Prepare a 1-ml syringe equipped with a 23-G needle with 0.5 ml of Euthanasia-III solution.

Inject the euthanasia solution

4. Once the ferret is anesthetized, insert the needle into the heart under the sternum, just lateral to the xiphoid process.
5. Check placement of the needle in the heart—pull back on the plunger to check for blood—if there is blood in the syringe the needle is in the heart (Fig. 15G.2.7).
6. Once the needle is in the heart, administer Euthanasia-III solution.
7. Check for lack of heartbeat.

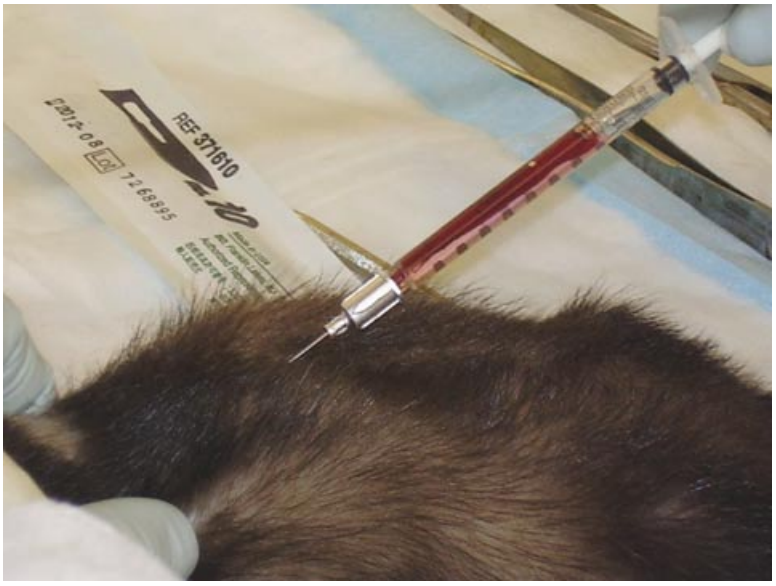

Figure 15G.2.7 Blood is seen in the syringe when the plunger is drawn back if the needle is properly placed in the heart.

FERRET TISSUE HARVEST

BASIC PROTOCOL 8

It is important to collect ferret tissue samples properly for analysis. In order to minimize the potential for cross-contamination, harvest tissues from the group of animals that are anticipated to have the lowest titer of virus first and the group that is anticipated to have the highest titer of virus last. Use new sets of instruments for each virus group. Use a separate set of instruments for each organ collected (i.e., one set for brain, one set for lungs, one set for nasal turbinates, etc.). Brain and spleen are collected for detection of systemic spread and may not be necessary if extrapulmonary replication of virus is not expected. Disinfect instruments in 70% ethanol between each animal/tissue, wiping any blood and tissue from instruments with gauze before placing in ethanol.

Harvested tissues should be placed on wet ice immediately if they are to be processed soon after or snap-frozen on dry ice for storage and later processing. Prelabeling and preweighing the sample collection tubes will save time. The average weight of sample collection tubes can be determined by weighing a representative sample of tubes.

Lung histopathology is best visualized when the lung is partially inflated, and this can be achieved by partially inflating the lungs with formalin at necropsy. Samples collected for histopathology should be <1-cm thick to allow adequate formalin penetration. Blot excess blood from tissues with gauze prior to placing the tissue in 10% neutral buffered formalin. The fixative volume needs to be at least 15 to 20 times greater than the tissue volume to ensure proper fixation (Carson, 2007). Samples from animals inoculated with highly pathogenic influenza viruses are placed in 10% formalin for at least 1 week and are transferred to fresh 10% formalin for several additional days before further processing.

CAUTION: Formalin is a potential carcinogen; appropriate personal protective equipment (PPE) should be worn and large volumes of formalin should be handled in a fume hood. Consult with your Chemical Waste Office for recommendations on proper disposal of used formalin.

Materials

70% ethanol
Dry ice (if freezing samples immediately to process later)
Wet ice (if processing samples that same day)
10% neutral-buffered formalin

15-ml conical tubes, prelabeled and preweighed
50-m conical tubes, prelabeled and preweighed
Tri-pour beakers or other containers to hold 70% ethanol to disinfect instruments
Racks to hold tubes
Surgical instruments including:
- 5-in. scissors
- Rongeurs, 5.5-in.
- Forceps
- Small spatula
- Sterile disposable scalpels
- Microdissecting curette
- Cut-resistant gloves (optional)

100-mm sterile petri dishes
Containers with a screw-top lid to hold at least 15 vol of 10% formalin and 1 vol of tissue
12-ml syringes
20-G needles
Suture material

Prepare for harvesting

1. Label preweighed conical tubes with animal ID and tissue to be collected.
2. Fill 1 liter plastic tri-pour beaker(s) with ~500 ml of 70% ethanol (one for each tissue collected e.g., brain, lung, and nasal turbinates)—enough to immerse the cutting surfaces of instruments.
3. Place instruments needed for each tissue collection into the beakers containing 70% ethanol to disinfect (Table 15G.2.3).
4. Organize prelabeled conical tubes in a rack.
5. Place conical tubes and (dry or wet) ice container in the biosafety cabinet.
6. Euthanize the ferret (see Basic Protocol 7).

Collect brain

As stated above, if systemic spread of virus is expected, the brain and spleen are collected for detection of extrapulmonary replication. These tissues should be collected before

Table 15G.2.3 Ferret Tissue Harvest Instruments

	Organ			
	Lungs	Nasal turbinates	Brain	Spleen
Instruments needed	Forceps Scissors Scalpel	Scalpel Microdissecting curette	Scissors Rongeurs Spatula Microdissecting curette (if removing olfactory bulb)	Forceps Scissors Scalpel

harvesting the lungs and nasal turbinates so that the risk of contamination from organs with high titers of virus is minimized; the titer of virus in the respiratory tract is usually higher than in extrapulmonary sites. If examination of extrapulmonary tissues is not warranted, proceed to the section on collection of lungs and nasal turbinates.

7. Place the euthanized ferret in ventral recumbency.
8. Clean the head with 70% ethanol.
9. Using scissors, remove the skin from the skull (Fig. 15G.2.8).
10. *Optional:* Using scissors, remove muscle and tissue from the cranium.

    It is easier to cut through the skull with the muscle removed but some users find the extra effort needed to cut the skull outweighs the extra time spent to remove the muscle.

11. Use rongeurs to make a transverse cut around the skull (Fig. 15G.2.9).

    Cut-resistant gloves may be worn on the hand holding the skull. Try not to insert tips of forceps too deeply or it will enter the brain.

12. Remove the top portion of skull to expose the brain.
13. While holding the skull over a sterile petri dish, use a spatula to gently tease the brain away from the skull and tip into petri dish (Fig. 15G.2.10).

Collect brain for virus titration

14. With a sterile scalpel and forceps, remove a coronal slice of front, middle, and hind section of the brain. We combine these sections into one tube and process them together.
15. Place brain sections into a labeled 50-ml conical tube.
16. Place on ice (wet or dry, depending on purpose).
17. *Optional:* After the brain is removed, use a curette to remove the olfactory bulb that is located in the skull ventral to the base of the frontal lobe.

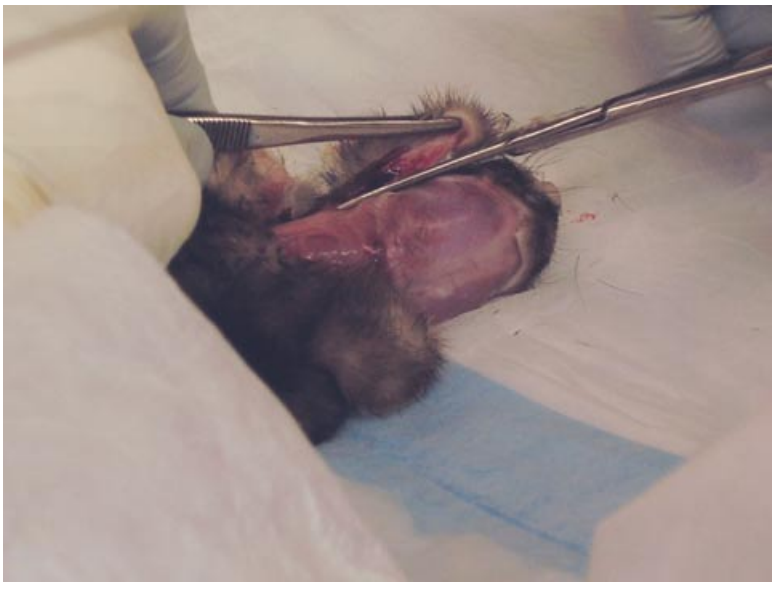

Figure 15G.2.8 Use forceps and scissors to remove skin from the skull.

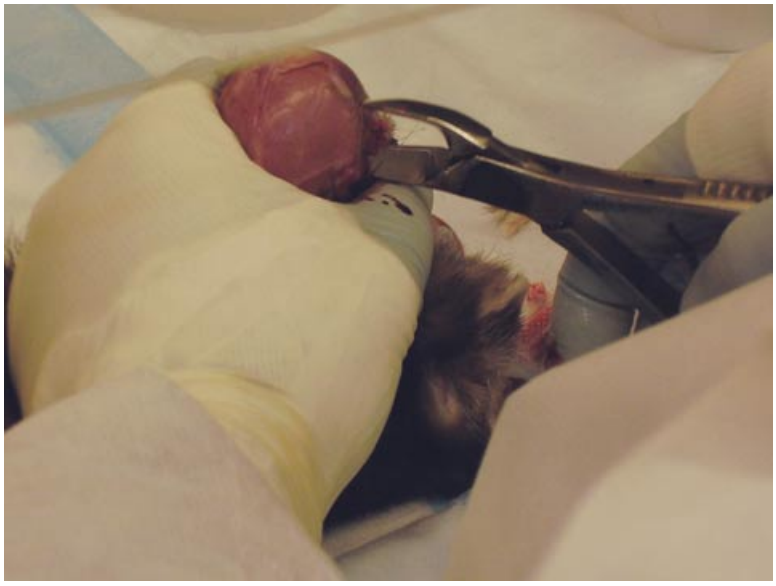

Figure 15G.2.9 Carefully cut through the skull with rongeurs.

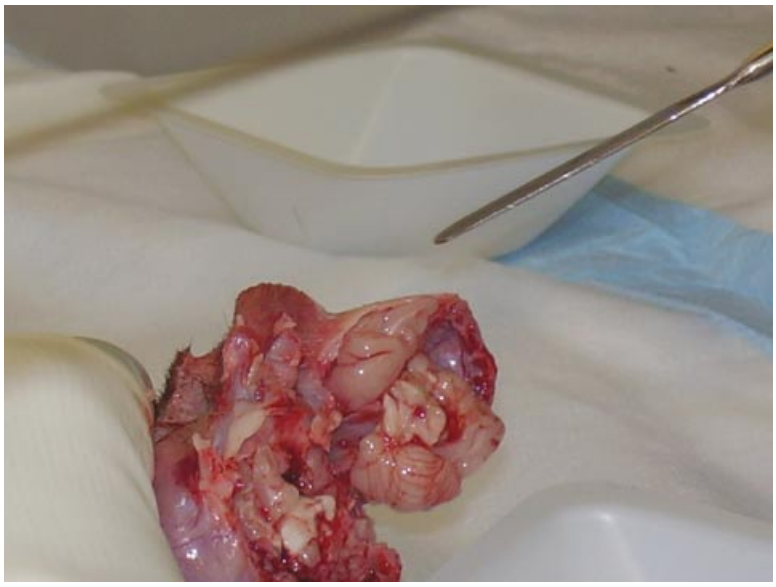

Figure 15G.2.10 Gently tease brain from the skull with a spatula.

Prepare brain sample for histopathology

18. Place the brain (1 vol) into an appropriate-sized container holding at least 15 vol of 10% formalin for proper fixation. Make sure the mouth of the container is wide enough to remove the sample later.

19. Store the sample at room temperature for at least 1 week before continuing with histology processing.

Collect the spleen

20. Clean the abdominal area with 70% ethanol.

21. Use forceps and scissors to remove the skin from the abdomen.

22. Use clean scissors to open the abdominal wall.
23. Remove the spleen with clean scissors and forceps.
24. Using a scalpel, cut the spleen into sections sized to fit into an appropriately sized labeled container.

Remove lungs

The lungs should be harvested before the nasal turbinates because there is usually more virus present in the nasal turbinates than in the lungs, and therefore, the lung tissue can become contaminated if the nasal turbinates are harvested first.

25. Clean the thorax area thoroughly with 70% ethanol.
26. Use scissors to remove the skin from the ribcage.
27. Use clean scissors to cut the ribcage and lift it away from the chest with forceps (Fig. 15G.2.11).
28. Use forceps and clean scissors to remove the lungs and heart and place onto a sterile petri dish.

    If you only need a portion of the lung, you can collect samples without removing the lungs from the chest cavity.

Lung collection for virus titration

29a. For virus titration, cut a portion of the left lower lobe and right lower lobe of the lung using a scalpel or scissors and forceps.

30a. Place each lung portion into an appropriately labeled tube.

31a. Place on ice (wet or dry, depending on purpose).

Lung inflation for histopathology

For histopathology, lung architecture is best visualized with the lung partially inflated with formalin.

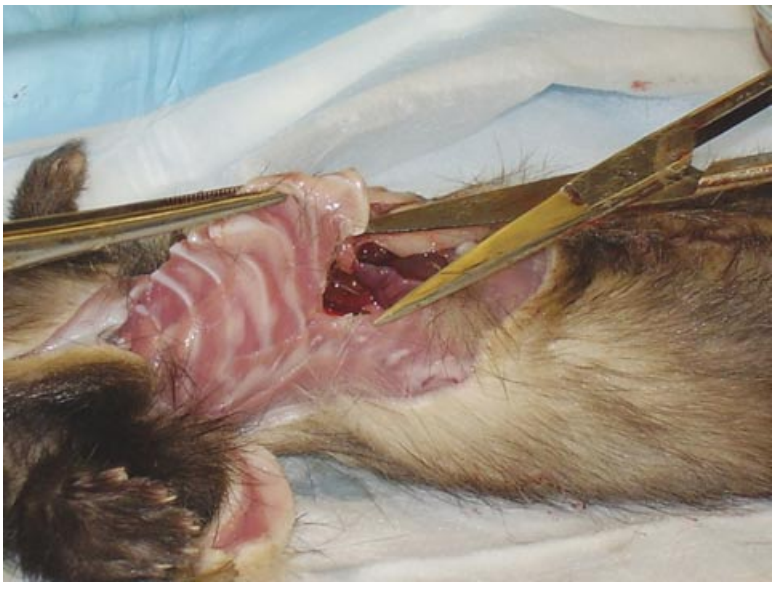

Figure 15G.2.11 Cut the ribcage with scissors and lift away with forceps.

29b. Fill a 12-ml syringe with 10% formalin and attach a 20-G needle. Insert the needle into the trachea. Tie suture material around the needle in the trachea, or pinch forceps around the trachea to prevent leakage.

30b. Slowly inflate the lungs with 10% formalin—do not overinflate! Remove the heart if it is not needed for histopathology.

31b. Place lung (1 vol) into an appropriately sized container holding at least 15 vol of 10% formalin for proper fixation. Store at room temperature for at least 1 week before processing.

Sampling lungs from the same animal for both virus titration and histopathology

If part of the lung is to be used for virus titration and part for histology, remove a portion for virus titration, then perfuse the remaining lung with 10% formalin.

29c. Use suture material to tie off a lobe of the lung for virus titration.

30c. Cut the lobe distal to the suture with scissors and place the sample in a tube on ice (wet or dry, depending on purpose).

31c. Leaving the suture in place, inflate the remainder of the lung with 10% formalin.

Lungs can be inflated with formalin without tying off the cut section of lung but this will result in significant formalin leakage.

Nasal turbinate collection

32. Cut away the cartilaginous and soft tissue of the tip of the nose with a scalpel.

33. Insert a curette into each nare and scoop out nasal turbinates into a sterile petri dish (Fig. 15G.2.12).

34. Place nasal turbinate fragments into a labeled 15-ml conical tube.

35. Place on ice (wet or dry, depending on purpose).

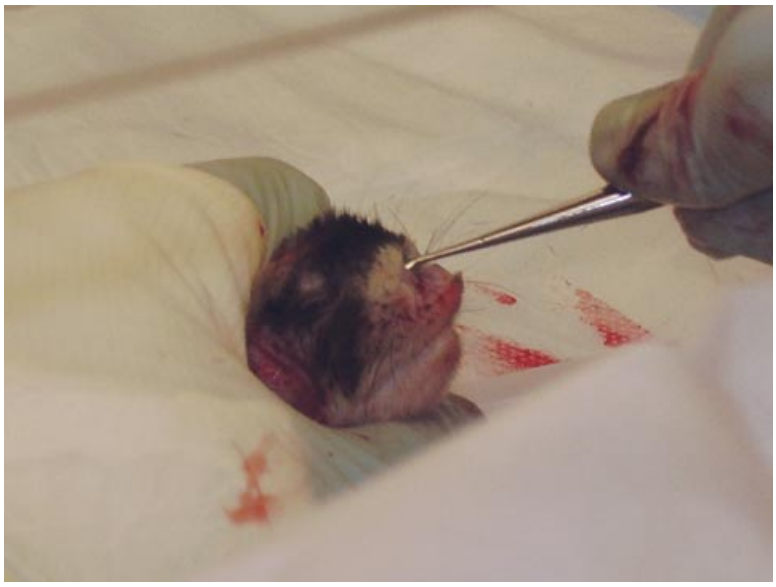

Figure 15G.2.12 Remove nasal turbinates with a curette.

TISSUE HOMOGENIZATION

Tissues are homogenized to make a 10% weight by volume (w/v) suspension (or 5% or 20% w/v for smaller or larger organs, respectively) for virus titration or to extract viral RNA for detection of viral nucleic acids. Tissue samples should ideally have only one freeze/thaw cycle between harvest and processing. We use one of the following scenarios in our laboratory: samples are harvested, homogenized, divided into aliquots, and snap-frozen on 1 day and thawed and titrated on another day, or samples are harvested, snap-frozen and stored at −70°C until a later date, when they are thawed, homogenized, and titrated in 1 day. Samples should be kept on wet ice or at 4°C at all times.

There are many different types of tissue homogenizers and tissue grinders available. In addition to the OMNI homogenizer that we routinely use in our laboratory, several other systems are listed below:

Alternative instruments and methods: (1) Disposable pestle system (Fisher Scientific, cat no. 03-392-106): a cordless battery-operated mixer and a small disposable pestle that fits into a microtube. (2) Kendall precision disposable tissue grinder (Lifeline Medical Inc., cat no. 3505SA for a 15-ml tube): A manual grinder with presterile units packed individually. (3) MagNA Lyser (Roche): The MagNA Lyser instrument can hold 16 samples for simultaneous processing. Using prefilled ceramic beads, it lyses tissue samples by creating a rapid oscillating reciprocal motion of the rotor that agitates the contents of the sample tubes.

Materials

Wescodyne solution (Steris)
Tissue samples in 15-ml or 50-ml conical tubes
Wet ice
L-15 tissue grinding medium (see recipe)
Dry ice

Cryotubes (Nunc, cat. no. 375418)
Autoclave
Hard disposable homogenizer tips in autoclavable bags (OMNI, cat. no. 34750-AC-16)
1-liter beakers for Wescodyne solution
Scale
5-, 10-, 25-, and 50-ml pipets
Pipettor
OMNI tissue homogenizer
Absorbent pads (blue pads)
Tube racks

Prepare equipment

1. Prelabel at least two Nunc cryotubes for each sample.
2. Prepare a spreadsheet to record sample IDs and organ weight to calculate the amount of medium needed to prepare a 10% (w/v) suspension.
3. Autoclave homogenizer tips in autoclavable bags.

Perform homogenization

Make a 1% solution of Wescodyne to inactivate the virus prior to disposal. Keep beakers of Wescodyne in the biosafety cabinet for used pipet tips and grinder tips. Leave all waste in Wescodyne for at least 10 min to inactivate virus before discarding. Decant the Wescodyne into the sink and discard consumables into autoclave bags.

4. Organize the organs to be titered on wet ice starting with the samples anticipated to have the lowest virus titer and ending with the samples anticipated to have the highest titer of virus.
5. Weigh each organ and record the weight on a spreadsheet.
6. Add L-15 tissue grinding medium to each tube; a minimum of 1.5 ml is needed.
   a. A 10% (w/v) suspension is adequate for most organs.

      For example, if a tissue weighs 0.54 g, add 5.4 ml L-15 medium for 10% (w/v) suspension.
   b. A 5% (w/v) suspension is appropriate for small organs like nasal turbinates.

      For example if a tissue weighs 0.12 g, add 2.4 ml L-15 medium for 5% (w/v) suspension.
   c. For large tissues such as a ferret brain, a 10% (w/v) results in a large volume.

      For example if a brain weighs 6.2 g, 62 ml will not fit in 50-ml conical tube.

      i. Add a set volume of L-15 grinding medium to grind (10 ml).

      ii. Then add enough L-15 grinding medium to bring up to 20% (10 ml + 21 ml = 31 ml).

      iii. Spin the homogenized sample.

      iv. Put 1 ml of the 20% homogenate into cryotube and add 1 ml of L-15 grinding medium to achieve a 10% (w/v) suspension and vortex well before use.
7. Set up the homogenizer and prepare beakers containing Wescodyne solution to disinfect tips. Also, prepare a 50-ml conical tube with Wescodyne solution.
8. Place absorbent pads in the work area.
9. Homogenize each organ using the tissue homogenizer.

   The amount of time needed to homogenize organs varies with the size and consistency of the tissue and the speed of the motor. There are six speed levels on the OMNI homogenizer and we typically use the maximum (6) speed. Soft tissues such as brain and lungs take less time (~10 to 20 sec) than harder tissues such as nasal turbinates (~30 sec to 1 min). Homogenize until tissue is no longer recognizable. Nasal turbinates will be pulverized but not homogenized.
10. Before removing the used tip from the grinder, immerse it in a 50-ml conical tube containing Wescodyne solution to disinfect the tip (prepared in step 7).
11. Remove the grinder tip and place it in a beaker containing Wescodyne solution. Put a new grinder tip on the homogenizer and continue until tissues are homogenized.
12. Centrifuge the homogenized samples to pellet cellular debris 10 min at ~524 × *g*, 4°C.
13. Organize the cryotubes in racks.
14. Aliquot 1000 µl (or half the volume if <2 ml total) of each tissue homogenate into duplicate cryotubes (300 µl minimum needed for titration).
    a. Place one cryotube on wet ice if titering immediately.
    b. Place duplicate cryotube(s) on dry ice to freeze.

TITRATION OF TISSUE HOMOGENATES ON MDCK CELLS

The amount of infectious virus in a sample is determined by determining the infectivity of the serially diluted sample on MDCK cells. The infectivity is determined by recording the presence of cytopathic effect (CPE) when an inoculum of 20 µl of the tissue homogenate is applied in quadruplicate on a 96-well tissue culture plate of MDCK cells and is serially diluted. The dilution at which 50% of the wells are infected ($TCID_{50}$) is computed using the Reed and Muench method (Reed and Muench, 1938). By taking into consideration the use of the initial 20 µl inoculum per well and the 10% (w/v) suspension of tissue homogenate, data can be expressed as $\log_{10}$ $TCID_{50}$/g of tissue. In order to increase the detection sensitivity of the assay, 100 µl of the sample can be inoculated into two wells of a 24-well plate (duplicate samples).

Materials

96-well plates of MDCK cells
24-well plates of MDCK cells
Homogenized tissue samples (see Basic Protocol 9)
Wet ice
Wescodyne solution (Steris)
Complete medium with TPCK trypsin (see recipe)
Dry ice

Absorbent pads
Multichannel pipettors
Pipet tips
1-liter beakers for Wescodyne solution
Rocker

Additional reagents and equipment for preparing MDCK cells (Support Protocol)

1. Calculate the number of plates needed to titer each organ and virus separately based on:

   a. Twelve samples per 24-well plate (2 wells/sample).
   b. Two samples per 96-well plate (serial dilution 10^{-1} to 10^{-10}, in quadruplicate per sample).
   c. If you expect the titer to be low, you can titer four samples per 96-well plate (10^{-1} to 10^{-6}).

   For example, if you have 20 lung homogenates to titer, you need ten 96-well plates and two 24-well plates; always make at least two extra of each plate type as backups.

2. Prepare MDCK cells as described in the Support Protocol.

Titrate tissue homogenates in 24-well plates

3. Inoculate two wells of a 24-well plate with each sample (kept on ice) so that the limit of detection for a 10% homogenate is $10^{1.5}$ $TCID_{50}$/g.
4. Label the lid of the 24-well plate for two wells per sample.
5. Flick off medium from the plate onto an absorbent pad.
6. Add 100 µl of homogenate per well, two wells per sample. Discard pipet tips into beakers containing Wescodyne solution.
7. Incubate 1 hr at room temperature on a rocker or gently rotate the plates every 10 to 15 min to ensure that parts of the monolayer do not dry out.

8. While tilting plate at an angle to collect the liquid in the well on one side, carefully remove inoculum from the wells with a multichannel pipettor and sterile tips. Do not touch the cell monolayer.

9. Re-feed the plate with 1 ml complete medium with TPCK trypsin per well.

10a. *For wild-type viruses:* Incubate 3 to 4 days at 37°C.

10b. *For temperature-sensitive viruses:* Incubate 6 days at 32°C or 33°C.

Titrate tissue homogenates in 96-well plates

11. Label the lid of 96-well plate for two samples per plate, 10^{-1} to 10^{-10} dilution.

    If lower titers are expected, four samples per plate can be titered from 10^{-1} to 10^{-6}.

12. Add 20 µl of homogenate (kept on wet ice) to each of the first four wells containing 180 µl of complete medium (10^{-1} dilution row). Mix contents of the well without touching the monolayer. Discard pipet tips into beakers containing Wescodyne solution.

13. Using a multichannel pipettor with sterile tips, draw up and transfer 20 µl to the next row. Change tips, mix the contents of the well without touching the monolayer, and transfer 20 µl to the next row. In this way, make serial 10-fold dilutions, changing tips after each transfer and at the last row discard 20 µl (Fig. 15G.2.13).

14. Mark the tube and freeze the remaining sample on dry ice.

15a. *For wild-type viruses:* Incubate 3 to 4 days at 37°C.

15b. *For temperature-sensitive viruses:* Incubate 6 days at 32°C or 33°C.

16. Read the $TCID_{50}$ results based on CPE and calculate using the Reed and Muench method (Reed and Muench, 1938).

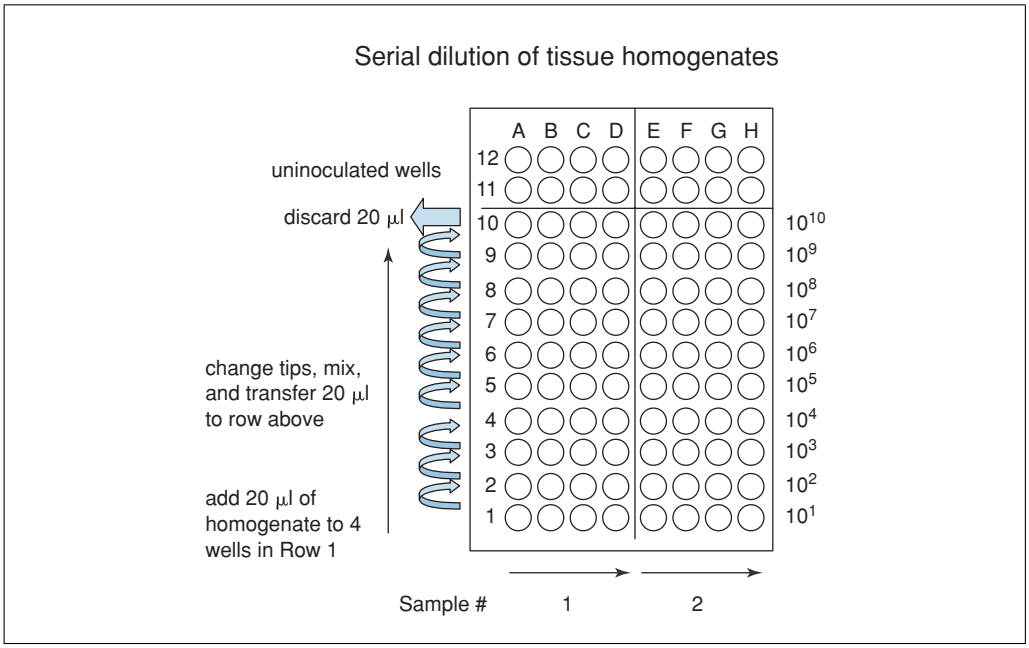

Figure 15G.2.13 Samples are diluted in 96-well plates containing a MDCK monolayer. The sample is inoculated into four wells and serial 10-fold dilutions are made in successive rows of the plate.

PREPARATION OF MDCK CELLS

SUPPORT PROTOCOL

Materials

MDCK cells
MDCK growth medium (see recipe)
MEM medium (Invitrogen, cat. no. 12360-038)
Complete medium with TPCK trypsin (see recipe)

96- and 24-well plates
Container lined with absorbent pads

1. Between 1 to 3 days before titration, plate MDCK cells in 96-well and 24-well plates containing MDCK growth medium so they will be confluent on the day of use. See *UNIT 15G.1* for details.
2. On the day of use, use plain MEM medium to wash growth medium containing fetal bovine serum from plates on the day of use, then add freshly prepared complete medium with TPCK trypsin.

   Do not save the complete medium with TPCK trypsin; it can only be used the day it is made.

3. Flick off medium from plates into a container lined with absorbent pads to prevent backsplash and contamination.
4. Wash MDCK cells with plain MEM.

   a. 96-well plate: 180 µl per well.
   b. 24-well plate: 1 ml per well.

5. Feed 96-well plates with complete medium with TPCK trypsin.

   a. 96-well plate: 180 µl per well.
   b. Do not add complete medium with TPCK trypsin to 24-well plates at this time.

TITRATION OF TISSUE HOMOGENATES IN EMBRYONATED EGGS

BASIC PROTOCOL 11

Ten- to eleven-day-old embryonated hen's eggs are usually used for cultivating influenza viruses. Serial dilutions of the tissue homogenates are inoculated into eggs and the titer of virus in the sample is expressed as the fifty percent egg infectious dose (EID_{50}). Eggs pre-incubated for 10 to 11 days can be purchased from local suppliers. Specific pathogen-free (SPF) eggs are available from Charles River Laboratory. Eggs should be kept with the air sac up. Always candle eggs before use to make sure the embryo is alive and healthy. The blood vessels should be thick and clear, and embryos should be visible and respond to light or slight agitation.

Materials

10- to 11-day-old embryonated hen's eggs
Dilution medium or buffer (L15 medium, PBS, or other appropriate buffer)
Virus or samples to be titrated
Disinfectant
Wet ice
70% ethanol
Washed red blood cells (0.5% turkey or chicken RBCS in PBS, or 1% horse RBCs in PBS and 0.5% BSA)

Egg candler (Brinsea, Egg Lume Candling lamp, cat. no. USF150)
1.5-ml microtubes (for preparation of dilutions)
Vortex

Repeat dispenser (with tips of appropriate size for 0.9 ml) or pipettor (with 5- to 10-ml disposable pipets)
Egg punch (We use sterile and disposable Lancet capillary blood sampling devices available from diabetic supply stores)
1-ml syringes
22-G, 1-in. needles
Incubator for eggs (35°C to 37°C)
Glue (Elmers)
Forceps
50- to 200-μl multichannel pipettor
50- to 200-μl pipet tips
96-well V-shaped plates

Prepare eggs

1. Candle eggs and select eggs with healthy embryos.
2. Aliquot 0.9 ml dilution medium into dilution tubes.
3. Add 0.1 ml of virus or sample into the first tube and vortex.
4. Discard the pipet tip into disinfectant and with a new tip transfer 0.1 ml sample from the first tube into the second tube.
5. Repeat steps 3 and 4 until transferring into the last tube.
6. Keep the diluted samples on ice.

Inoculate eggs

7. Use four eggs per dilution and mark the dilutions on the eggshells.
8. Spray surface of eggs with 70% ethanol.
9. Punch a hole on the top of each egg with a Lancet.
10. Inoculate 100 μl of the diluted sample into each of four eggs using a 1-ml syringe equipped with a 22-G, 1-in. needle.
11. Seal the hole using glue (e.g., Elmer's).

Incubate eggs

12. Incubate the eggs at 35°C (for human influenza viruses) or 37°C (for avian influenza viruses) for 2 to 3 days.

Chill eggs

13. At the end of the incubation period, chill the eggs overnight at 4°C overnight or 20 to 30 min at −20° to −30°C.

Determine hemagglutination endpoint

See *UNIT 15G.1* for more information and instruction about the hemagglutination assay.

14. Place the tray of eggs in the biosafety cabinet.
15. Spray the surface of the eggs with 70% ethanol.
16. With forceps cleaned with 70% ethanol, break and remove the shell over the air sac of the egg.
17. Harvest 50 μl of allantoic fluid from each egg and place into a 96-well plate.

Optional: Also, harvest 25 μl of allantoic fluid from each egg and place into the next well with 25 μl of PBS, resulting in a 1 in 2 dilution of allantoic fluid.

18. Add 50 μl red blood cells (RBCs) into each well that contains allantoic fluid.
19. Incubate 30 min at room temperature for chicken or turkey RBCs or 60 min for horse RBCs.
20. Observe the endpoint of hemagglutination in the well with undiluted and diluted allantoic fluid.
21. Determine the EID_{50} using Reed and Muench method as described previously (Reed and Muench, 1938).

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****.*

L-15 tissue grinding medium

500 ml (1 bottle) Leibovitz's L-15 medium (1×; Invitrogen, cat. no. 11415)
10 ml Antibiotic-antimycotic (100×; Invitrogen, cat. no. 15240-062)
Store up to 1 month at 4°C

MDCK complete medium with TPCK trypsin (add TPCK trypsin and use that day only!)

500 ml Minimum Essential Medium (MEM; Invitrogen, cat. no. 12360-038)
10 ml of 200 mM L-glutamine (100×; Invitrogen, cat. no. 25030-081)
10 ml Antibiotic/antimycotic (100×; Invitrogen, cat. no. 15240-062)
Appropriate volume of 1 mg/ml TPCK trypsin (0.5 to 2 μg/ml final concentration; see recipe)
Prepare fresh on day of experiment

Determine the optimal amount of trypsin to add to the MDCK complete medium by testing a range of concentrations (0.5, 1, 1.5, and 2 μg/ml final concentration in medium; 500 μl of TPCK trypsin in 500 ml medium = 1 μg/ml), with two or three different viruses in $TCID_{50}$ and plaque assays.

Choose the concentration of trypsin to use that gives the highest virus titer and does not damage the monolayer.

MDCK growth medium

500 ml Minimum Essential Medium (MEM; Invitrogen, cat. no. 12360-038)
50 ml fetal bovine serum, irradiated and heat inactivated (30 to 60 min @ 56°C)
10 ml of 200 mM L-glutamine (100×; Invitrogen, cat. no. 25030-081)
Store up to 1 month at 4°C

TPCK trypsin, 1 mg/ml

Dissolve 100 mg trypsin, TPCK-treated, irradiated (Worthington Biochemical, cat. no. LS003750) in 100 ml L-15 medium (Invitrogen, cat. no. 11415-064) plus 2 ml antibiotic/antimycotic (100×; Invitrogen, cat. no. 15240-062), so the final concentration of trypsin is 1 mg/ml. If needed, add 0.9 ml of 7.5% $NaHCO_3$ to solubilize trypsin. Divide into 0.5- to 1-ml aliquots and store at −20°C. Do no refreeze or reuse.

COMMENTARY

Background Information

Many of the seminal studies on epidemic influenza were conducted in human subjects. However, studies in humans are performed less frequently now than they were in the past. Therefore, it is important to consider the use of animal models for the evaluation of influenza pathogenesis, vaccines, and antiviral strategies. A major challenge for research on influenza is the selection of an appropriate

animal model that accurately reflects the disease and the protective immune response to influenza infection in humans.

Influenza A viruses are widely distributed in nature and can infect a wide variety of birds and mammals, including humans. Influenza A virus subtypes are classified based on the antigenicity of their surface glycoproteins, hemagglutinin (HA; *UNIT 15G.1*), and neuraminidase (NA) into 16 HA subtypes and 9 NA subtypes, and all of these subtypes have been found to infect birds. Waterfowl and shorebirds are the natural reservoirs of avian influenza viruses. In contrast, influenza B and C viruses only infect humans and different subtypes of these viruses have not been identified. Despite the diversity of mammalian species infected by influenza viruses in nature, only a few species are amenable to study in the laboratory.

Ferrets are exquisitely susceptible to infection with human influenza viruses and they remain the ideal small animal model for influenza research. The initial isolation of a human influenza virus by Smith and colleagues involved ferrets (Smith et al., 1933). Ferrets can be infected with nonadapted human influenza virus isolates and several avian influenza virus subtypes. Influenza virus infection in ferrets is primarily an upper respiratory tract infection, and infected ferrets exhibit clinical signs of infection similar to those seen in human influenza including fever, rhinitis, and sneezing. The disadvantages of the ferret as a model for studying influenza vaccines include expense, special housing requirements, a limited number of suppliers, the difficulty in obtaining animals that are seronegative for influenza virus, their exquisite sensitivity to other respiratory pathogens and ease of acquiring infection from their handlers, and the lack of species-specific reagents for evaluation of cellular immunity. In addition, the high body temperature of ferrets (average temperature of 38.8°C) may limit their utility in the evaluation of temperature-sensitive live attenuated influenza vaccines.

The use of mice for influenza research is discussed in a separate unit (*UNIT 15G.3*). Although they are not discussed in detail in this unit, hamsters (Abou-Donia et al., 1980; Heath et al., 1983; Subbarao et al., 1993), guinea pigs (Phair et al., 1979; Lowen et al., 2006), cotton rats (Niewisk and Prince, 2002; Ottolini et al., 2005), and rats (Teh et al., 1980) have been used for influenza research.

The choice of an animal model should be based on the scientific question and the utility of the model for the specific indication. Initial studies are often carried out in mice, and ferrets are used for subsequent follow-up studies. However, there may be specific indications, such as transmission studies, for which ferrets or guinea pigs are the appropriate models. The receptor for influenza viruses is sialic acid; human influenza viruses preferentially bind sialic acids with α2, 6 oligosaccharide linkages, while avian influenza viruses preferentially bind sialic acids with α2, 3 oligosaccharide linkages. While both types of receptors are present in the respiratory tract of mice and ferrets, the distribution of the receptors in the two species differs (van Riel et al., 2006), and this may be a consideration in selection of animal models.

Critical Parameters and Troubleshooting

As discussed in the unit on influenza propagation, quantification, and storage (*UNIT 15G.1*), virus samples must be kept on wet ice or at 4°C at all times. Tissues harvested for virus titration should be kept on wet ice or refrigerated and processed as soon as possible. If the samples are to be processed at a later time, they should be kept frozen below −70°C. Repeated cycles of freezing and thawing will destroy the infectivity of the virus. When tissue homogenates are titrated, the samples should be vortexed and pipet tips should be changed at each dilution. For virus titration or antibody assays, corresponding samples from all of the experimental groups in the study should be tested at the same time. For example, the lungs harvested on days 2 and 4 post-infection from vaccinated and mock-vaccinated animals should be titrated the same day using the same lot of MDCK cells. In order to minimize potential for cross-contamination, tissues from the group of animals that are anticipated to have the lowest titer of virus should be harvested first and the group that is anticipated to have the highest titer of virus last. Similarly, within a group, organs that are anticipated to have a lower virus load should be harvested first and respiratory tissues such as nasal turbinates and lungs should be harvested later to minimize the possibility of contamination of extrapulmonary organs with virus from the respiratory tract. A sample of the inoculum should be saved and titrated to confirm that the dose administered was accurate. If the study protocol requires administration of a similar inoculum on different days, the virus can be diluted and frozen in aliquots at −70°C, and a fresh aliquot can be used each day after one frozen vial is back-titered to confirm the titer.

The volume of inoculum administered intranasally can influence the outcome of infection and the extent to which the virus is distributed in the respiratory tract (Jin et al., 2007). If virus stocks are amplified in the allantoic cavity of embryonated eggs, the stock virus should be diluted before use as inoculum for animal studies because egg proteins in undiluted allantoic fluid can induce an inflammatory reaction. Whenever possible, viruses that have not been extensively passaged should be used because influenza viruses can acquire mutations with passage that may affect their biological characteristics. For example, stocks of highly passaged laboratory strains like influenza A/Puerto Rico/8/34 (H1N1) from different sources display variable virulence.

Ferrets should be prescreened for antibodies to circulating influenza viruses because they are exquisitely susceptible to influenza and can be infected by their caretakers. Prior infection with an influenza virus can affect the response of ferrets to subsequent infection with an influenza virus of the same or other subtype. Clinical parameters such as body weight and temperature should be measured at approximately the same time each day since there can be variation from circadian rhythm. Lung histology is best visualized when the lungs are partially inflated with formalin; mild inflammation can be missed if the lungs are partially collapsed.

When the amount of virus in tissue homogenates is measured in the presence of high levels of serum antibody (e.g., when vaccines are administered with adjuvant, or when immune serum or a monoclonal antibody are administered passively) the presence of virus should be measured by quantitative molecular methods. This is done to rule out the possibility of ex vivo neutralization of virus infectivity by serum antibody during tissue processing. Such ex vivo neutralization can account for a reduction in detectable virus (Subbarao et al., 2004). The use of nasal and bronchiolar wash samples, instead of tissue homogenates, for viral quantitation are also employed as a solution to this issue (Takiguchi et al., 1992).

Anticipated Results

Irrespective of whether an influenza virus induces morbidity or mortality in the experimental animal, the level of replication of influenza viruses in the upper and lower respiratory tract is an important and informative endpoint. It is often preferable to select two time points to evaluate the level of virus replication in tissues in case the intervention (e.g., antiviral treatment) delays the peak of viral replication. As in all experimental research, the inclusion of appropriate controls is critical. The level of viral replication in animals that have received an intervention e.g., vaccine or antiviral or antibody must be compared with a mock-treated group because the absolute value is not as meaningful as a statistically and biologically significant reduction in virus titer.

Animals immunized with influenza viruses or vaccines develop serum ELISA, HI, and neutralizing antibodies; the titers of HI and neutralizing antibodies correlate with protection from subsequent challenge.

Time Considerations

Peak viral replication in the respiratory tract of ferrets usually occurs 2 to 4 days following virus administration. If the kinetics of replication of the virus being studied is not known, a pilot experiment should be conducted to determine the ideal time to harvest tissues. In a typical experimental infection with influenza, antibodies are detectable in the serum as early as 7 days post-infection and are often assayed 14, 21, or 28 days post-infection.

There are two options to minimize the number of times a sample is frozen and thawed. The first option is that the samples can be homogenized on the day they are collected and aliquots frozen at −70°C until the samples for all of the time points have been collected and processed. Another option is to freeze the tissues when they are harvested and to homogenize and titrate all of the samples together. Sixty to seventy samples can be homogenized and titrated in 1 day. It is important to allow sufficient time to wash the MDCK 96-well plates before use because the medium is changed twice on these plates. If the samples containing wild-type influenza viruses are titrated in MDCK cells, cytopathic effect should be scored 3 days later and finalized on day 4. Samples that are incubated at lower temperatures (e.g., 32°C that is suitable for growth of temperature-sensitive viruses) should be incubated for 6 or 7 days. If samples are titrated in embryonated eggs, the eggs are incubated for 2 or 3 days and chilled overnight. The allantoic fluid is then harvested and tested for hemagglutinating activity.

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Queensbury, K.E. and Carpenter, J.W., eds. 2004. Ferrets, Rabbits, and Rodents Clinical Medicine and Surgery, 2nd edition. Saunders, St. Louis, Mo.

This book discusses the anatomy, husbandry, physiology, and diseases of small mammals including ferrets, rabbits, guinea pigs, and other small rodents.

van der Laan et al., 2008. See above.

This review article discusses various animal species that can be experimentally infected with influenza.

Luke and Subbarao, 2008. See above.

This chapter discusses the evaluation of influenza virus vaccines in animal models.

Internet Resources

http://www.cdc.gov/od/sap
CDC Select Agent program.

http://www.bmds.com
Use of ferret microchips.

http://www.avma.org/resources/euthanasia.pdf
Euthanasia guidelines from American Veterinary Medical Association.

http://www.searo.who.int/LinkFiles/CDS_CDS-Guidelines-Laboratory.pdf
WHO Guidelines on Laboratory Diagnosis of Avian Influenza (2007).

http://www.wpro.who.int/NR/rdonlyres/EFD2B9A7-2265-4AD0-BC98-97937B4FA83C/0/manualonanimalaidiagnosisandsurveillance.pdf
WHO Manual on Animal Influenza Diagnosis and Surveillance.

http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm
5th edition of Biosafety in Microbiological and Biomedical Laboratories.

http://www.cdc.gov/flu/pdf/h2n2bsl3/pdf
Biosafety guidelines for noncontemporary or non-human influenza viruses from the 5th edition of Biosafety in Microbiological and Biomedical Laboratories.