# Chapter 26

# Mouse Models of Viral Infection

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## Abstract

Viral respiratory tract infections are common in both children and adults. Mouse models of viral infection enable the characterization of host immune factors that protect against or promote virus infection; thus, mouse models are essential for interrogation of potential therapeutic targets. Moreover, they serve as critical models for the development of novel vaccine strategies. In this chapter, we describe methods for establishing mouse models of respiratory syncytial virus (RSV) and H1N1 influenza A virus infection. Protocols are provided for viral culture and expansion, plaque-forming assays for viral quantification, and infection of mice. Alternate modifications to the models are also described, and their potential impact is discussed.

**Key words** Respiratory syncytial virus (RSV), Influenza A virus, H1N1, Mouse model, Infection, Lung injury

## 1 Introduction

Lower respiratory tract infections, including bronchitis and pneumonia, affect over 100 million individuals in the United States each year and are a leading cause of health-care encounters, particularly in the late fall and winter [1]. Bronchitis results from infection of the bronchial mucosa. It has been estimated that over 60% of cases are caused by viruses, of which the most common are influenza A and B, respiratory syncytial virus (RSV), parainfluenza, coronaviruses, rhinoviruses, and human metapneumovirus [2, 3]. In comparison to bronchitis, pneumonia affects the more distal portions of the lung including the alveoli. Although it is widely accepted that the majority of pneumonia cases are caused by bacteria, recent evidence suggests that respiratory viruses are also common, either as the single cause or as a coinfecting pathogen [4–6]. Common viral pathogens in adults include influenza A and B, parainfluenza virus, rhinovirus, and coronavirus [4–7]. These pathogens are also common causes of pediatric pneumonia, as is RSV [7, 8].

Murine models of pulmonary viral infection have significantly advanced the characterization of immune targets, our understanding

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of viral evasion mechanisms, and the development of vaccines and therapeutics [9–11]. Despite the significant contributions of these models, inconsistencies among published reports call into question the impact of variable model approaches and how they affect outcome measures. Moreover, the lack of published methodologic details makes it impossible to address the impact of variability among murine models of viral infection [9, 12–14]. Here we provide detailed methodologic procedures for generation of RSV and influenza A virus working stock, and efficient viral plaque purification. Moreover, we provide methods for inducing RSV and H1N1 influenza A infection in adult mice and quantification of viral lung titers. Alternative approaches to the described methods are included where available.

## 2 Materials

RSV and influenza A are BSL2 level organisms. Proper precautions should be followed and proper personal protective equipment should be worn when working with RSV and influenza A viruses and virus-infected animals.

### 2.1 RSV Master Stock Preparation (see Note 1)

1. Hep-2 media: To a 500 mL bottle of 1× MEM + L-glutamine + Earle's salts (with phenol red) (10% EMEM), add 5 mL of penicillin-streptomycin (p-10,000 U/mL, s-10,000 μg/mL), 0.5 mL of Fungizone (amphotericin B, 250 μg/mL), and 50 mL of heat-inactivated fetal bovine serum (HI-FBS). To heat inactivate the FBS, thaw at room temperature, and then place in a 56 °C water bath for 30 min (*see* **Note 2**).
2. 1× phosphate-buffered saline (PBS).
3. 0.25% trypsin (Gibco).
4. 1% agarose: To a 400 mL of deionized water, add 4 g of agarose. Autoclave the mixture for 30 minutes at 120 °C.
5. 2× Hep-2 media = 1× Hep-2 media with 2× additives: To a 500 mL bottle of 1× MEM + L-glutamine + Earle's salts (with phenol red), add 100 mL of HI-FBS (do not remove 50 mL of media), 1 mL gentamicin (50 mg/mL concentration), 1 mL Fungizone, 10 mL penicillin-streptomycin, and 5 mL of L-glutamine (*see* **Note 3**).
6. Dry ice/alcohol bath: Dry ice should be broken into small pieces. To this, add 2-propanol; replenish with dry ice and 2-propanol as needed to maintain bubbles (will look as though it is boiling). Note that it is important to wear appropriate protective gloves and eyewear to prevent thermal injury from the ethanol-dry ice solution.

7. Plastic/vials: T-162 tissue culture flasks, 6- and 12-well tissue culture plates, autoclaved 4 mL 1-dram glass vials with printed labels and assembled cap and septum, electrical tape.

### 2.2 RSV Working Stock Preparation

1. Plastics/glass: T-162 tissue culture flasks, 50 autoclaved 4 mL 1-dram glass vials with printed labels and assembled cap and septum, 50 mL conical centrifuge tubes, 15 mL conical centrifuge tubes, sterile glass flasks, 10 mL pipettes.
2. 10% EMEM: To a 500 mL bottle of EMEM, add 10 mL of HI-FBS.
3. Dry ice/alcohol bath: Dry ice is broken into small pieces and placed in a Styrofoam container. Add 100% isopropyl alcohol to cover the dry ice; the alcohol will bubble as though boiling. A rack to hold the glass vials must be placed in the dry ice bath such that the bottoms of the vials are immersed but the caps are not (*see* **Note 4**).
4. Electrical tape.
5. 70% ethanol.
6. Sonicator/cell disruptor with probe and pulse operation.

### 2.3 RSV Hematoxylin and Eosin Plaque Assay

1. Hep-2 media (10% EMEM) as described in Subheading 2.1.
2. 10% EMEM+0.75% methyl cellulose: Put 3.75 g of methyl cellulose and a stir bar in a 500 mL bottle and sterilize in the autoclave. When cool, put on a stir plate and add 500 mL of room temperature Hep-2 in a sterile hood. Stir mixture for 1–2 days at room temperature. Make sure to monitor its progress. Store at 4 °C, dissolves better at cooler temperature.
3. Gill modified hematoxylin solution 1 (Fisher).
4. Eosin yellowish solution 1% w/v (Fisher).
5. 10% buffered formalin solution.
6. 1× phosphate-buffered saline (PBS).
7. Trypsin/EDTA (0.25%), phenol red.
8. Plastic/glass wear: 12-well tissue culture plates, T175 tissue culture flasks, 5 mL polystyrene tubes.

### 2.4 Preparation of H1N1 Influenza A Stock

1. Influenza A, strain A/PR/8/34 (American Type Culture Collection, ATCC, VR-95).
2. Freshly fertilized specific pathogen-free (SPF) chicken eggs (Charles River Laboratories).
3. Humidified egg incubator, preferably with automatic egg turner.
4. BSL-2 biosafety hood.
5. Egg candler.
6. 70% ethanol.

7. Glue gun or paraffin wax.
8. Sterile 18-guage needle.
9. Sterile 22-gauge needle, 1 in. long.
10. Sterile spatula or spoon.
11. Sharp sterile scissors.
12. 50 mL conical tube, sterile.
13. Sterile 0.5 mL microfuge tubes.
14. Liquid nitrogen for flash-freezing microfuge tubes.

### 2.5 Influenza A Plaque Assay

1. MDCK cells (American Type Culture Collection, ATCC).
2. PBS without $Ca^{2+}$ and $Mg^{2+}$.
3. Complete Eagle's minimum essential medium (cMEM): Mix 432.5 mL MEM, 50 mL heat-inactivated fetal bovine serum, 12.5 mL 1 M HEPES, and 5 mL penicillin/streptomycin (5000 U/mL each). Mix thoroughly and then filter using a 0.2 μm filtration system. Store at 4 °C.
4. 2× MEM: Mix 325 mL deionized water, 100 mL 10× MEM, 40 mL 7.5% BSA, 25 mL 1 M HEPES, and 5 mL penicillin/streptomycin (5000 U/mL each). Mix thoroughly and then filter using a 0.2 μm filtration system. Store at 4 °C.
5. 2% agarose solution: Add 2 g low melting point agarose powder to 100 mL deionized water. Mix in a 250 mL Erlenmeyer flask and autoclave at 120 °C, 20 psi for 30 min. Store at 4 °C.
6. TPCK-trypsin (Thermo Fisher Scientific).
7. 10% formalin.
8. 12-well tissue culture plates.
9. Sterile 1 mL microfuge tubes.

### 2.6 Viral Lung Titers

1. Screw thread, 15 × 45 mm 1 dram vials with rubber-lined lids.
2. Ceramic mortar and pestle that has been autoclaved and stored at 4 °C.
3. Glass beads.
4. Hep-2 media.
5. Electrical tape.
6. Histologic grade 2-propanol.
7. Dry ice.
8. 12-well tissue culture plates.
9. 10% EMEM + 0.75% methyl cellulose.

### 2.7 Murine Infection

1. For RSV infection, use female or male BALB/c mice 8–10 weeks old (Jackson Laboratory, Bar Harbor, ME) (*see*

**Note 5**). For H1N1 influenza A infection, mice on any genetic background can be used.

2. Balance: mice should be weighed at baseline and then daily following infection to assess/document weight loss from baseline values (*see* **Note 6**).
3. BSL-2 hood.
4. Anesthesia vaporizer.
5. Isoflurane *or* ketamine/xylazine cocktail: Mix 30 mL bacteriostatic sodium chloride injection (30 mL vials), 3 mL ketamine (100 mg/mL, store at room temperature), and 0.54 mL xylazine (100 mg/mL—AnaSed Injection—store at room temperature). Inject 100 μL intramuscular (I.M.) per 20 g mouse using 1 mL syringes and 26-gauge 3/8 needles. Allow 2–3 min for drug to take effect. Monitor animals until they are mobile again.
6. Oxygen tank.
7. Induction chamber.
8. Charcoal filter.
9. Pipette.

## 3 Methods

### 3.1 Generation of RSV Master Stock

*Day (−1): Plating Cells*

1. Grow Hep-2 cells to ~80% confluence in T-162 flasks in 50 mL of Hep-2 media (*see* **Note 7**).
2. Remove old media and wash cells aggressively twice with 10 mL of 1 × PBS pre-warmed to 37 °C.
3. Add 2 mL of 0.25% trypsin and aggressively rock back and forth to allow removal of adhered cells from the flask (*see* **Note 8**).
4. Neutralize the 0.25% trypsin by adding 8 mL of Hep-2 medium (total volume of 10 mL) and pipette up and down several times.
5. Take an aliquot for cell counts, and then dilute the cell suspension in Hep-2 media to achieve a concentration of $5 \times 10^5$ cells/mL (*see* **Note 9**).
6. Aliquot 2 mL of diluted Hep-2 cells into each well of a 6-well tissue culture plate to achieve $1 \times 10^6$ cells per well, and immediately shake the plate front to back and side to side to disperse the cells evenly (*see* **Note 10**).
7. Place the plate in the 37 °C 5% $CO_2$ incubator to be ready the following day.

*Day (0): Infecting Cells*

8. Cells should be 80% confluent before infection with RSV.
9. Make RSV dilutions in 1× Hep-2 media warmed to 37 °C. Prepare tenfold serial dilutions (1:1–1:1,000,000) using 1× Hep-2 media; be sure to vortex between each dilution. Vortex again before adding 100 μL to each well of a 6-well plate in duplicate.
10. Rock the plates on a plate rocker, at 37 °C at a fast speed for 1 h; rock the plates by hand in the opposite direction of the plate rocker every 20 min during the 1 h incubation to ensure even distribution of virus.
11. After the 1 h incubation, mix 1% agarose and 2× Hep-2 media (warmed to 37 °C) immediately before adding 2 mL to each well; add to the sides of the wells overlaying the inoculum that is already in the wells (*see* **Note 11**).
12. Place the 6-well plates in a 37 °C, 5% $CO_2$ incubator for 2–6 days until syncytia are visible; monitor closely (*see* **Note 12**).

*Day 4–6: Picking Plaques*

13. When virus is ready, pick at least five *well-isolated plaques*. Circle the plaques under an inverted microscope (4× objective works best) (*see* **Note 13**).
14. Remove plaque and agarose plug using a 1 mL or 2 mL pipette without sliding the pipette tip across plate. After removing the plaque, check the plate under the microscope to ensure plaque was successfully removed.
15. Place plaques in individual cryotubes filled with 0.5 mL of 1× Hep-2 media; pipette up and down several times to break up the agarose. Reserve a well-isolated plaque for the next round of plaque purification and freeze the rest at −80 °C.
16. Initiate the next round of plaque purification with the well-isolated plaque from **step 15**, making tenfold serial dilutions as described in **step 9**. Add 100 μL of each dilution to each well of 6-well plate in duplicate (cells should be 80% confluent). Overlay with agarose as described above.
17. Perform four rounds of plaque purification by repeating the steps above.
18. To generate master stock when the fourth round of plaques are picked, place the plaques in 0.5 mL of 1× Hep-2 media. Be sure to break up the agarose by pipetting up and down, and use the entire solution to infect an 80% confluent T-162 flask of Hep-2 cells. Pick several fourth round plaques that can be frozen as well.
19. Grow and harvest stock as per working stock protocol.

### 3.2 Generation of RSV Working Stock

*Day 0: Splitting Cells*

1. Split Hep-2 Cells into two T-162 flasks so that they will be 80% confluent on Day 0.
2. Infect two 80% confluent, T-162 flasks of cells. Remove the media from flasks, leaving 3 mL/flask; add the complete contents of one vial of RSV master stock (about 0.5 mL) to each of the two flasks (one vial of RSV master stock should be used for each flask).
3. Incubate both flasks for 1 h at 37 °C on a rocker table at a fast setting, rocking the flasks in the opposite direction every 20 min during the 1 h incubation to distribute virus (*see* **Note 14**).
4. Add 46 mL of 10% EMEM to each flask, and then incubate at 37 °C + 5% $CO_2$. Different viral subtypes induce cytopathology at different rates; check for cytopathic effects (CPE) 2–3 days after infection (Fig. 1).

*Day 3: Viral Harvest*

5. First thing in the morning: Set the centrifuge temperature to 4 °C; put dry ice in the dry ice/alcohol bath; put all refrigerator-cooled supplies on ice (except 10 mL pipettes).
6. Scrape flasks with cell scraper (do not remove media). The cell scraper does not need to be cold. Rinse the cells by pipetting media up and down and detach any remaining cells.

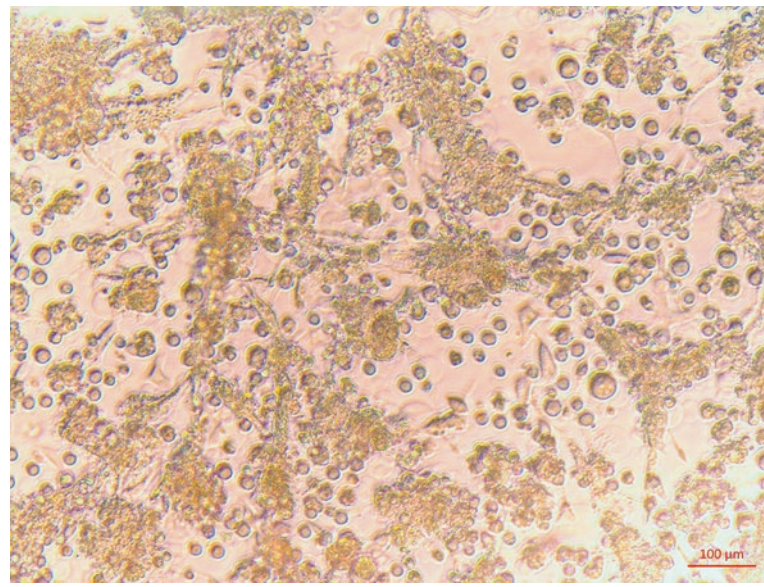


**Fig. 1** Syncytia formation in RSV-infected Hep-2 cells. RSV A2 syncytia formation Day 2 after Hep-2 infection using a 20× objective. Syncytia extensions and fusion of cells can be observed

7. Harvest the entire contents of each flask by dividing into two 50 mL conical centrifuge tubes/flasks (on ice). There will be approximately 25 mL per tube.
8. Sterilize the sonicator overnight (or on the day of use) under a UV lamp, and clean the sonicator probe with 70% ethanol. Rinse probe three times with 10% Hep-2 media and then submerge probe tip in ice-cold media in a 15 mL conical tube until ready for use.
9. Sonicate each tube (on ice) at an amplitude of 50% with one pulse per second for each ml of volume (25 times). Be sure to keep the virus suspension cold at all times.
10. Centrifuge at 800 × *g* for 10 min at 4 °C.
11. Pool the contents of the four tubes into a sterile glass flask on ice; mix by swirling.
12. Aliquot 1.0 mL into each precooled (on ice) glass dram vial. To one of the glass dram vials, add only 500 μL to be used later for viral titers.
13. Tighten each vial carefully and seal with electrical tape before placing in the dry ice/alcohol bath for approximately 5 min. Store vials at −80 °C and quick thaw when ready to use.
14. Follow the RSV hematoxylin/eosin plaque assay protocol (Subheading 3.3) to titer viral stocks. Prepare eight dilution tubes (450 μL each) and two 12-well plates with $1 \times 10^5$ Hep-2 cells per ml per well. Quick thaw the 500 μL vial of viral stock and inoculate 50 μL/well using dilutions ranging from $10^{-2}$ to $10^{-8}$, leaving the last row of wells for control. After 5 days, fix, stain, and count plaques. Calculate the virus titer using the dilution having at least five plaques per well (Subheading 3.3).
15. As an infection control, live virus is placed in a petri dish approximately 4 in. from a 40-W UV light on ice for 4 h. To ensure inactivation, UV-inactivated virus should be titrated on Hep-2 cells as described in Subheading 3.3 (*see* **Note 15**).

### 3.3 RSV Hematoxylin and Eosin Plaque Assay

*Day (−1): Plating Cells*

1. Allow Hep-2 cells to become about 80% confluent in a T-162 flask, and check under microscope using a 10× objective to verify. When cells are ready, remove media using a Pasteur pipet; wash the flask twice with 10 mL 37 °C 1× PBS.
2. Add 2 mL of trypsin/EDTA, incubate until cells start to detach, and give the flask a tap occasionally. Once cells are detached, neutralize the trypsin with 8 mL of Hep-2 media, and pipet up and down to assure all cells are detached and in solution (*see* **Note 8**).

3. Count cells and dilute the cell suspension to a concentration of $1 \times 10^5$ cells/mL (*see* **Note 9**). Add 1 mL of cell suspension to every well of a 12-well plate. Gently mix the cells in the plate in an (+) shaped pattern (15–20 times) to prevent the cells from settling in the middle of the plate. Label plate with date and contents (*see* **Note 16**).
4. Cells should be ready (70–80% confluent) the next day. Be sure to monitor the confluence several times a day so that they do not become over-confluent.

*Day (0): Infecting Plates*

5. Thaw virus sample quickly in a 37 °C water bath and place on ice. Keep samples on ice during assay.
6. Make three dilution tubes/sample (Falcon #2054 polystyrene); add 450 μL of 37 °C 10% EMEM/tube.
7. To titer viral stocks: Prepare eight dilution tubes (450 μL) each and two 12-well plates of 80% confluent Hep-2 cells. Quick thaw a 500 μL vial of viral stock and inoculate 50 μL/well using dilutions from $10^{-2}$ to $10^{-8}$, leaving the last row of wells for control.
8. To titer infected lungs: Prepare three dilution tubes of lung homogenate (*see* Subheading 3.7) with 450 μL each and one 12-well plate of 80% confluent Hep-2 cells. Inoculate with 50 μL/well using dilutions from neat to $10^{-3}$.
9. Decant media from 12-well plate into biohazard bag and add dilutions beginning with the most dilute samples.
10. Incubate for 1 h at 37 °C on a rocker table at a fast speed; hand rock every 20 min in the opposite direction.
11. After 1 h incubation, overlay cells with 1 mL of 10% EMEM + 0.75% methyl cellulose pre-warmed to 37 °C; do not remove inoculum.
12. Incubate in 5% $CO_2$ incubator at 37 °C for 5 days.

*Day (5): Staining Plates*

13. Do not remove the media. Add 1 mL of 10% formalin, and then wait 1 h.
14. Invert plates and dump formalin out. Wash plate three times in room temperature water: run water in a bucket and let it continually overflow so that the water is refreshed. Dunk the plate vertically in the water bucket and scoop water to fill all wells and dump. Repeat this two more times and dump out the water.
15. Add 1 mL hematoxylin to every well with a 10 mL pipette and let sit for 20 min. Then invert plate and dump liquid.
16. Wash the plates three times as in **step 14**.

17. Add 1 mL of eosin Y to each well, wait 3 min, invert plate and dump, and then wash wells as in **step 14**. Shake excess water from plates and let dry upside down overnight.
18. Count plaques at the dilution with no less than five plaques/well. Total the number of plaques counted at that dilution in each replicate well and add them together, divide by the number of replicates (3), multiply by total volume in the stock vial (1000 μL) divided by the amount added to each well (50 μL), and then multiply by the dilution. This will give you the number of plaque-forming units per ml. Calculate the $\log_{10}$ pfu/mL = [(Avg count) × (total volume)]/[(volume added/well) × (dilution)].

### 3.4 Preparation of H1N1 Influenza A Stock

H1N1 influenza A virus may be purchased from a commercial vendor or obtained from a trusted collaborator. If the H1N1 is obtained from a collaborator, it most likely has already been propagated and ideally has been sent to you in aliquots that can be used for each set of experiments. If this is the case, proceed to Subheading 3.5 for methods to quantify the virus. It is critical that aliquots remain frozen at −80 °C until use. Each freeze-thaw cycle results in loss of virus.

#### 3.4.1 Egg Inoculation

1. Obtain freshly fertilized specific pathogen-free (SPF) chicken eggs from a commercial vendor.
2. Immediately place the eggs in a humidified egg incubator at 37 °C with 55–60% humidity. Most incubators have an automatic egg turner. If yours does not have one, gently rotate the eggs several times each day.
3. Incubate the eggs for 7–8 days.
4. Candle the eggs to ensure that they are fertilized. Egg candlers can be purchased from poultry supply vendors or can be homemade (*see* **Note 17**) and consist of a bright light that is held against the egg for transillumination. Clean the candler with 70% ethanol to avoid contaminating the eggs. Remove the eggs from the incubator and place them in an empty egg carton. Turn off the lights; the room must be very dark. Hold the egg by the larger end and place it against the candle to transilluminate the contents. Fertilized eggs will have a thin network of blood vessels that lead to a pea-sized embryo. Unfertilized eggs will not have blood vessels, and a yolk will be readily apparent. Return the eggs to the incubator. Do not leave the eggs outside the incubator for more than 20 min.
5. Continue to incubate the eggs until Day 10 or 11.
6. Prepare virus for inoculation. Remove the virus from the freezer and thaw on ice. If the virus warms or sits for an extended period of time, it will degrade. Working in a BSL-2

biosafety hood, dilute virus stock to $10^3$–$10^4$ PFU/mL in sterile DPBS. The ideal volume for injection is 1 mL. Keep the diluted virus on ice at all times.

7. Inoculate the allantoic cavity of each egg as outlined in **steps 8–16**.
8. Candle each egg to ensure that the embryo is viable by checking for movement. Discard eggs with dead embryos.
9. Select three eggs and mark the margins of the air sac with a permanent marker. The air sac will be on the fat end of the egg and is readily identifiable as a translucent area when you are candling the egg.
10. Put the eggs in a container with the air sac up and place the container in a BSL-2 biosafety hood.
11. Clean the egg shell above the airspace with 70% ethanol.
12. Punch a small hole in the shell of each egg over the air sac. This can be achieved using a sterile 18-gauge needle. Some investigators prefer to use a high-speed rotary drill (such as a Dremel). Take care not to insert the needle too deeply to avoid damage to the yolk or embryo.
13. Draw up the diluted influenza virus in a sterile 1 mL syringe.
14. Attach a sterile 22-gauge 1 in. long needle to the syringe and advance the needle through the hole at a 45° angle into the allantoic cavity, the large fluid filled space just below the air sac.
15. Inject 0.2 mL of the virus into each egg.
16. Seal the holes in the eggs. This can be achieved using a small drop of glue from a glue gun, melted paraffin wax, or sterile Parafilm.
17. Place the eggs back in the incubator with the air sac pointing up.
18. Incubate the infected eggs for an additional 48 h.

#### *3.4.2 Influenza Virus Harvest*

1. Chill the eggs at 4 °C overnight, or for a minimum of 2 h. This kills the embryo, constricts the blood vessels, and prevents erythrocytes from absorbing the virus.
2. Transfer the eggs to a biosafety hood and wipe their shells with 70% ethanol.
3. Carefully open the shell around the air sac using sharp sterile scissors.
4. Holding the egg upright, gently open the allantoic membrane with sterile forceps.
5. Use a sterile spatula or small spoon to gently move the yolk to the side without rupturing it.
6. Gently aspirate the clear allantoic fluid using a sterile transfer pipette or sterile pipette tip. You may need to tip the egg slightly to the side to maximize the yield of fluid.

7. Place the fluid into a 50 mL conical tube. Place the conical tube in ice.
8. Pool the fluid from all three eggs.
9. Centrifuge the fluid at 1500 × *g* at 4 °C for 10–12 min.
10. Working in the hood, transfer the supernatant to a fresh 50 mL conical tube and then aliquot the fluid into sterile microfuge tubes.
11. Immediately snap freeze the fluid in liquid nitrogen.
12. Store aliquots of the virus at −80 °C for future use.

### 3.5 H1N1 Influenza A Plaque Assay

1. Make a solution of MDCK cells in cDMEM.
2. Seed 12-well plates with MDCK cells in cDMEM at a concentration of $2.5 \times 10^5$ cells/well. 16 wells will be needed in total.
3. Incubate at 37 °C in 5% $CO_2$ for 24 h.
4. Look at cells under a microscope. Cells are ready to be infected when they are 90–100% confluent.
5. Prepare serial virus dilutions ($10^{-1}$ to $10^{-8}$): Fill eight sterile microfuge tubes with 1080 μL of DMEM *without* FBS. Thaw virus stock on ice. Remove 120 μL of virus stock and add to the first tube. This has a dilution of $10^{-1}$. Mix the contents of the tube thoroughly by vortexing. Remove 120 μL and add to the next tube. This one will have a dilution of $10^{-2}$. Repeat steps until serial dilutions ($10^{-1}$ to $10^{-8}$) have been prepared for all tube.
6. Infect monolayers: Aspirate the media from each well. Wash twice with PBS. It is important to get all of the FBS-containing media out of the wells. Add 500 μL of each virus dilution to each well. Do this in duplicate for each dilution. Gently shake the plates back and forth to make sure the virus is evenly distributed in each well. Incubate monolayers at 37 °C in 5% $CO_2$ for 1 h.
7. Prepare agarose solution: Warm 2× DMEM to 37 °C. Melt 2% agarose in a microwave or using a hot plate. Do not overheat, just melt. Once liquefied maintain at 45 °C. Mix 2× DMEM and 2% agarose in 1:1 ratio in a sterile container. You will need a minimum of 8 mL. Cool DMEM-agarose mixture at room temperature for 5–10 min. Add TPCK-trypsin (final concentration: 1 μg/mL) to the DMEM-agarose mixture. Maintain at 37 °C until ready to use, but do not allow the solution to sit too long, since the activity of the TPCK-trypsin will wane over time.
8. Aspirate media from cells.
9. Rinse cells with DPBS.
10. Add 2 mL of the agarose-DMEM-trypsin mixture to each well.
11. Let plates sit for 15 min with the lids off to allow the agarose to solidify.

12. Turn plates upside down and return them to the incubator at 37 °C, 5% $CO_2$.
13. Check for plaques daily. They will be present after 48–72 h.
14. Once plaques appear, fix cells by adding 10% formalin on top of the agarose for 1–2 h at room temperature.
15. Aspirate formalin and agarose. Discard in accordance with institutional safety measures.
16. Wash plates under water.
17. Stain 5 min with crystal violet (0.5%).
18. Wash plates thoroughly to remove dye.
19. Allow plates to air dry.
20. Count plaques at the dilution with no less than five plaques/well. PFU/mL (of original stock) = # plaques/(dilution factor x ml of inoculum/well).

### 3.6 Infection with RSV or Influenza A

Virus should always be handled in a BSL2 biosafety cabinet, including when creating murine infections. Virus should be rapidly thawed immediately prior to use and stored on ice throughout the infection period.

1. Use the appropriate number of mice (8–10 weeks of age) per experimental and control groups (*see* **Notes 18** and **19**) for each experiment. BALB/c mice should be used for RSV infections. Any strain may be used for influenza A infection.
2. Prior to viral inoculation, weigh all mice to accurately determine inoculum required.
3. Lightly anesthetize the mice with 2% isoflurane in an induction chamber equipped with a charcoal filter. To avoid over- or under-sedation, it is recommended that no more than four mice be placed in the induction chamber at one time. Initially the mice will demonstrate rapid breathing. They are ready to inoculate when they establish a slowed, rhythmic breathing pattern (~6 min) (*see* **Note 20**).
4. Remove one mouse from the induction chamber at a time by grabbing the nape of the neck. Hold the mouse vertically, then slightly tip it back to a 20-degree angle, and deliver the calculated dose intranasally using a standard 100–200 μL pipette. Keep the mouse at the 20-degree angle until the entire inoculum is inhaled, and then return it to the cage. Monitor mice for 30 min to assure breathing and activity returns to normal.
5. For RSV infection, inoculate BALB/c mice with $5 \times 10^5$ PFU of RSV per gram of body weight (inoculum for an average 20 g mouse is $1 \times 10^7$ PFU) in 100 μL of MEM (*see* **Notes 15** and **21**).
6. For influenza A infection, use 50 PFU per mouse for BALB/c and C57BL/6 mice. The dose will need to be titrated to achieve the desired effect (*see* **Note 22**).

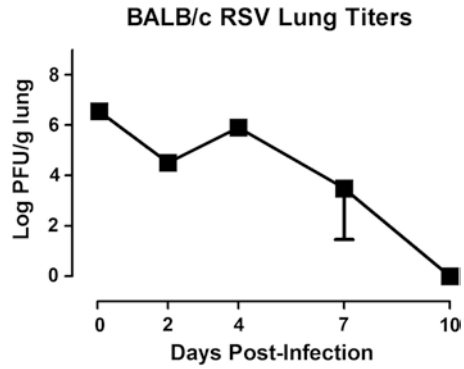


**Fig. 2** Viral kinetics in adult BALB/c mice. RSV Line 19 was delivered intranasally in 100 μL of vehicle. Viral lung titers were quantified by H&E plaque assay at indicated times postinfection

7. To confirm successful infection, monitor mice for weight loss and harvest left lungs to quantify viral lung titers as described in Subheading 3.7 on various days postinfection (Fig. 2).
8. Perform viral quantification by hematoxylin and eosin plaque assays as described in Subheadings 3.3 or 3.5. Expected findings at the following times postinfection for RSV are listed below. Findings for influenza A are generally similar but vary by mouse strain.
   (a) 1 h: Estimates viral titer delivered to the airway.
   (b) 2–4 days: Captures ongoing viral replication.
   (c) 4 days: Peak viral titers.
   (d) 5–7 days: Captures viral clearance.
   (e) 10 days: Below limit of detection.

### *3.7 Harvesting Lungs for Viral Plaque Assay*

1. Label two 1-dram glass 4 mL sample vials with rubber-lined lids per mouse and add 2 mL of 1× MEM to each vial. Weigh each vial before and after adding left lung lobes.
2. Animals are euthanized with pentobarbital at 250 mg/kg or 3× the anesthetic dose followed by cervical dislocation and exsanguination.
3. Collect and weigh harvested lungs, and then seal the cap with electrical tape. Snap freeze in the 2-propanol/dry ice bath for 5 min, and then place in −80 °C freezer.
4. Quick thaw lungs in 37 °C water bath when ready to process.
5. Use small, ceramic mortar and pestle that has been autoclaved and stored at 4 °C.
6. Add a small amount of sterile glass beads to the mortar.

7. When grinding lung with mortar and pestle, add both the lung and media from the glass vials.
8. After grinding, add contents to a 15 mL conical tube and spin at 8000 × *g* for 15 min at 4 °C.
9. After centrifuging, remove supernatant and return to original glass vials.
10. Proceed to Subheading 3.3 for RSV plaque assay or Subheading 3.5 for influenza A plaque assay. Fewer dilutions (3–4) will be required for viral lung titers than when titering stock virus. Thus, one 12-well plate should be prepared for each lung sample

## 4 Notes

1. Plaque purification is important for purifying a clonal population of virus or "master stock."
2. FBS is heat inactivated at 56 °C for 30 min to reduce the concentration of heat-labile complement proteins which contribute to the serum's hemolytic activity [15]. Though some studies have shown that heat inactivation does not impact in vitro studies with cultured lymphocytes [16]. Data published by Smith and colleagues [17] suggests that complement activation promotes the lysis of RSV-infected Hep-2 cells. Thus, heat inactivation of serum is recommended for growing RSV in Hep-2 cells.
3. Only add L-glutamine if it is not included in the base MEM media.
4. Vials must remain in the dry ice bath for no less than 5 min. A peak formed in the center and yellow color will indicate successful snap freezing of each vial. When vials are thawed, the color of the medium should be pink. If the medium is yellow after thawing, this usually indicates that ethanol contaminated the contents of the vial and that the vial should not be used for infection as the virus will not infect cells or mice.
5. With the surge of genetically modified mice on a C57BL/6 background, many investigators now are establishing RSV infection models using C57BL/6 mice. The key difference between the two mouse strains relevant to RSV host immunity is that BALB/c mice express a predominantly Th2 phenotype whereas C57BL/6 mice have a more balanced Th1/Th2 response.
6. In the adult mouse model of RSV infection, weight loss is used as a measure of illness. A bimodal weight loss pattern is typically observed at 2 and 6 days postinfection [18].
7. The use of early passage Hep-2 cells improves viral growth and produces higher viral titers.

8. If aggressive rocking of the trypsin over the cells does not elicit cell detachment within 1–2 min, placing at 37 °C may promote trypsin-mediated removal of adherent cells.
9. When the T-162 flask is at 80% confluence, cell counts are typically performed at a 1:10 dilution.
10. Hep-2 cells adhere to the plate quickly; thus, the shaking step must be done immediately after plating.
11. When preparing agarose, start by microwaving 1% agarose until completely melted (may boil), let cool on bench slightly, and then maintain at 55–60 °C in a water bath or dry oven. Make sure agarose has cooled enough so as not to kill the virus during the overlay as RSV is very temperature sensitive. If the agarose cools too much before addition to the plate, the agarose will solidify too early.
12. Agarose will solidify while in the incubator. When grown in agarose, syncytia look more rounded and clumpy, with little to no fusions compared to growing in a flask with medium alone.
13. To ensure optimal plaque isolation, circle and pick plaques from plates with less than 50 plaques.
14. To prepare for the viral harvest, print labels with ink that won't smudge in alcohol with date and place on glass dram vials. Precool supplies in refrigerator over the weekend (e.g., vials, sterile glass flasks, 10 mL pipettes, six 50 mL centrifuge tubes).
15. Various RSV infection controls have been reported in the literature, including the use of cell lysate [13], vehicle [19], PBS [20], or UV-inactivated virus [21]. In our hands, the use of cell lysate elicits early, subtle increases in macrophage MHC class II expression, whereas MEM (vehicle) does not. Depending on the question being asked in your model, UV-inactivated virus may serve as a more suitable control to determine disease pathology associated with live, replicating virus.
16. This step is critical for even distribution of the cells. If the cells are not distributed evenly throughout the well, areas of cell accumulation and overgrowth will occur. Hep-2 cells tend to accumulate in the center of the wells creating a characteristic "donut-hole" appearance and making viral purification and titers impossible (Fig. 3).
17. A candler can be constructed from a small LED flashlight (lamp size approximately 1.5 in. diameter), a small medicine cup, and electrical tape. Using a sharp blade, cut the bottom off the base of the medicine cup. Place the base of the cup over the lamp of the flashlight and cover it completely with electrical tape. The open end of the medicine cup should fit snugly over the end of the egg. Light should pass freely from the flashlight through the medicine cup and transilluminate the

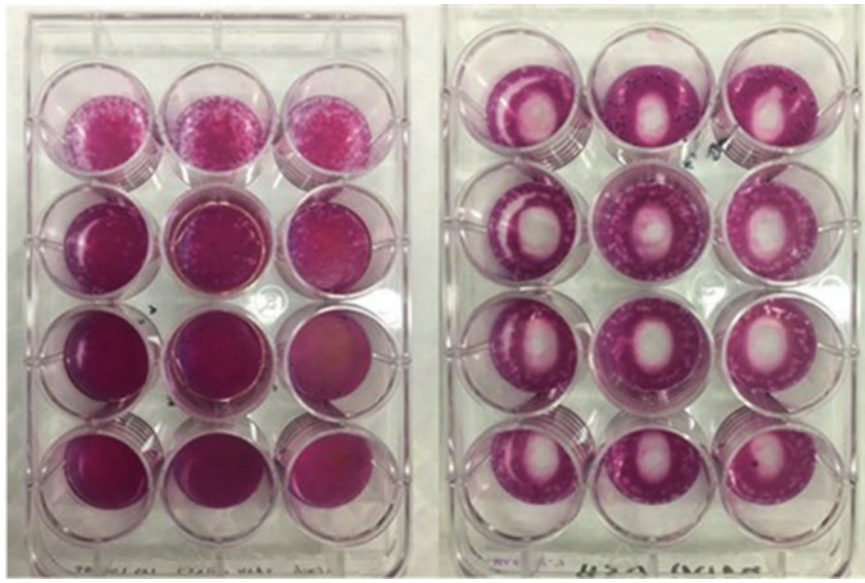

**Fig. 3** The effects of poorly dispersed Hep-2 cells on RSV Line 19 plaque formation. When Hep-2 cells are plated evenly, H&E staining will be uniform, and plaques will be countable; indicated here by plaque dots (left). If Hep-2 cells are overgrown in the middle of the well, they will stain with a "donut hole" or "crescent moon" making plaques impossible to count (right)

egg. If a medicine cup is not available, cardboard may be used but this is less sturdy and more difficult to keep clean.

18. BALB/c and C57BL/6 mice are both commonly used in mouse models of RSV infection. BALB/c mice display a more Th2-type cytokine response compared to C57BL/6 mice warranting their use in RSV-based studies. However, C57BL/6 mice have a wider variety of genetically modified mice, enabling more mechanistic-based questions.
19. Pilot experiments using at least five mice per group should be performed to determine the effect size of a particular intervention, allowing for a power analysis to be performed to determine the appropriate number of mice per group for future experiments.
20. Anesthesia: In order to deliver 2% isoflurane/oxygen, a vaporizer and induction chamber are required. Isoflurane induces a light level of sedation to overcome the animals' resistance to inhaling the inoculum and allows for a rapid recovery time. Alternatively, ketamine (50 mg/kg)/xylazine (9 mg/kg) delivered intramuscularly provides a deeper level of sedation than isoflurane and is good option if you do not have an induction chamber and vaporizer.
21. In adult mice (6–12 weeks of age), a variety of RSV viral strains, inoculum size, volumes, and routes of delivery are reported.

(a) Viral strain: The RSV A2, available through American Type Culture Collection (ATCC), is a commonly used viral strain for established murine models of RSV infection. The A2 strain has been used extensively in murine studies to model the immune response to infection, disease pathogenesis, and to establish critical vaccine parameters. Alternatively, RSV Line 19, originally isolated from an infant at the University of Michigan Hospital [22], has been used to establish murine models of severe RSV disease with significant mucus production and development of airway hyperresponsiveness [23]. Unlike RSV A2 or Long strains, RSV Line 19 elicits extensive IL-13 and mucin production, which are critical in assessing the role of RSV in allergic asthma [24].

(b) Viral inoculum: Reported viral inoculums range from $1 \times 10^5$ PFU to $1.5 \times 10^7$ PFU. The size of the viral inoculum will depend on the desired model. Despite reduced viral replication of RSV Line 19 compared to A2 in vivo, a dose-dependent increase in Gob5 and Muc5A expression along with increased IL-13 has been reported, suggesting the use of lower Line 19 inoculums in models designed to understand the role of RSV in allergic asthma [23]. Alternatively, higher doses may be warranted in models designed to assess disease pathology during acute primary infection.

(c) Route: Inhaled and intratracheal viral inoculums are the most common forms of delivery reported. Inhaled viral delivery is easy and quick and achieves viral inoculation in the nose and airway [9]. Intratracheal delivery may be used to deliver virus to the lower airway [25].

(d) Volume: The viral inoculum is most frequently delivered in 100 μL based on work published by Graham et al. [9] demonstrating more efficient delivery of 99m technetium sulfur colloid to the lungs upon inhalation of higher volumes of the inoculum.

22. The pathogenicity and lethality of H1N1 influenza A vary between mouse strains and may also vary with the sex of the animals. A dose of 50 PFU per mouse is a good starting dose; however, before proceeding with a full experiment, a pilot experiment should be performed to determine the optimal dose. Several doses, varied by two- to tenfold, may be tried. For studies of acute inflammation and lung injury, we chose a dose that induces a 10–15% drop in body weight. Weight loss is generally greatest 7 days postinfection. Weight loss greater than 20% is commonly premorbid.

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