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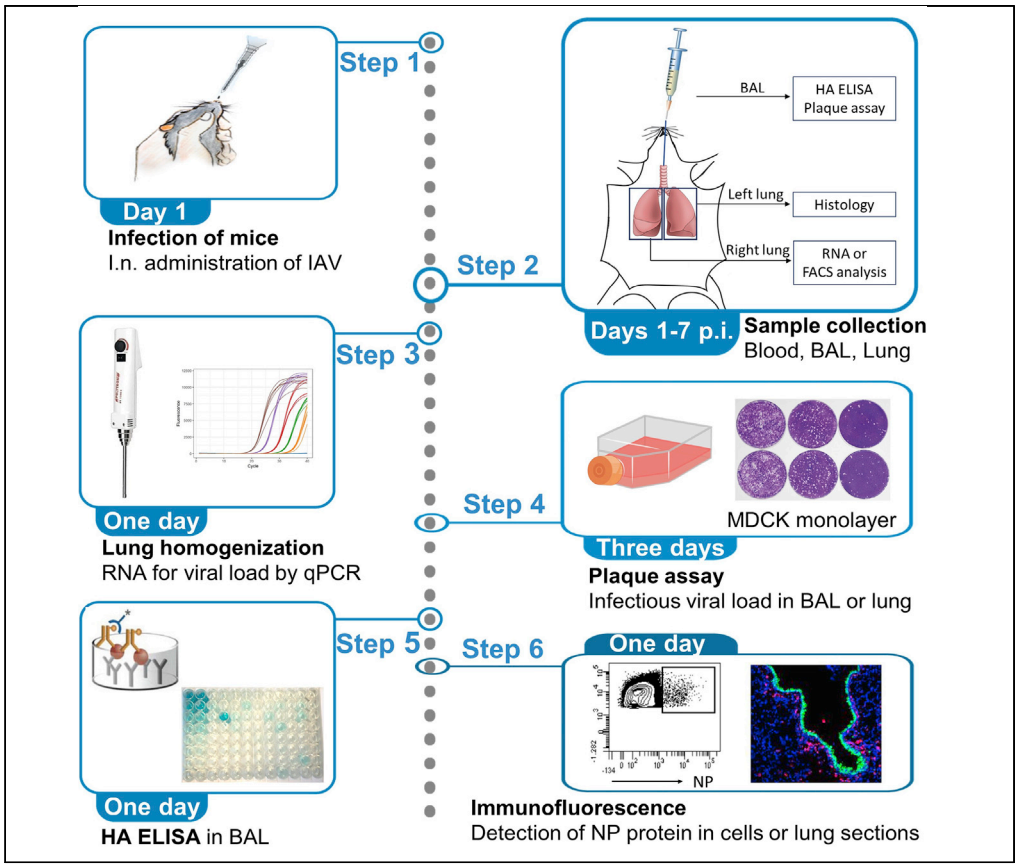
Protocol for influenza A virus infection of mice and viral load determination



Influenza A viruses (IAVs) are common respiratory viruses. Mouse models of IAV infection are valuable to study the mechanisms of IAV infection and pathology. Here, we present a detailed protocol for IAV infection of mice via intranasal administration. We detail the processing of mouse lung tissue and then describe the determination of viral load by several approaches including RNA, protein, or plaque-forming unit assays. This protocol may be adapted to other influenza strains or respiratory viruses.

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Highlights

Infection of mice by intranasal administration of IAV PR8 strain

Differential processing of lung tissue for molecular, protein, or histological assessment

Determination of viral load by RNA, protein, or plaque-forming unit assays

Analysis of virus localization by FACS analysis and histology

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Protocol for influenza A virus infection of mice and viral load determination

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SUMMARY

Influenza A viruses (IAVs) are common respiratory viruses. Mouse models of IAV infection are valuable to study the mechanisms of IAV infection and pathology. Here, we present a detailed protocol for IAV infection of mice via intranasal administration. We detail the processing of mouse lung tissue and then describe the determination of viral load by several approaches including RNA, protein, or plaque-forming unit assays. This protocol may be adapted to other influenza strains or respiratory viruses.

For complete details on the use and execution of this protocol, please refer to Galani et al. (2017).

BEFORE YOU BEGIN

Acquire appropriate licenses

Timing: up to several weeks

1. As this protocol describes work on experimental animals, appropriate licenses for animal experiments from institutional and regional ethical review boards should be obtained before performing experiments.
2. IAV is a biosafety level 2 pathogen and investigators may require institutional approvals before performing experiments.

Alternatives: The protocol below describes the specific steps for infecting wild-type (WT) C57BL/6 mice with the IAV strain A/PR/8/34. However, we have used the same pipeline for infecting several knockout mouse strains as well, and also with the Influenza strain A/WSN/33. Moreover, in this protocol, we have used commercially available purified Influenza A/PR/8/34 (H1N1) virus stock from Charles River. Alternatively, an in-house prepared virus stock may be used, as described elsewhere (Cottey et al., 2001; Eisfeld et al., 2014).

IAV dilution preparation for infection of mice

Timing: 30 min

Dilution	1:100	1:20,000 (LD50)	1:200,000 (0.1LD50)
IAV	1 µL stock	10 µL (1:100)	100 µL (1:20,000)
Saline	99 µL	1990 µL	900 µL

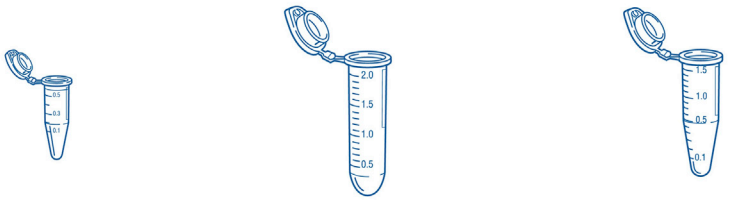

Figure 1. Schematic representation of the serial dilutions performed to reach desirable IAV doses LD50 and 0.1LD50

3. Thaw IAV stock on ice, and make aliquots of 10–20 µL in 200 µL tubes. Store aliquots at −80°C for up to several years.

   ⚠ CRITICAL: Avoid repeated freeze-thaw cycles of the aliquots to avoid loss of virus infectivity.

4. Make serial dilutions of IAV stock in saline to reach LD50 (lethal dose) and 0.1LD50 (sublethal dose).

   Note: Each IAV stock may vary in infectivity, so it always has to be titrated beforehand to determine optimal dose. An indicative dilution for Charles River's Influenza A/PR/8/34 (H1N1) purified virus stock is approximately 1:20,000 dilution for LD50 and 1:200,000 for 0.1LD50, corresponding to 100 pfu and 10 pfu of IAV, respectively, when infecting 6- to 8-week-old WT C57BL/6 mice. If an IAV stock from another source is used, a thorough titration by in vitro plaque assay and in vivo survival has to be performed to optimally determine the LD50 dose.

   a. Perform the following serial dilutions (Figure 1, as an example).

   Note: Make sure to vortex well when diluting a small volume in a bigger one.

Plate MDCK cells

Timing: 1 h

5. One day before performing the plaque assay, plate 4 × 10^5 MDCK cells/well in 1 mL/well MDCK cell growth medium in 12-well plates to form a confluent monolayer (~95% confluency). Incubate plates overnight (16–18 h) at 37°C, 5 % CO_2 incubator.

   Note: After thawing MDCK cells, you should passage them 2–3 times before using them for the plaque assay. It is not recommended to use MDCK cells that are kept for long in culture, e.g. over 7–8 passages, because it affects the uniformity of the monolayer. Usually, one confluent T75 cm^2 flask of MDCK cells will make 3–4 confluent 12-well plates the following day. Cells are harvested after treatment with 1× solution of Trypsin-EDTA (0.5%).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody (1:100)	BioLegend	Cat# 156604; RRID: AB_2783138
Mouse monoclonal anti-Influenza A NP, FITC-conjugated (clone D67J) (1:10–1:20)	ThermoFisher Scientific	Cat# MA1-7322; RRID: AB_1017747
Rat monoclonal anti-CD45, APC/Cy7 conjugated (clone 30-F11) (1:100)	BioLegend	Cat# 103116; RRID: AB_312981
Rat monoclonal anti-CD326 (EpCAM), PE/Cy7 conjugated (clone G8.8) (1:100)	BioLegend	Cat# 118216; RRID: AB_1236471
Bacterial and virus strains		
Influenza A/PR/8/34 (H1N1)	Charles River	Cat# 10100374
Chemicals, peptides, and recombinant proteins		
Sodium chloride injection, USP	Fresenius Kabi	Cat# C88B1
Atropine sulfate	Demo S.A.	n/a
Ketamine (Narketan)	Vetoquinol	Cat# Vm 08007/4090
Xylazine (Rompun)	Bayer AG	Cat# 85972596
Phosphate-Buffered Saline (PBS 1×), pH:7.4	ThermoFisher Scientific	Cat# 10010-015
Fetal bovine serum (FBS)	ThermoFisher Scientific	Cat# 10500
Paraformaldehyde (PFA) tablets 1 g	Merck	Cat# 104004
Tissue-Tek® O.C.T. Compound	Sakura	Cat# 4583
Sucrose	Sigma-Aldrich	Cat# S7903-1KG
Collagenase type IV from Clostridium histolyticum	Merck	Cat# C5138-1G
Red Blood Cell Lysing Buffer Hybri-Max™	Merck	Cat# R7757
TriReagent	Sigma-Aldrich	Cat# T9424
Chloroform	CARLO ERBA	Cat# 438603
2-propanol HPLC	LabChem	Cat# PROL-2GH-2K5
Ethanol absolute ≥99.8%	VWR	Cat# 20821.365
Agarose	Merck	Cat# A9539
UltraPure™ DNase/RNase-Free Distilled Water	ThermoFisher Scientific	Cat# 10977-035
RQ1 DNase	Promega	Cat# M6101
Random primers	ThermoFisher Scientific	Cat# 48190011
dNTP mix	Promega	Cat# U151B
M-MLV reverse transcriptase	Promega	Cat# M1701
RNaseOUT™ Recombinant Ribonuclease Inhibitor	ThermoFisher Scientific	Cat# 10777019
Trizma® base	Merck	Cat# T1503
Sodium Chloride (NaCl)	Lach-Ner	Cat# 30093-AP0
Bovine Serum Albumin (BSA)	Merck	Cat# A9647
Tween® 20	AppliChem	Cat# A4974
TMB Solution	ThermoFisher Scientific	Cat# 00-4201-56
Sulfuric acid (H_2SO_4)	Merck	Cat# 258105
DMEM, high glucose, pyruvate	ThermoFisher Scientific	Cat# 41966-029
Penicillin-Streptomycin (5,000 U/mL)	ThermoFisher Scientific	Cat# 15070-063
MEM Non-essential Amino Acid Solution (100×)	Merck	Cat# M7145
Trypsin-EDTA 0.5% (10×)	ThermoFisher Scientific	Cat# 15400-054
MEM 10×	ThermoFisher Scientific	Cat# 21430
Bovine Albumin Fraction V (7.5% solution)	ThermoFisher Scientific	Cat# 15260-037
L-glutamine (200mM)	ThermoFisher Scientific	Cat# 25030
Sodium Bicarbonate 7.5% solution	ThermoFisher Scientific	Cat# 25080
HEPES (1M)	ThermoFisher Scientific	Cat# 15630
DEAE-Dextran hydrochloride	Merck	Cat# D9885
Distilled water	ThermoFisher Scientific	Cat# 15230-001
Oxoid™ Purified Agar	ThermoFisher Scientific	Cat# LP0028B
Trypsin from bovine pancreas, TPCK treated	Merck	Cat# T1426

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Crystal violet solution	Merck	Cat# HT901
Trypan Blue solution	Merck	Cat# T8154
DAPI	Merck	Cat# 268298
Saponin from quillaja bark	Merck	Cat# S7900
ProLong™ diamond antifade mountant	ThermoFisher Scientific	Cat# P36961
Critical commercial assays		
RNeasy Micro kit	Qiagen	Cat# 74004
PrimeScript RT reagent Kit	Takara	Cat# RR037A
iTaq™ Universal SYBR Green Supermix	Bio-Rad	Cat# 172-5124
Influenza A H1N1 (A/Puerto Rico/8/1934) Hemagglutinin/HA ELISA Pair Set	Sino Biological	Cat# SEK11684
Experimental models: Cell lines		
Madin Darby Canine Kidney (MDCK) cells	Dr. Ross Walton, Imperial College London	ATCC: CCL-34; RRID: CVCL_0422
Experimental models: Organisms/strains		
Mouse: Male or female C57BL/6J 6–8 weeks old	The Jackson Laboratory	RRID: IMSR_JAX:000664
Oligonucleotides		
See qRT-PCR primers table	This paper	N/A
Software and algorithms		
LightCycler 96 application software	Roche	N/A
Prism version 8	GraphPad	N/A
FACSDiva™	BD Biosciences	N/A
Other		
Heating pad	Imetec	Type 73806
PCR tubes 0.2 mL	Capp	Cat# 5100200
PCR tubes 0.5 mL	Merck	Cat# GN682201
Microtubes 1.5 mL	Axygen	Cat# 311-08-051
Microtubes 2.0 mL	Axygen	Cat# MCT-200-C
Vortex	Labnet	Model: VX100
Refrigerated benchtop centrifuge	ThermoFisher Scientific	Model: Heraeus Biofuge Fresco
1 mL syringe with needle 27G	Pic Solution	Cat# 103601
Abbocath-T Catheter IV 16gx5-1/2"	Hospira	Cat# 1173782
−80°C freezer	Thermo Scientific Forma	Model: 706
Hot plate magnetic stirrer	Schott	Model: SLR
Surgical suture	Ethicon	Cat# W212
Polystyrene bijou containers	ThermoFisher Scientific	Cat# 11399133
10mL disposable syringe	BD	Cat# 307736
Needle, 70 × 30, 22GX1	B. Braun	Cat# 4657624
Scalpel	B. Braun	Cat# 5518091
Hybridization incubator	UVP	Model: HB-1000
100 µM cell strainer	Corning	Cat# 352360
Homogenizer	Kinematica	Model: Polytron PT1600 E
Block Heater	Wealtec	Model: HB-2
Spectrophotometer	Thermo Electron	Model: BioMate 3
LightCycler® 480 Multiwell Plate 96, white	Roche	Cat# 04729692001
LightCycler® 480 sealing foil	Roche	Cat# 04729757001
Real-time PCR machine	Roche	Model: LightCycler 96
Clear Flat-Bottom Immuno Nonsterile 96-Well Plates	ThermoFisher Scientific	Cat# 442404
Sealing tapes	ThermoFisher Scientific	Cat# 236366
Syringe filter 0.2 µm	Ahlstrom	Cat# 760548
96-well polystyrene microwell plates	ThermoFisher Scientific	Cat# 163320
Shaker for 4 multi-well plates	Kisker Biotech	Model: LD-45

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Absorbance microplate reader	BioTek	Model: Elx800
Class II Laminar Flow Biological Safety Cabinet	NuAire	Model: NU 437-400E
Steri Cycle CO2 Incubator	Thermo Scientific Forma	Model: 381
Heraeus Multifuge 3SR Plus	ThermoFisher Scientific	Cat# 75004371
Inverted microscope	Leica	Model: DM IRE2
Tissue culture treated flasks 75cm^2	Merck	Cat# C7106
Tissue culture 12-well plates, flat bottom	Merck	Cat# M8687
Polystyrene test tube with cell strainer cap	BD	Cat# 352235
Flow cytometer	BD	Model: ARIA III
Water bath	Memmert	Model: WB10
Cryotome	Leica	Model: CM1950
Polysine adhesion slides	Menzel Thermo Scientific	Cat# J2800AMNZ
Hydrophobic barrier PAP pen	Vector laboratories	Cat# H-4000
Confocal laser scanning microscope	Leica	Model: TCS SP5

MATERIALS AND EQUIPMENT

Anesthesia mix 1

Reagent	Final concentration	Amount
Ketamine (100 mg/mL)	20 mg/mL	0.3 mL
Xylazine (20 mg/mL)	1.3 mg/mL	0.1 mL
Atropine sulfate (1 mg/mL)	0.06 mg/mL	0.1 mL
dH_2O	n/a	1.0 mL
Total	**n/a**	**1.5 mL**

Always prepare and use fresh.

Note: As ketamine/xylazine often require veterinary approval before use, make sure to obtain one before performing the experiment.

Anesthesia mix 2

Reagent	Final concentration	Amount
Ketamine (100 mg/mL)	20 mg/mL	0.3 mL
Xylazine (20 mg/mL)	1.3 mg/mL	0.1 mL
dH_2O	n/a	1.1 mL
Total	**n/a**	**1.5 mL**

Always prepare and use fresh.

4% PFA solution

To make 100 mL of PFA 4% w/v, in a glass beaker placed on a heating magnetic stirrer set at 60°C, add 4 tablets of 1 g PFA in 86 mL ddH_2O, and additionally 2 drops of NaOH 10N. Stir until the tablets completely dissolve. During that time, the beaker should be covered with plastic wrap, in order to avoid evaporation, and aluminum foil, as PFA is light sensitive. Let the solution cool and add 10 mL 10X PBS. Adjust pH to 7.4, and add ddH_2O up to 100 mL. If the solution is not clear, filter with Whatman #4 filter paper. After preparation, 4% PFA solution should be aliquoted in 1–2 mL aliquots, and can be kept frozen at −20°C for up to one year.

⚠ CRITICAL: Prepare the stock in a fume hood.

Collagenase IV stock

To make 50 mg/mL collagenase IV stock solution, dissolve 1 g collagenase IV in 20 mL PBS. Store at −20°C for at least one year in aliquots.

Note: You should always check the enzymatic activity of collagenase, as it may vary by the Lot #.

Digest solution

Prepare 3 mL of digest solution for one mouse by adding 30 µL collagenase IV stock (final concentration 0.5 mg/mL) into 3 mL PBS supplemented with 1% FBS. Always prepare fresh.

FACS buffer

Reagent	Final concentration	Amount
PBS 1×	n/a	986 mL
FBS	1 %	10 mL
EDTA (500 mM)	2 mM	4 mL
Total	**n/a**	**1000 mL**

Store at 4°C for up to 1 month.

DNase treatment mix

Reagent	Final concentration	Amount
RQ1 DNase 10× Reaction Buffer	1×	1 µL
RQ1 RNase-Free DNase (1000 Units/mL)	1 U/µg	2 µL
RNA (in RNase-/DNase-free ddH_2O)	0.2 µg/ µL	2 µg in volume up to 7 µL
RNase-/DNase-free ddH_2O	n/a	Add to the final volume up to 10 µL
Total	**n/a**	**10 µL**

Always prepare and use fresh.

Reverse transcription mix

Reagent	Final concentration	Amount
M-MLV Reverse Transcriptase 5× Reaction Buffer	1×	5 µL
dNTP mix (10 mM)	2 mM	5 µL
RNaseOUT (100 mM)	4 mM	1 µL
M-MLV Reverse Transcriptase (200 u/µL)	8 u/µL	1 µL
RNA solution	n/a	12 µL
RNase-/DNase-free ddH_2O	n/a	1 µL
Total	**n/a**	**25 µL**

Always prepare and use fresh.

qPCR mix

Reagent	Final concentration	Amount
iTaq™ Universal SYBR Green Supermix 2×	1×	10 µL
Primer Forward (100 pmol/µL)	2.5 pmol/µL	0.5 µL
Primer Reverse (100 pmol/µL)	2.5 pmol/µL	0.5 µL
cDNA dilution	10 ng/reaction	4 µL
RNase-/DNase-free ddH_2O	n/a	5 µL
Total	**n/a**	**20 µL**

qRT-PCR primers

Gene	Primer	Sequence (5′-3′)
Gapdh	Fwd	AGGTGGTCTCCTCTGACTTC
	Rev	CTGTTGCTGTAGCCAAATTCG
Ppia	Fwd	CGCTTGCTGCAGCCATGGTC
	Rev	CAGCTCGAAGGAGACGCGGC
PR8 *NS1*	Fwd	TGTCAAGCTTTCAGGTAGATTG
	Rev	CTCTTAGGGATTTCTGATCTC

qRT-PCR reaction

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Denaturation	95°C	15 s	40 cycles
Annealing	59°C	40 s	
Plate read	n/a	n/a	
Final extension	59°C	10 min	1
Melting curve	55°C → 95°C	0.5 s	

Alternatives: This protocol describes the qPCR procedure using a Roche LightCycler 96 Real-Time PCR Detection system and corresponding reagents, in 20 μl reaction volumes. Any other qPCR machine can be used as well.

HA ELISA capture antibody

Aliquot upon arrival and store at −80°C for several months.

Tris-buffered saline (TBS) solution

Reagent	Final concentration	Amount
Trizma Base	20 mM	2.4228 g
NaCl	150 mM	8.766 g
1M HCl	pH 7.4	As much required
dH_2O	n/a	Up to 1 L
Total	**n/a**	**1 L**

Store at room temperature (15°C–25°C) for several months.

HA ELISA wash buffer

To make HA ELISA wash buffer (0.05 % Tween 20 in TBS, pH 7.2–7.4), add 0.5 mL of Tween 20 to 1 L of TBS and mix gently not to create foam. HA ELISA wash buffer can be stored at RT for several months.

Alternatives: Instead of TBS, PBS can be used.

HA ELISA blocking buffer

To make HA ELISA blocking buffer (2% BSA in wash buffer), dissolve 2 g of BSA in 100 mL wash buffer. HA ELISA blocking buffer can be stored at 4°C for up to one month.

HA ELISA sample dilution buffer

To make HA ELISA sample dilution buffer (0.1 % BSA in wash buffer, pH 7.2–7.4), dissolve 0.1 g of BSA in 100 mL wash buffer or dilute the blocking buffer 20 times. Filter with 0.2 μm filter. HA ELISA sample dilution buffer can be stored at 4°C for up to one month.

HA ELISA detection antibody dilution buffer

To make detection antibody dilution buffer (0.5 % BSA in wash buffer, pH 7.2–7.4), dissolve 0.5 g of BSA in 100 mL wash buffer or dilute the blocking buffer 4 times. Filter with 0.2 μm filter. HA ELISA detection antibody dilution buffer can be stored at 4°C for up to one month.

HA ELISA standard

Each vial contains 120 ng of lyophilized recombinant H1N1 (A/Puerto Rico/8/1934) HA. Reconstitute with 1 mL detection antibody dilution buffer. After reconstitution, store in aliquots at −80°C for up to several months.

HA ELISA stop solution

To make HA ELISA stop solution (2 N H_2SO_4), add 27.6 mL of condensed H_2SO_4 in 472.4 mL dH_2O to have 500 mL of 2 N final concentration. HA stop solution can be stored at RT for several years.

Note: Caution is required in its use as this is caustic.

MDCK cell growth medium

Reagent	Final concentration	Amount
DMEM	n/a	440 mL
FBS	10%	50 mL
Pen/strep (5,000 U/mL)	50 U/mL	5 mL
Non-essential amino acids (100×)	1×	5 mL
Store at 4°C for up to 2 months.		

MDCK infection medium

Reagent	Final concentration	Amount
DMEM	n/a	98 mL
Pen/strep (5,000 U/mL)	50 U/mL	1 mL
Non-essential amino acids (100×)	1×	1 mL
Store at 4°C for up to 2 months.		

1% dextran DEAE

Dilute 0.5 g of dextran in 50 mL dH_2O, filter through 0.2 μm filter.

Overlay medium

Reagent	Final concentration	Amount
MEM 10 X	0.7 X	50 mL
BSA fraction V (7.5%)	0.3 %	14 mL
L-glutamine (200 mM)	2.8 mM	5 mL
Sodium Bicarbonate (7.5%)	0.2 %	10 mL
1M HEPES	14.3 mM	5 mL

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Reagent	Final concentration	Amount
DEAE-Dextran (1%)	0.007 %	2.5 mL
Pen/strep (5,000 U/mL)	143 U/mL	10 mL
dH_2O	n/a	253.5 mL
Total	n/a	350 mL

Agar

Dilute 2 g of agar in 100 mL dH_2O to make 2 % w/v agar.

Crystal violet stain

Dilute 1% crystal violet in 20% methanol and dH_2O. Crystal violet solution can be stored at RT for several months.

Note: As crystal violet stain contains methanol, its waste should be collected and disposed of by licensed waste solvent disposal company.

Saponin

Dilute 1 g of saponin in 10 mL dH_2O to make 10 % w/v saponin, and sterile filter. This solution can be stored in 4°C. To make the working solution, dilute further with FACS buffer to a final concentration of 0.5 % w/v saponin. Always prepare the working solution fresh.

STEP-BY-STEP METHOD DETAILS

Infection of mice with IAV

Timing: 1 h

This step describes how to anaesthetize mice and infect them with IAV PR8.

1. Prepare a heating pad with small inclination to place mice while under anesthesia.
2. Weigh the mice.
3. Anaesthetize mice by intraperitoneal (i.p.) injection of 0.05 mL/10 g bodyweight of anesthesia mix 1.

Alternatives: Instead of using ketamine/xylazine anesthesia, isoflurane anesthesia may be used. However, as mice wake up faster with isoflurane anesthesia, mice need to be handled individually, as opposed to ketamine/xylazine anesthesia where several mice can be anesthetized simultaneously.

4. Holding the anaesthetized mice in an upright position, pipette 40 µL of the IAV dilution.
   a. Pipette the solution in a slow continuous steady movement and only in one nostril (Figure 2).

⚠ CRITICAL: As mice are obligate nasal breathers, it is important to administer the inoculum only in one nostril, in order not to block breathing. The pipette tip should not touch the mouse nostril, but rather the inoculum should be delivered at the opening of the nostril, making sure that it is properly inhaled.

   b. Immediately after administration, pinch the footpad so that the mouse takes a deep breath. This allows the viral solution to reach the lower respiratory system.

5. Place the mouse on the heating pad until it wakes up.
   a. Periodically check breathing and reflexes of the mouse.

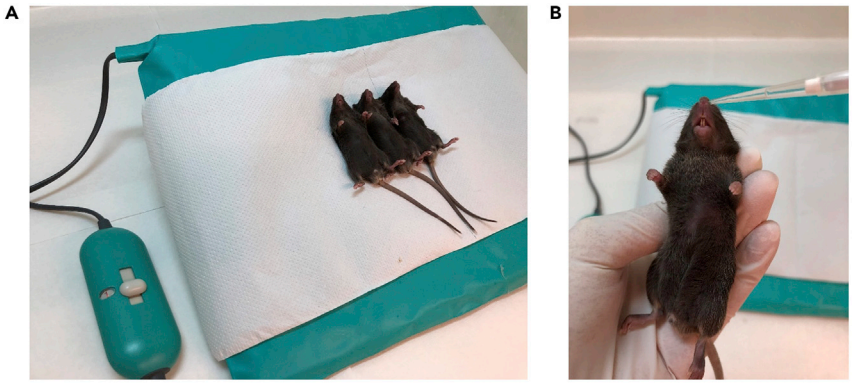

Figure 2. Intranasal administration of IAV in anesthetized mice
(A) Mice are placed on a heating pad with small inclination while under anesthesia.
(B) The viral solution is pipetted in a steady movement and only in one nostril, while holding the mouse in an upright position.

b. Place the mouse back in the cage only after it is able to flip from supine position and move its hind legs.

Note: If the anesthesia lasts longer than 5 min, you should apply an ophthalmic ointment to the eyes.

Sample collection

Timing: 1–2 h

This step describes how to terminally anaesthetize mice, and collect the samples at a desirable time-point post infection (p.i.).

Note: Peak of viral load is detected on days 3–4 p.i. for LD50, and on days 4–5 p.i for 0.1LD50.

6. Terminally anesthetize mice by i.p. injection of 0.1 mL/10 g of anesthesia mix 2.

⚠ CRITICAL: It is important to avoid using an inhaled anesthesia, such as isofluorane or CO_2, as it may influence the bronchoalveolar lavage (BAL) fluid content.

7. Collect blood by retro-orbital bleeding to a 1.5 mL tube.

Note: It is important to use non-heparinized capillary tubes for the bleeding, in order to allow the blood to clot in allotted time. Alternatively, sterile Pasteur pipettes may be used instead.

a. Allow the blood to clot by leaving it undisturbed at room temperature (RT) for 15 min.
b. Centrifuge at ~ 8,500×*g* for 10 min in a table centrifuge at 20°C.
c. Collect the serum into clean 0.5 mL tubes in aliquots.

Pause point: The sera can be stored at −80°C for at least several months before performing viral HA ELISA.

⚠ CRITICAL: It is important to avoid freeze-thaw cycles of aliquoted sera samples to avoid degradation of analytes.

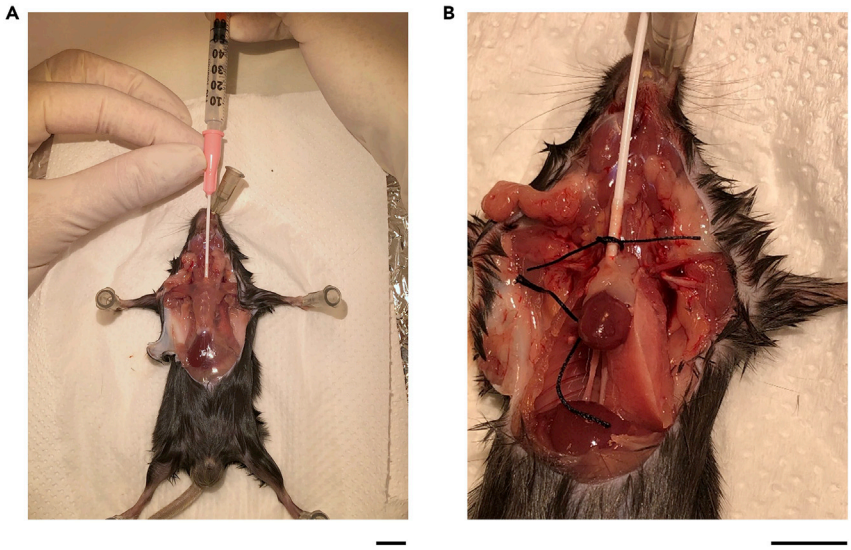

Figure 3. Collection of samples in euthanized mice

(A) BAL collection with catheter.

(B) Inflation of left lung for histology. The scale bars represent 1 cm.

8. Dissect the mouse.
   a. Place the animal on its back on a surgical plate and fix it by pinning down the limbs.
   b. Spray the skin with 70% ethanol to disinfect, and dissect the skin.
   c. Expose the trachea by removing the salivary glands and the sternohyoid muscle.
   d. Make a small incision in the trachea with scissors.

⚠ CRITICAL: It is important to be careful not to cut off the trachea completely, otherwise BAL will not be feasible.

9. Perform BAL.
   a. Load 500 μL of PBS in a 1 mL syringe with detachable needle.
   b. Assemble a catheter with the syringe (Figure 3A).
   c. Insert the catheter into the trachea and gently inject the PBS.
   d. Gently aspirate the solution back to the syringe.
   e. Transfer the recovered BAL fluid into a 1.5 mL tube placed on ice.
   f. Repeat the procedure once again. Averagely, 800 μL of BAL fluid is recovered from 1 mL of injected PBS.

⚠ CRITICAL: Be careful that the catheter is not inserted too far down into the trachea, as it may lead to damage of the lung structure.

⚠ CRITICAL: If no fluid is aspirated back in the syringe, try to carefully move the catheter a little bit up and down into the trachea.

10. Separate the cellular and noncellular components of the BAL fluid.
    a. Centrifuge at ~ 230×*g* for 10 min in a table centrifuge at 4°C.
    b. Collect the BAL supernatant (S/N) into clean 0.5 mL tubes in aliquots.

⚠ CRITICAL: The BAL S/N should be used immediately fresh for viral determination by plaque assay.

Pause point: The BAL S/N can be stored at −80°C for at least several months before performing viral HA ELISA.

c. Resuspend the cell pellet in 0.5 mL of PBS + 1% FBS, and use it for downstream applications (determination of inflammatory cell influx in the lung, FACS analysis and determination of infectious viral load in sorted cells by plaque assay).

11. Dissect the right lobe for RNA or viral load assessment by plaque assay.
    a. After performing the BAL, make an incision in the thorax to make the lung visible.
    b. Make a tight surgical knot around the right main airway, and remove the right lobe of the lung, leaving the right airway tightly sealed.
    c. Transfer immediately the right lobe in a 2 mL tube and snap-freeze in liquid N_2.

Pause point: The frozen lung tissues can be stored at −80°C for at least several months before performing RNA isolation as stated in the next section.

12. Dissect the left lobe for histology (Figure 3B).
    a. With a piece of suture, make a loose surgical node around the trachea.
    b. Load 350 μL of 4% PFA/Optimal Cutting Temperature (OCT) embedding medium mix in a 2:1 ratio in a 1 mL syringe with detachable needle.
    c. Assemble a catheter with the syringe.
    d. Insert the catheter into the trachea and the left main airway, and gently inject the mix to inflate.
    e. Remove the catheter, while tightening the surgical node around the trachea, until removing completely the catheter.
    f. Dissect the left lung together with the trachea and place in 7 mL polystyrene bijou bottle filled with 5 mL of 4% PFA. Leave O/N at 4°C.

Note: As residual air may cause the lung to float, hook the suture as you close the lid of the bijou, and place the bijou upside-down. In this way, the lung will be completely submerged in PFA.

    g. The next day rinse the tissues briefly in PBS and place in a fresh 7 mL polystyrene bijou bottle filled with 5 mL of 30% sucrose in PBS. Leave O/N at 4°C.

Note: Sucrose is necessary for cryopreservation.

    h. The next day tissues can be embedded in OCT medium to perform cryosectioning.

Pause point: The OCT-embedded frozen lung tissues can be stored at −80°C for at least several months before performing cryosectioning.

Note: Tissues may be stored in 30% sucrose solution for several days, before being embedded in OCT.

13. Alternative to steps 11 and 12, the lung may be used for FACS analysis.
    a. Perfuse the lung with 10 mL PBS administered via the right heart ventricle.
    b. Remove the lung and cut in small pieces with a scalpel.
    c. Transfer in 3 mL digest solution, and place in a rotator set at 37°C for 1 h. Perform occasional trituration with a 1 mL pipette.
    d. At the end of the incubation, pass through a 100 μm cell strainer, and wash with FACS buffer.
    e. Centrifuge at 200×*g* for 10 min at 4°C.

f. Resuspend in FACS buffer, and use single cell suspension for FACS analysis. Keep cells on ice until staining.

Note: Although perfusion of the lung has been performed, residual red blood cells may remain. In this case red blood cell lysis may be performed in the cell pellet with the addition of 1 mL red blood cell lysing buffer and incubation for 15 min on ice. At the end of the incubation, cells are washed with 20 mL of FACS buffer, followed by a centrifugation at 200×*g* for 10 min at 4°C. Cells are subsequently resuspended in FACS buffer.

Viral RNA determination

Timing: 1 day

This step describes how to isolate RNA from the lung, and determine RNA viral load.

14. Homogenize frozen lungs collected in step 11.
    a. Add 1 mL of TriReagent in the frozen lung.
    b. Immediately homogenize with a mechanical homogenizer for ∼10 s in maximum speed (30,000 rpm).
    c. Centrifuge homogenized lung samples at ∼ 10,000×*g* for 10 min at 4°C.
    d. Transfer the S/N to new 1.5 mL tube.

⚠ CRITICAL: Make sure that the homogenizer is autoclaved before use. If more than one sample is analyzed, wash the homogenizer with DEPC-treated dH_2O between samples.

15. Perform phase separation.
    a. Add 200 μL of chloroform and shake vigorously by hand for 15 s.
    b. Incubate for 2–3 min at RT.
    c. Centrifuge at ∼ 10,000×*g* for 15 min at 4°C.
    d. Collect the upper transparent phase and transfer into a fresh 1.5 mL tube.
    e. Add 500 μL of isopropanol to precipitate the RNA, and incubate at RT for 10 min.
    f. Centrifuge at ∼ 10,000×*g* for 10 min at 4°C.
    g. Remove the S/N and dissolve the pellet in 1 mL 75% EtOH.
    h. Mix by hand and centrifuge at ∼ 3,300×*g* for 5 min at 4°C.
    i. Remove completely the S/N.
    j. Air-dry RNA pellets.
    k. Dissolve RNA in 50 μL of RNase DNase-free ddH_2O.
    l. Incubate at 57°C in a heat block for 10 min.
    m. Store RNA samples at −80°C until use.
16. Perform quantitative and qualitative control of RNA.
    a. Measure O.D. (1:50) on a spectrophotometer at 260 nm to calculate concentration.
    b. Run 1 μg of RNA in 1.5% agarose gel to monitor RNA integrity.

Note: Intact RNA appears as two sharp bands corresponding to the 28S and 18S rRNA subunits in 2:1 ratio. Degraded RNA will appear as a smear in lower molecular weights.

17. Perform cDNA synthesis.
    a. Treat 2 μg of RNA with RQ1 DNase.
    b. Incubate at 37°C for 30 min.
    c. Stop the reaction with 1 μL RQ1 Stop Solution at 65°C for 10 min.
    d. Add 1 μL of random primers at 70°C for 5 min.
    e. Perform reverse transcription with M-MLV reverse transcriptase in the presence of RNaseOUT at 37°C for 60 min.

Table 1. Estimated guide for sample dilution depending on the initial viral dose used and the day p.i. of analysis.

	Day 1 p.i.	Day 2 p.i.	Day 3 p.i.	Day 4 p.i.	Day 5 p.i.	Day 7 p.i.
0.1LD50	1:2	1:2	1:40	1:40	1:40	1:40
LD50	1:2	1:50	1:100	1:100	1:100	1:50

18. Determine viral load by real-time qPCR analysis.
    a. Prepare qPCR master mix, use primers for IAV PR8 *NS1* gene.
    b. Run reaction at 59°C for 40 cycles.
    c. Calculate relative amounts of NS1 RNA expression normalized to *Gapdh* or *Ppia* according to the ΔΔCT method (Livak and Schmittgen, 2001).

Viral HA protein determination

Timing: 1 day

This step describes how to detect hemagglutinin (HA), one of the major glycoproteins found on the surface of IAV, in the BAL, with the use of a commercially available sandwich ELISA pair set.

⚠ CRITICAL: Bring all buffers to RT before use.

19. Coat a plate with capture antibody.
    a. Dilute capture antibody (stock concentration: 1 mg/mL) to working concentration (2 μg/mL) in PBS.
    b. Coat a 96-well microplate with 100 μL per well of the diluted capture antibody.
    c. Seal the plate and incubate overnight (16–18 h) at 4°C.
20. Wash the plate.
    a. Wash the plate with 300 μL wash buffer per well. Three washes of the plate are recommended.
    b. After the last wash, remove any remaining wash buffer by inverting the plate and blotting it against clean and dry paper towels.
21. Block unspecific binding.
    a. Add 300 μL of blocking buffer to each well.
    b. Incubate at RT (on a plate shaker) for 1 h.
    c. While blocking takes place, prepare the sample dilutions and the standard.
22. Prepare the sample dilutions.
    a. Use U-bottom 96 well plates to perform the dilutions. See Table 1 for estimated sample dilutions.

⚠ CRITICAL: Serial dilutions should be performed to achieve consistency. For example, in order to have a final dilution of 1:100, perform two dilutions of 1:10 each.

23. Prepare the HA standard by serial 2-fold dilutions.
    a. Prepare 7 tubes, one for each dilution of the standard curve.
    b. Add 500 μL of sample dilution buffer to six of the tubes.
    c. Calculate the volume of the stock standard needed to have a starting concentration of 3000 pg/mL in 1000 μL of sample dilution buffer and mix well.
    d. Transfer 500 μL of the prepared working standard to the second tube to have a concentration of 1500 pg/mL, mix well by pipetting and continue the same procedure until the lowest recommended concentration (46,875 pg/mL) is achieved.
24. Repeat wash step #20 after blocking.
25. Pipette 100 μL per well of samples or standards. Seal the plate and incubate for 2 h at RT on a plate shaker.

26. 10 min before the end of the incubation, prepare the detection antibody.
    a. Dilute detection antibody (stock concentration: 0.2 mg/mL) to working concentration (0.5 μg/mL) in HA ELISA detection antibody dilution buffer.
27. Repeat wash step #20 after incubation.
28. Pipette 100 μL of the detection antibody to each well. Seal the plate and incubate for 1 h at RT on a plate shaker.
29. Repeat wash step #20.
30. Pipette 200 μL of substrate solution to each well. Incubate for ~20 min at RT, by monitoring the development of blue color.

⚠ CRITICAL: Avoid placing the plate in direct light.

31. At the end of the incubation, pipette 50 μL of stop solution to each well. Gently tap the plate to ensure thorough mixing.

Note: If the blue color has not been sufficiently developed at the end of the 20 min incubation with the substrate solution, the incubation time may be prolonged. You should however balance that to the development of blue color in the zero standard (blank control) wells.

32. Measure the OD immediately in a microplate reader set to 450 nm.
    a. Calculate the mean absorbance for each set of duplicate standards and samples. Subtract the mean zero standard absorbance from each.
    b. To create the standard curve, use the mean absorbance for each one of the seven standards at the y-axis and their concentration at the x-axis and construct a curve through the seven points of the graph.
    c. Use the absorbance of the unknown samples to calculate the corresponding concentration through the standard curve. If samples are diluted, the concentration must be multiplied by the dilution factor.

Note: The procedure described here is in accordance to the latest protocol provided by the manufacturer. During our experiments we used more than one kit that had different specifications regarding, for example, the storage of the various kit components or the concentrations of the antibodies. So, make sure to always read carefully the manufacturer instructions (www.sinobiological.com/pair-set/hemagglutinin-ha-sek11684).

Infectious viral load assessment by plaque assay

Timing: 3 days

This step describes how to determine infectious virus in the BAL, based on the susceptibility of the established canine kidney cell line MDCK to IAV infection.

33. Aspirate the medium of the 12-well plates with MDCK cells from step #5 in the 'before you begin' section, wash the monolayers twice with PBS and leave the cells in PBS until required.
34. Prepare serial dilutions of the infectious samples (BAL) in 1.5 mL tubes using MDCK infection medium as follows:
    a. Add 405 μL of medium in each tube.
    b. Add 45 μL of sample to the first tube and vortex well.
    c. Perform 10-fold serial dilutions throughout all tubes. The tubes will have the following effective dilutions of virus: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, etc.
    d. The final volume of inoculum used for the infection is 200 μL per well.
35. After completing the serial dilutions, remove the PBS from the cells and add 200 μL of inoculum in each well.

Note: When performing the assay for the first time, it is essential to test the undiluted sample as well. Following infection with 0.1LD50, plaques can hardly be detected at days 1–3 p.i. in the undiluted samples, whereas at day 5 p.i viral titers are usually determined at the 10^{-2} dilution. On the other hand, after LD50 viral titers can be determined at dilutions 10^{-2} and 10^{-4} at days 3 and 5 p.i., respectively.

⚠ CRITICAL: It is essential to plate samples at least in duplicates and in every assay to use an uninfected control sample (MDCK infection medium) to independently ensure cellular viability as well as a positive control (Influenza A virus).

36. Infect the cells for 1 h at 37°C. Gently rock the plates every 20 min to ensure even coverage and prevent cells from drying out.
37. During this incubation time, prepare the overlay medium. Estimate that for each 12-well plate, 9.1 mL of overlay medium is needed. Aliquot overlay medium in 15 mL tubes and leave them in a 37°C incubator. Prepare the solution of 2 % agar in dH_2O by melting it in a microwave oven for about 2 min and cooling it to 65°C in a water bath until used.
38. After the 1 h of infection, remove the inoculums from the 12-well plates. Mix 3.9 mL of agar solution with the pre-warmed overlay medium and add 2 μg/mL of TPCK-treated trypsin. Gently add 1 mL of the agar overlay medium to each well.

⚠ CRITICAL: It is crucial to test and optimize the amount of trypsin used because the activity of each batch may vary.

Note: TPCK-treated trypsin is added in the overlay medium to allow the cleavage of the IAV hemagglutinin, facilitating thus the viral binding to sialic acid residues, and the subsequent viral entry.

Alternatives: Low-viscosity overlay media, such as Avicel, may be used instead of agar (Matrosovich et al., 2006).

39. Allow agar overlay to dry for 10 min in the laminar flow hood and place the plates upside-down in the incubator at 37°C, 5 % CO_2 for 3 days to enable plaques to form.

Note: Plaque formation will not be affected if the plate is placed in an upright position in the incubator. However, in our experience, this leads to humidity formation on the agar that affects the following steps of plaque visualization.

40. Visualize plaques.

Note: Plaques will be visible from day 2 post plating, mainly in the undiluted samples, but it is recommended to allow their development until 3 days post plating.

a. Count the plaques with naked eye by viewing the plate under a light source.
b. Carefully remove agar from the plates, trying not to disturb the cells, using a small spatula.
c. Fix and stain cells by adding 1 mL of crystal violet stain/well for at least 10 min.
d. Gently wash off the stain with running water. The monolayer will appear purple, while plaques will appear as clear areas.
e. Leave the plates to air-dry at RT and recount the number of plaques.

Note: Once fixed, stained and dried, plates can be stored indefinitely for future analysis.

41. Determine viral titers.

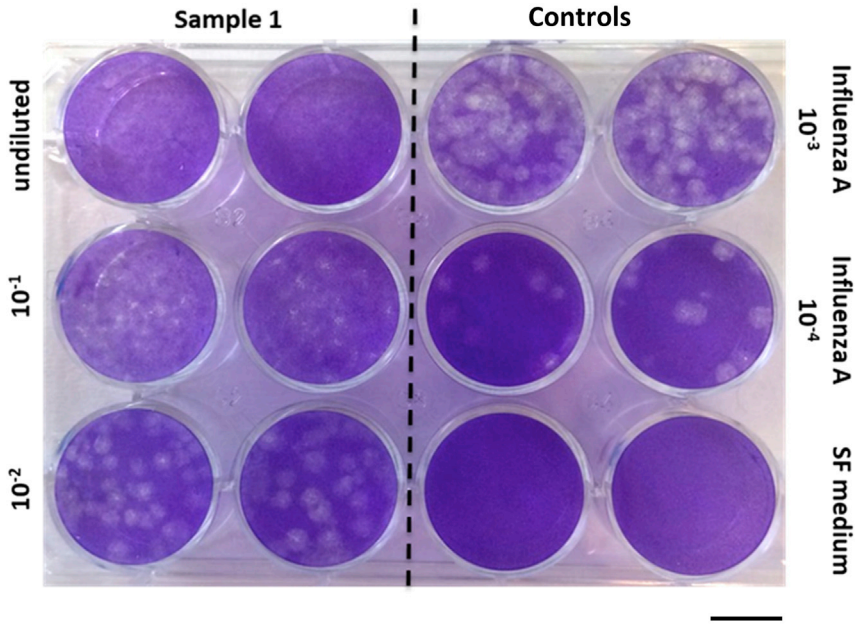

Figure 4. Representative plaques visualized by crystal violet staining

In this example, 40 and 45 plaques were counted for replicates of the 10^{-2} dilution of Sample 1. Based on the above equation, 42.5 (average)/10^{-2} (dilution) * 0.2 mL (inoculum) would yield a titer of 21.25*10^3 pfu/mL. Influenza A dilutions (10^{-3}, 10^{-4}) are used as positive control for plaque formation and serum-free (SF) medium as negative control. The scale bar represents 1 cm.

a. Count the plaques in each well and calculate the average of every dilution. The negative control should have a uniform monolayer (Figure 4).

Note: Ideally count the wells with 10–100 plaques.

b. Determine the viral titer of the initial viral suspension according to the following formula: Pfu/mL = (average number of plaques/$10^{-\text{dilution}}$) * (1mL/volume of sample (in mL) used for the infection)

Infectious viral load assessment by plaque assay in cells

Timing: 3 days

This step describes how to determine infectious virus in sorted cells from the lung, based on the susceptibility of the established canine kidney cell line MDCK to IAV infection.

42. Count viable cells of the BAL from step #10 or the lung single cell suspension prepared in step #13 using trypan blue for dead cell exclusion.

Note: Trypan blue cannot penetrate an intact cell membrane, so it will stain blue only the dead cells.

43. Adjust cell concentration to 1 × 10^7 cells/mL in FACS buffer.
44. Minimize unspecific antibody binding by incubation with anti-mouse CD16/32 antibody (Fc blocking 1:100 diluted) for 15 min at 4°C.
45. Stain cells with cell-specific antibodies.
    a. Add appropriate cell-specific antibodies, e.g., against the epithelial marker EpCAM and the leucocyte marker CD45 in 1:100 dilution directly in the cell suspension.

b. Incubate for 30 min at 4°C in the dark.
c. After the end of the incubation, wash with 10× volume of FACS buffer.
d. Resuspend in 1 mL of FACS buffer, and filter in a polystyrene test tube with cell strainer cap.
e. Add DAPI at 0.25 μg/mL concentration to discriminate live/dead cells.

46. Sort for the desirable cell population(s), e.g., the $CD45^+$ leucocytes and the $EpCAM^+$ epithelial cells, in a BD ARIA III flow cytometer or equivalent.

47. Lyse the cells to release the virus.
    a. Centrifuge sorted cells at ~ 230×*g* for 10 min at 4°C.
    b. Resuspend pellets in serum-free DMEM medium by adjusting the concentration to 10^6 cells/mL.
    c. Freeze and thaw cells twice by alternate immersing the samples in liquid N_2 and a water bath set at 37°C.

Note: Alternate freezing and thawing leads to disruption of cells through the formation of ice crystals, and allows for the liberation of the cytoplasmic content that contains the virus.

    d. Centrifuge lysates at ~ 10,000×*g* to remove debris.

48. Use the S/N, undiluted or 10-fold serially diluted, as the inoculum for performing the plaque assay according to steps #33–41.

Infectious viral load assessment by plaque assay in lung tissue

Timing: 3 days

This step describes how to determine infectious virus in whole lung tissue, based on the susceptibility of the established canine kidney cell line MDCK to IAV infection.

49. Homogenize frozen lungs collected in step 11.
    a. Add 1 mL of serum-free DMEM medium in the frozen lung.
    b. Immediately homogenize with a mechanical homogenizer for ~10 s in maximum speed (30,000 rpm).
    c. Centrifuge homogenized lung samples at ~ 10,000×*g* for 10 min at 4°C.
    d. Transfer the S/N to new 1.5 mL tube.
50. Use the S/N, undiluted or 10-fold serially diluted, as the inoculum for performing the plaque assay according to steps #33–41.

Note: In our experience, performing plaque assay in whole lung tissue leads to a smaller number of plaques, when compared to BAL S/N and BAL cells.

Analysis of viral presence in cells

Timing: 3 h

This step describes how to monitor IAV presence in cells through the immunofluorescent detection of IAV nucleoprotein (NP).

51. Prepare samples as described in steps 42–45c.
52. Fix the cells by resuspending the pellet in 100 μL of 4% PFA, vortex the tubes, and incubate for 10 min at RT.
53. Wash the cells with 1 mL of FACS buffer.
54. Permeabilize the cells by resuspending the pellet in 100 μL of 0.5% saponin.

55. Minimize intracellular unspecific antibody binding by incubation with an anti-mouse CD16/32 antibody (Fc blocking diluted 1:100) for 15 min at 4°C.
56. Stain the cells intracellularly by the addition of FITC-conjugated influenza A anti-NP antibody (10 μg/mL) directly in the cell suspension for 30 min at 4°C in the dark.
57. At the end of the incubation, wash the cells once with permeabilization buffer and then with FACS buffer.
58. Analyze on a BD ARIA III flow cytometer or equivalent. Unstained samples and single-stained samples are used to set appropriate PMT voltages for the positive and negative populations.

Analysis of virus localization in tissues

Timing: 1 day

This step describes how to monitor IAV localization in the lung tissue through the immunofluorescent detection of IAV NP.

59. Use OCT-embedded tissues collected in step #12 to perform 10 μm-thick cryosections in a cryotome. Ideally, collect sections in the sagittal plane of the left lung lobe at the level of the respiratory tree. You can place up to 3 sections on the same slide.

Note: You can store cryosections until further use in a slide box at −20°C in the presence of some silica gel to absorb moisture.

60. Let slides air-dry at RT.
61. Define areas around sections with a hydrophobic pen.
62. Fix sections with 4 % PFA for 10 min at RT.
63. Wash the slides 3× with PBS.

⚠ CRITICAL: Do not allow sections to dry from this point on.

64. Minimize unspecific antibody binding by blocking with 5 % goat serum for 30 min at RT.

Note: Blocking of unspecific binding is usually performed with serum from the host animal that the secondary antibody originates from. If no secondary antibodies are used, any available serum may be used for blocking.

65. Stain sections with FITC-conjugated IAV anti-NP antibody (5 μg/mL) for 1 h at RT in the dark.

Note: In parallel to NP staining, you may stain for cell-specific markers. In case unconjugated antibodies are used, add the anti-NP antibody together with the secondary antibodies.

66. Wash the slides 3× with PBS.
67. Stain nuclei with 2.5 μg/mL of DAPI diluted in PBS for 3 min at RT in the dark.
68. Rinse the slides briefly in PBS.
69. Mount sections with Prolong Diamond mounting medium and coverslip.
70. Visualize with a Leica TCS-SP5II Confocal Microscope.

EXPECTED OUTCOMES

Based on this protocol, we developed a strategy for collecting the maximum information regarding virus infection from a single mouse. During the progression of virus infection in mouse models, viral titers can be determined in several ways. In the case of RNA viruses, such as Influenza viruses, quantification of RNA of non-structural genes, such as NS1, can be a direct way of determining viral load.

By detecting viral proteins, such as HA, is a validation of transcribed and packaged virus. However, for assessing functional, i.e., infectious virus, plaque assay that is based on the ability of the virus to infect an epithelial cell line, should be used. Further, immunofluorescent detection of the virus can provide further information on the localization of the virus within cells or in the tissue.

In this protocol, we have used two viral doses, the lethal dose LD50, and a sublethal dose 0.1LD50, and we propose specific time-points for analysis based on these doses. If other doses are used, the timing of analysis should be appropriately adjusted.

In summary, by employing a detailed approach that is summarized in this protocol, we were able to detect and quantify viral load by several approaches during Influenza A virus infection in mice. Most importantly, our protocol provides an approach to take advantage of the maximum amount of information out of a single mouse, reducing the number of experimental animals used and maximizing the information obtained. This is an advantage over other protocols that have focused on one or two methodologies (Eisfeld et al., 2014; Pan et al., 2013; Tse et al., 2013).

LIMITATIONS

This protocol provides a detailed approach to determine viral load after infection of mice with the Influenza A viral strain PR8, which has been commonly used in the literature. Although, we have used the same protocol in several knockout mouse strains as well, and also after infection with another viral strain A/WSN/33, adjustments should be made when applying the protocol in other models of infections. Adjustments may include the timing of analysis post infection, the dilutions of samples used for the various assays, and the use of specific reagents, such as primer pairs for molecular analysis, and antibodies for immunofluorescent detection.

TROUBLESHOOTING

Problem 1

Mice do not recover from anesthesia (step 5).

Potential solution

Make sure that you weigh the mice and you administer the correct volume of anesthesia mix/mouse weight. The mice should be placed on a heating pad during recovery from anesthesia to prevent hypothermia. The mice must be monitored throughout the recovery time for normal cardiac and respiratory rate and pattern, by observing chest wall movements. Occasional toe pinching allows controlling for potential loss of reflex stimulation.

Problem 2

There is blood contamination in the BAL (step 9).

Potential solution

The presence of blood cells in the BAL is observed in later time points p.i. after LD50 dose due to diffuse alveolar hemorrhage as a result of the viral infection and the subsequent inflammation. It can be used as a parameter of disease severity. That is why it is crucial to avoid causing bleeding while dissecting the mouse. The skin dissection and the exposure of the trachea should be carefully performed in order not to damage any blood vessels. If bleeding occurs, try to place some paper tissue, trying to avoid the contamination of the BAL. Blood contamination also creates a problem by mistakenly counting blood leukocytes as BAL inflammatory cells.

Problem 3

No viral load is detected in any of the assays, or inconsistent results are obtained between experiments (steps 18, 32, 40, 58, 70).

Potential solution

It is possible that the virus stock was not well handled. It was either left too long at RT or came in direct contact with dry ice that may compromised its viability, or an aliquot that had undergone several freeze and thaw cycles was used. It is absolutely critical to store the virus stock at −80°C without any fluctuations in the storage temperature, in order to maintain the virus infectivity intact. Moreover, make sure to always use the same set of pipettes to perform the dilutions, as the protocols described involve small volumes and serial dilutions that should be performed as consistent as possible between experiments.

Problem 4

Inconsistent results are obtained among experimental animals of the same experiment (steps 18, 32, 40, 58, 70).

Potential solution

Make sure that when performing the i.n. administration, the mouse is properly anaesthetized, so that the entire volume of the viral inoculum is inhaled, but the mouse should not be under too deep anesthesia, so that it is able to take a deep breath and the viral solution is able to reach the lower respiratory system.

Problem 5

No plaques were formed in the plaque assay (step 40).

Potential solution

Make sure that when pipetting the overlay medium onto the MDCK cells, that it is not too hot, so that it does not kill the cell monolayer.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Evangelos Andreakos (vandreakos@bioacademy.gr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol does not generate or analyze any datasets or codes.

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AUTHOR CONTRIBUTIONS

All the authors helped to conceive and/or optimize the protocols. I.E.G., V.T., and E.E.E. conducted the experiments; I.E.G., V.T., E.E.E., and E.A. analyzed the data; I.E.G. wrote the paper; and E.A. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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