
*Accelerating COVID-19 Therapeutic Interventions and Vaccines (ACTIV) Public-Private Partnership*
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Viruses

Choosing a Strain
For SARS-CoV-2 studies in small animal models, investigators should select a virus strain and set parameters that address viral passaging, cell line selection for viral propagation, and viral sequencing techniques.

Primary Isolates
- Primary isolates should always be considered for research because they are the naturally circulating viruses that vaccines and therapeutics aim to combat.
  - Aim to test multiple isolates representative of the diverse clades known to be in circulation, and do not assume that all are identical (i.e., it may be difficult to select one isolate that adequately represents all the others). ¹
  - Recognize that isolates may not be identically represented in different geographical regions (i.e., some clades might be more prevalent in one location than in others).
  - As the virus continues to change and evolve, consider updating the tested viruses with more recent isolates that appear and become more prevalent (e.g., compared to Spike 614D, the Spike 614G variant increased in frequency in many locations throughout the world, suggesting a selective advantage for this variant).
- Ensure that the virus stock used has as uniform a genotype and phenotype as possible.
  - A primary isolate preparation can include a mix of strains, therefore confirming genotype is important. (See Passaging and Sequencing Viruses section on page 4).
- Select strains that have been used in other small animal model studies to maximize comparability of results.
  - Sequence data should be stored, and accession numbers should be associated with publications.

Genetically Engineered Viruses
- Consider making synthetic, infectious clones of SARS-CoV-2 for the following purposes:
  - to match the sequence of any circulating virus of known sequence
  - to generate low cell culture passage stock generation
  - to introduce specific mutations and assess their impact on virus replication or disease phenotypes
  - to leverage reporter genes such as green fluorescent protein (GFP) and nanoluciferase (NanoLuc) to improve the performance of a particular assay
- Advantages: Genetically engineered viruses allow testing the appearance of mutations that could be associated with resistance in antiviral or vaccine assays, by allowing the study of the impact of single or combined mutations in the virus phenotype

¹ https://nextstrain.org/ncov/global
• Challenges: There may be a limited number of labs capable of this engineering the virus.

Animal-Adapted Viruses

• Animal-adapted virus strains can help investigators better replicate human COVID-19 by increasing SARS-CoV-2’s infectiousness and pathogenicity in model animals (e.g., by introducing mutations to enhance mouse ACE2 binding and virus entry).

• Consider generating an animal-adapted virus strain to achieve greater infectivity or virulence in specific animals relative to wild type counterparts that may have been generally resistant to infection. (Or use a strain that has already been developed.)
  o For example, administering mouse-adapted viruses to wild type mice or mouse mutants that do not express the human interferon-stimulated gene ACE2, or that have a particular genetic susceptibility to obesity or hypertension, could increase the stock of mice that are eligible for inclusion in a COVID-19 study.

• Use mouse-adapted SARS-CoV-2 strains to test experimental vaccines and therapeutics such as antivirals and neutralizing antibodies.

• Note the potential pitfall that adapting SARS-CoV-2 to mouse models may alter the virus’s immunogenicity in mice as well as its sensitivity to specific antivirals, although unlikely.

• Consider potential risks of gain of function mutations and minimize these risks by conducting thorough assessments of risks and benefits independently validated by regulatory bodies.

• Consider that disease phenotype in the animal model will be affected by the following parameters:
  o virus strain
  o virus amplification procedure
  o mouse strain
  o route of infection
  o virus titer/volume
  o anesthesia
  o animal age or comorbidities
  o sex of animal
  o virus tropisms
  o host genetics

• Choose mouse strains carefully, because different mouse strains—and even sister strains (e.g., substrains of BALB/c mice)—can vary by thousands of nucleotides that can affect the characteristics of adapted virus strains in vivo.
  o Recognize that animal age can dramatically impact animal-adapted virus and disease models and so must be carefully considered when generating a new model or using an existing one.

2 https://europepmc.org/article/med/33031744
3 https://www.nature.com/articles/s41586-020-2708-8
4 https://www.nature.com/articles/s41586-020-2708-8
• Minimize complexity of the infection protocol (e.g., virus titer/volume and route of infection, such as with or without anesthesia), because any variability can affect the model’s reproducibility.
• Assess all of the following:
  o replication site in the animal’s body
  o infected cell types
  o resemblance of the infection process and resulting disease characteristics (including cytokine levels and other inflammatory responses) to those seen in humans
  o effect of host genetics on any of the virus or model characteristics
  o full range of pathogenic outcomes
  o lethal viral dose

**Example: Developing the SARS-CoV-2 MA10 Mouse Model**

**Background:** Mouse-adapted SARS-CoV-2 strains have been generated using both BALB/c and C57BL/6 mice, with pathogenesis ranging from mild to severe to lethal outcomes, especially as a function of dose and increasing age. One strain—called MA10—was developed by serially passaging SARS-CoV-2 in young BALB/c mice (10 passages total, with selection of viral plaques to establish a lineage after passage 10).

**Methods:** Researchers used the following methods to develop the MA10 Mouse Model:

- Sequence selected plaques to identify mutations present in the passage 10 strains, and then sequence the strains after the remaining five passages to chart the kinetics of each viral strain’s evolution. It is best to obtain the recombinant version of SARS-CoV-2 MA10 because it is at a lower passage number and reproduces disease phenotypes in vivo.
- Select the adapted strain most suited to the model.
- Identify a cell line in which the selected strain grows well
  - In this case, the selected strain
    - grew well in Vero E6 cells
    - grew less well in human airway epithelial cell culture than wild type SARS-CoV-2
    - led to an approximately 15% mortality rate in host young BALB/c mice at a $10^4$ dose, 90 percent mortality in 1-year-old animals at a $10^3$ dose.
    - led to clinical disease with recovery in C57BL/6 mice
    - exhibited fewer mutations than the other adapted strains
    - reintroduced the genetic changes into a molecular clone, recapitulating severe and lethal disease phenotypes in young and aged mice
Passaging and Sequencing Viruses

Once a viral strain is selected, investigators must produce a virus preparation for their assays. In developing a preparation, researchers should consider potential risks of passaging the virus (e.g., any unwanted viral adaptations can undermine reproducibility across batches) and should quality control each batch preparation.

Passaging Considerations

- Recognize that some virus specimens will mutate during each passage, and that those variants better adapted to the tissue culture in the preparation will be selected for at each passaging stage. This process may undesirably change the genetics of the strain, potentially causing altered disease phenotype in the animal model.
- Serial passaging of a virus strain could enrich the virus stock for defective interfering particles, which cannot infect the small animal host cells and are therefore less valuable for the study (see Reproducibility Across Batches section on page 5). DIs also may induce stronger innate immune responses that attenuate viral pathogenesis.
- Do not assume that infectious titers will remain consistent across different cell lines: plaque-forming unit titration measures infectiousness within the tissue culture system.
used but varies across tissue culture systems (e.g., from in vitro to small animal model testing).
  o Rely on titration measures derived from the model used in the study, ensuring that virus titrations are appropriate.
• Use the earliest passage possible of virus stock.

Reproducibility Across Batches: Avoiding Unwanted Adaptation
• Limit dilution or plaque purification to establish a clonal virus population to begin amplification.
• Passage the virus isolate at very low multiplicities of infection to avoid accumulation of DI particles and other undesired genetic diversity, but not so low as to allow bottlenecks that could amplify an aberrant variant.
  o In general, the recommendation is to perform less than 3 serial number of passages in cultures possible (limited to 3 passages) to conserve the genetic sequence
• Minimize the number of passages to limit variability.
• Use a highly permissive amplification (i.e., tissue culture) system to minimize selective pressure.
  o Vero E6 cells are commonly used, as are Huh7, A549, and Calu3 cells (Vero and Huh7 cells in particular produce high titers of virus).
  o Regardless of cell line used, researchers should closely monitor them for mutations of the furin cleavage site.
• Return to a previous passage to make new batches of virus when possible instead of continuing to passage virus from a finished batch, which could increase variability.
• Confirm the genotype and phenotype of your preparation (see Quality Control section on page 5).
• Sequence all virus stocks that go into the animal. Look for spike gene mutations.

Investigators should recognize that even if all precautionary measures are followed, variability across laboratories may occur and thus virus preparations must be carefully quality controlled. It is recommended that BEI supply reference stocks for cross-lab comparisons.

Quality Control
Given the inevitable risk of undesired virus variability, all virus preparations should be quality controlled using methods such as the following:

• Obtain viral stocks from BEI or another reputable repository. When choosing a repository or getting viral stock from a repository, confirm whether their team has done genetic quality control.
• Use a healthy tissue culture that is free of adventitious agents (e.g., mycoplasma).
• Confirm passaged virus’ genotype by determination of consensus sequence, which should match the primary isolate of interest.
  o Suggested methods for virus genome analysis include (1) whole genome sequencing (e.g., using Illumina, Oxford Nanopore [ARTIC Network protocols for
SARS-CoV-2 sequencing]), and (2) polymerase chain reaction (PCR) to amplify regions of interest (e.g., Sanger sequencing).

- Check for overrepresentation of sequences at the ends of genes when using RNA sequencing.
- Consider conducting nanopore sequencing or RT-PCR in search of internal deletions.
- Confirm the virus’s expected phenotype in vitro.
  - Virus preparations should exhibit infectious titers in Vero E6 cells of more than $10^6$ PFUs per milliliter. Remember that a PFU titer measures infectious titer in the tissue culture system used but varies across different cell lines.
- Consider calculating the ratio of viral RNA load to PFU titer measures taken in appropriate cell lines.
- Confirm that virus’s in vivo replication and phenotype match expectations for the selected animal model.
- Challenges:
  - Lab-to-lab variability is almost inevitable, due to animal differences, lab conditions, number of animals (statistical differences), etc. Lab-to-lab studies should be considered to achieve consensus.
  - Sources of variability when confirming phenotypes not only depend on virus preparation, but also of the reproducibility of the phenotypic model systems. For example, the phenotype of a particular virus strain in mice might vary according to the host microbiome of the animals.
  - Isolating high quality RNA from slough cell contamination is difficult
  - If viremia is present, low level of RNA detection in multiple tissues is common

### Compliance and BSL3 Best Practices

**BSL2 Best Practices**

- Ensure that mice are immunocompetent.
- House mice in either ventilated racks or cages outfitted with static filter tops.
- Supply housing units with adequate food, water, and mouse enrichment supplies.
- Use specific pathogen-free mice for vaccine (or vaccine challenge) studies.
- Consider using sentinel mice if conducting a study in a well-established and diverse colony of mice.
  - Sentinel mice may be less suitable if conducting an accelerated experiment on Mouse-Adapted SARS-CoV-2 Pathogenesis

**BSL3 Best Practices**

Consider the following best practices, in addition to those above, when working in a BSL3 lab:

- Ensure that all cage, water, and food changes, and other manipulations, are conducted in biological safety cabinets.
- Autoclave cages, bedding, and other waste products for disposal.
- Bag and autoclave mouse carcasses for disposal.
• Use only BSL3-approved disinfectants.
• Properly decontaminate all animal blood and tissues before removing them from the BSL3 facility (e.g., using internally validated ultraviolet radiation or heat inactivation protocols; ensure that these protocols do not affect the analytes of interest in the samples).
• Consider that simple tasks take much longer than normal in a BSL3 facility, and it also takes time to enter and exit these facilities because of safety precautions.
  o For example, in a 50-hamster BSL3 study, inoculation might take two people 3 hours (4 hours if also collecting baseline blood) and weighing the animals might take one person 2.5 hours.
• Plan studies carefully to ensure that planned tasks (e.g., two- or three-times daily dosing of test articles can be executed within the BSL3’s light period.
• Use a light cycle in the BSL3 for running a hamster study (e.g., 7am to 7pm).

IACUC and IBC Standards
To ensure that study designs align with Institutional Animal Care and Use Committee (IACUC) standards, researchers should adhere to the following best practices:

• All research using animal-adapted viruses should be conducted in accordance with the US Department of Health and Human Services (HHS) Framework for Guiding Funding Decisions about Proposed Research Involving Enhanced Potential Pandemic Pathogens (HHS P3CO Framework).
• Refer to publications that IACUC has already approved, using these publications to inform study parameters such as the following:
  o doses (PFU/volume)
  o routes of administration
  o endpoints (e.g., lethargy, isolation, huddling, weight loss of 20% or more)
  o group sizes
  o observation frequencies (e.g., once or more per day)
• Ensure that studies align with Institutional Biosafety Committee (IBC) standards.
• Establish protocols as early as possible for both viral inactivation and animal containment (e.g., to guard against viral shedding and aerosol transmission).

As of September 2020, SARS-CoV-2 is classified as a Risk Group 3 non-select agent and is not considered a dual use research of concern agent. Facilities used for SARS-CoV-2 studies do not need prior Centers for Disease Control and Prevention [CDC] approval.

**Mouse Models**
Wildtype mouse models generally do not show clinical signs of SARS-CoV-2 infection and thus are best used for studying heterogeneity of responses to infection, which has also been observed in humans. However, several varieties of ACE2 transgenic mice do offer effective models of moderate to severe infection (such as K18-hACE2 K18C57BL/6 mice transgenic\(^5\)\(^6\)\(^7\)\(^8\)\(^9\), and hACE2 knock-in C57BL/6 mice) as well as lung-specific infection and recovery (such as adenovirus ACE2 and hACE2 Mouse ACE2 promoter in ICR mice). Some transgenic models may also be useful for studying viral replication.

Choice of mouse model affects the quality as well as the comparability of experimental results and should reflect the experimental question being asked by the researcher. Researchers must choose whether to use adapted virus in wild type mice, to use transgenic mice, or to transfect mice with the *ACE2* gene using an adenovirus. Regardless of model selection, researchers must also determine protocols for housing and caring for their animals and should generally follow best practices for research conducted in biosafety level-2 or -3 (ABSL2 and ABSL3, respectively) laboratories (see Compliance and BSL3 Best Practices section on page 6).

**Designing Mouse Model Experiments**

**Study Design Best Practices**
- Consult with biostatisticians using standard statistical methodologies to inform power calculations. Also, use published studies for power calculations.
- Include sufficient infected and mock-infected control groups. Both groups should undergo identical procedures throughout the study to optimize comparability and control for environmental influences as well as procedural stressors.
- Study design and group size should be determined prior to study start.
  - Use a dedicated set of animals to establish a survival curve (i.e., do not repurpose these animals for timed necropsy studies, because doing so could affect statistical analyses).
- A typical baseline infection study generally requires
  - **Necropsy time points for viral load determination and/or weight loss**: 5 or 6 mice per sex/group
  - **Survival group**: 10 or more mice per sex/group for a survival study

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\(^9\) [https://www.biorxiv.org/content/10.1101/2020.07.18.210179v1.full](https://www.biorxiv.org/content/10.1101/2020.07.18.210179v1.full)
Replication experiments: additional mice may be required to ensure rigor through replication experiments (e.g., one study with 30 mice is less robust than 3 studies with 10 mice)

- Track each mouse (e.g., using tags, ear punches, microchips, or other), particularly for studies involving intranasal delivery.
- Allow mice a 1- to 2-week acclimation period within the facility to allow for dissipation of stress hormones that could impact results.
- If breeding transgenic animals, confirm the animals’ genotype using standard genotyping protocols supplied by the mouse vendor (e.g., The Jackson Laboratory has a published protocol for genotyping the K18- HuACE2 mouse: https://www.jax.org/Protocol?stockNumber=034860&protocolID=38276) or found in the primary literature. Note: Newly developed transgenic mouse models should be sufficiently monitored for stable transgene expression over time.
- Genotyping after transfection is important to confirm desired protein presence (e.g., Confirming ACE2 gene expression after transfection) can be conducted either pre- or post-infection. Stable transgene expression is a prerequisite for conducting these experiments.

Existing SARS-CoV-2 Mouse Infection Models
Researchers must generally select either a wild type or transgenic mouse model. Both model types present distinct advantages and drawbacks.

Wild Type Mice
- The most common wild type mouse models (i.e., C57BL/6 and BALB/c mice) show no clear evidence of susceptibility to infection by SARS-CoV-2 at 10^5 plaque forming units (PFU). However, at this concentration some wild type mice do exhibit increased IL-10, IL-27, and chemokine production in the brain (it remains unknown whether a very low-level brain infection is established and quickly resolved. More data are needed to understand the increased chemokine levels).
- Although currently untested, outbred mice may be valuable for studying pathogenesis, and until mouse-adapted virus strains become available, studying SARS-CoV-2 infections in outbred mice may help researchers study heterogeneity of responses to infection, which has been observed in humans.
- Existing mouse adapted strains (SARS-CoV2 MA2 or SARS-CoV2 MA10) require the use of BALB/cAnNHsd mice obtained from Envigo (strain 047). C57BL/6J mice were obtained from the Jackson Laboratory (strain 000664). Note that sister strains (sub-strains) may encode novel mutations that alter disease severity and outcome.

Transgenic Mice
Unlike wild type mice, some initial strains of ACE2 transgenic mice from the SARS era (described below) have shown evidence of moderate to severe SARS-CoV-2 infection. Additional transgenic mouse models will continue to become available.
**K18-hACE2, K18 promoter in C57BL/6 mice transgenic**\(^{10,11,12,13,14}\)

- C57BL/6 mouse, containing a hemizygous human ACE2 (hACE2) transgene under the transcriptional control of the cytokeratin 18 (K18) promoter to drive hACE2 protein expression in epithelia.
- Intranasal administration of SARS-CoV-2 at a high dose of \(10^5\) PFU in this model has resulted in 20-25% weight loss and an average of 6-day survival.
  - Diffuse pneumonitis is significant along with cytokine storm associated with lethality in these mice.
- Virus has been detected in nasal turbinates, lungs, and brain using both PFU measurements, viral RNA loads, polymerase chain reaction (PCR), and immunohistochemistry.
- Cytokine and chemokine expression are elevated first in the lungs and later in the brain, with milder changes occurring in the spleen (unlike in the lungs and brain, no virus is detected in the spleen).
- Morbidity and mortality responses vary with dose; moderate \((10^4)\) to high \((10^6)\) PFU infection in this model closely reflects lung disease features of humans requiring admission to intensive care units (ICUs) and may therefore be suitable for testing therapeutics aimed at treating severe disease; moderate titers that still result in severe disease are preferred in this model.
- Lower doses of virus \((less than 10^5)\) result in infection with recovery; the degree of body weight loss and mortality is dose dependent.
- This transgenic mouse is available as a standardized model for purchase from the Jackson Laboratory in Maine (Stock #34860).

**hACE2 Mouse ACE2 promoter in ICR mice**\(^{15}\)

- ICR mouse transfected with a mouse ACE2 promoter to drive targeted hACE2 expression in animals' lungs, kidneys, and intestines.
- Intranasal administration of SARS-CoV-2 at \(10^5\) PFU has resulted in 10% weight loss by day 5, followed by recovery.
  - The virus produces \(10^6\) copies in the lung and intestine, causing moderate interstitial pneumonia but no pathologies in other organs (i.e., pathology is lung-specific and serves as a good model for disease recovery).

**hACE2 CAG promoter C57BL/6 mice transgenic**\(^{16}\)

- Deliver CAG promoters into transgenic mice to drive ubiquitous hACE2 expression.
- In these mice, lungs and brain are infected by SARS-CoV-2, leading to 35% weight loss, elevated cytokine and chemokine levels, and 100% mortality within 8 days.

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\(^{14}\) [https://www.biorxiv.org/content/10.1101/2020.07.18.210179v1.full](https://www.biorxiv.org/content/10.1101/2020.07.18.210179v1.full)

\(^{15}\) [https://www.nature.com/articles/s41586-020-2312-y](https://www.nature.com/articles/s41586-020-2312-y)

\(^{16}\) [https://jvi.asm.org/content/81/3/1162](https://jvi.asm.org/content/81/3/1162)
• This model, similar to the K18 promoter model described above, could serve as a valuable system for studying disease profiles similar to those in humans with severe COVID-19.
• This model is available from Taconic.

**hACE2 knock-in C57BL/6 mice**

• The human ACE2 cDNA replacing the endogenous mouse Ace2 sequences.
• The endogenous mouse Ace2 regulatory elements direct expression of the authentic human receptor (hACE2).
• Studies with 10-13-week-old hACE2-KI hemizygous females are susceptible to SARS-CoV-2, and the virus replicates efficiently in respiratory tissues using 1x10^5 PFUs.
• High viral RNA loads in the nasal conchae and lungs were observed; virus RNA was also detected in olfactory bulbs and at lower levels in brain tissues; low to undetectable levels of virus were observed in spleen, small intestine, kidneys and feces.
• Hemizygous females did not exhibit morbidity or mortality (e.g., no weight loss) when infected with typical wildtype SARS-CoV-2 viruses.
• The hACE2-KI mice have loxP sites flanking the human ACE2 cDNA sequence. Upon exposure to Cre recombinase, the human ACE2 cDNA will be deleted, generating a knock-out allele with the ability to eliminate expression in selected tissues if desired.
• This transgenic mouse is available as a standardized model for purchase from the Jackson Laboratory (Stock # 35000).

**Adenovirus ACE2**

• Use a serotype 5 adenovirus to transduce respiratory epithelial cells to express human ACE2 receptors.
• Administer replication-deficient adenovirus (AAV5), either intranasally or intravenously (at 10^8 focus-forming units [FFU]), to induce expression of human ACE2 in the animals’ lungs.
• Viral titers and cytokines and chemokines are measured in the lungs, with viral persistence to day 10 with limited clinical disease.
• Use this platform to study lung-specific infections.

**Selecting Route of Administration and Dose Ranging**

• Generally, administer SARS-CoV-2 either intranasally (most common) or intravenously.
  • Consider using aerosol particles instead (not yet attempted) to deliver the virus more uniformly into the lung tissue.
  • Consider experimenting with intratracheal administration.
• Administer 30 to 50 microliters of virus containing solution to an anesthetized mouse held in an upright position. Anesthesia options include ketamine/xylazine, isoflurane (e.g., a mouse-specific isoflurane vaporizer and anesthetic circuit).

17 [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7284254/](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7284254/)
Nonhuman primate (NHP) studies have sometimes administered the virus rectally or through the eyes; these routes might also be considered in mice or other small animals.

For general guidance on anesthesia, researchers can refer to *Anesthesia and Analgesia in Laboratory Animals* (Fish et al., 1997).

- Because each of the mouse models have differing susceptibility to SARS-CoV-2 infection, and routes may impact lethality, median lethal dose ($LD_{50}$) will vary depending on mouse strain choice and publications should be referenced.
- Viral stocks used for rodents include USA-WA1/2020 strain (Gen Bank: MN985325.1), and each strain/batch of and virus stock should be tested for $LD_{50}$ prior to study.
- Dose ranges should be established in mice based on viral batch

**Determining Age and Sex Differences**

- Most studies using transgenic or transfected mice have used animals aged 4 to 10 weeks.
  - More experiments are required to determine how or whether age may impact results. Aged mice may be susceptible to more severe disease.
  - It is known that 18-month-old C57BL/6 and BALB/c mice are not susceptible to SARS-CoV-2 infection and exhibit no weight loss (although doses of $10^5$ PFU administered to older animals have resulted in some weight loss; researchers have yet to confirm viral load in these older animals).
- Modest sex differences have been observed in K18 ACE2K18-hACE2 transgenic mice: males lost weight slightly faster than females and exhibited higher cytokine levels in the brain (the female mice exhibited more of a Th2 phenotype in the brain than did male mice).
  - More research is needed, particularly at lower doses (e.g., $10^3$ PFU), to better understand these and other potential sex differences.

**In-Life Measurements**

When conducting small animal model studies of SARS-CoV-2 infection, researchers should use in-life measurements of body weight, general health, conditioning scores, disease severity, and chemokine and cytokine levels.

Past studies in both wild type and transgenic mice have identified the levels of virus and of chemokines and cytokines summarized below, which may be used to inform prospective small animal studies of SARS-CoV-2 infection.

**Viral Load**

Researchers evaluating viral load in K18 hACE2 mice following administration of $10^5$ PFU of Washington strain SARS-CoV-2 (USA-WA1/2020) found that viral titers taken in nasal turbinates remained fairly steady through day 6, rose markedly by day 2 and declined sharply by day 4 in the lungs, and rose markedly in the brain by day 4, continuing to rise at day 6.

- No significant sex differences in viral load were observed.
• Despite the decline in viral titers in the lungs by day 4, all the infected K18 hACE2 mice eventually died from infection, likely from peripheral inflammation.

In-life viral load is assessed using swabs; see also “Viral Load” within the Post-Mortem Measurements section on page 14.

**Nasal Lavage under Anesthesia**
Some investigators have used nasal lavage to recover virus from sedated animals. Sample protocols include the following:

- **Anesthesia:** some have sedated mice using 100 mg/kg of ketamine (or 12.5 mg/kg of xylazine)
- **Cholinergic agonist:** some administer 100 µl of carbachol, a cholinergic agonist, via intraperitoneal injection to stimulate secretion of virus from animals’ submucosal glands.
- **Collection:** after approximately 15 minutes, use a gel-loading pipette tip to collect 20 µl of virus secretions per mouse, and measure PFUs of infectious viruses.
- **RNA Isolation:** then immediately add these nasal secretions to 500 µl of Trizol, mixing and storing the solution at -80°C until conducting RNA isolation.
  - The RNA isolation results are then quantified by PCR in terms of cycle threshold (Ct) value per volume lavage.

**Chemokine and Cytokine Levels**
Using proteomics panels developed by Luminex Corporation, investigators have assessed both chemokine and cytokine levels in K18 hACE2 mice at 2 and 4 days post infection. Note that results of chemokine and cytokine level assessments may vary between proteomics and RNA sequencing (RNA Seq) approaches.

**Chemokines**

**Cytokines**
Cytokine expression appears generally weaker than chemokine expression. Common cytokines measured in lung: IL-12p70, IL-2, IFN-γ, IL-4, IL-13, IL-33.

Common cytokines measured in brain: IL-4, IL-14, IL-10, IL-6, IL-12p70, IFN-γ.

In contrast to the K18 hACE2 mice, wild type control mice exhibit no evidence of viral load, yet they do exhibit inflammatory cytokine responses in their brains (e.g., expressing IL-1 and type I IFN); investigators do not know whether these cytokines were expressed within the brain or entered the brain from outside.
Post-Mortem Measurements

When conducting small animal model studies of COVID-19, investigators can use various kinds of post-mortem measurements to characterize the effects of SARS-CoV-2 on the animal being studied, including measuring viral load (e.g., using PCR or nasal lavage), conducting histopathological analyses, studying gross pathological features, and analyzing cytokines.

Viral Load

To assess virus titers in small animal models, investigators can conduct quantitative real-time (qRT) PCR assays on viral DNA and then use the amplified viral DNA sequence to measure virus titers (including PFUs) across various tissues (post-mortem viral load is assessed via tissue homogenates; see also “Viral Load” within the In-Life Measurements section on page 12). Whenever assessing virologic endpoints in animal model tissues, it is crucial that investigators isolate high-quality RNA (to protect against potential contamination from sloughed cells), and recognize that if viremia has occurred in the animal they may detect low levels of RNA across multiple tissue systems. Investigators should consider quantifying subgenomic viral mRNAs, which is more indicative of actively infected cells than genomic RNA.

Histopathological Analyses

- Researchers interested in conducting histopathological analyses of post-mortem animal tissues should collaborate with a board-certified pathologist who has specialized training in veterinary medicine and animal models, if possible.
- However, to preserve blinding and avoid bias, it is critical that the pathologist not know which experimental condition a tissue sample came from.
- Histopathological analyses should be conducted in consultation with investigators knowledgeable about the human disease state, to aid identification of similarities and differences across species that may be relevant to interpreting the animal model results.

Previous histopathological analyses on wild type mice using adapted virus and K18 hACE2 transgenic mice have identified several kinds of pathology in the lungs and in other tissues, as summarized in the table below:

<table>
<thead>
<tr>
<th>Type of Mice</th>
<th>Day (Post-Infection)</th>
<th>Tissue</th>
<th>Pathology Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>Day 2</td>
<td>Lung</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Mixed mononuclear inflammation (minor)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hepatocellular necrosis (minor)</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>Nasal turbinate</td>
<td>Mild rhinitis (minor)</td>
</tr>
</tbody>
</table>
### Day 2

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td></td>
<td>Alveolar histiocytosis</td>
</tr>
<tr>
<td>Liver</td>
<td>Mixed mononuclear inflammation (minor)</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular necrosis (minor)</td>
</tr>
</tbody>
</table>

### K18 hACE2

**Day 4**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Inflammatory cellular accumulations and hemorrhage in alveolar spaces and interstitium</td>
</tr>
<tr>
<td></td>
<td>Vasculitis</td>
</tr>
<tr>
<td></td>
<td>Edema</td>
</tr>
<tr>
<td>Nasal turbinate</td>
<td>Neutrophilic rhinitis (minor)</td>
</tr>
<tr>
<td>Brain</td>
<td>Mild meningoencephalitis with vasculitis (minor)</td>
</tr>
<tr>
<td>Small intestine and liver</td>
<td>Small cellular aggregates (minor)</td>
</tr>
</tbody>
</table>

**Histochemistry**

Histochemistry, often with hematoxylin and eosin (H&E) staining, has been used to analyze the tissues of deceased model organisms. Researchers have also used the nucleoprotein (N protein) as a target to localize antigens in small animal model tissues, using the following procedure:

1. Incubate slides with blocking reagent (10% normal goat serum, 30 minutes).
2. Deliver rabbit monoclonal antibody (#40143-R019 from Sino Biological US Inc., Wayne, PA, USA) against SARS-CoV-2 N protein (1:20,000 dilution, 60 minutes).
3. Incubate tissue with Rabbit Envision (Dako) and diaminobenzidine (Dako) as chromogen.

Investigators utilizing histochemistry to study animal model tissue samples should also consider the best practice guidelines captured in the bulleted lists below.
General Best Practices

Making In-Life Preparations for Tissue Necropsy
- Create detailed plans for preparation and analysis before beginning a histochemical study; this attention to detail will greatly aid subsequent evaluation, interpretation, and examination of tissue.
- Maintain all animals (including controls) in one facility and avoid stressing animals by transferring them (e.g., to BSL3 facilities).
- Control for as many parameters as possible using closely matched positive and negative control conditions in all animal model experiments (e.g., match euthanasia conditions and tissue fixation protocols).

Preparing Tissues
- Prepare quality tissue samples to optimize preservation of fine tissue architecture and, in the case of immunostaining, antigenicity of target epitopes.
- Harvest samples as soon as possible following death to minimize autolysis.
- For tissues that will be paraffin-embedded, fix sections in either 10% neutral buffered formalin or in 4% paraformaldehyde (note that other fixatives may also be appropriate depending on endpoints of interest).
- Place samples in a minimum of 20-to-1 (fixative-to-tissue) volume with maximal tissue thickness of approximately 5 mm, with at least one dimension of tissue consistently fixed.
- In some cases, it may be possible to use cryopreservation instead of tissue fixation to preserve specific antigens. However, this process typically results in suboptimal retention of cellular and tissue architectural detail.

Testing and Presenting Results
- Maintain awareness of normal anatomy and morphology to recognize changes; when using animal models of human disease, know differences between species (e.g., lungs have different lobe configurations in humans versus in mice).
- Recognize that the severity and character of tissue damage can vary even within the same organ of the same animal (i.e., pathology can be “patchy”).
- Enlist a qualified veterinary pathologist, if possible, to photograph, tabulate, and grade post-mortem tissue.
- Ensure that the pathologist is blinded when photographing tissue samples to avoid bias (note that the pathologist can be unblinded to select photographs that highlight findings).
- Prioritize flow cytometry over other methods when quantifying different inflammatory cell types in small animal models.
  - Single-cell RNAseq might also be considered.
- Report absolute numbers (not percentages) when using flow cytometry to measure B-cell or T-cell infiltration.
- In publications, show tables capturing results from all tested animals, side-by-side with selected pictures (e.g., of encephalitis) to help readers interpret the pictures in context.
**Animals: Best Practices**

- Recognize that inbred mouse strains can exhibit biased (e.g., Th1 vs. Th2 immune responses) or deficient immune signaling pathways that might influence infection susceptibility or severity.
- Consider age and sex of model animals when designing experiments, because these parameters can influence factors for infection.
- Include wild type mice, as well as uninfected control mice, for comparison whenever using transgenic mouse strains for histopathological analyses.
- Husbandry depends on caging and facility.

**Lungs: Best Practices**

- Recognize that the lung, unlike other organs, undergoes dynamic size changes during normal respiration.
- Select a method of euthanasia that does not target the lungs, such as an intravenous agent, to avoid undesired changes to the lung prior to conducting post-mortem lung tissue analysis (overdose of inhalational agents such as CO₂ introduces minor edema and hemorrhage).
- Standardize handling of postmortem lung tissue to prevent postmortem atelectasis or variable inter-animal insufflation.
- Avoid post-mortem atelectasis and ensure standard tissue preservation procedures to enable comparisons among animals.
- Define sample collection methods in publications and include collection site (standardized versus lesions sites) and total number, the latter of which depends on the size of the tissue, distribution of tissue lesions, and overarching goals of the study (e.g., for mouse studies, it is typical to evaluate the entire lung).
- Use right ventricular perfusion of a fixative into the lungs before removing them.
- Weigh extracted lungs and then convert other measurements (e.g., cytokine levels and PFUs) to weights for more exact assessment.
- Inflate the lungs via intratracheal instillation of fixative to best preserve lung morphology and reduce artifactual atelectasis.
- Fully inflate and formalin-fix lungs for better visibility of features.
- Insufflate airspaces without dislodging inflammation or mucocellular debris.
- Place individual lungs in cassettes, and track which lungs came from which animals to enable linkage of histopathology results with other measurements in that animal (e.g., chemokine and cytokine levels).
- Optimize and validate each new antibody to be used in immunostaining, utilizing appropriate positive and negative controls to ensure accurate staining results.
- Evaluate controls in a standardized manner to identify antemortem or euthanasia-related variables affecting lung evaluation.
- Present histopathology photographs of the entire lung to visualize pathological features (e.g., abnormal density, swelling, or cellular influx) across the entire tissue, and to avoid biased selection of photographs that misrepresent the overall pathology in the lungs.
**Immunohistochemistry**

- Two antibodies are now available for use in small animal SARS-CoV-2 infection model studies: (1) Anti-SARS-CoV-1/2 NP monoclonal mouse antibody, clone 1C7C7\(^\text{18}\); and (2) Rabbit polyclonal NP cross-reactive antibody.\(^\text{19}\) Other antibodies will undoubtedly be reported in coming publications.

- When conducting brain immunohistochemistry analyses, especially if staining for multiple proteins, it is important that researchers serially section the tissue to aid colocalization of proteins of interest as well as potential double staining.

- Previous immunohistochemistry studies in brain tissue of mouse models have reported that SARS-CoV-2 is detectable only in specific cells within the brain, although follow-up studies are needed to identify these cells.

- If conducting immunohistochemistry along with histopathological analyses, snap freeze and section the tissues in the BSL3 facility and then fix and stain them afterward, or else fix and stain the tissues in the BSL3 facility.

- Use of RNA scope for viral detection and quantification is also a technique under development.

Several immunohistochemistry studies of mouse SARS-CoV-2 infection models have reported upregulation of fluorescent antibodies (Abs) that bind to both the SARS-CoV-2 nucleocapsid and to the hACE2 protein in k18 hACE2 mice (upregulation was assessed in comparison to wild type control mice). Generally, among k18 hACE2 mice, upregulation of the nucleocapsid Ab was mild in the nasal turbinates, strong in the lung, and medium in the brain, whereas upregulation of the hACE2 Ab was medium in the nasal turbinates, strong in the lung, and mild in the brain. Nucleocapsid Ab fluorescence patterns in the lungs of k18 hACE2 mice appeared to indicate that the viral nucleocapsids were coating the alveolar sacs.

**Quantitative Morphometry**

- To improve the rigor of histopathological analyses, investigators may also examine tissues microscopically using quantitative morphometry to identify structural changes (i.e., lesions) that could indicate pathology.
  - For example, severe SARS-CoV-2 infection often causes diffuse alveolar damage, and various kinds of lung lesions (e.g., necrosis, edema, hyaline membranes, thrombi, hemorrhage, syncytia) can be evaluated and scored using quantitative morphometry techniques.

- All quantitative morphometry results should be correlated with virologic endpoints for validation of measurements.


\(^{19}\) [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7103305/](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7103305/)
Semiquantitative Scoring

Scoring can be done using semiquantitative approaches that apply predetermined morphologic criteria to transform qualitative tissue changes into numerical scores. Scoring best practices for several lung lesion types are provided in the table below, based on previous K18 mouse studies:

<table>
<thead>
<tr>
<th>Lesion Type(s)</th>
<th>Scoring Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema</td>
<td>0. none</td>
</tr>
<tr>
<td>Hyaline Membrane Formation</td>
<td>1. uncommon detection in &lt;5% lung fields (at 200x magnification)</td>
</tr>
<tr>
<td>Necrotic Cellular Debris</td>
<td>2. detectable in up to 33% of lung fields</td>
</tr>
<tr>
<td></td>
<td>3. detectable in 33-66% of lung fields</td>
</tr>
<tr>
<td></td>
<td>4. detectable in &gt;66% of lung fields</td>
</tr>
<tr>
<td>Neutrophil Infiltration</td>
<td>0. within normal limits</td>
</tr>
<tr>
<td></td>
<td>1. scattered polymorphonuclear leukocytes (PMNs) sequestered in septa</td>
</tr>
<tr>
<td></td>
<td>2. #1 plus solitary PMNs extravasated in airspaces</td>
</tr>
<tr>
<td></td>
<td>3. #2 plus small aggregates in vessel and airspaces</td>
</tr>
<tr>
<td>Mononuclear Infiltrates</td>
<td>0. none</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>1. uncommon detection in &lt;5% lung fields (at 200x magnification)</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>2. detectable in up to 33% of lung fields</td>
</tr>
<tr>
<td></td>
<td>3. detectable in up to 33-66% of lung fields</td>
</tr>
<tr>
<td></td>
<td>4. detectable in &gt;66% of lung fields</td>
</tr>
</tbody>
</table>

Quantitative Scoring

- Compared to semiquantitative methods, quantitative scoring methods typically achieve higher precision and sensitivity.
- Quantitative scoring approaches may require high-quality images and specialized software to analyze tissue samples properly, which can increase costs.
  - Advances in automation and artificial intelligence software are increasing both efficiency and cost-effectiveness of quantification of tissue parameters, especially for larger projects, making these methods more accessible to more researchers over time.

Macroscopic Analysis of Gross Pathological Features

- Macroscopic evaluation and scoring can complement histopathological evaluation of lesions.
- Tissues (e.g., heart or lungs) can be removed in situ and photographed for scoring.
Assessment of tissue for macroscopic indicators of disease (e.g., tissue color, consistency, surface texture) must be conducted immediately upon removal from the animal’s body, and prior to tissue fixation (i.e., fixation will affect the physiological parameters being scored).

- Investigators should maintain dedicated cameras in their BSL3 facilities to photograph gross tissue samples upon removal from an animal.
  - Equipment cannot be carried in and out of a BSL3 facility.
- Image files should be transferred electronically from computers inside the BSL3 facility to other external systems for analysis.

**Cytokine Analyses**

- Post-mortem animal model analyses can also assess the presence of cytokines or various other elements of host response.
  - For example, researchers may conduct transcript profiling at either the tissue- or the cell-specific level (e.g., using techniques such as RNA-Seq, single-cell RNA-Seq, qRT PCR or various commercial platforms such as NanoString).
  - They might also conduct protein-level cytokine profiling, for example using ELISAs, Luminex Corporation panels, or NanoString panels.

**Hamsters**

Hamsters have commonly been used as models for infection from a variety of different agents, including paramyxoviruses, flaviviruses, and filoviruses (using adapted strains), as well as SARS-CoV agents. A 2019 paper by Miao et al., published in *Frontiers in Immunology*, provides a good reference for how to use hamsters to identify antibodies against host proteins, and several 2020 papers have reported results using wild type Golden Syrian hamsters as SARS-CoV-2 infection models (e.g., Imai et al., *PNAS*; Sia et al., *Nature*; Brocato et al., *JVI*; Rogers et al., *Science*).

In SARS-CoV-2, hamsters are effective models of human transmission, viral replication, and severe disease. Transmission among hamsters appears to occur both by direct contact and by aerosol. The virus replicates efficiently in the trachea as well as in the lungs and nasal turbinates, with minor replication in the intestine, resulting in severe pathological lesions that resolve by day 10. Hamsters simulate severe disease characteristics seen in humans including viral replication in the lung, focal inflammatory cell infiltration in the interstitium and the alveolar cavity, pulmonary edema, and alveolar hemorrhage.

Unlike in humans (and ferrets), hamster infections do not seem to remain in the upper respiratory tract (possibly due to intranasal virus administration protocol). Hamsters can show mild clinical signs of infection (e.g., ruffled fur and rapid breathing) and experience weight loss from day 1 to 2 post-infection, with recovery by day 10 to 12, whereas the clinical course of human infection commences with an incubation period (median of 5.1 days) before illness then develops by 11 days post-infection.
Hamsters are a species regulated by the U.S. Department of Agriculture (USDA) and covered under the Animal Welfare Act Regulations, from which mice of the genus *Mus* are excluded. Researchers and their institutions should ensure that all individuals working with hamsters follow these regulations.

**Best Practices for Using Wild Type Golden Syrian Hamster SARS-CoV-2 Models**

**Choosing Animals**

- **Age of animal**: Animals typically range from either 4 to 12 weeks or 7 to 8 months of age.
- **Sex of animal**: Both male and female Golden Syrian hamsters have been used to model SARS-CoV-2 infection.
  - Use 3- to 4-week-old animals because they gain weight rapidly.
  - Some researchers have reported issues with females fighting (this could require more cages to do the same size study with female animals).
  - Cost tip: if ordering animals from Charles River, ordering by age rather than by weight reduces cost (e.g., researchers who want ~120 to 160 kg animals can order 10- to 12-week-old animals, which will provide animals of the desired weight but at less cost).

**Housing and Handling**

- **Cage sizing**: Caging requirements are provided in the Animal Welfare Act regulations. If a study requires use of cages that differ from those specified in the regulations, for special procedures such as aerosol exposures, then these variations will require IACUC approval.
  - Select cages whose dimensions fit into biological safety cabinets that are necessary to work with the animals.
- **Pairing guidelines**: Consider the following pairing guidelines:
  - More than two hamsters should not generally be housed together (thus housing costs for hamsters are higher than they are for mice).
  -Male animals can be pair housed, but they will likely fight (e.g., stomach bites) and researchers will have to budget for extra observation.
    - Females are even more likely to fight.
- **Required skillset**: Technicians should be trained specifically for working with hamsters; skills with other animals, such as mice, are not necessarily transferrable to hamster work.
  - During the week before hamsters enter the high biosafety lab, animal caretakers should pet and handle them two or three times so that the animals are tame and easy to handle once in the facility.
  - Puncture-resistant gloves should be worn by anyone handling non-sedated hamsters, especially post-inoculation.
• **Timing of experiments:** Hamsters are nocturnal, so all procedures conducted during the day may have physiological effects (e.g., increased viral titers, weight loss).
  - Handling disrupts circadian rhythms, increases stress, and leaves hamsters less able to cope with insults or infections; hamsters may need one week to reach physiological baseline after a cage change procedure.

• **Enrichment:** When working in a BSL3 facility, it is recommended to limit large enrichment (e.g., running wheels) that may cause difficulty in removing animals from caging and increase chances of exposures to technicians.

• **Food:** Most studies do not add supplemental food enrichment beyond standard study protocols.
  - If food enrichment is added, details should be included in resulting publications, because food changes can affect weight loss endpoints.

**Agent Administration**
- Use the following routes of administration for specific agents and purposes:
  - Inoculation: Intranasal or catheter
  - Test article (e.g., antivirals, Abs): intranasal, intraperitoneal, oral gavage, catheter, etc.
  - Infection: intranasal (10^3 to 10^6 PFUs; microsprayer achieves similar results), catheter

**Considerations for anesthetic agents**
- Administer ketamine/xylazine intraperitoneally, sometimes in combination with isoflurane.
  - Note that animals respond variably to ketamine/xylazine anesthesia. Even after receiving identical doses, some animals remain anesthetized for 1 hour while others may remain anesthetized for only 15 minutes. Researchers should adapt their anesthetic plan according to length of anesthesia needed for required procedures. Researchers should consult with their institutional laboratory animal veterinarians if they lack experience with anesthesia in hamsters.
  - Doses of anesthesia will also differ between hamsters and mice.
  - It is not recommended to administer ketamine/xylazine once animals have been infected for two or more days, as this can lead to respiratory depression in an animal with developing respiratory pathology, potentially leading to death
    - At two days post-infection or after, administer isoflurane only (e.g., using an induction box and nose cone).
    - Isoflurane can potentially be used on its own, but because it takes longer to anesthetize animals than ketamine/xylazine, it increases the time window needed for dosing, creating logistical challenges in large studies with a lot of procedures.

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20 Fish, et al. (1997) *Anesthesia and Analgesia in Laboratory Animals*
Considerations for virus (see also Viruses section on page 1)

- Inoculate each animal with 200 µL of virus.
  - Up to $1.0 \times 10^5$ TCID50 per animal
  - Example: 100-hamster study = 20mL of virus (budget for 25 mL to account for losses and characterization)

Considerations for investigational agents

- Note that an experimental agent will likely have different dosing requirements, as well as a different pharmacokinetics profile, in hamsters compared to mice. These should be investigated in non-virus exposed pilot studies prior to entering an ABSL3 study.
- When unsure of an experimental agent’s potential mechanism of protection or expected dosing in humans, consider conducting at least two studies in parallel: one to administer the agent prophylactically and the other(s) to test the agent as a treatment.

Measurements

- Timing of measurements:
  - 4 to 5-day models work well for assessing viral load and clinical endpoints
  - Useful metrics at this point are: Weight loss, lung weight gain, viral titers in the lungs and/or nasal cavity, pulmonary histopathology, and others
  - 14-day or longer models may be useful for some test articles.
- It is recommended to take blood draws (i.e., venipuncture) through the cranial vena cava. This method is reliable and safe if researchers are well-trained, allows for repeat draws, and easily enables collection of blood volumes of 200 µL.
  - Avoid blood draws via the lateral saphenous, which has a small volume and is difficult to puncture in a biological safety cabinet. Drawing blood through the lateral saphenous can also lead to hemolysis.
  - Cardiac puncture can also be used to draw blood, but this procedure requires highly skilled personnel.

Disease Severity, Virus Titers, and Viral RNA Levels

Consider the following findings and best practices:

- Neither high nor low doses of virus have caused death in wild type hamsters, although infection with higher doses appears to cause increased weight loss; weight loss of 5 to 10% for low doses, or of 10 to 20% for high doses, occurs for approximately the first 5 days post-infection, followed by weight recovery.
  - During the same 4 to 5-day period, virus titers are high in the trachea, as well as in the lungs and nasal turbinates.
  - Both low and high passage virus (with or without furin site cleavage mutation) replicate well in the lungs, but low passage virus gives more fulminant lung pathology (Boudewijns et al., Nature Communications, in press)
  - By day 7, viral titers may have declined below the level initially administered.
    - However, viral RNA levels (both genomic and sub-genomic levels) may reach as high as $10^{10}$ copies (N) per milliliter of lung homogenate on day 2
post-infection and remain as high as $10^8$ per milliliter by day 7 (Sia et al., 2020). (Viral RNA can vary from $10^6$ to $10^8$ copies of (N) per milligram of lung at day 5 [Rogers, et al., 2020]).

- Investigators wanting to use a range of methods to assess viral titers (e.g., qRT PCR and TCID50) should divide an animal’s tissues (e.g., their lungs) into small pieces and run different assays on each piece. Existing data indicate that viral titers are correlated across different areas of infected hamsters’ lungs.\(^{21}\)

**Pathology Findings**

- Consider using micro-computed tomography (micro-CT), if accessible, to detect lung abnormalities post-infection (Boudewijns et al., *Nature Communications*, in press).
  - Imai et al., 2020 used this technique to detect patchy ground glass opacities (GGO) with a central, peribronchial distribution, which progressed from mild at day 2 post-infection to more severe, peripherally distributed, rounded, multilobe GGO with partial lung consolidation by days 7 and 8.
  - Other studies have confirmed that at day 7, infected hamster lungs appear mottled and hemorrhagic, with edema/failure to collapse.
  - Manifest pulmonary consolidations were present in SARS-CoV-2 infected hamsters (but not in STAT2/− hamsters) as well as an increase of the non-aerated lung volume (Boudewijns et al., *Nature Communications*, in press).
- Consider using hamster models to study long-term lung damage in human COVID-19.
  - CT findings showed that all infected hamsters developed a pneumomediastinum 4 to 6 days post-infection, which resolved by day 8 to day 10. This finding was unexpected and likely secondary to severe pulmonary damage, micropulmonary rupture, and gas tracking into the mediastinum.
    - Improvement of CT lung abnormalities began 8 to 10 days post-infection, with gradual decrease in GGO and lung remodeling.
    - Minimal residual lung abnormalities/remodeling (including ill-defined GGO and linear bands) remained in ten of eleven infected animals imaged on day 14 post-infection, and in six to ten infected animals imaged on day 20.
- Higher doses of SARS-CoV-2 cause more severe lung abnormalities in hamsters than lower doses.

**Histopathology**

Conducting histopathological analyses can add time and cost to a study (i.e., the tissue must be fixed, trimmed, embedded, cut, stained, and examined by a qualified pathologist), but can also provide valuable insight into a hamster model. Below are summarized results from previous histopathological analyses of hamster SARS-CoV-2 models.

\(^{21}\) Based on personal communications with investigators.
Results from Example Study:

- Severe lung lesions on day 3 post-infection, extending across larger areas for hamsters infected with high doses compared to those infected with low doses.
- At 6 days post-infection, no differences were observed in the histological changes between the lungs of animals administered high and low doses (viral RNA levels exhibit the same patterns).
- Focal inflammatory cell infiltration in the interstitium and alveolar cavity were prominent, and pulmonary edema and alveolar hemorrhage were evident in some areas of the lungs of animals infected with the high dose on day 3 post-infection, and in the lungs of animals infected with either dose on day 6.
- Viral antigen-positive cells were detected in the bronchi or the lungs—or both—of all eight animals infected with either dose on days 3 and 6 post-infection; more virus antigen-positive cells were detected in the lungs of virus-infected animals on day 3 than on day 6 post-infection.
- By contrast, at day 10 post-infection, pathological changes were mild, and no viral antigen-positive cells were detected in the lungs of animals infected with either dose. This result was consistent with that of the previously described micro-CT analysis.
- Inflammation tends to be concentrated in lung areas with higher cell densities.

At 4 days post infection, lung disease was characterized by a multifocal necrotizing bronchiolitis, massive leukocyte infiltration (Boudewijns et al., Nature Communications, in press)

Researchers who do not want to conduct histopathological analyses on their hamster models (e.g., to avoid the extra time and cost) should strongly consider measuring lung weights post-mortem as a proxy for some of the same endpoints (see Hamsters In-Life section on page 26).

Immunosuppressed Golden Syrian Hamster Model for SARS-CoV-2
Immunosuppressed Golden Syrian Hamster models, described below, have also been used.

Cyclophosphamide Transient Model
A cyclophosphamide (CyP) transient, partial immunosuppression model has been used to test wild type female Golden Syrian hamsters, aged 6 to 8 weeks (Brocato et al., JVI, 2020).

- Isoflurane was administered along with intranasal challenge of virus (10^2 to 10^5 PFUs) and intraperitoneal CyP injections.
- CyP injections continued for approximately 21 days post-infection, during which time animals lost weight, which was gradually recovered after CyP injections ceased (measured up to day 35 post-infection).
- Viral RNA levels measured using pharyngeal swabs were relatively high but did not generally vary with dose in this model.
**Rag2 Knockout Model**
A Rag2 knockout Golden Syrian hamster model with no functioning B or T cells has also been used to study SARS-CoV-2 infection using $10^4$ PFU intranasal injections.

- In this model, animals died a median of 6 days post-infection.
- Viral replication occurred mainly in animals’ tracheas and lungs.

**STAT2 Knockout Model**
Virus administered intranasally to STAT2 knockout hamsters resulted in substantially reduced tissue pathology in the lungs compared to wild type hamsters administered the same strain of SARS-CoV-2, despite the fact that viral replication in the lungs of the STAT2 knockout hamsters was substantially elevated compared to that in wild type hamsters.

**Measurements Used in Hamster Models**
Investigators using hamster models to study SARS-CoV-2 infection have used the following in-life and post-mortem measurements to characterize outcomes:

**Hamsters In-Life**
- Body weight
- Nasal washes (with anesthesia)
- Nasal swabs for viral load (qRT PCR, TCID50)
  - Maxapplicator’s™ Ultra Fine swab (0.5 mm) fits into the nose of a hamster (but not of a mouse).
- CT (non-invasive imaging with anesthesia)
  - The ability to conduct CT imaging in BSL3 facilities is limited
- Clinical observations (limited use)
- Blood samples for pharmacokinetics (PK)
- Body temperature changes (not recommended: very limited use)
- Note that many of the inflammatory cytokine panels that are commonly used in rat and mouse models do not work in hamsters.\(^{22}\)

**Hamsters Post-Mortem**
- Viral titer
  - TCID50
- Viral RNA levels (qPCR)
  - Predominantly using N levels to also assess sub-genomic RNA
- Histopathology
  - Hematoxylin and eosin staining
  - Cell marker staining
  - Viral antigen staining
  - In situ hybridization for viral RNA
  - Immunohistopathology

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\(^{22}\) [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3085612/](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3085612/)
• Gross necropsy
  o Lung weights
    ▪ A good proxy metric for hemorrhage and inflammation. Healthy hamster lungs compose approximately 0.5% of body weight, but 7 days post SARS-CoV-2 infection, this percentage can double or nearly triple.
• Bronchoalveolar lavage fluid (BALF)
  o Inflammatory cytokines
  o Cell type profiles
• Tissue for test article (TA) quantification

Example: A Hamster Model Used to Test Oral Antivirals against a Milder SARS-CoV-2 Strain

Background: Investigators using a Belgian isolate of SARS-CoV-2, which originated in a person traveling from Wuhan, passaged the strain and found a deletion just upstream of the furin cleavage site on the sixth passage. They used virus from this sixth passage, which was less pathogenic for hamsters than virus from previous passages, for antiviral studies because no highly efficacious antivirals are yet available for testing against SARS-CoV-2. After administering $10^5$ PFUs of this strain to hamsters, researchers observed forms of lung pathology such as peri-bronchial inflammation, necrotizing bronchiolitis, bronchopneumonia, apoptotic bodies in bronchi walls, peri-vascular inflammation and edema, and bronchopneumonia. Replication of this viral strain was highest within the lungs, but some replication was also observed in both the ileum and the stool; and although hamsters lost weight for 2 days post-infection, their weight more than recovered by day 4 (i.e., right when viral replication in the lungs peaked).

Methods: Investigators have used this model to test both hydroxychloroquine and Avigan (an antiviral) in combination with azithromycin:

• Hamsters were simultaneously infected intranasally with SARS-CoV-2 and administered first treatment.
• Hamsters were treated twice daily before being euthanized on day 4.
• Investigators collected lung, ileum, and stool samples from the euthanized animals.

Results:

• In comparison to hydroxychloroquine, high doses of Avigan (i.e., 1,000 mg/kg) led to substantially reduced viral titers and RNA copies, and low doses (i.e., 600 mg/kg) led to reduced viral titers.
  o The higher Avigan dose also led to a 172-fold reduction in relative infectivity, whereas the lower dose led to a 136-fold reduction in relative infectivity compared to untreated hamsters or those treated with hydroxychloroquine.
• Progressively higher doses of Avigan in hamsters is associated with more robust pharmacokinetics, as well as milder lung pathology (based on cumulative lung scores, measured using histopathological analyses).
  o Hydroxychloroquine, by contrast, had no effect.
• Another study of this model using 1,000 mg/kg doses of Avigan indicates that the agent offers sentinel hamsters complete protection against transmission of SARS-CoV-2 from infected animals.
• Note that 600 and 1,000 mg/kg doses of Avigan were administered via intraperitoneal injection, whereas a lower dose of 300 mg/kg was administered orally.
  o Oral administration of lower doses led to increased weight loss during the first 4 days post-infection, compared to higher doses administered via intraperitoneal injection.
    ▪ Investigators hypothesized that the volume of agent administered orally may have inhibited eating behavior.
• This model has also been used successfully to evaluate the therapeutic efficacy of neutralizing antibodies.23

Ferrets
Relative to other animal models, ferrets present both advantages and disadvantages for study of respiratory viruses such as influenza and SARS-CoV-2. In general, two advantages of using ferrets rather than hamsters or mice are that (1) ferrets are larger, so pathologies of interest may be more visible, and (2) SARS-CoV-2 infection in ferrets is relatively contained to the upper respiratory tract, which could be desired for certain studies.

Ferrets are generally better suited as models of virus transmission or replication than of clinical disease or of response to therapeutics. However, ferret models are suited for testing the efficacy of mucosal vaccines and therapeutics aimed at preventing upper respiratory tract replication and transmission of SARS-CoV-2, and they are also generally good model systems for studying mild COVID-19.

Ferrets are a U.S. Department of Agriculture (USDA)-regulated species, which means that more paperwork is required to initiate a ferret model study than to initiate a mouse model study (i.e., standards regulating the care of ferrets are higher than they are for mice)

The lists below contain transferable insights from influenza research in ferrets, as well as known advantages and disadvantages of using ferrets to study SARS-CoV-2.

Advantages in the Context of Influenza Research
• Ferrets are susceptible to natural infection with human influenza viruses.

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23 [https://science.sciencemag.org/content/early/2020/09/23/science.abe3354](https://science.sciencemag.org/content/early/2020/09/23/science.abe3354)
Ferrets must be acquired from specific vendors that adequately restrict the animals’ contacts with humans, because if a ferret acquires influenza from a human before a study, it may seroconvert and then be more difficult to infect.

For COVID-19 research, investigators should test ferrets for the presence of SARS-CoV-2 antibodies before conducting their experiments.

- Ferrets can be experimentally infected with virtually any strain of influenza virus (contra mice).
- Ferret models resemble humans in several crucial ways:
  - Ferrets’ susceptibility to influenza virus strains of different virulence appears to correlate with that of humans (e.g., avian highly pathogenic influenza viruses and 1918 strains are lethal to ferrets, whereas seasonal human strains are generally non-lethal).
  - Ferrets’ upper and lower respiratory tract resembles that of humans in its distribution of sialic acid receptors.
  - The ability of ferrets to model human SARS-CoV-2 infection will depend on the distribution of ACE2 receptors within the animal’s respiratory tract.
- Ferrets sneeze and transmit influenza viruses by direct contact and by aerosol.
  - Cages have been designed to help measure the transmission of influenza viruses from infected to noninfected ferrets.
- Ferrets transmit mammalian influenza viruses easily, but not avian influenza viruses; they are used together with guinea pigs as a mammalian transmission model for influenza.
  - Researchers have used ferrets to determine how well-adapted an avian influenza virus strain is to transmission among mammals, and thereby to assess the degree of risk, from a given influenza strain, of a pandemic.
- Ferrets have traditionally been used to generate strain-specific antisera for influenza virus antigenicity studies.

Disadvantages in the Context of Influenza Research

- Ferrets are more expensive than other small animals such as mice and hamsters.
- Ferrets are more complicated and expensive to use than other small animals, and therefore the number of animals per experiment (typically 3 to 6) is usually smaller.
- Ferrets require larger and more specialized laboratory spaces as well as more experienced personnel.
- Ferrets exhibit substantial individual variability; no inbred strains are currently available.
- Limited reagents exist for use in ferret studies (e.g., although consortia are working to develop reagents for immunological characterization of ferrets, no reagents now exist for evaluation of ferret mycobacterium avium complex [MAC] or tetramer staining).

Known Advantages in the Context of SARS-CoV-2 Research

- Ferrets can be experimentally infected with SARS-CoV-2 (contra mice, which require either ACE2 receptor transgenes or mouse-adapted virus strains to become infected).
- Results of ferret-based SARS-CoV-2 studies have been consistent across laboratories.
• Ferrets transmit SARS-CoV-2 to research naïve animals, making them a suitable model for viral transmission.
• Infection of ferrets with SARS-CoV-2 results predominantly in upper respiratory tract infection, with viral replication also present in the lungs and the gastrointestinal tract (mimicking the pattern of viral replication in humans).
• Virus is detectable in nasal washes of infected ferrets, allowing viral material to be reliably removed without euthanasia and thus enabling longitudinal measurement of viral replication.

Known Disadvantage in the Context of SARS-CoV-2 Research
• Clinical alterations in ferrets, following mucosal exposure to SARS-CoV-2, are mild or undetectable.

Additional Considerations when Using Ferret Models
Below are brief descriptions of important considerations for investigators using ferret models to study respiratory viral infections such as SARS-CoV-2.

Age, Sex, and Number
• Select what ages of ferrets to use, especially because age affects variables such as susceptibility to disease.
  o In both influenza and SARS-CoV-2 infection, older animals appear to be more susceptible.
  o Young adult ferrets (i.e., ferrets that have just become sexually active) are most often used.
  o Researchers interested in studying aged ferrets typically use animals that are between 1 and 2 years of age.
• Conduct ferret experiments using castrated males and spayed females; ferrets may also be descended through removal of their anal scent glands, but this practice is not standardized.
  o Some vendors also offer (for an extra charge) ferrets that have already been castrated or spayed, or descended, or both.
• Weigh the tradeoffs between the increased cost and complexity of using more ferrets versus the need to obtain robust evidence for endpoints such as viral replication and infiltration into the lungs.

Route of Administration
• Most ferret respiratory viral disease models administer virus intranasally, although some use either intratracheal or aerosol administration, largely based on the previous experience of the facility conducting the study.
• Intranasal infection of SARS-CoV-2 appears to result in consistent infection profiles in ferrets, though aerosol administration may result in a more robust infection phenotype by delivering virus deeper into the lower respiratory tract.
**Tissue Collection**

- Ferret tissue should be collected by removing small tissue samples (as opposed to removing whole lungs, as is done with mice).
  - Challenge: This smaller sampling approach could lead to artifactual pathology differences (e.g., between laboratories or between animals) that would not appear in assessments of viral titers or other endpoints in whole lungs.

**Vaccinations**

Some U.S. states and research facilities prohibit the importation or use of ferrets that have not been vaccinated against certain infections (e.g., canine distemper). Researchers should learn of such restrictions and identify ferret vendors that can supply approved animals. However, they should also consider the possibility that a previous vaccination for any viral infection may result in an enhanced immune response that could affect the outcomes of ferret studies of SARS-CoV-2.