ANIMAL CARE AND USE STANDARD

The Animal Care & Use Standards are designed to provide guidance regarding good practice to institutional animal users and carers, as well as Animal Ethics Committees (AECs), on the care and use of animals for scientific purposes such as research and teaching. The Standards are evidence-based, reflecting current or accepted good practice and allow for the flexibility that is required in research and teaching activities using animals.

INFECTION OF MICE WITH INFLUENZA VIRUS

This standard has been developed by the University of Melbourne Animal Care & Use Standards Committee, and endorsed by the University of Melbourne Animal Welfare & Ethics Committee.

V1 Date of Approval:	11 July 2016	**Date of Review:**	11 July 2016

1. ASSOCIATED STANDARDS

This standard should be read in conjunction with the following University of Melbourne Animal Care & Use Standards:

- General anaesthesia of mice and rats

2. SUMMARY

Influenza virus is a highly contagious respiratory pathogen causing outbreaks each winter, frequently of epidemic proportion, and, much more rarely, pandemics. Mice are not the natural host of influenza virus but can be infected with some influenza strains and have proven to be very useful models for the study of viral immunity and pathogenesis, and in the first stages of vaccine and drug testing.

3. BENEFITS & RISKS

3.1 Not all strains of influenza can replicate in mice and so testing of recent human isolates, with the exception of the 2009 pandemic (pdmH1N109) virus, can be problematic.

3.2 Drug and vaccine manufacturing companies now require data in other animal models before deciding to take products forward for development but mice can be used by the researcher as a first assessment tool.

3.3 The ferret is the animal of choice to study influenza as it has the same receptor for the virus in its respiratory tract and therefore can support the replication of all human strains of virus. Ferrets also show similar disease signs to humans. This animal model is restricted, however, by the availability of animals and of reagents suitable for immune cell assessment, although these are gradually becoming available.

3.4 Mice, in contrast to ferrets, generally do not transmit influenza virus through the air, although contact transmission is observed with some viral strains. This lessens the human safety risk of working with the virus in mice but it should be noted that the virus will survive on fomites for long periods. This Standard does not address safety and procedural aspects of influenza in ferrets.

3.5 Highly pathogenic strains of virus of avian origin that replicate outside the respiratory tract (eg H5N1) should never be used in mice at this University as they require BSL3+ containment.

4. PROCEDURE/PROTOCOL

4.1 Infection procedure

4.1.1 Infection should be performed only by persons trained to a suitable level of competency.

4.1.2 To establish a productive infection, infectious virus is delivered by the intranasal route with a micropipettor, dropping the virus directly onto the nares of a firmly scruffed mouse in a horizontal position. This is typically done under light isofluorane anaesthesia with virus in a maximum volume of 50 µl and results in exposure of the virus to the entire respiratory tract, resulting in pulmonary infection. Mice should be anaesthetised sufficiently and the procedure performed quickly so that the virus dropped onto the nares is breathed in rather than expelled as the mouse wakes.

4.1.3 A robust assay for the measurement of infectious units of influenza virus, such as a plaque assay, should be used in order to accurately determine the appropriate dose noting that a commonly used sublethal dose may be only two-fold less than a lethal dose when given by this route.

4.1.4 Alternatively, virus infection can initially be limited to the upper respiratory tract by delivery of virus in a maximum volume of 10 µl to an animal when awake. Some virus strains will then progress down the respiratory tract.

4.1.5 Virus can also be delivered by alternative routes, such as intraperitoneally, which results in an abortive infection, with at most only one round of replication in cells outside the respiratory tract. This is typically used to prime immune responses in the absence of clinical infection.

4.2 Diversity of outcomes according to virus strain and mouse strain

4.2.1 The effect of influenza infection will vary with strain of virus, strain of mouse, age of mouse, dose of virus, mode of infection.

4.2.2 Respiratory tract infection is acute and, depending on viral strain and dose, virus may be cleared in immunocompetent mice within 7-10 days. In immunodeficient animals this period may be longer and a sub-lethal dose may be lethal.

4.2.3 If using unfamiliar mouse strain/viral strain combinations, a pilot experiment should be performed where mice are monitored at least daily and clinical signs and weights determined. The frequency of monitoring must be escalated in line with Section 5 - Intervention Criteria for mild-moderate influenza infection. Data from such experiments will improve confidence of animal wellbeing impacts and support adjustments to monitoring frequency if appropriate.

4.2.4 Some infections can be subclinical but most can be classified as mild-moderate or severe for the purposes of intensity of monitoring. The following Table of clinical signs must be used when determining the need for interventions and the degree of severity of an influenza strain in the particular mice under study.

	Mild to Moderate Influenza Infection	Severe Influenza Infections
Appearance	Slight to moderate piloerection with no dehydration (skin tenting); slight evidence of decreased grooming, slight ruffled or greasy appearance.	Piloerection with clear evidence of dehydration (skin tenting); poor coat condition (ruffled and greasy fur); not grooming; hunched.
Behaviour, activity, response to provocation	Subdued but responsive, decreased activity or decreased interaction with peers.	Lethargic/dull; inactive; unresponsive or minimally responsive to activity and provocation.
Altered respiratory pattern/depth	Very slightly altered rate and depth of respiration without other signs consistent with pain or distress.	Obviously altered rate and depth of respiration; may be accompanied by signs consistent with pain or distress.
Weight changes	<20% compared to weight at start of experiment.	Significant weight decrease of ≥20% compared to weight at start of experiment.

4.2.5 Typical influenza viruses used for research purposes include:

i) A/Puerto Rico/8/34 (PR8): H1N1 subtype, originally a human isolate but mouse adapted; lethal to BALB/c mice at doses above 100 plaque forming units (pfu) unless mice prevaccinated or infected. Note that PR8 is lethal to C57Bl6 at doses <100pfu due to the greater susceptibility of these mice to this strain.

ii) X-31 virus: a virus with surface antigens from an H3N2 subtype virus with all internal proteins from PR8; much less severe than PR8 but causes significant weight loss in some mouse strains

iii) Early H3N2 viruses: non-lethal infections ranging from subclinical to moderate severity, typically used at a dose of $10^{4.5}$ pfu.

iv) 2009 pandemic virus (pdmH1N109): severe infection in mice and some humans, must be used with caution as researchers not necessarily immune despite vaccination.

4.2.6 Mice can lose considerable body weight after infection, as much as 25-30% for some virus-mouse strain combinations, and yet still recover once infection starts to be cleared. Weight loss correlates with disease severity and, in mice that are otherwise active and engaged in normal mouse behaviours, the AEC has agreed humane endpoints for weight loss criteria alone of 20% maximum loss for mild-moderate influenza infections and 25% maximum loss for severe influenza infections when compared with pre-experimental body weight. It should be noted that this weight loss of >25% is only acceptable under specific conditions and would only be permitted with appropriate scientific justification and consideration by the AEC.

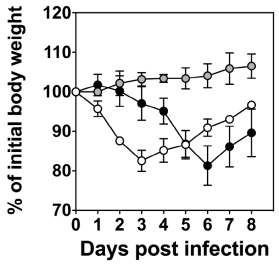
Figure 1. Weight loss in BALB/c mice following infection with 3 strains of influenza virus

Grey symbols = a mild seasonal H3N2 influenza at high dose ($10^{4.5}$ pfu)

Black symbols = sublethal dose of PR8 (50 pfu). Note >100pfu is lethal.

White symbols = X-31 virus at high dose (10^{4} pfu)

Virus delivered under anaesthetic to the total respiratory tract.



5. MONITORING & INTERVENTION

5.1 Monitoring

5.1.1 Once infected with influenza virus, mice should be monitored daily and observations recorded on a monitoring sheet appropriate for either mild-moderate infections or severe infections. It is not necessary to weigh mice daily during the course of mild-moderate infections but a starting weight should be taken in case the infection becomes more severe than expected and intervention is called for. Mice must be weighed daily during severe infections and must be monitored twice daily around the expected humane endpoint.

5.1.2 Monitoring score sheets should include the following clinical signs. Degrees of severity of some signs may also be used for severe infection monitoring.

Table 1. Clinical signs to be included on Monitoring sheets

Q	Inactive
H	Hunched
R	Ruffled
G	Greasy fur
LB	Laboured breathing
A	Isolated
D	Appears dehydrated
WL	Weight loss

5.1.3 Once the virus has been cleared and weight has been regained (>10 days post infection) monitoring frequency may be reduced.

5.2 Humane endpoints

5.2.1 The following humane endpoints have been agreed to by the AEC for adoption for all experimentation where severe infections are expected or the outcome is unknown. It should be stated in the AEC application that mice can lose considerable body weight after infection and yet still recover once infection starts to be cleared. Where doubt exists regarding the humane endpoint classification of an animal, a second opinion must be sought from the Animal Facility Manager (AFM) or Animal Welfare Officer (AWO).

5.2.2 The following text can be copied and pasted into monitoring sheets and used in relevant applications:

"Humane endpoints and actions:

1. *Euthanasia of mice with laboured breathing or where their condition has lead to an inability to eat or drink (eg. severe hunching, inactivity), irrespective of weight loss.*
2. *Euthanasia of mice where weight loss of 25% of their pre-experimental body weight is detected, or absolute weight has dropped to 15 grams.*
3. *Euthanasia of mice where weight loss of 20-24% of their pre-experimental body weight is detected over a 2 day period and is accompanied by one or more other clinical signs comprising isolated, inactive, hunched, very greasy or ruffled fur.*
4. *Mice must be monitored twice daily around the time of expected endpoint for that strain of mouse and virus."*

5.2.3 A clinical score sheet can also be used to capture additional information on the degree of disease severity for research purposes but should not be used to determine humane endpoints.

5.2.4 Humane endpoint 3 is relevant where mice are undergoing antiviral treatment as these can remain otherwise well despite weight loss of 20-25%.

5.2.5 As weight loss is an important and reliable measure of humane endpoint, researchers should endeavour to use mice of matched weights of at least 20 grams in their experiments to provide consistent results.

5.2.6 Mice with laboured breathing must never be killed by CO_2 inhalation, due to an unacceptable risk of continued and prolonged distress in animals with already compromised respiratory function. Alternatively, these mice should be killed by injection of sodium pentobarbitone (intraperitoneal or intravenous). See the "Humane killing of mice and rats" Standard for additional information.

5.2.7 These endpoints serve as the minimum welfare standard for severe influenza infection models. Earliest possible endpoints must always be applied. Individual projects may warrant the establishment of additional humane endpoints.

Table 2. Recommended Intervention criteria for Influenza studies using mice

Intervention criteria for mild-moderate influenza infections	
Observations	***Action required***
Normal (√ on monitoring sheet)	None (daily observations)
Slightly greasy or ruffled fur but otherwise fully active	None (daily observations)
Decreased activity combined with slightly greasy or ruffled fur	Daily weighing of mice
Weight decrease of ≥15% compared to weight at start of experiment	Increase frequency of observations to twice daily
Weight decrease of ≥20% compared to weight at start of experiment OR appears dehydrated	Unexpected adverse event: euthanasia if meets criteria for humane endpoint or euthanasia on advice of animal facility manager (AFM) or animal welfare officer (AWO)
2 or more of the following: inactive, hunched, ruffled, isolated or has greasy fur	Unexpected adverse event: euthanasia if meets criteria for humane endpoint or euthanasia on advice from the AFM or AWO

Intervention criteria for severe influenza infection	
Observations	***Action required***
Normal (√ on monitoring sheet)	None (daily observations including weight)
1 or more moderate signs or weight decrease of ≥15% compared to weight at start of experiment	Increase frequency of observations to twice daily
Weight loss of 20% or more of their pre-experimental body weight detected over a 2 day period and is accompanied by one or more other clinical signs comprising isolated, inactive, hunched, very greasy or ruffled fur	Euthanasia if meets criteria for humane endpoint. If in doubt about clinical signs seek a second opinion from the AFM/AWO.
Weight loss of 25% of their pre-experimental body weight is detected or absolute weight has dropped to 15 grams	Euthanasia
Laboured breathing, irrespective of weight loss	Euthanasia. If in doubt seek a second opinion from the AFM/AWO.
Inability to eat or drink (eg. severe hunching, inactivity), irrespective of weight loss	Euthanasia. If in doubt seek a second opinion from the AFM/AWO.

6. ADDITIONAL INFORMATION

- N/A

7. ENFORCEABLE REQUIREMENTS

7.1 Appropriate monitoring for disease severity.

7.2 Determination of disease severity for new virus strains or mouse strains if unknown.

7.3 Adherence to humane endpoints.

7.4 Mice with laboured breathing must never be killed by inhalation of gaseous agents (e.g. CO_2)

8. EXEMPTIONS

Where adherence to this Standard conflicts with proposed work, the University's AECs may grant exemptions to all or part of the Standard. To seek exemption, applications should clearly outline how the proposed work deviates from the Standard, and justify the need for this. Before seeking exemption, it is recommended that you consult with the University's AWO.

9. UNEXPECTED ADVERSE EVENTS

An unexpected adverse event is any event, which impacts negatively on the wellbeing of animals, and which was not anticipated, or has occurred at a frequency or severity in excess of what was anticipated in line with the AEC approval. This can be a single or cumulative event, and will normally involve unexpected mortality, morbidity or injury. Anyone identifying an unexpected adverse event must act to remove and/or minimise any immediate risk to animals. Immediately thereafter, the University's AWO and relevant AFM must be notified of the event. The AWO will advise researchers of the appropriate response.

10. GLOSSARY

Scientific Term	Lay Description
Immunodeficient	Having a deficit in one or more innate or adaptive immune components
Immunosufficient	Having a normal immune response
Fomites	An inanimate object that can carry infectious biological agents
Pandemic	A world wide epidemic due largely to lack of pre-existing immunity
Strain	A variant of the virus in common circulation

11. REFERENCES & RESOURCES

- Beausoleil, N.J. and Mellor, D.J. (2015). Introducing breathlessness as a significant animal welfare issue. *New Zealand Veterinary Journal,* 63(1): 44-51 (DOI: 10.1080/00480169.2014.940410).