 <p>THE UNIVERSITY OF QUEENSLAND AUSTRALIA CREATE CHANGE</p>	<p>UQ Animal Ethics Committee - Standard Operating Procedure  <b>LAB_008 Euthanasia - Carbon Dioxide Asphyxiation in Mice and Rats</b>          Institutional author: <b>UQ Biological Resources</b>          AEC Reviewed &amp; Approved: 18/02/2021</p>	<p>Version #3</p>
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## LAB\_008 Euthanasia - Carbon Dioxide Asphyxiation in Mice and Rats

### I. OBJECTIVE

To promote safe and humane euthanasia of mice and rats by carbon dioxide asphyxiation, as per Clause 3.3.45 of the Australian Code for the care and use of animals for scientific purposes.

**NB: The use of (\*) indicates this statement is dependent on the facility procedures**

**NB: The use of (\*\*) indicates this statement is dependent on AEC Approvals**

### II. COMMENTS / RECOMMENDATIONS

- Consideration must be taken as to the potential for stressful auditory, visual or olfactory stimuli that may be perceived by other animals. Efforts must be made to isolate these potential stressors:
  - Euthanasia or laboratory rodents should only occur in “terminal procedure rooms”(\*),
  - The terminal procedure room must have sufficient ventilation, else biosafety cabinets or fume hoods should be used for this procedure,
  - Ensure the area is cleaned prior to use, and between animals,
  - Different species (i.e. rats and mice) should not be euthanised in the same area at the same time.
- Wherever possible this procedure should be performed with the animals remaining in their home cage, rather than transferring them into an unfamiliar euthanasia chamber.
- The rate of carbon dioxide (CO<sub>2</sub>) exposure to rodents (i.e. the flow rate) must be controlled.
  - Low CO<sub>2</sub> flow rates, relative to chamber volume (e.g. <20% displacement/minute), must be avoided because they will result in a prolonged periods of respiratory distress (i.e. “air hunger”). Therefore it is important to know the delivery chamber volume and to use a flow regulator.
  - Placing rodents directly into “pre-filled” high concentrations of CO<sub>2</sub> (e.g. >90%) must be avoided because it will cause pain to the animals. Therefore it is important to ensure that any waste gas is discarded when cleaning the chamber between animal groups.
- This procedure is appropriate for use in pregnant dams, and will result in the humane death of foetuses.
- Adverse events must be managed as per LAB\_022 UQBR Veterinary Care Program

#### In relation to human safety:


- Carbon dioxide should never be used without adequate ventilation/air flow
- The operator must ensure they are aware of all relevant safety procedures (including facility and procedure specific PPE requirements)
- All accidents, injury or near misses are to be reported immediately to the Facility Manager and recorded on a UQ OHS Incident Report Form.

### III. EQUIPMENT

- PPE(\*)  
*Although PPE is facility dependent, minimum expectations include: disposable gloves, clean log-sleeved laboratory gown, hair bonnet, eye protection, face mask.*
- Home cage enclosure (\*)
- Paper towel
- CO<sub>2</sub> delivery chamber:
  - This may be the home cage using a modified lid (that permits CO<sub>2</sub> delivery);
  - or a specific euthanasia chamber (of a defined volume)
- CO<sub>2</sub> supply/outlet unit (with flow regulator)
- Cadaver bag

#### Conditions:

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## IV. PROCEDURE

### Adult rodents

1. Remove the home cage lid.
2. If delivering CO<sub>2</sub> to rodents in their home cage replace the home cage lid with the modified home cage lid. Alternatively, retrieve the rodent(s) from the home cage and place them into the specific euthanasia chamber, securing the lid.

*Overcrowding must be avoided - rodents should be able to freely move around within the delivery chamber.*

*Refer to the following SOPs when handling rodents: LAB\_006 Handling and Restraint in Mice and Neonates and LAB\_039 Handling and Restraint in Rats and Neonates.*

3. Start CO<sub>2</sub> delivery at a flow rate  $\geq 20\%$  of the chamber's volume per minute, but not greater than 40% (see table 1. Guiding information relative to CO<sub>2</sub> flow rates in rodents, within V. REFERENCE INFORMATION). Assess the flow regulator to ensure gas is flowing at the appropriate pre-determined rate.

*For example, when using a 6L container this means the flow rate of CO<sub>2</sub> entering the chamber should be set to at least 1.2L/min (i.e. 6L x 0.2/min = 1.2L/min).*

4. Monitor the animals while the chamber is filling for normal behavioural responses. If the progression of behavioural responses is considered abnormal, e.g. if animals have protracted responses immediately ensure the lid is secure and that gas is flowing at the appropriate rate (as per steps 2-3). If no obvious cause for the abnormal response can be identified and resolved promptly, implement an alternative humane method of killing rodents (e.g. LAB\_007 Euthanasia - Cervical Dislocation in Mice and Rats; LAB\_011 Euthanasia - Lethal Injection in Mice and Rats).

*Normal behavioural responses, that should be expected to occur in succession: unremarkable behaviour, excitability (increased activity), elevated respiratory rate, ataxia (staggers), ventral recumbence with elevated respiratory rate, unconsciousness (unresponsive to stimuli), recumbence with reduced respiratory rate, erratic respiratory rate (agonal breathing), loss of spontaneous respiration. These changes should occur within 5 minutes of CO<sub>2</sub> supply.*

5. Once the chamber has reached 100% CO<sub>2</sub> concentration do not reopen the cage for at least 5 minutes.
6. Following this period, remove the chamber lid and confirm death in the rodents, as per Table 2. Indicators of death in laboratory rodents (see V. REFERENCE INFORMATION). If there is any hesitation in confirming death, repeat steps 3-5, alternatively immediately implement an alternative humane method of killing rodents (e.g. LAB\_007 Euthanasia - Cervical Dislocation in Mice and Rats; LAB\_011 Euthanasia - Lethal Injection in Mice and Rats).
7. Once death is confirmed, ensure CO<sub>2</sub> has been turned off, place the cadaver(s) into the cadaver bags and dispose of them as appropriate(\*)
8. Discard the home cage for cleaning, as appropriate (\*). If euthanasia was performed using a specific euthanasia chamber ensure the chamber is cleaned and that any potential remaining waste gas has been removed prior to reuse.

*Carbon dioxide is heavier than the ambient air and will pool in undisturbed, deep spaces. This must be considered when cleaning a specific euthanasia chambers to ensure all waste gas is removed.*

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## Neonatal rodents




Neonate procedures are to be followed as per adult mice, however, there are few notable differences:

- Sealed bags may be used as an alternative option to the home cage or specific euthanasia chamber
- Once the chamber is filled to 100% CO<sub>2</sub>, neonates must be left undisturbed within the high concentration gas chamber for a prolonged period, relative to adults. These prolonged periods are represented in Table 3. Minimum time of 100% carbon dioxide exposure to confirm death in mice and rats (within V. REFERENCE INFORMATION).

## V. REFERENCE INFORMATION

**Table 1. Guiding information relative to CO<sub>2</sub> flow rates in rodents.**

*The rate of carbon dioxide (CO<sub>2</sub>) exposure to rodents (i.e. the flow rate) must be controlled. CO<sub>2</sub> flow rate should be administered ≥20% or more, but not greater than 40% (chamber volume displacement per minute). [It is noted that some recent literature supports a much wider range of acceptable CO<sub>2</sub> flow rates, however, the number of studies and the limitations of their findings does not provide sufficient evidence to be confident of the associated welfare impact to rodents].*

Type of carbon dioxide delivery chamber used	Delivery chamber volume (approximate)	Minimum CO <sub>2</sub> flow rate to ensure ≥20% chamber volume displacement/ minute
<b>Conventional wire top mouse cage</b> 	<b>Variable(*), usually these are 4-7L</b>	<b>1-1.5L/minute</b>
<b>OptiMICE cage</b> 	<b>6L</b>	<b>1.5L/minute</b>
<b>Tecniplast Greenline mouse IVC</b> 	<b>7L</b>	<b>1.5L/minute</b>

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<p><b>Conventional wire top rat cage</b></p>		<p><b>Variable(*), usually these are ~20L</b></p>	<p><b>4L/min</b></p>
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**Table 2. Indicators of death in laboratory rodents: all indicators must be observed to confirm death**

Criteria	Confirmation
<b>Absence of eye reflexes</b>	<p>Absence of a corneal reflex: Place gentle pressure directly to the eyeball over the cornea. The eye should be unresponsive and the eyelids should not blink. NB: This should only be performed in an unconscious animal.</p> <p>Additionally, the eyes and eyelids must be unresponsive, and the eyes should appear “glazed” with fixed-dilated pupils.</p>
<b>Absence of spontaneous, rhythmic breathing</b>	<p>There is a complete lack of breathing and respiratory movements.</p> <p>Deeply anaesthetised animals may exhibit shallow and irregular breathing, which must not be confused with a lack of spontaneous breathing. Thus, confirmation of a lack of spontaneous breathing requires astute monitoring and must not be used as sole criteria for confirming death.</p>
<b>Absence of a rhythmic heart beat</b>	<p>Asystole is confirmed via direct thoracic auscultation or palpation.</p> <p>This judgement may be assisted via observation of mucosal membrane discolouration, absence of ECG or pulse oximetry conduction.</p>
<p>If there is any hesitation in confirming the above criteria a secondary method of euthanasia must be performed. For example lethal injection, cervical dislocation, decapitation, *bilateral thoracotomy, *resection of the heart and or lungs, *exsanguination and or *cardiac perfusion.</p> <p>*indicates techniques that are not appropriate in a conscious animals – they require that the animal has lost its righting reflex (e.g. unconscious, lying on its side) AND withdrawal reflexes (e.g. toe pinch withdrawal).</p>	

**Table 3. Minimum time of 100% carbon dioxide exposure to confirm death in mice and rats.**

AGE	MICE	RATS
<b>Non-haired pups 0–6 days</b>	60 minutes	40 minutes
<b>Haired pups, eyes closed 7–13 days</b>	20 minutes	20 minutes

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<b>Haired pups, eyes open, pre-weaning 14–20 days</b>	10 minutes	10 minutes
<b>Weanlings and adults 21+ days</b>	5 minutes	5 minutes

## VI. BIBLIOGRAPHY

AVMA. (2020). AVMA guidelines for the euthanasia of animals: 2020 Edition. American Veterinary Medical Association (AVMA) Retrieved from <https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf>

Boivin, G. P., Bottomley, M. A., Dudley, E. S., Schiml, P. A., Wyatt, C. N., & Grobe, N. (2016). Physiological, Behavioral, and Histological Responses of Male C57BL/6N Mice to Different CO<sub>2</sub> Chamber Replacement Rates. *Journal of the American Association for Laboratory Animal Science* : JAALAS, 55(4), 451-461.

Boivin, G. P., Bottomley, M. A., Schiml, P. A., Goss, L., & Grobe, N. (2017). Physiologic, Behavioral, and Histologic Responses to Various Euthanasia Methods in C57BL/6NTac Male Mice. *Journal of the American Association for Laboratory Animal Science* : JAALAS, 56(1), 69-78.

Charbonneau, R., Niel, L., Olfert, E., von Keyserlingk, M., & Griffin, G. (2010). CCAC guidelines on: euthanasia of animals used in science. Canadian Council on Animal Care (CCAC). Retrieved from <https://www.ccac.ca/Documents/Standards/Guidelines/Euthanasia.pdf>

Chisholm, J. M., & Pang, D. S. J. (2016). Assessment of Carbon Dioxide, Carbon Dioxide/Oxygen, Isoflurane and Pentobarbital Killing Methods in Adult Female Sprague-Dawley Rats. *PLOS ONE*, 11(9), e0162639. doi:10.1371/journal.pone.0162639

Donovan, J., & Brown, P. (2006). Euthanasia. *Current protocols in immunology*, Chapter 1, Unit 1.8. doi:10.1002/0471142735.im0108s73

Moody, C. M., Chua, B., & Weary, D. M. (2014). The effect of carbon dioxide flow rate on the euthanasia of laboratory mice. *Lab Anim*, 48(4), 298-304. doi:10.1177/0023677214546509


Muñoz-Mediavilla C, Cámara JA, Salazar S, et al. (2016). Evaluation of the foetal time to death in mice after application of direct and indirect euthanasia methods. *Laboratory Animals*. 50(2), 100-107. doi:10.1177/0023677215600626

NHMRC. (2008). Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes: The Assessment and Alleviation of Pain and Distress in Research Animals. National Health and Medical Research Council (NHMRC).

NHMRC. (2013). Australian code for the care and use of animals for scientific purposes, 8th edition. National Health and Medical Research Council (NHMRC).

Pritchett, K., Corrow, D., Stockwell, J., & Smith, A. (2005). Euthanasia of Neonatal Mice with Carbon Dioxide. *Comparative Medicine*, 55(3), 275-281.

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Pritchett-Corning, K. R. (2009). Euthanasia of neonatal rats with carbon dioxide. *Journal of the American Association for Laboratory Animal Science* : JAALAS, 48(1), 23-27.

Turner Patricia V., Hickman Debra L., van Luijk Judith, Ritskes-Hoitinga Merel, Sargeant Jan M., Kurosawa T. Miki, Agui Takashi, Baumans Vera, Choi Woo Sung, Choi Yang-Kyu, Flecknell Paul A., Lee Byeong H., Otaegui Pedro J., Pritchett-Corning Kathleen R., Shimada K. (2020). Welfare Impact of Carbon Dioxide Euthanasia on Laboratory Mice and Rats: A Systematic Review. *Frontiers in Veterinary Science*, 7(1), 411.

Uni. Cal. (2019). Euthanasia of Research Animals. University of California (Uni. Cal.) Retrieved from <https://research.uci.edu/compliance/animalcare-use/research-policies-and-guidance/euthanasia.html>

Uni. Melb. Animal Care & Use Standards Committee (2019). Humane Killing of Mice and Rats. University of Melbourne (Uni. Melb.) Retrieved from [https://staff.unimelb.edu.au/\\_\\_data/assets/pdf\\_file/0011/1974341/STANDARD-Humane-killing-of-mice-and-rats-FINAL.pdf](https://staff.unimelb.edu.au/__data/assets/pdf_file/0011/1974341/STANDARD-Humane-killing-of-mice-and-rats-FINAL.pdf)

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