

 <b>LEHIGH</b> UNIVERSITY	<b>Animal Care and Use Committee (IACUC)</b> SOP: Carbon Dioxide Euthanasia of Rodents		
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The intent of this Institutional Animal Care and Use Committee (IACUC) standard operating procedure is to outline the process for euthanizing rodents with carbon dioxide (CO<sub>2</sub>).

### Introduction and Background

To the greatest extent possible, research animals being euthanized should not experience pain, fear, or other significant stress prior to or during their death. CO<sub>2</sub> is a frequently used euthanasia agent for small laboratory animals, including rodents, due to its rapid onset of action, safety, low cost, and ready availability. The Lehigh University IACUC has prepared the following standard operating procedure in order to:

1. Assist the research community by clarifying the specific procedures relating to the use of CO<sub>2</sub> for euthanasia.
2. Promote best practices and ensure that pain and distress are minimized in euthanasia of laboratory rodents.
3. Ensure compliance with federal regulations and the Guide for the Care and Use of Laboratory Animals (the Guide).

This guidance is derived from the Public Health Service (PHS), Office of Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH), the American Association of Veterinary Medicine (AVMA), peer institutions, and attending veterinary consultation.

This guidance includes the following topics:

1. Species
2. Administering CO<sub>2</sub>
3. Confirming death
4. Exemptions

### Species

Any rodent used for research at Lehigh University may be euthanized by CO<sub>2</sub> by following the process described below. The euthanasia method must be described in the IACUC protocol and approved in advance by the LU IACUC. Examples of species that may be euthanized according to this guidance include mice of the genus *Mus*, rats of the genus *Rattus*, hamsters, and gerbils. Although the AVMA considers CO<sub>2</sub> an acceptable form of euthanasia for other species, the LU IACUC will require specific justification for CO<sub>2</sub> use in non-rodents.

### Process for Administering CO<sub>2</sub>

1. All personnel administering CO<sub>2</sub> to rodents must be properly trained. Principal Investigators (PIs) are responsible for ensuring that their research staff, including student investigators, are properly trained and adhere to all applicable animal care and use protocols, policies, and guidelines. Training on the use of the CO<sub>2</sub> equipment (e.g. automated CO<sub>2</sub> euthanasia chamber) and appropriate methods of euthanasia is available from Central Animal Facility (CAF) staff and/or the Attending Veterinarian. A digital training video and a copy of the user manual for the automated chamber is also available.
2. Compressed CO<sub>2</sub> in cylinders is the only acceptable source of CO<sub>2</sub> for euthanizing rodents. The use of dry ice, fire extinguishers, and other sources of CO<sub>2</sub> is prohibited.
3. The euthanasia chamber is constructed of clear material (e.g. Plexiglas) to allow ready visibility of the animals. The chamber must be kept clean to minimize odors that might distress animals subsequently euthanized. Gas must be delivered in a predictable and controlled fashion, at a low-flow rate. Do not overcrowd the chamber. All animals in the chamber must be able to make normal postural adjustments. In order to facilitate compliance with these requirements, the automated euthanasia chamber available in the CAF must be used when following this SOP for euthanizing laboratory rodents.
4. Euthanasia must occur in a procedure room or laboratory, away from other rodent housing. Euthanasia must not be performed in the animal room.
5. When using the automated chamber, rodents must be euthanized in their home cages. **Mixing or combining of animals from multiple cages into a single cage for euthanasia is prohibited.**

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6. The on/off switch on the back of the automated chamber is left in the “on” position at all times. The white “ready” light on the front of the unit will be lit when the chamber is ready for use. Turn the three knobs on the front of the chamber counterclockwise to open the door. The door will open when the knobs are in a horizontal position. The red “fault” light will be on and gas cannot flow while the door is open. Home cages can be loaded into the automated chamber stacked into two rows. Cages must be slightly offset to allow for CO<sub>2</sub> circulation to bottom cages. Do not attempt to place more cages in the chamber than will comfortably fit. Close the door and turn all three knobs clockwise to seal. The red “fault” light will indicate if the door is open.
7. Inspiration of high concentrations of CO<sub>2</sub> is both aversive and painful. The automated euthanasia chamber is pre-programmed to ensure that the chamber is slowly filled with CO<sub>2</sub> over several minutes and that the chamber is purged of CO<sub>2</sub> when a cycle ends. The gas flow meter connecting the CO<sub>2</sub> to the chamber is left on at all times. To start a cycle, after loading cages and closing the door, flip the switch to choose the adult or the neonate cycle, and press the green “start” button on the front of the chamber. The white “ready” light will remain on, and the green “run” light will indicate when a euthanasia cycle is running.
8. CO<sub>2</sub> first renders the animal anesthetized and then, with adequate exposure time (see table below), will result in death by CO<sub>2</sub> narcosis. The automated euthanasia chamber is pre-programmed to ensure that CO<sub>2</sub> is delivered for a sufficient amount of time; including additional time to confirm death. Once a euthanasia cycle is running in the automated chamber, the system will cycle through three stages: the gas will flow for 8 minutes to charge the chamber with CO<sub>2</sub>; the gas flow will stop and the chamber will remain fully charged for 5 minutes (60 minutes for the neonate cycle), and; the exhaust blower will activate and purge the chamber for 4 minutes.
9. Animals must remain in the chamber until clinical death has been ensured. When the exhaust blower automatically switches off, animals may be safely removed from the chamber. After removing animals from the automated chamber, death must be confirmed by protocol personnel qualified to recognize the cessation of vital signs in rodents (see **Confirming Death**).

Resistance to hypoxia results in prolonged time to unconsciousness when CO<sub>2</sub> inhalation is used as a euthanasia agent in neonatal rodents. The duration of exposure to carbon dioxide varies with the age of the neonate compared to the adult rodent. The following guidance should be followed:

AGE	Minimum time in 100% CO <sub>2</sub>	
	MICE, HAMSTERS, GERBILS (Pritchett <i>et al.</i> 2005)	RATS (Pritchett-Corning 2009)
Non-haired pups 0-6 days	60 minutes	40 minutes
Haired pups, eyes closed 7-13 days	20 minutes	20 minutes
Haired pups, eyes open, preweaning 14-20 days	10 minutes	10 minutes
Weanlings and adults 21+ days	5 minutes	5 minutes

**Note: the automated CO<sub>2</sub> euthanasia chamber in the CAF has been pre-programmed with two cycles to meet these standards; one cycle for neonates and one for adults.**

Fetuses also require extended exposure to inhalant anesthetics, including CO<sub>2</sub>. When fetuses are not required for study, the method chosen for euthanasia of a pregnant mother should ensure cerebral anoxia to the fetus and minimally disturb the uterine milieu to minimize fetal arousal. A recommended method for euthanasia of the mother is CO<sub>2</sub> exposure followed by cervical dislocation.

### Confirming Death

Since the anesthetic effects of CO<sub>2</sub> are reversible, animals that are prematurely removed from the chamber prior to death can recover. Death must be confirmed by the protocol’s PI or personnel who have

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been trained by the PI to recognize the cessation of vital signs in rodents. If needed, additional training is available through the Attending Veterinarian. A combination of criteria is most reliable in confirming death, including lack of pulse, breathing, corneal reflex and response to firm toe pinch, inability to hear respiratory sounds and heartbeat by use of a stethoscope, graying of the mucous membranes, and rigor mortis. None of these alone, except rigor mortis, confirms death.

Rodents, especially neonates, are particularly resistant to euthanasia by overdose of inhaled agents such as CO<sub>2</sub>. Therefore, all animals being euthanized with CO<sub>2</sub> must also receive a confirmatory method of euthanasia to ensure death. These confirmatory methods, to be performed after carbon dioxide overdose, include:

- Exsanguination
- Decapitation
- Cervical dislocation (Cervical dislocation may be used for adult mice, rats, and gerbils **only**. **Cervical dislocation may not be used in hamsters of any age or other rodents prior to weaning.**)
- Bilateral thoracotomy
- At least 50% additional time in the euthanasia chamber filled with 100% CO<sub>2</sub>. **Note that the automated euthanasia chamber has been pre-programmed for cycles that include this additional time in the chamber. None of the additional physical methods listed above are required when the automated chamber is used for CO<sub>2</sub> euthanasia.**

Death of the animal must be ensured prior to disposal of the carcass. Failure to confirm the death of a euthanized rodent comprises serious noncompliance, reportable to the appropriate regulatory, accrediting, and funding agencies.

### Exemptions

Methods of euthanasia used will be consistent with the recommendations of the AVMA Guidelines for the Euthanasia of Animals (2020), unless a deviation is justified for scientific reasons in writing by the PI. Any departures from these guidelines will require the PI to provide strong scientific and clinical justifications for the exemption. These justifications must be included in the IACUC protocol and will be reviewed by the IACUC in consultation with the attending veterinarian.

### References

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