I. **Purpose:** The Institutional Animal Care and Use Committee (IACUC) developed this guidance to ensure proper and humane procedures are employed when euthanizing rodents using carbon dioxide (CO₂). This guidance document is intended to (1) assist the Brown research community by outlining the specific procedures to be used when euthanizing laboratory rodents with CO₂ as a primary method, and (2) promote best practices and ensure that pain and distress are minimized in euthanized laboratory rodents by clarifying the IACUC’s expectation that a secondary method will be used.

II. **Background:** This guidance is derived from regulatory requirements set forth by the Public Health Service (PHS), Office of Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH), American Association of Veterinary Medicine (AVMA), and practices used by peer institutions. CO₂ inhalation is a common method of euthanasia for rats and mice and must be included in a researcher’s IACUC-approved protocol. In accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition (the Guide, p. 124) and the NIH, personnel administering CO₂ to rodents must be properly trained in this technique and adhere to the corresponding IACUC-approved protocol and institutional policies. CO₂ is an anesthetic and its effects are reversible; therefore, upon completion of CO₂ euthanasia, death must be confirmed for each animal by one or more of the secondary methods listed below. Disposal of an animal that has not been properly euthanized is a serious animal welfare concern and a significant breach of the conditions of Brown University’s approved PHS Animal Welfare Assurance and could result in reportable non-compliance.

**Other Species and Neonates:** Any rodent used for research may be euthanized by CO₂ in accordance with the guidance below. This includes mice of the genus *Mus*, rats of the genus *Rattus*, hamsters, gerbils, and other laboratory rodents. Researchers must consult Animal Care (AC) veterinary staff prior to submitting a protocol requesting to use CO₂ for any species other than rodents, and for assistance with CO₂ euthanasia for neonatal (up to 21 days of age) rodents. Neonatal rodents are resistant to CO₂ induced hypoxia resulting in a prolonged time to unconsciousness (up to 60
minutes) and therefore it is recommended that neonatal rodents be placed into the CO2 chamber for 10-15 minutes (until fully anesthetized evidenced by no observable movement and no response to a toe pinch) and then euthanized by decapitation with a sharp blade or scissors.

III. Procedures:

1. Only compressed CO2 from a cylinder affixed with a flowmeter regulator may be used. All other sources of CO2 are prohibited (e.g., dry ice, fire extinguishers, or chemical means).

2. Animals should be euthanized in their homecage whenever possible. Animals in the euthanasia chamber should be readily visible and personnel must remain present for the entire euthanasia procedure. Animals must not be overcrowded in the chamber by combining multiple cages and must not be mixed with unfamiliar or incompatible animals (e.g., animals from different cages must not be mixed in the chamber during euthanasia). All animals in the chamber must be able to make normal postural adjustments. If euthanizing in OptiMice cage, attach the OptiMice inhalation nozzle and cap so the delivery hose can be attached properly to the cage.

3. The euthanasia chamber must not be pre-filled with CO2 prior to placing rodents in the chamber. Sudden exposure of conscious animals to CO2 concentrations of 70% or greater has been shown to be distressful and potentially painful. CO2 is heavier than room air, therefore always empty (by turning it over) and clean the chamber with dilute chlorhexidine between uses to remove residual CO2 (to avoid a pre-fill situation).

Start with room air then slowly fill the chamber with CO2 over several minutes. To ensure that conscious rodents are exposed to an appropriate level of CO2, the flow rate should be set to displace 10-30% of chamber volume, as follows:

- 1L/minute when using the small chamber or a split Thoren mouse cage
- 3L/minute when using the large chamber or a solid Thoren mouse or rat cage.
- 1.5L/minute when using the OptiMice cage

4. CO2 exposure first anesthetizes the animal, then results in CO2 narcosis following adequate exposure time. Animals should be left in the euthanasia chamber for at least one minute after spontaneous movements have ceased, with CO2 continuing to flow at the same rate. Animals must be continuously monitored for adverse reactions for the duration of the euthanasia procedure. It is
important to contact Animal Care staff promptly if the procedure appears to produce excessive agitation or other complications.

<table>
<thead>
<tr>
<th>Age</th>
<th>Minimum Time in 100% CO₂</th>
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</thead>
<tbody>
<tr>
<td>Non-haired pups (0-6 days old)</td>
<td>60 minutes</td>
</tr>
<tr>
<td>Haired pups, eyes closed (7-13 days old)</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Haired pups, eyes open, preweaning (14-20 days old)</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Weanlings and adults (21+ days old)</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

5. Death of the animal must be confirmed via a secondary method prior to disposal of the rodent carcass. CO₂ is an anesthetic and the effects are reversible. Failure of the animal to move or show a reflex response is not sufficient to confirm death. One of the secondary methods below must be used to ensure that the rodent will not recover:

- Bilateral thoracotomy
- Exsanguination
- Cervical dislocation
- Decapitation

IV. Noncompliance: As stated in the PHS Policy, “Unintended recovery of animals after apparent death from CO₂...constitute[s] serious noncompliance with the PHS Policy and serious deviation from the provisions of the Guide...the IACUC, through the Institutional Official, must promptly provide OLAWith a full explanation of the circumstances and actions taken.” AAALAC International reporting requirements include inappropriate euthanasia techniques and/or failure to confirm euthanasia. Failure to confirm death of a euthanized rodent is therefore a significant noncompliance at Brown, will be reported to the appropriate regulatory and accrediting agencies, and will require corrective actions (i.e., re-training) and possible additional steps (i.e., prohibition of certain personnel from performing CO₂ euthanasia) depending on the specific circumstances of the noncompliance.