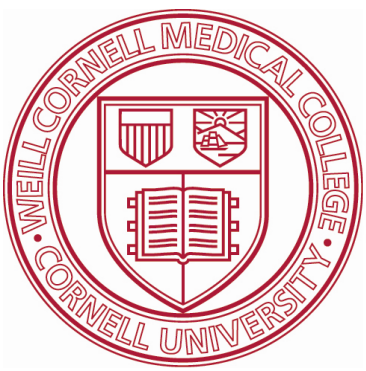
RECOMMENDED METHODS OF EUTHANASIA FOR LABORATORY ANIMALS



INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
MEMORIAL SLOAN KETTERING CANCER CENTER
WEILL MEDICAL COLLEGE OF CORNELL UNIVERSITY

CURRENT REVISION DATE: 05.06.15

RECOMMENDED METHODS OF EUTHANASIA FOR LABORATORY ANIMALS

Euthanasia derives from the Greek *eu* meaning good, and *thanatos* meaning death. Euthanasia is the act of painlessly terminating life. The criteria that have been used for determining that death is painless are rapid loss of consciousness followed by cardiac and respiratory arrest. There are additional criteria that should be considered when euthanizing research animals including the emotional effect the technique has on the person(s) performing the method, and the resultant effect on the tissues or samples that may be collected post mortem. There are a variety of acceptable methods that can be used for each species. The advantages and disadvantages of each technique should be considered in selecting the most appropriate method for a particular project. These guidelines review techniques which are recommended by both Memorial Sloan-Kettering's (MSKCC) and Weill Cornell Medical College's (WCMC) Institutional Animal Care and Use Committees (IACUC) and the American Veterinary Medical Association (AVMA). Additional methodologies are available. Consult the "*2013 AVMA Guidelines on Euthanasia*,"https://www.avma.org/kb/policies/documents/euthanasia.pdf which is available in RARC's administrative offices in Zuckerman 920 (MSKCC) and E-700 (WCMC). RARC's technical and veterinary staff is available to demonstrate and/or discuss these techniques. Contact RARC's Education & Quality Assurance Section at 646-962-6703, or Veterinary Services at 646-888-2430 (MSKCC) or 212-746-1079 (WCMC) for training or consultation.

Suggested Techniques:

1. **Carbon dioxide (CO_2)** - the preferred technique for euthanizing rodents, small birds, and other small laboratory animals. For rodent euthanasia, in an effort to reduce stress, compatible groups should not be disrupted when possible. CO_2 must be used with a gaseous source and a sanitizable chamber. Dry ice CANNOT be used as a source of CO_2. 100% CO_2 should be utilized for euthanasia. It is imperative that the animals be exposed to the gas for sufficient time, as short duration exposure to CO_2 only induces anesthesia causing the animal to become unconscious and unresponsive. You should verify, by observation, that the animal is not breathing and subsequently confirm, by palpation, the lack of a heart beat. Neonates require prolonged exposure to the gas because their hemoglobin has a higher affinity for oxygen. Neonates may require up to 60 minutes or more of exposure to ensure death. Preanesthesia with CO_2, followed by decapitation with a sharp scissors, is recommended for euthanasia of neonatal rodents. Third trimester rodent embryos derived from dams euthanized with CO_2 must be sacrificed by decapitation prior to use. Please refer to the Institutional Animal Care and Use Committee's "*Guidelines for the Euthanasia of Mouse and Rat Fetuses and Neonates*" (Appendix 1) for complete details. Appendix 2 contains an age identification chart. CO_2 is **not** recommended for euthanasia of amphibians and reptiles. CO_2 may be used for euthanasia of fish if the CO_2 is bubbled through the water, followed by a physical method of euthanasia after loss of consciousness.

   All RARC facilities contain euthanasia stations which deliver CO_2 via adjustable flow meters. These stations can be attached to a stainless steel euthanasia lid which is placed over the animals' cage. There are several types of euthanasia stations in the vivaria depending on the source of carbon dioxide. CO_2 may be piped in from reservoirs serving the entire building or from individual pressurized tanks which contain pressure reducing regulators and flow meters. PLEASE REVIEW THE SPECIFIC INSTRUCTIONS FOR THE EUTHANASIA STATION YOU ARE USING. Instructions are provided on or near each euthanasia station (see Appendices 3 and 4 for sample instructions). Alternatively, you can request assistance from RARC's Education & Quality Assurance and/or Veterinary Services staff. If animals are euthanized with carbon dioxide in a laboratory, pressure reducing regulators and flow meters are required to deliver a gas displacement rate of 10% to 30% of the chamber volume/min.

   It is essential that the appropriate procedures are followed precisely. This is necessary to avoid delivering the gas at a high velocity which is stressful to the animals. Rapid exposure to CO_2 results in asphyxiation. The animal may remain conscious until respiratory and cardiac arrest occurs. Slower CO_2 administration results in anesthesia

before cardiorespiratory arrest. When utilizing a chamber, animals are removed from their home cage and placed within. When utilizing a euthanasia lid for CO_2 delivery, the lid is placed on top of the cage containing animals**.** Independent of the system, the cage(s) or chamber should remain covered after they are "flooded" with CO_2 and left undisturbed for AT LEAST 15 MINUTES for mouse euthanasia. Rats and all other non-mouse rodent species approved for CO_2 euthanasia must include a secondary physical or chemical method of euthanasia as described below. Regardless of the method used, it is imperative that animals are not overcrowded (greater than 5 adult mice or greater than 900 grams (g) combined body weight for rats).

Euthanasia of rats, and all other non-mouse rodent species, by investigative staff must include an additional physical or chemical method of euthanasia to assure against failed euthanasia. One of the following IACUC approved methods must be described in the animal use protocol and employed at the time of euthanasia:

- Decapitation while under CO_2 anesthesia. Decapitation can be accomplished by utilizing a large sharp scissor or guillotine.
- Bilateral thoracotomy while under CO_2 anesthesia. Requires the use of a sharp scissor or scalpel blade by trained personnel.
- Barbiturate overdose via intracardiac (IC) injection while under CO_2 anesthesia or intraperitoneal injection. Minimal recommended dose of Sodium pentobarbital is 300 mg/kg.
- Cervical dislocation (CD) while under CO_2 anesthesia. **Only** permissible for **rats** weighing less than 200 g and is only to be performed by trained personnel
- Confirmation of rigor mortis by physical exam

For species not requiring the addition of a physical method of euthanasia, death should be verified by lack of breathing and heartbeat (by placing your thumb and index finger on opposite sides of the animal's chest). As described above, neonates require considerably longer periods of gas exposure and lose their pink color before death. For additional assurance, the plastic bag used for carcass disposal of adults or neonates should be filled with CO_2 prior to closure.

RARC's Husbandry and Operations section provides routine humane euthanasia of mice and rats at no cost. Cages containing animals to be euthanized are placed on special "euthanasia" racks located in each animal facility[1]. Importantly, animals left on these racks must be afforded the same conditions as all mice housed in this facility, specifically:

- **Do not** compromise ventilation by stacking cages of live animals one on top of the other
- **Do not** overcrowd cages (greater than 5 adult mice, or greater than 900 g combined body weight for rats)
- **Do not** leave cages without food and water adequate for 24 hours
- **Do not** leave sick/distressed animals
- **Do not** leave pre-weanling animals without the nursing female.

Suckling pup(s) without their dam, sick animals, any cage with a *Health Check Card* and/or animals in pain or distress must be euthanized by the investigative staff or brought to the direct attention of the Facility Manager or a member of the Veterinary Services staff for euthanasia.

2. **<u>Barbituric Acid Derivatives</u>** - Sodium (Na) pentobarbital is the most common barbiturate agent for euthanasia. Barbiturates depress the CNS producing sequential unconsciousness, deep anesthesia, apnea, and cardiac arrest. Concentrated formulations which contain muscle relaxants and anti-convulsants are available and are recommended for euthanasia. Dosage for euthanasia (300 mg/kg Na Pentobarbital) in mammals is usually 3 times the amount required for anesthesia. The following chart provides recommended routes for administration by species:

[1]These procedures are applicable for animals in standard holding rooms only. Procedures for animals housed in hazardous material areas and xenograft rooms are different and should be followed.

Species	Administration
mouse	IP
rat	IP
guinea pig	IP
hamster	IP
rabbit	IV (lateral ear vein)
ferret	IV (cephalic or saphenous veins)
cat	IV (cephalic or saphenous veins)
dog	IV (cephalic or saphenous veins)
swine	IV (ear vein)
primate	IV (after tranquilization with ketamine IM)
reptiles	intracoelomic
amphibians	intracoelomic, dorsal lymph sac
chickens	IV (brachial vein)

Sodium pentobarbital is a Schedule II drug which is regulated by the Federal and New York State (NYS) Drug Enforcement Agency (DEA). Federal and NYS regulations require maintenance of records including the date, purpose, and amount of agent used. A DEA license obtained for clinical practice **can** be used to obtain drugs for research use. Investigators can obtain euthanasia solution from RARC's Veterinary Services at MSKCC @ 646-888-2430 or WCMC @ 212-746-1079. Euthanasia agents must be administered before their expiration date. All expired agents, if acquired from RARC, must be returned to RARC to be disposed as instructed by the DEA.

3. **Isoflurane and Other Inhalational Anesthetics** - Overdose with isoflurane or other types of inhalational anesthetics in an appropriate bell jar or induction chamber is an acceptable, although expensive, method of euthanasia for rodents and other small animals. Additionally, since rabbits hold their breath when exposed to unfamiliar odors, the use of inhaled methods is difficult unless used after sedation. Inhalational anesthetics should be used in a chemical hood or with appropriate scavenging as they may be toxic to personnel. As with CO_2, death should be confirmed by verifying the absence of respiration and a heartbeat. In rats and all other non-mouse rodent species approved for euthanasia by inhalant agent, euthanasia must include a secondary physical or chemical method as described in *section 1 Carbon Dioxide*.

4. **Cervical Dislocation** - Is an acceptable technique for euthanizing mice and rats weighing less than 200 g when scientifically or clinically justified. This technique must be approved by the IACUC during protocol review. Scientific necessity must be clearly documented. Personnel must demonstrate proficiency on anesthetized and/or euthanized animals.

5. **Decapitation** - Is an acceptable technique for euthanasia of rodents when scientifically or clinically justified. This technique must be approved by the IACUC during protocol review. Scientific necessity must be clearly documented. Personnel must also demonstrate proficiency on anesthetized and/or euthanized animals. Decapitation is a potential safety hazard to personnel. Additionally, many animal species react adversely to the smell of blood. Animals should not be decapitated in the presence of other animals and the investigator should wash his/her gloved hands and the guillotine between animals. The blade on the guillotine or scissors should be sharpened regularly. Please review the IACUC Policy at your institution as regularly scheduled maintenance is recommended for all guillotines used based on frequency of use. Decapitation may also be used for euthanasia of amphibians and fish following anesthesia or sedation. Use alone must be scientifically justified in the animal use protocol and approved by the IACUC. Sharp equipment (guillotine or heavy scissors) of the appropriate size for the species should be used to ensure that the head is separated from the body rapidly and completely. Decapitation should be followed by pithing of the brain as brain death may not be immediate in poikilotherms.

6. **Potassium chloride** - Overdosage with KCl is permissible **only** in anesthetized animals by intravenous or intracardiac injection. Rapidly rising serum potassium levels result in cardiac arrest.

7. **Exsanguination** - Laboratory animals may be exsanguinated during perfusion and/or to collect large volumes of blood or blood products. Animals must be anesthetized to reduce the distress associated with hypovolemia prior to exsanguination and death must be verified**.**

8. **Pithing –** Pithing may be used for euthanasia of amphibians and fish following anesthesia or sedation. Use alone must be scientifically justified in the animal use protocol and approved by the IACUC. Species specific procedures must be followed to ensure that both the brain and the proximal spinal cord are destroyed by rapidly moving the rod/needle back and forth to transect the cord. In fish, pithing should be followed with decapitation.

9. **Hypothermia -** Is the recommended technique for euthanasia of zebrafish. An ice bath is made up of a 50:50 mixture of crushed ice:water and a physical barrier or other method is utilized to ensure the fish are not in direct contact with the ice. A thermometer must be used to measure the temperature of the water and fish are placed into the water when the temperature is 2-4ºC. Adult fish (> 90 dpf) are left in the water for a minimum of 10 minutes following loss of opercular movement and fry, 4 to 90 days post fertilization [dpf], for a minimum of 40 minutes. If hypothermia exposure is shorter than these recommended durations, an adjunct method of euthanasia should be used. When used in embryos/larvae < 4 dpf, hypothermia should be followed with an adjunct method such as placement in diluted sodium hypochlorite solution (6.15%). Hypothermia may also be used for altricial rodents. Details are provided in Appendix 1.

10. **Tricaine Methanesulfonate (MS-222) -** Is a chemical agent that may be used for euthanasia of amphibians and adult fish. MS-222 is supplied as a powder that is dissolved in water to the desired concentration. Only FDA approved products should be used. Solutions with a concentration of > 500 mg/L are acidic and therefore, must be buffered to a pH of 7.0-7.5. For the euthanasia of adult fish (≥ 90 dpf), concentrations of at least 500 mg/L must be used. Fish must be left in the bath for a minimum of 10 minutes; alternately, gills may be flushed with the solution for at least 10 minutes. Death must be verified by monitoring for absence of opercular movement for at least 3 minutes. For the euthanasia of fish <90 dpf, anesthesia with MS-222 must be followed with an adjunct method such as sodium hypochlorite (fish <7 dpf), hypothermia, or a physical method. For the euthanasia of amphibians, a concentration of 5 g/L with immersion for at least 1 hour is necessary to achieve euthanasia. Immersion in a lower concentration or immersion for shorter periods of time requires a secondary physical method of euthanasia. Intracoelomic administration is not recommended. Death must be verified by monitoring for absence of respiration or opercular movement, as well as the absence of heart beat (can be visualized at the ventral midline beneath the sternum). Individuals handling MS-222 must receive specific training and must follow RARC's "*Guidelines for the Proper Handling, Use, and Disposal of Tricaine Methanesulfonate (MS-222)*."

11. **Benzocaine and 2-phenoxyethanol** - Are additional methods of euthanasia for amphibians and fish. Please contact a RARC veterinarian for additional information.

13. **Dilute sodium hypochlorite solution (5-10%) –** Is used as an alternative or as an adjunct to hypothermia for euthanasia of larval zebrafish (4-7 days post-fertilization [dpf]). For embryos/larvae < 4 dpf, dilute sodium hypochlorite with or without hypothermia is used. Bleach solution (sodium hypochlorite 5-10%) is added to housing system water at 1 part bleach to 5 parts water. Animals should remain in this solution for at least five minutes to ensure death.

Special Considerations:

Death Verification - Regardless of the technique utilized, it is **essential that death is confirmed**. Many of the agents utilized induce deep anesthesia prior to death. Therefore the animal must be carefully evaluated for absence of respiration, heartbeat, corneal reflex, and response to firm toe pinch, as well as the graying of mucous membranes and rigor mortis. Alternatively, a secondary method of euthanasia, such as thoracotomy or exsanguination, can be used to ensure death once the animal is unconscious. Additional signs which can be used to assess death in larger animals include dilated and fixed pupils. RARC′s policy must be followed for carcass disposal. Animals must be placed in leak-proof bags and placed in an appropriate refrigerator. Animals exposed to hazardous agents, including biological

agents, chemicals, and radionuclides must be placed in an appropriate color-coded bag, identified with a special label, and must be disposed of or stored at designated locations. Embryos and larval zebrafish < 1 cm in size that are euthanized with dilute sodium hypochlorite solution may be disposed of down the drain with the euthanasia solution followed by 3-5 minutes of running water. Larger zebrafish, and larval zebrafish euthanized by other methods are disposed of as described for other species above. Please contact RARC for additional information.

Separation of Animals - *The Guide for the Care and Use of Laboratory Animals* indicates that "Euthanasia should be carried out in a manner that avoids animal distress". The IACUC recommends that live animals be separated from animals being euthanized. When possible, keep live animals or cages of animals separated in different rooms or areas and avoid disruption of compatible groups. Alternatively, animals may be euthanized in chemical fume hoods, biosafety cabinets or under snorkels. Please contact RARC's veterinary staff for advice and /or consultation on this matter.

Summary of recommended methods by species:

Species	Acceptable Method	Agent	Comment
Mouse	1,2,3,4,5,7	CO_2,Barbiturates, Isoflurane	
Rat	1,2,3,4,5,7	CO_2, Barbiturates, Isoflurane	Secondary method of euthanasia required
Guinea Pig	1,2,3,5,7	CO_2, Barbiturates, Isoflurane	Secondary method of euthanasia required
Hamster	1,2,3,7	CO_2, Barbiturates, Isoflurane	Secondary method of euthanasia required
Rabbit	2,3,6,7	Barbiturates, Isoflurane, KCl	KCL only with anesthesia
Ferret	2,6,7	Barbituates, KCl	KCL only with anesthesia;
Dog	2,6,7	Barbiturates, KCl	KCL only with anesthesia
NHP	2,6,7	Barbiturates, KCl	KCL only with anesthesia
Swine	2,6,7	Barbiturates, KCl	KCL only with anesthesia
Amphibians	2, 10	Barbiturates, MS-222	A secondary method is recommended due to prolonged time to euthanasia
Fish	9, 10, 12	Hypothermia, sodium hypochlorite , MS-222	Method varies by age

APPENDIX 1

Institutional Animal Care and Use Committee
Guidelines for the Euthanasia of Ferret, Mouse and Rat Fetuses and Neonates

The *AVMA Guidelines for the Euthanasia of Animals* provides specific recommendations for the euthanasia of prenatal or neonatal animals. The following guidelines* have been adopted and approved by the Institutional Animal Care and Use Committees at the Weill Cornell Medical College and Memorial Sloan Kettering Cancer Center to be utilized in reviewing proposals which involve the use of rodent and ferret fetuses or neonates.

FETUSES:

a) Fetuses **UP TO 14 DAYS** in gestation:

Neural development at this stage is minimal and pain perception is considered unlikely. Euthanasia of the mother or removal of the fetus should ensure rapid death of the fetus due to loss of blood supply and non-viability of fetuses at this stage of development.

b) Fetuses **15 DAYS IN GESTATION TO BIRTH**:

The literature on the development of pain pathways suggests the possibility of pain perception at this time. Whereas fetuses at this age are not sensitive to inhalant anesthetics, euthanasia may be induced by:

- **skillful injection of chemical anesthetics**,
- **decapitation with surgical scissors,**
- **cervical dislocation, or**
- **rapid freezing** (immersion in liquid nitrogen)

When chemical fixation of the whole fetus is required, fetuses should be anesthetized prior to immersion in or perfusion with fixative solutions.

Anesthesia may be induced by-

- **HYPOTHERMIA[2] of the fetus,**
- **INJECTION OF THE FETUS with a chemical anesthetic, or**
- **DEEP ANESTHESIA OF THE MOTHER with a chemical agent that crosses the placenta, e.g., pentobarbital**.

A RARC veterinarian should be consulted for considerations of fetal sensitivity to specific anesthetic agents. When fetuses are not required for study, the method chosen for euthanasia of a pregnant mother must ensure rapid death of the fetuses.

NEONATES (RODENTS)[3,4]:

a) Neonates **Days 1-6[4]:**

Acceptable methods for the euthanasia of neonatal mice and rats include:

- **injection of chemical anesthetics** (e.g., pentobarbital),
- **decapitation (with sharp surgical-grade scissors)**, or
- **cervical dislocation**

NOTE: The above methods may be used following induction of anesthesia with CO_2 or inhalant anesthetics.

Immersion in liquid nitrogen may be used **only for newly born pups (i.e. within 24 hrs of birth);**

Pups **older than one day should be anesthetized prior to freezing** with liquid nitrogen.
Similarly, **anesthesia should precede immersion or perfusion with chemical fixatives**.

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[2]Phifer CB, Terry LM. 1986. Use of hypothermia for general anesthesia in preweanling rodents. Physiol & Behav 38:887-890.

[3]Pritchett, K. 2009. Euthanasia of Neonatal Rats with Carbon Dioxide. JAALAS 48(1):23-27.

[4]National Institutes of Health Intramural Guidelines for the Euthanasia of Mouse and Rat Fetuses and Neonates. Revised January 2010.

Anesthesia may be induced by inhalant or injectable anesthetics; the RARC veterinarian should be consulted for appropriate agents and dosages.

Alteratively, **<u>when adequately justified</u>,**

- **hypothermia**[2] may be used to induce anesthesia in pups **younger than six days**.

b) **Days 7-14**[4]**:**

- CO_2 followed by decapitation
- CO_2 alone—animals must remain undisturbed in the CO_2 flooded chamber for 15 minutes (mice) or 30 minutes (rats) and then left an additional 30 minutes in room air to ensure they do not recover

c) **Days 15-21**[4]**:**

- CO_2 alone—animals must remain undisturbed in the CO2 flooded chamber for 15 minutes (mice) or 30 minutes (rats)

In all cases, the person performing the euthanasia must be fully trained in the appropriate procedures.

NEONATES (FERRETS) [5]**:**

a) Neonates **Days 1-7:**

Acceptable method for the euthanasia of neonatal ferrets (kits) include:

- **decapitation (with sharp surgical-grade scissors)**

NOTE: The above method may only be used following induction of anesthesia with CO_2 or inhalant anesthetics.

b) **Days 7-56:**

- **injection of chemical anesthetics** (e.g., pentobarbital) intravenously (IV) or intraperitoneally (IP) if venous access is not feasible,

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In all cases, the person performing the euthanasia must be fully trained in the appropriate procedures.

[5] AVMA Guidelines for Euthanasia for Animals: 2013 Edition. p. 46-47.

APPENDIX 2

Determination of mouse pup age based on visual appearance[6]:

Appearance	Relative Age
Nonhaired pups	0 to 6 d
Haired pups, eyes closed	7 to 11d
Haired pups, eyes open, (preweaning)	12 to 20 d
Weanlings and adults	21 d of age and older

Determination of rat pup age based on visual appearance[3]:

Appearance	Relative Age
Nonhaired pups	0 to 6 d
Haired pups, eyes closed	7 to 13 d
Haired pups, eyes open, (preweaning)	14 to 20 d
Weanlings and adults	21 d of age and older

Determination of ferret kit age based on visual appearance[7]:

Appearance	Relative Age
Finely haired kits	0 to 14 d
Haired kits, teeth erupted	14 to 30 d
Haired pups, eyes and ears open	30 to 35 d
Weanlings and adults	56 days of age and older

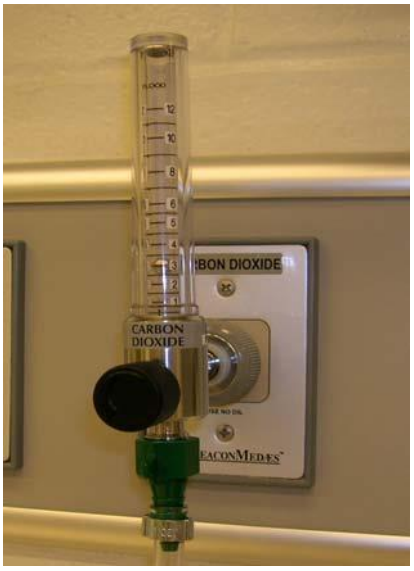
[6]The Jackson Laboratories. 2010. JAX Mice Pup Appearance by Age (Poster) http://jaxmice.jax.org/images/literature/pupsposter-large.jpg

[7]Fox, J. G., Anderson, L. C., Loew, F. M., & Quimby, F.W. eds. 2002. Laboratory Animal Medicine. Academic Press: San Diego, CA.

APPENDIX 3

Recommended Procedures for Euthanizing Rodents Using a CO2 Flow Meter

1. The wire bar lid containing the feed and water bottle may be removed or left in place on the cage containing the rodents you plan to sacrifice.
    1. Ensure that the CO2 tubing is attached to one of the two sizes of euthanasia lids, rat or mouse, via the quick release fitting at the center of the lid; rat cages will require use of the larger of the 2 lids.
    2. Place the lid over the cage so that the exhaust holes in the lid are over the cage.
    3. Animals should not be overcrowded. There should be sufficient space on the cage floor to accommodate all animals so that they can move freely.



4. **Administer CO2 slowly**
    1. CO2 flow is initiated by turning the black knob on the CO2 flowmeter. Adjust the flow rate to **3 liters/minute for mouse cages** and **6 L/minute for rat cages**. The rate is confirmed by centering the silver ball next to the liter number on the flowmeter.
    2. Allow the CO2 to flow for at least 3 minutes before turning off the flow meter.
    3. Mice should be exposed to CO2 for **15 minutes.**
    4. **Rat users**- Please note that an additional physical method of euthanasia is required

5. **Death must be verified by monitoring the animal(s) for lack of breathing and absence of heart beat**. *(Animals which have stopped breathing but still have a heartbeat may revive!)*
    1. Death can be verified by observing for the absence of respiration and palpation of the heart by placing your thumb and index finger on opposing side of the animal's chest.
    2. Neonates (hairless pups) require considerably longer periods of gas exposure because their blood has a higher affinity for oxygen. They lose their pink color before death is recognized.

3. Dead animals should be removed from the cage and placed in a sealed plastic bag for disposal into a RARC carcass refrigerators or freezers.

4. Verify that the system has been turned off by checking that the silver ball in the flowmeter is at ZERO.

Please Note: Contact RARC personnel for assistance, training, or questions regarding the procedures described.

APPENDIX 4

Recommended Procedures for Euthanizing Rodents Using a CO2 Flow Gauge

1. The wire bar lid containing the feed and water bottle may be removed or left in place on the cage containing the rodents you plan to sacrifice.
    1. Ensure that the CO2 tubing is attached to one of the two sizes of euthanasia lids, rat or mouse, via the quick release fitting at the center of the lid; rat cages will require use of the larger of the 2 lids.
    2. Place the lid over the cage so that the exhaust holes in the lid are over the cage.
    3. Animals should not be overcrowded. There should be sufficient space on the cage floor to accommodate all animals so that they can move freely.

4. **Administer CO2 slowly**
    1. CO2 flow is initiated and adjusted by turning the black **Flow control knob** on the CO2 regulator clockwise.
    2. Observe the **Flow gauge** and adjust the flow rate to **3 liters/minute for mouse cages** and **5-6 L/minute for rat cages**.
    3. Allow the CO2 to flow for **at least 3 minutes** before turning off the Flow control knob.
    4. Animals should be exposed to CO2 for **15 minutes for mice** and **30 minutes for rats.**
    5. **Rat users**- Please note that an additional physical method of euthanasia is required. (see IACUC Guideline for details)

6. **Death must be verified by monitoring the animal(s) for lack of breathing and absence of heart beat**. *(Animals which have stopped breathing but still have a heartbeat may revive!)*
    1. Death can be verified for observing for the absence of respiration and palpation of the heart by placing your thumb and index finger on opposing side of the animal's chest.
    2. Neonates (hairless pups) require considerably longer periods of gas exposure because their blood has a higher affinity for oxygen. Preanesthesia with CO2 followed by decapitation is recommended.

3. Dead animals should be removed from the cage and placed in a sealed plastic bag for disposal and placed into a RARC carcass refrigerator or freezer.

NOTE: **TURN THE SYSTEM OFF** by turning the **Flow control knob** counterclockwise (-) until it stops.

Please note: Contact RARC personnel for assistance, training, or questions regarding the procedures described.

APPENDIX 4
continued

Euthanizing rodents using a CO_2 flow gauge set-up

Flow Gauge
Close-up

Adjusting the **Flow-control knob** for either the "mouse" or "rat" setting on the ***Flow gauge***

DO NOT TOUCH THE HANDLE ON THE TANK!!!