

Guidelines for the Humane Euthanasia of Laboratory Animals

The University of Texas at Austin
Institutional Animal Care and Use Committee

These guidelines have been written to assist faculty, staff, and students in performing vertebrate animal procedures in a humane manner and complying with pertinent regulatory requirements. Under some circumstances deviations from these procedures may be indicated but such variances must be approved in advance by the IACUC.

This document provides information to be used when planning and performing euthanasia of vertebrate animals used for biomedical research or teaching at The University of Texas at Austin. It is organized into three parts:

Section A – General Background
Section B – Recommended Methods
Section C – Technical Comments

Section A – General Background

Definition

The *Guide for the Care and Use of Laboratory Animals* defines euthanasia as “...the act of humanely killing animals by methods that induce rapid unconsciousness and death without pain or distress” (p. 123). Techniques used for euthanasia must be chosen to assure that a rapid loss of consciousness will occur, followed shortly by death without pain or significant distress being perceived by the animal.

Humane Considerations

There is a wide variety of animal species used in biomedical research, and specific methods used for each species must be considered based on their anatomy and physiology. However, the general principles for humane euthanasia in all species have been summarized by the International Council for Laboratory Animal Science (2006):

Principles for Animal Euthanasia

<http://www.sciencemag.org/content/312/5774/700.full.pdf>

1. Whenever an animal's life is to be taken, it should be treated with the highest respect.
2. Euthanasia should place emphasis on making the animal's death painless and distress-free. The method likely to cause the least pain and distress to the animals should be used whenever possible.
3. Euthanasia techniques should result in rapid loss of consciousness, followed by cardiac or respiratory arrest and ultimate loss of brain function.
4. Techniques should require minimum restraint of the animal and should minimize distress and anxiety experienced by the animal, before loss of consciousness.
5. Techniques used should be appropriate for the species, age, and health of the animal.
6. Death must be verified following euthanasia and before disposal of the animal.
7. Personnel responsible for carrying out the euthanasia techniques should be trained:
 - (i) to carry out euthanasia in the most effective and humane manner;
 - (ii) to recognized signs of pain, fear, and distress in relevant species; and
 - (iii) to recognize and confirm death in relevant species.
8. Human psychological responses to euthanasia should be taken into account when selecting the method of euthanasia, but should not take precedence over animal welfare considerations.

9. Ethics committees should be responsible for approval of the method of euthanasia (in line with any relevant legislation). This should include euthanasia as part of the experimental protocol, as well as euthanasia for animals experiencing unanticipated pain and distress.
10. A veterinarian experienced with the species in question should be consulted when selecting the method of euthanasia, particularly when little species-specific euthanasia research has been done.

Gentle, careful handling of subject animals is of the utmost importance during the procedure in order to minimize distress to the animal. Measures should be taken to ensure that euthanasia is performed in a way that minimizes reactions among other animals that may be present. Euthanasia should be performed quickly and efficiently in a procedural area that is separate from rooms in which animals are housed.

When considering the impact of euthanasia on animal well-being, it is important to note that an unconscious animal does not perceive pain. Appropriately conducted procedures that render the cerebral cortex nonfunctional eliminate the perception of pain. Once this initial unconscious state is reached, reflex motor activity may still be observed, but pain is not perceived. This concept can be utilized in two-step approaches that combine an initial anesthetic event (e.g., general anesthesia via isoflurane or tricaine) with a secondary physical method (e.g., decapitation or exsanguination).

Protocol Requirements

Euthanasia is generally performed at the end of a project or, in some cases, at a point where animals would otherwise experience severe or chronic pain or distress that cannot be relieved. Because euthanasia may be needed as a means to relieve pain or distress that cannot be alleviated by analgesics, sedatives, or other treatments, protocols should include criteria for monitoring and initiating an early endpoint. This type of pre-planning for potential adverse outcomes will enable a prompt decision to be made by the research staff in conjunction with the veterinarian to ensure that the studies are humane and the objective of the protocol is achieved.

Even when the planned experiment does not include euthanasia, there may be a need to humanely euthanize animals for unanticipated reasons. For this reason, at least one method must be documented for each species used in a protocol.

Euthanasia techniques must be reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) during review and approval of the submitted protocol application form. Any subsequent change in euthanasia techniques must also be reviewed and pre-approved by the IACUC. The Office for Laboratory Animal Welfare (OLAW) characterizes the method of euthanasia as a significant component of the animal use protocol. **Use of a euthanasia technique that is not described in the approved protocol may be considered significant noncompliance, which can result in protocol suspension and mandatory reporting to the federal funding agencies that support the Principal Investigator.**

Training and Personnel Requirements

Euthanasia must be carried out by personnel properly trained in the procedure being used. This is especially important when physical methods such as decapitation, cervical dislocation or pithing are used, since these methods require a certain amount of expertise to assure a humane outcome. It is the PI's responsibility to assure that all persons performing euthanasia are properly trained and supervised to assure a high degree of technical competency. **All individuals performing euthanasia as part of a research project must be listed on the approved protocol.**

The clinical staff of the Animal Resources Center (ARC) is available to demonstrate and/or discuss euthanasia techniques.

Verification of Death

Proper euthanasia technique will include a physical examination or close observation to assure that the animal is dead prior to disposal. Death should be confirmed by personnel who can recognize cessation of vital signs in the species being euthanized. A combination of criteria is most reliable, including for most species the lack of a palpable pulse or heartbeat, breathing or opercular movements, reflex responses to noxious stimuli, inability to hear respiratory sounds and heartbeat by use of a stethoscope, graying of the mucous membranes, and rigor mortis. However, laboratory animals often possess characteristics making it difficult to confirm their death by observation alone, such as small body size, ability to tolerate hypothermia/hypoxia, slow metabolism, and/or being euthanized in groups. For this reason a secondary means to confirm death might be accomplished by methods such as thoracotomy, decapitation, pithing, vital organ harvest, exsanguination, freezing, or fixation. Verification of death is especially important when CO₂ or anesthetic gases are used and the animal is discarded intact, i.e., it is not used for tissue harvest or other invasive postmortem procedures.

Equipment Used for Physical Methods

Physical methods may include the use of instruments that are blunt (e.g., cervical dislocation), or sharp (e.g., decapitation or pithing). The Principal Investigator must assure that the choice of instrument is appropriate for the size and the anatomical conformation of the animal involved, with input from the Attending Veterinarian as needed. In many cases the use of specialized equipment such as a custom guillotine or enterotomy scissors will perform better than conventional scissors, knives or scalpels. Each lab must provide for the proper periodic evaluation and sharpening or replacement of equipment to assure proper function.

The IACUC has prepared guidance and recommendations for the operation and maintenance of guillotines: http://www.utexas.edu/research/rsc/iacuc/guillotine_requirements.html

Best Practice Information

The primary source document for appropriate euthanasia practices is the *American Veterinary Medical Association Guidelines for the Euthanasia of Animals*, last updated in 2013. However, the committee writing that report recognized that it cannot be considered an all-encompassing document, and the language allows the use of professional judgment based on other current literature sources. The following reference list includes some of the most useful and readily available sources to be used when euthanasia methods are being considered.

U.S. Guidance

- *AVMA Guidelines for the Euthanasia of Animals (2013)*
American Veterinary Medical Association
<https://www.avma.org/KB/Policies/Pages/Euthanasia-Guidelines.aspx>
- *Guide for the Care and Use of Laboratory Animals (2011)*
Institute for Laboratory Animal Research
http://www.nap.edu/openbook.php?record_id=12910

International Sources

- *CCAC Guidelines on Euthanasia of Animals Used in Science (2010)*
Canadian Council on Animal Care (CCAC)
<http://www.ccac.ca/Documents/Standards/Guidelines/Euthanasia.pdf>

- *Recommendations For Euthanasia Of Experimental Animals Part 1 (1996) and Part 2 (1997)*
European Commission
<http://www.lal.org.uk/>
Part 1: la.rsmjournals.com/content/30/4/293.full.pdf
Part 2: la.rsmjournals.com/content/31/1/1.full.pdf

Species-Specific Information

- *Report of the ACLAM Task Force on Rodent Euthanasia (2005)*
American College of Laboratory Animal Medicine
http://www.aclam.org/content/files/files/public/active/report_rodent_euth.pdf
- *Guidelines For Use Of Live Amphibians And Reptiles In Field And Laboratory Research (2004)*
American Society of Ichthyologists and Herpetologists
<http://www.asih.org/files/hacc-final.pdf>
- *Guidelines To The Use Of Wild Birds In Research (1999)*
The Ornithological Council
http://www.nmnh.si.edu/BIRDNET/documents/guidelines/Guidelines_August2010.pdf
- *Fish Research and the Institutional Animal Care and Use Committee (2003)*
Institute for Laboratory Animal Resources
http://dels-old.nas.edu/ilar_n/ilarjournal/44_4/v4404Borski.pdf
- *Guidelines for the Use of Fishes in Research (2004)*
American Fisheries Society
http://fisheries.org/docs/policy_useoffishes.pdf
- *Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research (2011)*
American Society of Mammalogists
<http://www.mammalsociety.org/articles/guidelines-american-society-mammalogists-use-wild-mammals-research-0>

Study Considerations and Alternatives

It must be recognized that it is extremely important for experiments be planned and performed in a way that ensures the validity of the data produced. If the euthanasia method used interferes with the ultimate goals of the research study and makes the data unusable, then the lives of the animals may have been wasted. Careful consideration of the possible adverse effects of the various options available must occur. There may occasionally be situations in which options that are not listed in this document might be considered acceptable. The 2013 AVMA guidelines list large-scale ecological sampling and open-ocean specimen collecting as types of studies that may require special humane killing considerations. Another unique situation arises when collection and analysis of brain tissue is the object of study, in which case an adjunct method such as pithing may be contraindicated. These exceptions must be carefully considered by the investigator and the IACUC to assure the best outcome for the animals as well as the study.

Section 2 – Recommended Agents and Methods of Euthanasia Listed By Species

The selection of specific agents and methods for euthanasia will depend on the species involved and the objectives of the protocol. Generally, inhalant or non-inhalant chemical agents (such as barbiturates, inhalant anesthetics, or CO₂) are preferable to physical methods (such as cervical dislocation or decapitation). However, scientific considerations might

preclude the use of chemical agents for some experimental studies. All methods of euthanasia must be reviewed and approved by the IACUC. Specific justification will be required when physical methods are used as the sole method on fully conscious animals.

***Asterisks denote recommended methods**

AMPHIBIANS

- *Barbiturates
- *Tricaine methane sulfonate (MS-222) - buffered
- *Benzocaine hydrochloride - buffered
- *Sedation prior to decapitation followed by pithing
- Inhalant anesthetics
- Carbon dioxide (CO₂)
- Stunning prior to decapitation followed by pithing

BIRDS

- *Barbiturates
- *Sedation prior to decapitation or cervical dislocation
- Inhalant anesthetics
- Carbon dioxide (CO₂)
- Small birds: cervical dislocation or decapitation

CATS & DOGS

- *Barbiturates
- *Injectable anesthetic drug overdose (e.g., Ketamine/Xylazine)
- Inhalant anesthetics
- Carbon dioxide (CO₂)
- Potassium chloride or exsanguination (under general anesthesia)

FISH

- *Tricaine methane sulfonate (MS-222) - buffered
- *Benzocaine hydrochloride - buffered
- *Quinaldine
- *2-phenoxyethanol
- *Barbiturates
- *Sedation prior to decapitation (or cervical transection) followed by pithing
- *Rapid chilling in icewater (zebrafish or other very small tropical/subtropical fish)
- *Inhalant anesthetics (administered in water)
- Clove oil, eugenol or isoeugenol
- Decapitation (or cervical transection) followed by pithing
- Stunning followed by pithing

NONHUMAN PRIMATES

- *Barbiturates
- *Injectable anesthetic drug overdose (e.g., Ketamine/Xylazine)
- Inhalant anesthetics
- Carbon dioxide (CO₂)
- Potassium chloride or exsanguination (under general anesthesia)

RABBITS

- *Barbiturates
- *Injectable anesthetic drug overdose (e.g., Ketamine/Xylazine)
- Inhalant anesthetics
- Carbon dioxide (CO₂)
- Potassium chloride or exsanguination (under general anesthesia)
- Cervical dislocation (< 1 kg)
- Decapitation

REPTILES

- *Barbiturates
- *Injectable anesthetic drug overdose (e.g., Ketamine/Xylazine)
- *Sedation prior to decapitation followed by pithing
- Inhalant anesthetics (in appropriate species)
- Carbon dioxide (CO₂) (in appropriate species)
- Physical methods such as decapitation after sedation
- Stunning prior to decapitation followed by pithing

RATS, MICE AND OTHER SMALL MAMMALS

- *Barbiturates
- *Injectable anesthetic drug overdose (e.g., Ketamine/Xylazine)
- *Sedation prior to decapitation or cervical dislocation
- Inhalant anesthetics
- Carbon dioxide (CO₂) – slow fill from a gas cylinder using a flowmeter
- Potassium chloride or exsanguination (under general anesthesia)
- Cervical dislocation (< 200 g)
- Decapitation

SWINE

- *Barbiturates
- Potassium chloride or exsanguination (under general anesthesia)
- Inhalant anesthetics
- Carbon dioxide (CO₂)

Section C – Technical Comments on Agents and Methods

Inhalant Anesthetics

Because most inhalant anesthetics act as topical irritants in their liquid state, animals should be exposed to the vapors of the anesthetic only. Chambers must be designed to assure the animals do not come into contact with the wicking material that is saturated with the liquid anesthetic. Sufficient air or oxygen must be provided during the induction period to avoid hypoxia prior to unconsciousness. All agents are given “to effect” until respiratory and cardiac arrest occurs. Care should be taken to minimize personnel exposure to vapors.

Isoflurane has a rapid mode of action and is readily available as a pharmaceutical grade product, so it is the most commonly used agent, delivered either via an anesthetic machine vaporizer or by using an open drop method in a sealed chamber. Other agents such as halothane and methoxyflurane are not currently available in the US in an appropriate form so they have become obsolete.

Ether had historically been used as a euthanasia agent, but it is highly flammable, can form explosive peroxides after exposure to air and light, and is known to be a distressful irritant when administered to animals. **Ether must not be used for euthanasia** of laboratory animals.

Non-Anesthetic Gases

Carbon dioxide has long been the preferred technique for euthanizing rodents and other small laboratory animals. Use of a sealed chamber (or a special adapter to fit the top of a home cage), a compressed gas cylinder, a regulator, and a flowmeter is required. The proper CO₂ flow rate should displace 10-30% of the chamber volume per minute. CO₂ generated by other methods, e.g., dry ice, is unacceptable because the gas flow cannot be adequately regulated. Chambers must not be overcrowded to avoid distress during the procedure. Because CO₂ can act as a reversible anesthetic, it is imperative that the animals be kept in the chamber for several minutes after respiratory arrest. In order to assure death after CO₂ in those circumstances where the animal is discarded intact (i.e., it is not used for tissue harvest or other invasive postmortem procedures), a physical means to assure death **MUST** be performed after CO₂ exposure. Examples of acceptable physical methods include cervical dislocation (for mice or rats no larger than 200 grams), thoracotomy (making a stab incision into the chest to open up the lung cavity), or decapitation.

Due to physiologic characteristics, neonates require prolonged exposure to the gas. For more detailed information, refer to IACUC guidance document “Guidelines for the Use of Carbon Dioxide (CO₂) for Rodent Euthanasia.”

Nitrogen, argon or carbon monoxide may be acceptable under specific and unique situations but have no clear advantages and are rarely if ever used in biomedical research.

Pharmacological Agents

Drugs used for euthanasia must be obtained as pharmaceutical grade compounds whenever possible. Please see related guideline “Guidelines for the Use of Drugs and Chemicals in Animal Research.”

Use of these agents requires adequate restraint and mastery of appropriate injection techniques. Barbiturates are acceptable for all species, but are most commonly used for mammalian species and birds. These drugs should be administered intravenously (IV) whenever possible, but intraperitoneal (IP) or intracoelomic administration is acceptable for rodents, amphibians, reptiles and fish. Intracardiac injection is an alternative, but this should be done only on animals that are already sedated or anesthetized. **Sodium pentobarbital** is the most common barbiturate agent for euthanasia, used either alone or in commercially available euthanasia mixtures. The dosage is usually at least three times that required for anesthesia, and ranges from 85 mg/kg for larger species to 200 mg/kg for some rodents. A dosage of 120 mg/kg is sufficient for most species, but more should be given if death

does not ensue. Commercial euthanasia formulations should be used following label directions (e.g., 1 ml/lb for Beuthanasia-D). Sodium pentobarbital and pentobarbital mixtures are controlled substances (Class II or III). Investigators using these agents must have current federal (DEA) and state (DPS) registration approval and are required to store the drug in a locked location and maintain detailed daily use records. For more information, contact the Environmental Health and Safety (EHS). Euthanasia using **potassium chloride** (KCl) is permissible only in an anesthetized animal. 75-150 mg/kg KCl should be given rapidly IV to effect, until rising serum potassium levels result in cardiac arrest. NOTE: The concentration of a saturated KCl solution is in the range of 200-300 mg/mL.

Tricaine methane sulfonate (MS-222) is a useful agent for aquatic species. It can be used as an injectable agent with IACUC approval in some specialized situations (200-300 mg/kg of a 1% solution in physiologic saline) but is most commonly administered as an immersion bath (500 mg/liter in H₂O) for amphibians and fish. The pH of the solution should be tested and buffered to neutrality as needed with sodium bicarbonate, especially when used in freshwater or low Ca⁺ seawater. The immersion time needed to assure death can range from 20 minutes to several hours, so it may be advantageous to use MS-222 as an initial anesthetic step followed by a physical method of euthanasia. Note: Cutaneous exposure to MS-222 can cause retinal toxicity in personnel. Chemical resistant gloves (e.g., nitrile) should be worn when using MS-222. There are currently two pharmaceutical grade forms of MS-222 available on the market. **Benzocaine hydrochloride** (250-500 mg/liter immersion) can be used as an alternative for amphibians and fish, but this agent also requires buffering. Use of benzocaine gel applied directly to the skin can be an acceptable method for some species of amphibian.

Another useful immersion agent for fish is **quinaldine** (100 mg/liter). **Clove oil** contains eugenol as the active compound, and the current literature states that these compounds (including isoeugenol) are acceptable agents for fish euthanasia (400 mg/liter). A pharmaceutical grade eugenol is being marketed to the fisheries field. Clove oil is also available in a USP grade which is used in human dentistry. **2-phenoxyethanol** can be used (0.6 ml of the liquid compound per liter) but it is not a preferred method because it can be slow acting in some species and adverse reactions can occur prior to unconsciousness.

NOTE: The poor aqueous solubility of some of the agents used for immersion methods may require the initial preparation of a concentrated stock solution in an alternative solvent. Acceptable protocols for such preparation are available in the literature and should be used.

Anesthetic overdose with some non-barbiturate injectable anesthetics (e.g., ketamine/xylazine) is acceptable in some species, using a dose that is typically 3-5X higher than the normal anesthetic dose. Alternatively, standard doses can be used to sedate or anesthetize animals prior to the use of a physical method in a two-step procedure.

Physical Methods (NOTE: These methods require that the user have experience and skill in the techniques to be used.)

Exsanguination is acceptable for all species if the animal is first rendered unconscious by another method. Rapid removal of blood can be accomplished by severing major vessels or (in smaller animals) by cardiac venipuncture. Exsanguination is also the ultimate cause of death during terminal perfusion procedures that include a saline or buffer flush prior to injection of fixative.

Cervical dislocation is acceptable for mice, birds, rats (< 200 gm) and rabbits (< 1 kg), but proper technique is essential. It is therefore recommended that animals be first sedated with another agent (carbon dioxide, pentobarbital or halothane are suggested). If specifically approved by the IACUC, it can be used as a sole means of euthanasia on conscious animals under circumstances where tissues uncontaminated by chemical agents are required. For more detailed information, refer to IACUC guidance document "Guidelines for the Use of Cervical Dislocation for Rodent Euthanasia." Investigators responsible for the use of this method must ensure that personnel performing cervical dislocation have been properly trained and consistently apply it humanely and effectively. Personnel should be trained on anesthetized and/or dead animals to demonstrate proficiency.

Decapitation with proper equipment may be performed on small mammals, birds, fish, amphibians, and reptiles after the animal has been sedated or lightly anesthetized (carbon dioxide, pentobarbital, or isoflurane are suggested). If specifically approved by the IACUC, it can be used as a sole means of euthanasia on conscious animals under circumstances where tissues uncontaminated by chemical agents are required. Investigators responsible for the use of this method must ensure that personnel who perform decapitation have been properly trained to do so and are monitored for competence. Personnel should be trained on anesthetized and/or dead animals to demonstrate proficiency. Decapitation of non-sedated fish, amphibians, and reptiles should be followed by **cranial pithing** (see below) to assure rapid loss of brain function. Decapitation should generally be used only when study design requires it due to the potential hazard to personnel and the possibility of operator error leading to a prolonged or distressful death. Many species react adversely to the smell of blood, so animals should not be decapitated in the presence of other animals and the person performing decapitation should change gloves and/or wash hands between animals. Adult rodents should be decapitated with a commercially available guillotine unless the procedure is performed under anesthesia or after cervical dislocation.

The IACUC has prepared guidance and recommendations for the operation and maintenance of guillotines: http://www.utexas.edu/research/rsc/iacuc/guillotine_requirements.html

Cervical transection is used as an alternative to decapitation in some fish species. It is performed using a knife or other sharp instrument inserted caudal to the skull to sever the spinal cord and cervical vertebrae. When performed in non-sedated fish, it should be followed by pithing.

Pithing is the insertion of an instrument (a rod or stiff wire) into the (brain case) and using a sweeping motion to physically disrupt the brain and brainstem. It is an adjunct method that should be performed immediately after decapitation of amphibians, fish, and reptiles to assure rapid loss of brain function in these species due to the potential tolerance of their brains to low oxygen concentrations.

Other physical methods. Stunning (a blow to the head sufficient to cause momentary unconsciousness) can be used as an alternative to sedation as the first step in the euthanasia of amphibians, fish, and reptiles, where it must be followed by decapitation and pithing. **Rapid chilling** using an ice-water slurry is a humane method for zebrafish and potentially other small tropical or subtropical fish species. Suitable fish must be no larger than 3.8 cm in length (tip of the snout to the posterior end of the last vertebra) and must be from a species that has a lower lethal temperature of above 4°C. **Flash freezing** by rapid immersion in liquid nitrogen may be indicated when unadulterated tissues are required but is only appropriate for small animals such as neonatal altricial rodents (less than 5 days old) and some fish species. Suitable animals must weigh less than 4 gm and must not be from a species that is known to physiologically tolerate freezing. Freezing can be used as a secondary method to assure death after an acceptable chemical or physical method has been used to kill the animal, but, these should not be performed until all signs of life have ceased (e.g., breathing, opercular movements, heartbeat, etc.) If an anesthetic gas or CO₂ has been used, another physical method should be used to assure death prior to placing animals in a freezer.