AUBURN UNIVERSITY

ANIMAL RESOURCES PROGRAM
CORE TRAINING MANUAL

Revised February 2013

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<th>Sections A, D, and E</th>
<th>should be read by Principal Investigators, Co-investigators, Students,* and Staff</th>
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<td>should be read by Principal Investigators</td>
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* Not required for students who are only exposed to animals in a classroom setting.
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A. Training Policy

The Office of Research Compliance (ORC) and Office of the University Veterinarian (OUV) will provide or coordinate training for faculty, staff, students and others (e.g., visiting faculty) engaged in the care and use of animals for research, teaching and/or demonstration purposes. Training will include review of institutional policies and procedures governing all activities involving live vertebrate animals, as well as species-specific technical training in animal husbandry, handling and related areas of animal care and use in research and education.

The Auburn University (AU) animal care and use training program has been developed in accordance with recommendations in *The Guide for the Care and Use of Laboratory Animals*, The Public Health Service Policy for the Care and Use of Laboratory Animals, The Animal Welfare Act (AWA), and *The Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching*. Specific elements of the training program have been defined in the context of and tailored to institutional needs at AU, and in light of federal policies governing animal use, all of which are likely to be dynamic.

The AU core training program will address:

a) Origins and evolution of institutional animal care and use programs and the regulations, policies and laws governing their operation.

b) Organization of the ORC.

c) The role of the Institutional Animal Care and Use Committee (IACUC) and procedures necessary for selection and completion of protocol review forms.

d) The “three R's” - Replacement, Reduction, and Refinement - as defined by Russell and Burch (*The Principles of Humane Experimental Technique*, 1959) and their application to the design of activities involving animals.

e) Requirements and procedures for identification and evaluation of alternatives to animal use.

f) Procedures for reporting animal welfare concerns and for responding to such reports.

g) Occupational health and safety issues.

h) Other such topics and issues as may be essential to satisfy regulations, policy or law governing animal use.

In addition, species-specific training addresses:

a) Basic biology, handling, husbandry and care of live vertebrate animals used in teaching, research and demonstration.

b) Basic techniques for intradermal, intraperitoneal, intramuscular and intravenous administration of drugs and for collection of blood and body fluid.

c) Methods of tranquilization, analgesia and anesthesia.
d) Principles and applications of surgery and guidelines for planning invasive procedures involving animals.

All principal animal users (i.e., principal investigators and/or course instructors; hereinafter referred to as PIs), as well as staff and students with assigned responsibilities for animal care and use in the context of research, teaching or demonstration activities, will receive and/or document training appropriate to their qualifications, experience and the specific circumstances of animal use proposed by them, in the case of PIs, or assigned to them, in the case of staff and students. PIs must be members of the Auburn University faculty with primary oversight responsibility for design and management of research, teaching and/or demonstration activities involving animals. PIs will be identified through Animal Use Protocol review by the IACUC. Each PI, as part of completing the Animal Use Protocol, will agree (by signature) to a certification statement assuring that all individuals performing animal procedures as a component of activities described in the Animal Use Protocol either are, or will be, prepared to perform their particular animal-related duties through documentable training and/or experience. Individuals to be engaged in activities involving animals must be apprised of relevant occupational health and safety risks before being allowed to work with the particular species. Moreover, all individuals working with animals must be enrolled in the AU Occupational Health and Safety Program (OHSP). This does not apply to students who are only exposed to animals in a classroom setting.

B. Origins and Evolution of Institutional Animal Care and Use Programs and the Regulations, Policies and Laws Governing their Operation

Animal and Plant Health Inspection Service (APHIS) implements the AWA through its Animal Care Policy Manual. (See also the Code of Federal Regulations Title 9, Chapter 1, Subchapter A, Parts 1, 2, and 3.) Standards are set forth in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, 1999). The Health Research Extension Act of 1985 greatly expanded provisions of the AWA, including a requirement that all institutions conducting biomedical and behavioral research under auspices of the AWA establish animal care committees.

In 1985, the White House Office of Science and Technology published the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. The United States Department of Health and Human Services (DHHS), Public Health Service implements these nine basic principles, developed by the Interagency Research Animal Committee, in its Policy on Humane Care and Use of Laboratory Animals. Guidance is provided in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1986). Whenever U.S. Government agencies develop requirements for testing, research, or training procedures involving the use of vertebrate animals, the following principles shall be considered; and whenever these agencies actually perform or sponsor such procedures, the responsible Institutional Official shall ensure that these principles are adhered to:

I. The transportation, care, and use of animals should be in accordance with the Animal Welfare Act and other applicable Federal laws, guidelines, and policies.

II. Procedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society.

III. The animals selected for a procedure should be of an appropriate species and quality and the minimum number required to obtain valid results. Methods such as mathematical models, computer simulation, and in vitro biological systems should be considered.

IV. Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings might cause pain or distress in other animals.

V. Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anesthesia. Surgical or other painful procedures should not be performed on unanesthetized animals paralyzed by chemical agents.

VI. Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure or, if appropriate, during the procedure.

VII. The living conditions of animals should be appropriate for their species and contribute to their health and comfort. Normally, the housing, feeding, and care of all animals used for biomedical purposes must be directed by a veterinarian or other scientist trained and experienced in the proper care, handling, and use
of the species being maintained or studied. In any case, veterinary care shall be provided as indicated.

VIII. Investigators and other personnel shall be appropriately qualified and experienced for conducting procedures on living animals. Adequate arrangements shall be made for their in-service training, including the proper and humane care and use of laboratory animals.

IX. Where exceptions are required in relation to the provisions of these Principles, the decisions should not rest with the investigators directly concerned but should be made, with due regard to Principle II, by an appropriate review group such as an institutional animal care and use committee. Such exceptions should not be made solely for the purposes of teaching or demonstration.

Animals covered by federal legislation/regulations and Guides are presented below.

**Animal Welfare Act/USDA Animal Care Policy**: any live or dead dog, cat, nonhuman primate, guinea pig, hamster, rabbit or any other warm-blooded animal, which is being used, or is intended for use for research, teaching, testing, experimentation, or exhibition purposes or as a pet. [It excludes birds, rats and mice bred for use in research, horses not used for research purposes, and other farm animals such as livestock and poultry used or intended for use as food or fiber or for improving animal nutrition, breeding, management, or production efficiency, or for improving the quality of food or fiber.]

**PHS Policy**: applicable to any live, vertebrate animal (e.g., traditional laboratory animals, livestock, poultry, wildlife, aquatic animals) used in research, research training, experimentation, biological testing, and related activities wherein the activity is supported by the PHS and conducted at Auburn University, or at another institution as a consequence of the subgranting or subcontracting of a PHS-conducted or supported activity by AU. Applicability also includes live vertebrate animals wherein the PHS does not support the activities but the animals are housed in the same facility as PHS-supported animal activities. The Policy requires that AU animal care and use programs be based on the Guide for the Care and Use of Laboratory Animals and that they comply, as applicable, with USDA regulations relating to animals. The PHS, through its Office for Laboratory Animal Welfare (OLAW), advises that the maintenance of uniform and consistent standards is an essential ingredient in the development and implementation of a quality animal care and use program. Only when the institution can document that the animal care and use program funded by a non-PHS source is entirely separate and distinct, physically and programatically, from PHS-supported activities will OLAW consider its exclusion from the AU Assurance of Compliance with PHS Policy on Humane Care and Use of Laboratory Animals. In any case, OLAW requires that non-PHS supported activities undergo IACUC review on a continuing basis.

**Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching**: any warm-blooded vertebrate animal used in agricultural research or teaching for which the scientific objectives are to improve understanding of the animal’s use in production agriculture and that may require a simulated or actual production agricultural setting consistent with the consideration of the animal’s well-being.
The cited federal regulations/legislation and standards are the basis for the Auburn University Policies for Use of Animals in Production, Maintenance, Teaching, Research and Demonstration. The AU Policy requires that the Institutional Animal Care and Use Committee (IACUC) oversee the use of all live vertebrate animals by AU faculty, whether for research, instruction, demonstration, production, or maintenance purposes and whether housed in facilities at Auburn or elsewhere. The goal is to apply a single standard of high-quality animal care for the benefit of overall animal health and well-being.

C. Auburn University Institutional Animal Care and Use Committee (IACUC)

IACUC Membership

The IACUC consists of at least fifteen members. The following shall serve continuing appointments: the Director of ORC—non-voting, University Veterinarian as the Institutional Attending Veterinarian (IAV); the Director of the Division of Laboratory Animal Health; and the Director responsible for Outlying Units, Alabama Agricultural Experiment Station (AAES). The following shall serve three-year rotating appointments: at least eight faculty members representative of animal-user disciplines (scientists, animal-users); at least one faculty member representing non-animal-user disciplines; and at least two members from the community who have no other current affiliation with the University and whose immediate families are not affiliated with the University. Rotating membership vacancies shall be filled for compliance with the regulations and, to the extent possible, with similarly qualified individuals. In addition to the qualifications noted above, one committee member must be a veterinarian. The chair of the committee shall be a faculty member with at least one year of experience as a member of the AU IACUC. The President of Auburn University appoints committee members and the committee chair. All committee members are required to sign and abide by a Confidentiality Agreement.

Membership of Auburn University’s IACUC meets the compositional requirements and recommendations set forth in the Public Health Service (PHS) policy, the AWA, and the Ag Guide, namely at least one: Doctor of Veterinary Medicine (DVM), with training or experience in laboratory animal science and medicine who has direct or delegated program responsibility for activities involving animals; practicing scientist experienced in research involving animals; scientist with experience in agricultural teaching and/or research using agricultural animals; animal, dairy or poultry scientist with training and experience in the management of agricultural animals; veterinarian with training and experience in agricultural animal medicine who is licensed to practice (or eligible for licensure); individual whose primary concerns are in a nonscientific area; individual not affiliated with the institution and not a laboratory animal user, who represents general community interests in the proper care and treatment of animals.
Federally Mandated IACUC Functions

a) Review, at least once every six months, the research facility’s program using the USDA Regulations/Guide as basis.

b) Inspect, at least once every six months, all of the animal facilities including animal study areas/satellite facilities, using the USDA Regulations/Guide as basis.

c) Prepare reports of IACUC evaluations and submit the reports to the Institutional Official.

d) Review and investigate legitimate concerns involving the care and use of animals at the research facility resulting from public complaints and from reports of noncompliance received from facility personnel or employees.

e) Make recommendations to the Institutional Official regarding any aspect of the research facility’s animal program, facilities, or personnel training.

f) Review and approve, require modifications in (to secure approval), or withhold approval of those components of proposed activities related to the care and use of animals.

g) Review and approve, require modifications in (to secure approval), or withhold approval of proposed changes regarding the care and use of animals in ongoing activities.

h) Suspend an activity involving animals when necessary, take corrective action, and report to the funding agency and the USDA.

The primary functions of the IACUC are to review and inspect all aspects of the institution’s animal care and use program (e.g. facilities, personnel training, occupational health and safety), review animal use protocols, review and investigate complaints about animal use, and make recommendations to the Institutional Official. The purpose of these reviews and inspections is to provide a mechanism that ensures compliance with all regulations and policies and to promote the welfare of animals without compromising valid scientific objectives that might benefit other animals and/or human beings.
IACUC Interactions

Protocol Reviews and Approval

Animal Use Protocol Forms and Animal Production/Maintenance Facility Standard Operating Procedures

The IACUC shall oversee the use of all live vertebrate animals by Auburn University, whether for research, instruction, demonstration, production, or maintenance purposes. Investigators/instructors using live vertebrate animals in such activities are required to submit a protocol review form for IACUC review. Two forms are available for submission. The choice of which form to use is determined as follows:

(1) To submit a protocol describing research, teaching, or demonstration activities, one should submit the “Animal Use Protocol Form.” There is a place on this form to indicate which type of activity is being described (e.g. research, teaching).

(2) To submit a protocol describing only the production and/or maintenance of animals (e.g. those animals being produced and/or maintained for the purposes of being used in various research or teaching activities), one should submit the form entitled “Animal Production/Maintenance Facility Standard Operating Procedures Form (SOP).”
It is understood that some protocols may involve more than one category of activity. For example, a protocol may involve birds being captured and released in the field for the purpose of data collection for a research project. However, this protocol may involve the simultaneous activity of teaching a class of students the technique of field capture and handling of birds. On such protocols it is necessary and permissible to check the activity as both research and teaching. Furthermore, if animals are being produced and/or maintained solely for a particular research or teaching project, then one may choose to submit the Animal Use Protocol Form and check the activity as both production/maintenance and research or production/maintenance and teaching, whichever is applicable.

Submission and Processing of Animal Use Protocol Form and SOP Form:
(1) Forms are available for download from the ORC forms website:
https://fp.auburn.edu/vpr/compliance/animalresources/?Forms

(2) One original form with all signatures is required for submission. Forms can be mailed or hand-delivered to:
Office of Research Compliance
IACUC Administration
115 Ramsay Hall Basement
Auburn University, AL 36849
IACUCadmin@auburn.edu
334-844-5978

(3) Forms received at least seven days prior to a scheduled meeting date (e.g. by 11:30 A.M. on Thursday of the week prior to a scheduled Thursday afternoon meeting) will be placed on the agenda. Forms received after the aforementioned deadline may be deferred to the following meeting.

(4) A primary and a secondary reviewer of each protocol are assigned in the IACUC office. Committee members are not assigned as reviewers of protocols on which they or a member of their academic department or unit are listed as the PI, co-investigator, faculty advisor, or departmental chairperson on the protocol.

Assessment of Scientific Merit
The IACUC in its review of animal care and use proposals considers general scientific merit by posing the following questions:
   (1) Is the research design sound?
   (2) Does the proposal contain appropriate rationale for involving animals?

Specific scientific merit (peer review) is beyond the scope of the IACUC’s responsibilities and qualifications.
IACUC Meetings
IACUC meetings are generally held on the first and third Thursdays of each month. They are open to the public unless proprietary and/or confidential information (e.g., trade secrets, methods and materials under transfer agreement) is under discussion. In such instances, the Chair may ask public attendees to leave the meeting for the duration of such discussions or deliberations.

Duration of Protocol Approval
A protocol will be approved for up to three years. Continuation of the project beyond three years requires submittal of another protocol review form for review by the IACUC. Notifications of impending expiration are sent to PIs and the relevant unit head at least 30 days in advance of the expiration date. Once a protocol has expired, a notification of expiration is sent to the PI, and copies of the notification are filed in the ORC.

Revision of an Approved Protocol
Changes in vertebrate animal use in research and teaching are inevitable, but federal regulations and AU policy require review and approval by the IACUC of significant changes in any activity involving the use of animals. All revisions to an approved protocol requested by the PI must be approved prior to being implemented. A Protocol Revision Form can be obtained on-line from the websites listed above. At the Chair’s discretion, the proposed revision may be referred to IACUC or to a subcommittee of IACUC. However, if any member of the IACUC requests that the proposed protocol revision(s) be reviewed at a convened meeting of the committee, then the revision must be deferred until such time that a meeting can be convened. In some cases, the IACUC Chair or the Director of the Office of Research Compliance may judge that changes are sufficiently different from the original protocol to warrant that a new protocol be submitted.

Policy: Significant changes include but are not limited to changes that result in:

- increased animal distress or pain or increased invasiveness of the procedures.
- changes in veterinary care, such as anesthetic agent(s), use or withholding of analgesics, and methods of euthanasia.
- using a different species.
- an increase in animal numbers.
- a change in the overall aims or objectives of the study.
- a switch from non-survival to survival surgery.
- performing additional procedures not described in the approved IACUC protocol.
- a change in hazardous substance use.
- allowing other investigators to use your animals on their protocols, or using animals approved for use on one of your protocols for use on another of your IACUC-approved protocols.
Annual Review of Approved Protocols
The ORC will forward to all PIs a reminder that an annual review is due at the end of each October. The reminder email will contain an annual review form for all PIs with protocols that were active during the previous fiscal year (October 1 to September 30). All changes to the approved protocol must be reported, and, if significant, will be reviewed by the IACUC at a convened meeting. Failure to submit an annual review report may result in closure of the protocol and activities being carried out.

Actions and Sanctions for Failure to Follow an Approved Protocol
In an instance of non-compliance with an approved protocol, the appropriate Dean (or designated Associate Dean), Director, or Vice President may utilize the standard disciplinary procedures set forth as a condition of each person’s employment with Auburn University. Other sanctions may be imposed in accordance with sponsor requirements and obligations or other applicable AU policies.

Appeal of IACUC Decisions
If a PI disagrees with the revisions required by the IACUC to obtain approval of a protocol, or with the disapproval of a protocol, the investigator may, with the concurrence of the appropriate Dean/Department Head or Chair, submit a written appeal to the IACUC stating the reasons for objecting to the required changes and/or proposing an alternative resolution. The PI may also request a meeting with the IACUC to discuss the differences of opinion and resolve them. If no satisfactory resolution is reached, the PI may submit a written appeal to the Associate Provost and Vice President for Research requesting assistance. The Vice President will attempt to mediate a solution to the situation. However, neither the Vice President nor any other administrative official can override disapproval by IACUC.

D. Completing the IACUC Protocol Form
Project Veterinarian

The PV’s signature on the protocol form documents the fact that “medical care for animals will be available and provided as indicated by a qualified veterinarian. By accepting this responsibility, the veterinarian is providing assurance that any personal interest he/she might have in the project will not conflict with his/her responsibility for the provision of adequate veterinary care for the animals. Furthermore, the veterinarian provides assurance of review and consultation on the proper use of anesthetics and pain relieving medications for any painful procedures.”

The USDA Animal Welfare Act regulations and standards stipulate that if procedures on animals are proposed that may cause more than momentary or slight pain or distress
to animals, consultation with the PV must occur in the planning of those procedures. The consultation can be in the form of a meeting or phone consultation between the PI and the veterinarian, or it can be in the form of a review of the protocol forms by the veterinarian before the IACUC meeting so that revisions to the animal form can be made, if needed, prior to IACUC review.

Criteria for Granting Approval of Protocols (per AWA Mandates and the Guides)

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<tr>
<th>Criteria</th>
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<tr>
<td>Activities</td>
<td>Must be in accord with USDA Regulations/PHS Policy.</td>
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<tr>
<td>Pain/Distress</td>
<td>Must avoid/minimize discomfort, distress, and/or pain. If pain/distress is caused, appropriate sedation, analgesia or anesthesia will be used. Project veterinarian (PV) must be involved in planning. Use of paralytics without anesthesia is prohibited. Animals with chronic/severe unrelievable pain will be euthanized.</td>
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<td>Surgery</td>
<td>Must meet requirements for sterile surgery and pre/postoperative care. Cannot use one animal for several major operative procedures from which it will recover, without meeting specified conditions.</td>
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<td>Euthanasia</td>
<td>Euthanasia method must be consistent with the American Veterinary Medical Association (AVMA) recommendations.</td>
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<td>Housing/Health</td>
<td>Living conditions for animals must be consistent with standards of housing, feeding and care directed by veterinarian or scientist with appropriate expertise.</td>
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<tr>
<td>Alternatives</td>
<td>Alternatives to painful procedures must be considered; must document consideration of alternatives if animals experience pain or suffering.</td>
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<tr>
<td>Rationale and Methods</td>
<td>Must provide written narrative of methods/sources, rationale for using animals, and the reasons for using the requested species and the number of animals.</td>
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<td>Duplication</td>
<td>Must provide assurance that activities do not unnecessarily duplicate previous efforts.</td>
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<td>Qualifications</td>
<td>Personnel must be appropriately qualified.</td>
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<td>Deviations from Requirements</td>
<td>Must be justified for scientific reasons, in writing.</td>
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Explaining Why the Use of Animals in Research is Important

"Study/activity justification and objectives." This is a very important issue because you are asking for the privilege of using animals for procedures that rarely will benefit them individually, and might result in their death. In general, there must be a compelling potential for benefit to human or animal health to warrant the use of animals. Points to consider:

- If you are studying a human or animal disease or health concern, it is helpful to carefully explain the disease, what causes it, what therapy is currently used to treat it, and how the proposed animal experiments might better prevent human or animal pain and suffering.

- Because there are non-scientists on the IACUC, your response should be written so that members of the general public (including the lay members on the IACUC) would readily understand why it is important to use animals for your work.

- Make sure you explain medical terms and define abbreviations the first time they are used.

Describing Your Animal Studies

In the description of the experimental design or activities involving animals, keep in mind that the IACUC needs to understand the proposed use of animals. To perform an appropriate review of your proposed animal work, IACUC members must understand what combination of procedures will be performed on individual animals. Details of procedures such as surgery and euthanasia are requested elsewhere, but the general types of surgery and the final disposition of the animals should be clear from your response. Keep these points in mind:

- For more complex experiments, it is very helpful to provide a flow chart to make the experimental design clear.
- The description of the animal procedures should stand by itself. The IACUC should not have to read another document such as a grant application to understand what you propose.
- Define all abbreviations the first time they are used to facilitate comprehension.
- Do not use technical language that only specialists in your field would understand. Not only is it difficult for trained professionals to navigate through technical jargon outside their fields, there are non-scientists and lay members serving on the IACUC.
Selecting the Species

The central theme evaluated by the IACUC is this - assuming that animals are indeed necessary, the least sentient ("aware") species capable of providing the needed data should be used. The hierarchy of sentient species can be a subject of disagreement, but generally is as follows:

- Apes (chimps, orangutans, gorillas)
- Monkeys (baboons, rhesus monkeys, marmosets, tamarins)
- Larger animals commonly kept as pets such as dogs and cats
- Larger animals such as pigs and goats commonly used as farm animals
- Rabbits
- Rodents (guinea pigs, hamsters, rats, mice)
- Non-mammalian vertebrates (poultry, amphibians, reptiles, fish)
- Invertebrates (crustaceans, slugs)
- Smaller life forms (insects, arachnids, worms)
- Single cell organisms (yeast, bacteria, etc.)

Justifying the Species

Justifications for using a particular species may include:

- The presence of previous work in the biomedical literature that validates the use of a particular species in an animal model of a human disease.

- The existence of a large body of previous laboratory data that would have to be repeated if another species was used instead.

- Characteristics of the species that render it uniquely suited to the proposed research.

- Size, availability, and cost.

- Availability of reagents or research tools unique to that species.

Cost savings alone is not an adequate justification for using a particular species. The justification should be based on sound scientific reasoning.

Justifying the Number of Animals Requested

You are asked to request a certain number of animals, and justify why you need that number. The IACUC realizes that it can be difficult to provide such information in advance.
Some important points:

- According to *the Guide*, a statistical analysis should be used to justify animal numbers. Commonly a power analysis is the most appropriate tool for justifying group sizes, but consult a biostatistician for the best tools for your particular studies. You might even discover that you need to request more animals per group than you thought would be necessary.

- It is acceptable to request animals that will be used to perfect surgical or other techniques prior to initiating planned experiments. This is preferable to beginning a large experiment that will experience technical problems that might cause pain or distress to the animals.

- Studies on cadavers from other approved protocols in advance of any procedure on a live animal are strongly encouraged. By doing this, techniques can be perfected as much as possible before any live animals are used.

- It is also acceptable to ask for animals that will be used in pilot experiments in addition to animals requested for more robust experiments. Pilot experiments can be used to perfect technique, demonstrate feasibility, or provide a justification for proceeding with larger, more tightly controlled experiments.

The “Three R’s” - Replacement, Reduction, Refinement

The concept of alternatives to animal use was first introduced in 1959 by the British scientists Russell and Burch (In: The Principles of Humane Experimental Technique, Methuen, London). A responsibility of the IACUC (mandated by federal policy and regulations) is to ensure that animal users make appropriate efforts to consider and/or fulfill the three R’s.

**Replacement** Substitute non-animal techniques for animal usage. Examples include:
- cell culture or tissue culture systems
- computer simulations
- in vitro assays such as immunologic bench assays to replace animal bioassays.

It is not very common for any of the above alternate systems to adequately replace animals in experiments. However it is possible and consideration should always be given to non-animal systems.

**Reduction** Decrease the number of animals used for a particular activity or project whenever appropriate. Examples include:

- Limiting group sizes to the minimum needed to obtain statistically significant data.
• Performing multiple experiments simultaneously so that the same control group can be used for all the experiments.
• Sharing tissues with other investigators so that additional animals are not needed.
• Designing experiments so that animals serve as their own controls.
• Using newer instrumentation that improves precision and reduces the number of animals needed per data point.

Reduction is usually more feasible than replacement. However, when considering how to reduce animal use, you must find a balance between causing more pain or distress on fewer animals and causing less pain or distress in more animals. For instance, if an investigator proposes to double the number of invasive surgical procedures on animals so that fewer animals are used, the increased pain and distress experienced by the remaining animals may not be justified by a simple reduction in animal use. This is a difficult area, and you should seek advice from your PV and the IACUC as needed.

Refinement Modify a technique or activity so as to reduce the pain and/or distress experienced by animals. Examples include:

• New anesthetics that allow rapid induction and reduced recovery times.
• New analgesics that provide more extended pain relief postoperatively with less frequent administrations.
• New bleeding and injection techniques that cause less tissue damage or distress.
• Improved surgical techniques that minimize trauma and the length of anesthesia.

Check literature and with your veterinarian concerning improved techniques that have evolved that reduce pain or distress on the animals.

U.S. animal welfare regulations define a painful procedure as one that “would reasonably be expected to cause more than slight or momentary pain or distress in a human being to which that procedure was applied, that is, pain in excess of that caused by injections or other minor procedures.”
Requirements and Procedures for Identification and Evaluation of Alternatives to Animal Use

In accordance with the Health Research Extension Act of 1985, scientists performing painful experiments on animals must document if there are alternative methods to the painful procedure and report this information to the IACUC when they submit their animal use protocol form for approval. It is then the responsibility of the IACUC to determine if the alternative methods should be used. To assist IACUC’s and investigators in complying with this portion of the law, Congress established the Animal Welfare Information Center (AWIC) at the National Agricultural Library (10301 Baltimore Avenue, Beltsville, MD USA 20705-2351, Tel: 301 504-6212, Fax: 301 504-7125, e-mail: awic@nal.usda.gov website: http://www.nal.usda.gov/awic).

The regulations require, as a minimum, that an investigator perform a search of the literature in an attempt to identify alternatives to painful procedures. A multi-database approach is usually necessary, as an alternative procedure or method may originate from outside the specific discipline being studied.

1) Directory/websites for alternatives (examples)

OLAW http://grants.nih.gov/grants/olaw/olaw.htm
Office of Laboratory Animal Welfare
National Institutes of Health (NIH)
RKL1, Suite 1050, MSC 7982
6705 Rockledge Drive
Bethesda, MD 20892-7982
Phone: (301) 498-7163
Fax: (301) 402-2803

Altweb http://altweb.jhsph.edu
Altweb is a site for news, information, discussion, and resources from the field of alternatives to animal testing. This site is a collaborative effort funded by the alternatives Research & Development Foundation, the Doerenkamp - Zbinden Foundation, the Humane Society of the United States, the Office for Protection from Research Risks at the National Institutes of Health, and the Procter & Gamble Company. It is being developed by the Center for Alternatives to Animal Testing at Johns Hopkins University in collaboration with the Altweb Project Team, to serve academic, industrial and government scientists, educators, the media, and the general public.

Altweb is intended to foster the development of scientifically acceptable in vitro and other alternatives to animal testing. Alternatives are defined as methods that reduce animal use, replace whole animal tests, or refine existing tests by minimizing animal distress.

Animal Welfare Information Center http://www.nal.usda.gov/awic
United States Department of Agriculture (USDA)
This site provides access to:

- Full-text versions of all pertinent Federal laws, regulations, guidelines and policies, and links to international laws,
- AWIC newsletters,
- AWIC publications,
- Links to databases, information on alternatives, farm animals, and organizations,
- Links to the National Agricultural Library, Animal and Plant Health Inspection Service, Office for Protection from Research Risks, and NetVet.

VETINFO, Veterinary Information Service  [www.vetinfo.com](http://www.vetinfo.com)

Compilation of Literature on the Assessment of Animal Welfare and Animal Distress

Extensive bibliography and links to full-text documents related to the assessment of pain in animals, animal welfare, and animal distress. Produced by Dr. J.D. Kuiper, Department of Laboratory Animal Sciences, Utrecht University, The Netherlands, and Tim Allen, Animal Welfare Information Center, U.S. Department of Agriculture.

Institute of Laboratory Animal Resources (ILAR) Journal  [http://dels.nas.edu/ilar/](http://dels.nas.edu/ilar/)

ILAR Journal is the quarterly, peer-reviewed publication of the Institute for Laboratory Animal Research, which is a unit of the National Research Council, National Academy of Sciences. ILAR Journal provides thoughtful and timely information for all those who use, care for, and oversee the use of laboratory animals. Provides access to online version of the journal and many back issues; a searchable index is available.

2) Databases

Major Databases Include:
- AGRICOLA 1970 to the present
- CAB-INTERNATIONAL DATABASES 1972 to the present
- MEDLINE 1984 to the present
- CSA LIFE SCIENCES 1985 to the present

Core databases include:
- AT ALTERNATIVES circa 1920s to the present
- BIOLOGICAL VALUES
- BIOMEDICAL DISSERTAIONS
- BOOKS
- CABLINE
- CURRENT CITATION 1995 to the present
- DRUG DOSAGES
- INSIDE CONFERENCES
- LABORATORY ANIMAL LITERATURE circa 1920s to the present
Unnecessary Duplication

The USDA Animal Welfare Act Regulations state that IACUCs must evaluate a written assurance that the proposed animal studies do not unnecessarily duplicate previous studies. You are asked to document that your proposed work is not unnecessarily duplicative on the IACUC forms.

The form of the written documentation is not specified by the Animal Welfare Act, but typically the same types of documentation used for the alternatives mandate do double duty here. Experience has shown that database searches are effective ways to document that work proposed is not unnecessarily duplicative.

Note that the critical concept is that unnecessary duplication is not allowed. Acceptance of new ideas in science is often dependent upon the ability of other scientists to duplicate published reports. The IACUC can allow duplication of previous work if you can convince them that it is important scientifically to do so.

USDA Pain/Distress Categories

Auburn University requires the PI to assign animals to pain/distress categories on the animal protocol form. The goal here is to help you correctly classify experimental procedures on laboratory animals into USDA pain/distress categories, upon which the institutional categories are based.

Defining Painful and Distressful Procedures

A simple yet useful definition of a painful or distressful procedure on an animal is this:

"A procedure that would cause pain or distress in a human."

It is important to understand that if multiple procedures will be performed on an animal, the animal is placed in the category appropriate for the most painful/distressful procedure. One animal cannot be placed in multiple categories.
Category “B”
Category B animals are those that are being "bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery but not yet used for such purposes." These animals have not been used for any research procedure, however minor. Category B is the correct category for breeders and other animals that are not undergoing any experimental procedures.

The availability of such a group of animals must be detailed on the AU IACUC protocol form entitled “Animal Production/Maintenance Facility Standard Operating Procedures.”

Category “C”
Category C animals are not subjected to procedures that involve pain or distress or would require the use of pain-relieving drugs. Routine procedures such as injections and blood sampling from veins that produce only mild, transient pain or discomfort are reported in this category. Another example of category C procedures is an observational study of animal behavior. Animals that are euthanized before tissue collection or other manipulations are also commonly placed in this category, if no other procedures are performed that would place them in a higher pain/distress category.

Category “D”
Category D animals are those subjected to potentially painful procedures for which anesthetics, analgesics, or tranquilizers will be used. The important concept is that animals are given appropriate anesthesia and/or pain relief to limit their pain and distress as much as possible.

Examples of category D procedures are:

- Surgery conducted with appropriate anesthesia and postoperative analgesia;
- Rodent retroorbital eye bleeding performed under anesthesia;
- Removal of a small tumor under local or general anesthesia, and use of analgesia after an animal's skin is exposed to ultraviolet light to cause a "sunburn"; and
- Terminal exsanguination (euthanasia by removal of blood) under anesthesia.

Category “E”
Category E animals are those that are subjected to painful or stressful procedures without the use of anesthetics, analgesics, or tranquilizers. Withholding of anesthetics, analgesics, or tranquilizers can only be allowed if it is scientifically justified in writing and approved by the IACUC. Examples of category E procedures are lethal dose studies (e.g. LD50 studies) that allow animals to die without intervention, pain studies that would not be possible if pain-relieving agents were administered, and psychological conditioning experiments that involve painful stimuli such as a noxious electrical shock that cannot immediately be avoided by an animal.
Category E studies are given increased scrutiny by the IACUC because it must be satisfied that less painful or stressful alternatives are not available, or that less painful/stressful endpoints cannot reasonably be used. By law, the institution must annually report all category E procedures to the USDA and include a scientific justification supporting the IACUC's decision to approve them. Often, the justification given by the researcher on the animal forms submitted to the IACUC is used for the annual report.

It is important for information on category E procedures to be complete and accurate. Once submitted to the USDA, this information is available to the public.

Humane Endpoint Criteria

Federal regulations require that IACUCs determine that discomfort to animals will be limited to that which is unavoidable for the conduct of scientifically valuable research, and that unrelieved pain and distress will only continue for the duration necessary to accomplish the scientific objectives. The criteria used for intervention in research studies to prevent unnecessary pain and distress are called "endpoint criteria" because they describe when it is time to either:

- Euthanize an animal to prevent suffering;
- Discontinue a painful procedure; or
- Remove an animal from a study.

Common examples of endpoint criteria include a limit on weight loss as a percentage of body weight; anorexia for an extended time; sudden pain or distress that cannot be controlled with analgesics, sedatives or tranquilizers; or severe medical conditions that cannot be controlled with appropriate therapy (e.g. severe systemic infections, kidney or liver failure, heart disease).

More specific criteria are often used for certain types of studies. For example, endpoint criteria used for rodent cancer studies involving the growth of tumors under the skin often include maximum tumor volumes or tumor weight as a percentage of body weight, skin ulceration over the tumor, interference with normal gait or movement, and interference with normal feeding and drinking behaviors.

Endpoint criteria for mice used to produce ascites fluid rich in monoclonal antibodies will be discussed later.

Humane endpoint criteria should be addressed on the IACUC protocol form when it is anticipated that an animal will endure painful or distressful conditions.
Death as an Endpoint
The use of death as an endpoint in animal experiments is strongly discouraged.

Legal, regulatory, and moral guidelines require that animal pain and distress be minimized.

For these reasons, investigators are encouraged to administer euthanasia in death endpoint experiments prior to the actual death of the animal unless a compelling case can be made that experimental validity would be irrevocably compromised.

These objectives assume that investigators can differentiate between animals that are found morbid (i.e. affected with disease and illness), and those that are found moribund (i.e. in the state of dying).

The IACUC believes that the PI and the responsible PV can judge and should perform euthanasia on moribund animals. Their judgment should be based on their professional experience with the animal model used in the experimental protocol. They should professionally and objectively evaluate the signs of dying with the animal model and perform euthanasia accordingly.

Investigators are expected to justify experimental endpoints and to agree that they can judge and will perform euthanasia on animals found moribund in a particular protocol. Moreover, all investigators are expected to monitor experimental animals at least daily (including weekends and holidays) to achieve this objective.

If experimental death itself is the required endpoint, the investigator must first receive approval to conduct such studies by providing strong scientific justification to the IACUC. Inconvenience or increased costs alone are not justifiable reasons.

Federal law authorizes veterinary staff to euthanize animals in states of unauthorized, uncontrolled pain or distress. The PI is strongly encouraged to work closely with the PV in cases where uncontrolled pain or distress may develop.

Guidelines on morbundancy and times for intervention by euthanasia include: impaired ambulation (unable to reach food or water easily, inability to remain upright); evidence of muscle atrophy or other signs of emaciation; any obvious prolonged illness including such signs as lethargy (drowsiness, aversion to activity, lack of physical or mental alertness), prolonged inappetence; difficulty breathing; central nervous system disturbances; or chronic diarrhea or constipation.

Euthanasia

Euthanasia literally means a "good death." A more appropriate simple definition is a "gentle death."
The USDA AWA defines euthanasia as "the humane destruction of an animal accomplished by a method that produces rapid unconsciousness and subsequent death without evidence of pain or distress, or a method that utilizes anesthesia produced by an agent that causes painless loss of consciousness and subsequent death."

*The Guide* defines euthanasia simply as "the act of killing animals by methods that induce rapid unconsciousness and death without pain or distress." When it is necessary to euthanize animals as part of experimental protocols, it is very important to use appropriate euthanasia techniques.

**Training**

Because improper technique can cause pain and suffering to animals during euthanasia, you must be trained to properly and humanely perform euthanasia. Of course, the IACUC is interested in your training and ability to humanely perform any procedure on animals, but proper training for euthanasia is an area of emphasis because of the increased potential for harm to animals.

Do not perform euthanasia or any other procedure on an animal until a person experienced with the procedure has trained you and you feel confident performing the technique.

**Verifying Death**

It is very important that you make sure an animal is really dead before placing it in a bag and disposing of the bag. It is easy to mistake a deeply anesthetized animal for a dead animal, and you do not want the animal to experience the terror of waking up in a closed bag and slowly suffocating to death.

OLAW and USDA recently emphasized the importance of ensuring that euthanized animals are really dead, and further stated that unintended recovery of animals after euthanasia represents 1) serious noncompliance with the PHS Policy and 2) a serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals. Such incidents must be reported to OLAW by the IACUC with a full explanation of the circumstances and actions taken to prevent recurrence.

**E. Mechanism for Receipt and Review of Concerns Involving Care and Use of Animals at AU as Registered Via Public Complaints and by Employees or Students**

The ORC and/or the IACUC will review and/or investigate any concern relating to animal care and use brought to the attention of the Committee. This includes claims by the public concerning any aspect of the animal care and use program or by employees or students who report alleged instances of animal abuse, violation of approved protocols,
use of animals not covered by approved protocols, violation of any animal related regulation or standard (such as the AWA, PHS Policy or IACUC Policy), or complaints regarding the care received by animals housed in University laboratory animal, wild animal or agricultural facilities.

**STEPS IN THE PROCESS**

a) Concerns should first be addressed to the individual(s) or unit at whom/which the complaint is directed. If the concern cannot be handled directly and an emergency situation exists, the University Veterinarian (UV) should be contacted immediately (334/844-5667, 334/844-4622). The UV, or a designee, will take any necessary immediate action. If the concern is not an emergency, is not adequately addressed, and/or if there is fear of retribution, a formal complaint should be filed (see below).

b) A formal complaint is initiated by contacting one of the following individuals:
   - Director, ORC 334/844-5978
   - The Chair of the IACUC (Name and phone number may be obtained by calling ORC 334/844-5978.)
   - University Veterinarian 334/844-5667

c) Information to be provided in the formal complaint shall include:
   - Complainant’s name
   - Individual(s) or unit the complaint is against.
   - Description of the event or charges, including applicable dates of observations and documentation to substantiate the charges.
   - Signature of complainant.

d) A signed complaint must be submitted to an individual listed under b (above) for a formal review to be conducted.

While hearsay complaints cannot be formally filed, individuals who have serious concerns based on hearsay evidence can call any of the individuals listed in b (above). The individual contacted, or a designee, may follow-up on concerns by means other than the formal complaint process such as review of protocols, discussions with employees, or unannounced laboratory inspections. The process may lead to the filing of a formal complaint.

The signed formal complaint will be submitted to the Chair of the IACUC as soon as possible. A formal complaint should remain confidential to the extent possible to protect all concerned. The Chair, within three days of receiving the formal complaint, will appoint a subcommittee composed of three IACUC committee members to investigate the concern. In an initial inquiry phase, the subcommittee will focus on information gathering and fact-finding to confirm the concern as warranting a formal investigation and evaluation of all relevant facts versus a concern that may be based on mistaken allegations. At the inquiry phase, the individuals at whom the concern/complaint is directed will be informed of the nature of the concern/complaint and of the investigative procedures to be followed and given an opportunity to explain their side of the issue. As
much documentation as is reasonably needed to support or refute concerns involving care and use of animals will be collected. Such information may include, but not necessarily be limited to, interviews of all parties involved, inspecting facilities, collection of pertinent documents, on-site evaluation of animals, and detailed review of procedures with responsible personnel.

The subcommittee will prepare a report for the IACUC. The IACUC will immediately review the concern or complaint and will determine what action will be taken (majority quorum vote and minority opinions will be recorded). The Chair of the IACUC will immediately notify the individual(s) at whom the concern/complaint is directed, the relevant facility director, department/unit head and dean/director, the Director of ORC, and the complainant. The Director of ORC will inform the Office of the Vice-President for Research and others as directed by the OVPR.

Reports will be filed in the OVPR and in the OAR for documentation and investigation of the incident. No employee, student, IACUC member or laboratory personnel shall be discriminated against or be subject to any reprisal for reporting perceived noncompliance with any of the regulations or policies pertaining to animal care and treatment.

The IACUC, through the Institutional Official, shall file a report with appropriate federal or state agencies as dictated by the actions taken by IACUC and by applicable compliance standards.

F. Validation of Relevant Content of Grant Proposal vs. IACUC-approved Protocol

- For determination of consistency between the grant proposal and protocol, the IACUC staff must review a copy of the proposal in its final form (i.e. as it was submitted to the funding agency).
- The PI is informed by written notification of any discrepancies that would require protocol revision.
- When requests for protocol modification are submitted may depend on the funding agency (e.g. if IACUC approval is required prior to scientific peer review) or the PI’s preference.

Validation: PI’s Preference

- Option 1: Request for protocol modification is submitted to the IACUC office well in advance of the funding decision.
  - Advantage: PI should avoid delay in the establishment of an account for a funded project.
  - Disadvantage: Wasted effort if not funded.
- Option 2: PI does not request protocol modification request until after the
award announcement is received.
  - Advantage: Time-effective in preparation of request.
  - Disadvantage: May delay establishment of an account.

G. Additional Considerations Pertaining to Protocols

Surgery

Surgery on animals requires highly trained, conscientious individuals, and appropriate prior planning. To understand the issues involved, some important terms and concepts must be addressed. Surgery is addressed in some detail in the animal protocol forms.

Sterile or Aseptic Technique: This refers to a series of practices followed to prevent the contamination of the surgical site by microbes during surgery. If an animal will recover from surgery, sterile technique must be used.

General anesthesia: Simply stated, general anesthesia is a state of unconsciousness characterized by a complete lack of pain and sensory perception.

Regional anesthesia: In contrast to general anesthesia, regional anesthesia refers to preventing pain and sensory perception in one small part or a region of the body, such as a section of skin or an entire limb.

Prior to beginning surgery, you must ensure that the animal will not feel pain during the procedure. General or regional anesthesia must be provided.

Survival Surgery
It is important to distinguish between survival and non-survival surgery.

Survival surgery is surgery in which the animal regains consciousness after anesthesia. If an animal undergoes survival surgery, aseptic (sterile) technique must be used to prevent postoperative infections, no matter what vertebrate species is involved. The incision site must be properly prepared prior to the incision. The hair must be clipped and the skin must be disinfected, often with chlorhexidine or iodine solutions.

Non-Survival Surgery
Non-survival surgery is surgery in which the animal is euthanized while under anesthesia, and does not regain consciousness. If an animal undergoes non-survival surgery, sterile technique may not be required. Even though the animal will not survive beyond the end of surgery, at a minimum, the surgeon should wear gloves, the surgical site should be clipped, and the instruments and work area should be clean.
Major vs. Minor Surgery

Major surgery is defined as surgery that penetrates and exposes a body cavity such as the chest or abdomen or surgery that produces substantial physical or physiological impairment. Examples of major surgeries include laparotomy, thoracotomy, craniotomy, joint replacement, and limb amputation.

Minor surgery is less invasive surgery that does not meet the criteria for major surgery above.

Multiple Major Survival Surgeries

Multiple major survival surgeries on one animal are discouraged unless they can be justified scientifically by the investigator and approved by the IACUC. There are a number of justifications commonly accepted by IACUCs, based on guidance provided by the Animal Welfare Act Regulations and Guidelines and the Guide. These include:

- Scientific justification - if more than one major survival surgery must be performed to achieve research objectives;
- Conservation justification - if the animal species under consideration is rare, endangered, or in short supply;
- Clinical justification - if the animal must undergo multiple major survival surgeries to treat a medical condition, in consultation with a veterinarian;
- Other unusual justifications - if approved by the USDA Chief Administrator.

Cost savings alone is not an adequate justification for multiple major survival surgeries. Major survival operative procedures must not be performed a second time on an animal in a separate IACUC proposal. Animals surviving a major operative procedure must be identified (written documentation) to prevent their use in a second major survival operative procedure.

Location

The rooms that can be used for surgery vary depending on:

- The species.
- Whether a surgery is major or minor.
- Whether the surgery is survival or non-survival.

A dedicated surgical suite is required for major survival surgery on all non-rodent mammals (this includes rabbits).

In contrast, a clean area or portion of a room (along with the use of aseptic technique) is acceptable for:
• Major survival surgeries on rodents and lower vertebrates.
• All non-survival surgeries.
• All minor survival surgeries.

Postoperative Care for Survival Surgeries
Postoperative care must be provided after survival surgeries. The animal should be monitored to make sure it is recovering properly. If the surgical procedure would be expected to cause pain in a human, then it should be assumed that the procedure will be painful in an animal, no matter what the species, and appropriate postoperative analgesia should be provided unless nonuse is approved by the IACUC. The agent, dose, route, frequency, and duration of postoperative analgesia provided should be discussed with and approved by a veterinarian, preferably during the planning stages of the experiments.

Documenting Postoperative Care
Documentation of postoperative care is very important. A simple rule to follow is this – "if it isn't written down, it didn't happen." The USDA requires that health care records be maintained in a manner consistent with prevailing professional veterinary practice standards.

For animals larger than rodents, individual health care records are usually maintained, with records of daily observations and treatments during the postoperative care period.

For smaller animals like rodents, group records instead of individual records are usually kept. The veterinary staff or the research staff may maintain the records, but the records should always be accessible to the veterinary staff should complications arise. The records should be maintained at least a year after the death of the animal to meet USDA policy.

Postoperative Recovery Period
In the absence of complications, the postoperative period traditionally ends 7-10 days after surgery, when skin sutures are often removed. After that, routine daily monitoring can be resumed, and routine entries in the health records discontinued.

The USDA does not allow changes in animal ownership during the postoperative recovery period, and does not allow movement of the animal between facilities during recovery from anesthesia unless the IACUC approves it. These prohibitions are meant to help ensure continuity of care during the postoperative period. Appropriate health records must be maintained regardless of the animal's location.
Fasting

Animals are often fasted prior to surgery so that the risk of aspiration pneumonia is minimized. Aspiration pneumonia can occur if an animal vomits, then breathes (or "aspirates") the vomit into the lungs. For this reason, fasting is often recommended.

However, rodents and rabbits are unable to vomit because of their esophageal physiology, and thus they should not be fasted prior to surgery unless there are other medical or scientific reasons for doing so.

Methods of Euthanasia

PHS Policy and the Guide state that methods of euthanasia should be consistent with the recommendations of a panel sponsored by the American Veterinary Medical Association, unless the IACUC approves deviations for scientific reasons. This Report of the AVMA Panel on Euthanasia contains many guidelines used by the IACUC to evaluate methods of euthanasia. This document is available on the Animal Resources website, http://www.auburn.edu/research/vpr/animals/resources/res_index.htm and in the Journal of the American Veterinary Medical Association (Vol. 218, No. 5, Pages 669-696, 2001.

Euthanasia methods can be broadly separated into physical and nonphysical (or pharmacologic) methods.

Physical methods rely on trauma to the head or spine to cause rapid death, or death due to fatal loss of blood. Examples include cervical dislocation, decapitation, captive bolt pistols, and exsanguinations ("bleeding an animal out").

Non-physical or pharmacologic methods rely on drugs to cause loss of consciousness and death.
Hierarchy of Euthanasia Techniques
The various guidelines set up a hierarchy of euthanasia techniques, from most desirable to least desirable:

- Most desirable are nonphysical methods of euthanasia such as carbon dioxide inhalation and barbiturate overdose.
- Next are physical methods used in conjunction with sedation or anesthesia. Examples include exsanguination, decapitation, or cervical dislocation of an anesthetized animal.
- Less desirable are physical methods alone. Examples include exsanguination, decapitation, or cervical dislocation on a conscious animal without sedation or anesthesia. Such methods should not be used unless approved by the IACUC based upon scientific justification.
- Least desirable are methods of euthanasia disapproved by the Panel. Only under the most exceptional circumstances will the IACUC approve these methods.

Commonly Recommended Techniques for Euthanasia of Common Lab Species

- Rodents - carbon dioxide asphyxiation, barbiturate overdose by the intraperitoneal route
- Rabbits - barbiturate overdose via the lateral ear vein
- Dogs, cats, horses, other large animals - barbiturate overdose by injection in cephalic, jugular, or other vein.
- Cattle – barbiturate overdose, penetrating captive bolt
- Pigs – barbiturate overdose, carbon dioxide asphyxiation, penetrating captive bolt. Because pigs often become very stressed when handled and restraint can be difficult, they are often anesthetized prior to intravenous barbiturate overdose in an ear vein.
- Fish and amphibians – tricaine methane sulfonate (MS 222)
- Poultry – carbon dioxide asphyxiation

Disapproved Methods
Several injectable agents are condemned by the AVMA Panel on Euthanasia as not appropriate when used alone. They include strychnine, nicotine, caffeine, magnesium sulfate, potassium chloride, and all neuromuscular blocking agents.
In addition, the following methods of euthanasia should not be used alone without special justification and IACUC approval:

- Exsanguination - due to anxiety associated with low blood pressure.
- Decompression - numerous disadvantages.
- Rapid freezing - not humane.
- Air embolism - the injection of air intravenously, can be accompanied by convulsions, other neurological signs.
- Drowning - not humane.
- Strychnine - causes violent convulsions and muscle contractions.
- Nicotine, magnesium sulfate, potassium chloride - cause cardiac and/or respiratory arrest before unconsciousness.
- Chloroform - known toxicity for animals and humans.
- Cyanide - extreme danger to humans and other animals.
- Stunning by a blow to the head - may not cause death and aesthetically objectionable.

Intracardiac Injections
Administration of injectable euthanasia agents into the heart provides rapid loss of consciousness and death. But intra-cardiac injections should only be performed in heavily sedated, anesthetized, or comatose animals, unless the IACUC approves it after considering an extraordinary justification.

The same holds true for blood collection from the heart.

Decapitation
There are two especially important issues regarding euthanasia of rodents and small rabbits.

The first is the use of decapitation alone. The primary justification for using decapitation without sedation or anesthesia is the need to recover tissues and body fluids that are chemically uncontaminated by sedatives or anesthetic agents. Special commercial guillotines designed to accomplish decapitation in a uniformly instantaneous manner are available.

The advantages of decapitation are that it may induce rapid unconsciousness, it does not chemically contaminate tissues, and it is rapidly accomplished. The disadvantages are that the handling and restraint required to perform this technique may be distressful to animals, the guillotine blade is a hazard to personnel performing the technique, the technique may be aesthetically displeasing to personnel, and there is some experimental evidence that brain activity and sensory capabilities do not end immediately.
Consequently, the use of decapitation without prior anesthesia or sedation should be used in research settings only when scientifically justified by the user and approved by the IACUC.

Cervical Dislocation
The second issue is cervical dislocation. Cervical dislocation is used to euthanize poultry, other small birds, mice, and immature rats and rabbits. Because the ligaments holding vertebrae together are too strong in larger animals to allow effective physical separation, cervical dislocation should only be performed on:

- Mice and small birds.
- Rats weighing less than 200 grams.
- Rabbits weighing less than 1 kg.

Remember that cervical dislocation is a physical method, and you should anesthetize or sedate the animals first, unless there are scientific reasons for not doing so approved by the IACUC.

For mice and rats, the thumb and index finger are placed on either side of the neck at the base of the skull or, alternatively, a rod is pressed at the base of the skull. With the other hand, the base of the tail or hind limbs are quickly pulled, causing separation of the cervical vertebrae from the skull.

For immature rabbits, the head is held in one hand and the hind limbs in the other. The animal is stretched and the neck is hyper-extended and dorsally twisted to separate the first cervical vertebra from the skull.

Cervical Dislocation Advantages and Disadvantages
Advantages of cervical dislocation are that it may induce rapid unconsciousness, it does not chemically contaminate tissue, and it is rapidly accomplished.

Disadvantages are that it may be aesthetically displeasing to personnel and that there is some experimental evidence that brain activity and sensory capabilities do not end immediately after dislocation.

Exsanguination
Exsanguination, or the near-complete withdrawal of blood from an animal, can be used to ensure death in unconscious animals. Because anxiety is associated with very low blood pressure, exsanguination should not be used as a sole means of euthanasia.
Carbon Dioxide Inhalation

Carbon dioxide inhalation is an effective means of euthanizing adult rodents and poultry. Bottled, compressed carbon dioxide is recommended as the source of the gas because the rate of inflow into the euthanasia chamber can be regulated. Important points:

- Use of carbon dioxide generated by other methods such as from dry ice, fire extinguishers, or chemical means (e.g., antacids) is unacceptable.

- Carbon dioxide is not generally used for euthanasia on larger animals such as rabbits, dogs, cats, and swine because it appears to induce greater distress in these species.

- "Pre-charging" euthanasia chambers to a concentration of 70% carbon dioxide may induce death more rapidly, but some veterinarians advocate low gas flows such that conscious animals are never exposed to carbon dioxide concentration above 70% in the chamber. This is because high concentrations (>70%) can cause nasal irritation, discomfort, and excitability. Rather, the animals should first be placed into the chamber, followed by the addition of CO₂ at a low flow rate (e.g. a rate sufficient to displace approximately 20% of the chamber volume per minute) to complete the process. Rapid gas flows should be avoided since excessive noises ("winds") can develop and induce excitement in the animals. Gas flow should be maintained for at least 1 minute after apparent clinical death (e.g. at least one minute after the animal has quit breathing). It is important to confirm that an animal is dead after removing it from the chamber. Unintended recovery must be obviated by the use of appropriate CO₂ concentrations and exposure times or by other means.

- Only one species at a time should be placed into a chamber, and the chamber must not be overcrowded. When placed into the chamber, all animals must have floor space. Euthanasia should always be done in cohorts (live animals should not be placed in the chamber with dead animals). Chambers should be kept clean to minimize odors that might distress animals prior to euthanasia. Animals must not be euthanized in animal housing rooms, except under special circumstances such as during quarantine for infectious disease agents.

- Neonatal rodents and one-day-old chickens: Since the time period for euthanasia is substantially prolonged in neonatal animals due to their inherent resistance to hypoxia, CO₂ narcosis must be followed by a physical means of euthanasia after the animals lose consciousness to ensure irreversibility of the procedure (e.g. decapitation, cervical dislocation, or thoracotomy).

Pithing

Pithing is the destruction of the central nervous system by mechanical means. Either the brain, the spinal cord, or both may be destroyed, depending on the species and
additional methods of euthanasia used. Pithing is a physical means of euthanasia, and thus should be used only if nonphysical methods are not appropriate.

Accordingly, pithing is generally used as an adjunctive procedure to ensure death in an animal that has been rendered unconscious by other means. For some species such as frogs, with anatomic features that facilitate easy access to the central nervous system, pithing may be used as a sole means of euthanasia, but anesthetic overdose is a more suitable method.

Pithing requires knowledge of anatomic landmarks and requires great skill. Like all methods of euthanasia, it should only be performed by trained personnel.

Reducing Animal Anxiety During Euthanasia

When animals are euthanized, other animals should not be present because vocalization and release of pheromones in urine and feces can occur during euthanasia that induce anxiety in other animals. Similarly, euthanasia chambers should be cleaned well between uses to reduce animal anxiety caused by exposure to alarm pheromones in urine and feces.

Use of Hazardous and Toxic Agents

Using Hazardous and Toxic Agents in Animals

The AU Institutional Biosafety Committee (IBC) and appropriate safety officers are charged with evaluating safe practices for using hazardous agents in animals.

Guidelines for performing infectious disease work with animals are found primarily in the Centers for Disease Control and Prevention (CDC)/NIH publication entitled "Biosafety in Microbiological and Biomedical Laboratories," or "BMBL." The BMBL has guidelines for working with a wide variety of infectious agents in both research laboratories and the animal facility.

If your animal work requires the use of hazardous or toxic agents, there are many important considerations. Such agents can be categorized in the following way:

- Infectious diseases.
- Toxic chemicals, including carcinogens, mutagens, biological toxins, and organic chemicals.
- Radioactive substances.
- Recombinant DNA.

Some points to consider when using such agents in animals:

- The risk of accidental human infection or exposure is usually reduced if animals are anesthetized or sedated before they are injected with agents using a hypodermic needle. When anesthetized, animals will not struggle
unpredictably - this helps prevent accidental redirection of the needle toward personnel handling the animals.

- If using an infectious agent, an antibiogram or other appropriate therapeutic panel should be developed on infectious strains before they are used in animals. If an accidental human exposure occurs, physicians will know immediately which antibiotic or other therapeutic agent to use to best treat the infection. Consult with your occupational health staff in advance of experiments.

- If using a toxic agent, know in advance what antidote or action to take if accidental exposure should occur through an injection, spill, or break in the skin. Have any necessary antidotes, decontamination kits, or spill kits readily available. Consult with the appropriate safety officer in advance of experiments.

- When administering hazardous agents to animals, it is best for personnel to work in pairs. If one person becomes contaminated, the second person can help decontaminate the person and the area quickly.

Biosafety and Animal Biosafety Levels
There are four levels of containment procedures for infectious agents recognized in the BMBL. The levels are designated Biosafety Levels 1, 2, 3, and 4. The containment and handling safeguards become more stringent as the biosafety level (or “BSL”) number increases. For each of the four biosafety levels, there are corresponding Animal Biosafety Levels 1, 2, 3, and 4 (or "ABSL") that provide guidelines for housing and manipulating animals infected with agents that require that level.

Before beginning any animal studies involving infectious agents, both research staff and personnel in the animal facility must understand how to safely conduct the study in the animal facility. Standard Operating Procedures (SOPs) should be written and approved by the Institutional Biosafety Committee before any infectious work begins, and you may be required to provide a detailed SOP prior to the IACUC review. An SOP should describe how animals will be handled and housed in the animal facility after they are infected. It is very important that the veterinarian, biosafety officer, biosafety committee, and animal facility manager be involved in the planning as needed to minimize the risk of exposing humans or other animals to the agent.

“Select Agents”
The Department of Health and Human Services enforces "Additional Requirements for Facilities Transferring or Receiving Select Agents" for certain infectious agents and biological toxins such as aflatoxin and tetanus toxin. These “select agents” are given additional regulatory oversight because of their potential use in biological ("germ") warfare. The list of select agents includes infectious agents like hemorrhagic fever viruses, and plague, brucellosis, and anthrax bacilli. It also includes a
number of biological toxins such as aflatoxin and botulinum toxins. An investigator must register with the Centers for Disease Control and Prevention and obtain approval before beginning any work with agents on the “select agent” list.

Studies Using Toxic Chemicals and Radioisotopes
As with infectious disease studies, the use of toxic chemicals or radioisotopes in animals requires careful coordination between many people, including:

- The research staff.
- The veterinarian and animal facility supervisor.
- The appropriate institutional units or committees (Department of Risk Management and Safety - DRMS, radiation safety) responsible for use of hazardous agents.

The IACUC should ensure that all research and animal facility personnel are trained to properly minimize the risk of accidental human or animal exposure. SOPs describing containment and handling procedures should be written and approved by appropriate committees before any animal work begins. It is critically important to also train the animal caretakers who will clean, feed, and water the animals. If highly specialized training is required to handle animals safely, then the research staff might have to assume husbandry duties for infected animals.

Studies Using Recombinant DNA
There are additional guidelines to consider if your work in animals includes:

- The inoculation of infectious agents or cells with recombinant DNA into animals, or
- The use of recombinant molecules in an animal's genome.

The purpose of the NIH Guidelines for Recombinant DNA and Gene Transfer are to specify practices for constructing and handling recombinant deoxyribonucleic acid (DNA) molecules and organisms and viruses containing recombinant DNA molecules.

Although the recombinant DNA inserted into many transgenic mice may not be covered, it is wise to check with the Institutional Biosafety Committee before you produce transgenic mice to determine what committee approvals will be necessary.

Experiments involving recombinant DNA must be reviewed by the IBC. In some cases an NIH committee called the "Recombinant DNA Advisory Committee" must also approve the experiments. Experiments involving very high risk, such as introducing novel antibiotic resistance into human pathogens, may also require NIH approval before initiation. Guidance can be obtained from the Office of Biotechnology Activities (OBA) at the NIH.
Risk Categories
The NIH Guidelines for Recombinant DNA and Gene Transfer document describes different levels of agent containment practices very similar to the levels described in the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories manual for infectious agents.

If recombinant nucleic acid is introduced into infectious agents, the level of laboratory and animal facility containment required is primarily based upon the "Risk Category" assigned to the agent involved. The Risk Categories are based upon disease potential in healthy humans and availability of therapy. For each Risk Category, there is a corresponding set of animal biosafety guidelines that must be used, as follows:

- **Risk Category 1 (RC-1).** Agents that are not associated with disease in healthy adult humans. Generally, if an RC-1 agent is used in animals, "Biosafety Level 1-N" (BSL1-N) animal containment measures are used.

- **Risk Category 2 (RC-2).** Agents that are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are often available. Generally, if an RC-2 agent is used in animals, BSL2-N animal containment measures are used.

- **Risk Category 3 (RC-3).** Agents that are associated with serious or lethal human disease for which preventive or therapeutic interventions may be available (high individual risk but low community risk). Generally, if an RC-3 agent is used in animals, BSL3-N animal containment measures are used.

- **Risk Category 4 (RC-4).** Agents that are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are not usually available (high individual risk and high community risk). Generally, if an RC-4 agent is used in animals, BSL4-N animal containment measures are used.

The containment practices recommended for each of the animal biosafety levels (BSL1-N through BSL4-N) are found in Appendix Q of the NIH Guidelines for Recombinant DNA and Gene Transfer document.

Using Explosive Agents in the Animal Facility
Think carefully before using explosive agents in research settings. In the AU animal facilities, the use of ether to anesthetize or euthanize animals, and the use of other explosive agents are prohibited unless there are compelling scientific reasons for not using non-explosive alternatives. Check with the DRMS and IACUC before using any explosive agents in the animal facility.
Special Considerations

If you are approved to use explosive agents such as ether to euthanize animals, DO NOT put the bagged carcasses in a refrigerator or freezer UNLESS you are absolutely certain that all of the agents have evaporated from the carcasses, and then only if the refrigerator or freezer is certified as explosion-proof. Sparks produced by non-explosion-proof refrigerators and freezers can ignite the fumes given off by the carcasses and cause a tremendous, deadly explosion.

Also, you must be aware that ether stored in metal cans will form highly unstable peroxides around the can lid over time. These peroxides can become so unstable that they can detonate if the can is jarred. If you have old cans of ether in the chemical storage vault in your laboratory area, consult with the DRMS to make sure that they do not represent an explosion hazard.

Weight Loss as an Endpoint

Immature animals: maximum weight loss is a deviation of 15% from recognized growth curves or age-matched control animals.

All protocols involving excessive weight loss will be evaluated on a case-by-case basis.

Background: Weight losses may occur in research animals in association with a variety of experimental regimens including studies where feed and essential nutrients are withheld, such as studies of nutritional deficiencies, toxicology or cancer. Weight loss also occurs in association with many spontaneous diseases and is a prime indicator of declining clinical condition. Moderate food restriction and weight loss, rather than being detrimental, has been shown to promote health and extend the life of laboratory rodents and other species. The IACUC has accepted weight losses of greater than 20% that can be scientifically justified.

Policy: The upper limit of acceptable weight loss in mature animals on experimental regimens shall generally be 20%. Written scientific justification must be provided to the IACUC for approval for a greater than 20% weight loss. In studies where weight loss is expected to occur, monitoring must be done by investigative staff trained and experienced in recognizing clinical signs of illness and distress in study animals. Weights must also be taken at least weekly under such circumstances and be readily available for review by the veterinary staff and the IACUC. In their protocol submissions, investigators must address situations where weight loss will exceed proposed limits and remedial measures that will be taken. Veterinary staff may intervene when such remedial measures prove ineffective or to address weight losses that occur in excess of 20% of pre-study body weight in any research animal, or when other limits approved by the IACUC have been reached or exceeded. Such intervention may include euthanasia. Exceptions to this policy will be allowed only if there is a veterinary determination that weight losses exceeding approved limits are not endangering animal health and well-being and a specific waiver is obtained from the IACUC.
Monitoring of Biological Materials

The injection of transplantable tumors, hybridomas, cultured cell lines, or other biological materials into rodents can pose a health risk to animals and personnel. These biological materials have been a source of mouse hepatitis virus, mousepox, and other significant disease agents at research facilities. Moreover, rodent pathogens can be carried and propagated by non-rodent (e.g. human) cell lines when these cell lines have been propagated in rodents or rodent biological materials.

Biological materials should be evaluated for rodent pathogenic microorganisms by polymerase chain reaction (PCR) or mouse antibody production (MAP) tests. The major disadvantage of MAP testing is the 6 to 8 weeks required to obtain results. The Research Animal Diagnostic and Investigative Laboratory (RADIL) at the University of Missouri offers a PCR-based alternative to MAP testing, the Infectious Microbe PCR Amplification Test or IMPACT, which is a panel of PCR assays that detects murine pathogens. Typically, IMPACT testing requires 2 vials of each sample with a minimum of $1 \times 10^7$ cells/vial and a turnaround time of 7-10 days.

If your protocol involves the injection of transplantable tumors, hybridomas, cultured cell lines, or other biological materials into rodents, please provide the Division of Laboratory Animal Health (DLAH) the name of the cell line(s), source, test, and results of tests performed to evaluate the presence of rodent pathogenic microorganisms. If the cells are not of rodent origin and have not been tested for the presence of rodent pathogens, please confirm that the materials (cells) to be used have not been propagated in rodents or rodent biological materials. Alternatively, please contact DLAH to make arrangements to have biological specimens tested before use. Approval for the use of biological materials in animals housed at DLAH facilities will only be given after the Director of DLAH has assessed the test results to determine their adequacy.

Food or Water Restriction

For the purposes of this policy, food or water deprivation is defined as any restriction in access to food or water. The rationale may be to establish food or water as reinforcers, to study caloric restriction, or to prevent obesity and protect the health of the animals. Restricted access to food is neither unusual nor undesirable. Nevertheless, restrictions must be conducted with care and tailored to the feeding patterns and nutritional requirements of the strain and species as well as the requirements of the study.

The delivery of food or fluids is commonly used to maintain extended sequences of behavior in studies with a wide range of animals. When restriction is used to establish food or water as reinforcers, a common rule of thumb has historically been to maintain body weight at 80% of free-feeding weight or to deprive of water for 12 to 23 hours. Such an all-encompassing definition of food restriction is inappropriate and cannot be applied to all species. Rodents are nearly continuous feeders and will continue to gain weight through their life, so an absolute "80%" target can never be identified. Moreover, appropriate body weights will depend on the age and strain of the animal. On the other
hand, an “80%” target would be too restrictive and may jeopardize the health of other species. Restricted access to food or water must be tailored to the species under study. When beginning work with a new species, consult with the PV as well as the literature when designing and describing protocols for fluid or food restriction.

Food and water consumption are interdependent, but species differ in their circadian or other patterns of drinking and their response to food restriction. Unless specific protocols require exemption, allowing most laboratory animal species to feed at least once per day is consistent with standards of humane care and is required for species covered by USDA regulations.

Constant access to water typically is provided under food control regimens, but requirements of the species and the scientific protocols may require different patterns of access. Conversely, water-deprived animals often have non-restricted access to food, but investigators should be aware that most food consumption occurs only when water is available. Water should be available long enough to maintain sufficient food intake.

Food-restricted animals typically are weighed frequently, usually 5-7 times per week. Species whose weights change slowly or for which sedatives or anesthetics must be used to determine body weights may be weighed less frequently. If so, other tactics to ensure appropriate caloric and nutritional intake must be specified. Where possible, highly desirable foods (fatty or sweet “treats”) may be used as reinforcers and this could reduce the degree of food or water restriction imposed, but even under these conditions, some restriction enhances their reinforcing efficacy. In all cases, records should be kept of measures taken to ensure appropriate nutrition or hydration.

Animals tolerate food restriction physiologically better than water restriction, so food restriction should be used if possible. Fluid reinforcers often have advantages, however, such as in procedures that must control the position of the subject’s head or limit jaw movements. When water, sweet drinks, or fruit-flavored drinks are used as a reinforcer, access to water outside the experimental session needs to be controlled. Determining parameters of water restriction, including especially the period(s) of access during the day, that do not produce dehydration or excessive weight loss requires careful consideration and sensitivity to the species. When this is done, animals need not be at risk. Careful observation of behavior, regular clinical monitoring of the animal’s health, and records of measures taken are critical for ensuring successful application of fluid control procedures.

**Blood Collection**

When collecting blood samples, the volume and frequency of collection must be carefully limited so that neither shock nor anemia result.

How much blood can be collected at one time? One simple guideline is to collect no more than 1% of the body weight at one time. Assume that a ml of blood weighs 1
gram. For example, for a 4,000 gram rabbit, 40 ml (0.01 x 4,000= 40 ml) could be safely collected. For a 20 gram mouse, 0.2 ml (0.01 x 20= 0.2 ml) could be safely collected.

How often can blood be collected? In general, multiple blood collections should be spaced far enough apart to prevent anemia and distress in animals. Blood collections of no more than 1% of body weight can usually be performed every 2-4 weeks without harm. More frequent collections might require hematocrit monitoring to prevent severe anemia in an animal.

Blood collection from the heart should only be performed on anesthetized animals as a terminal procedure.

**Housing Rodents on Wire Floors**

The Guide recommends that solid bottom caging with bedding be used preferentially for rodents. The Guide addresses this issue because there is some evidence that rodents prefer solid bottom caging with soft bedding over wire mesh flooring, and some limb pathology has been associated with prolonged housing of rodents on wire mesh floors. The IACUC is expected to address this issue during protocol review, and if you want to house animals on wire mesh flooring, you will be asked to provide a scientific justification on the protocol form.

Some toxicology projects in rodents are performed on wire mesh floors so that animals do not remain in contact with metabolites in urine and feces. Rodents in metabolism cages must usually be on wire mesh floors so that urine and feces can be collected under the cage.

**Dog Exercise Program**

The Animal Welfare Act regulations and standards require that institutions adopt a program of dog exercise. Dogs must be exercised outside of their cages if they are housed singly in an area less than two times the minimum space required. If the runs in a facility do not provide singly housed dogs with enough space, the dogs must be exercised unless a dog is exempted:

- By the PV for health reasons, or
- By the IACUC based upon a scientific justification provided by the investigator.
Exemptions must be evaluated by the PV every 30 days or less to comply with the Animal Welfare Act regulations.

Exemptions to the Dog Exercise Program
If you have concerns that exercise outside of the runs would adversely impact your dog studies, discuss the issue with your PV and the IACUC.

Prolonged Restraint

The Guide also has special language addressing prolonged restraint of animals while they are conscious. In general, restraint for all animals should be the least restrictive and for the shortest time necessary to complete research objectives. Prolonged restraint should be avoided unless it is essential for achieving research objectives. Examples of prolonged restraint include rodent restraint in inhalation chambers, and swine and dogs restrained in slings. Consider the following guidelines:

- Restraint devices are not to be considered normal methods of housing.
- Restraint devices should not be used simply as a convenience in handling or managing animals.
- The period of restraint should be the minimum required to accomplish the research objectives.
- Animals to be placed in restraint devices should be given training to adapt to the equipment and personnel.
- Provision should be made for observation of the animal at appropriate intervals, as determined by the IACUC.
- Veterinary care should be provided if lesions or illnesses associated with restraint are observed. The presence of lesions, illness, or severe behavioral change often necessitates temporary or permanent removal of the animal from restraint.

H. Antibody Production

The production of polyclonal and monoclonal antibodies in animals has been critical for biomedical research progress for many years. This section will help you understand the ethical and procedural concerns that must be addressed when planning antibody production in animals and completing IACUC forms.
Polyclonal Antibodies

In production of polyclonal antibodies, animals are typically immunized multiple times to elicit a strong antibody response, then bled so that immune serum can be collected and used in experiments. Two important considerations in producing polyclonal antibodies in animals are proper immunization technique and proper bleeding technique.

Immunization Technique

When producing polyclonal antibodies, adjuvants are usually mixed with antigens to augment the antibody response. The classic adjuvant is Freund's adjuvant, which is available in two forms:

- "Complete" (Complete Freund's Adjuvant, or "CFA"). CFA is a mixture of oils and water plus killed Mycobacterium tuberculosis. It typically elicits a very strong immune reaction. If used more than once, the immune reaction usually progresses to intense inflammation and sterile abscesses.

- "Incomplete" (Incomplete Freund's Adjuvant, or "IFA"). IFA is similar to CFA, but is missing the killed mycobacteria. This renders the IFA less effective as an immune stimulant, but it can be used safely multiple times without causing intense inflammation.

Use of CFA and IFA

To prevent inflammation and pain, CFA must only be used once. IFA is less inflammatory, and can be used multiple times. Typically, CFA mixed with antigen is administered to an animal the first time, then IFA is mixed with antigen for the second administration, then either IFA mixed with antigen or antigen alone is used for subsequent immunizations.

The USDA states that the injection of CFA may cause more than momentary or slight pain. This means that CFA injections might necessitate assignment of USDA pain category D (painful/stressful but relieved) requiring the use of post-injection analgesics or sedatives.

Reducing CFA Side Effects

To reduce inflammation when using CFA, consider the following measures:

- Choose or make preparations of CFA with a lower mycobacterial concentration, i.e., 0.05 to 0.1 mg/ml, rather than 1 mg/ml.
- Add a concentrated antigen solution to the adjuvant to obtain a more antigen-rich emulsion, thereby reducing the volume of emulsion injected.
- Use multiple injection sites to limit the volume injected at any one site.
• Separate the injection sites to avoid fusion of inflammatory lesions.
• Maintain sterility of the antigen solution.

CFA and IFA Injections
The quantity of CFA or IFA adjuvant injected should be limited. Typical limits on adjuvant use are around 1 ml of combined adjuvant/antigen per immunization for rabbits (typically up to ten divided 0.1 ml injections), and around 0.25 to 0.5 ml combined adjuvant/antigen per immunization for smaller animals (up to ten divided 0.05 ml injections). These amounts have been shown to produce high titer antibodies, yet limit inflammation.

CFA as a Health Hazard to Humans
Beware: CFA is a health hazard to humans.

If you are already sensitized to mycobacterial antigens by a previous exposure to CFA or through a natural infection of tuberculosis, you are likely to experience severe inflammation if you splash CFA into your eye or accidentally inject yourself with it. The inflammation and pain may be so severe that surgical removal of the site may be necessary. Protect your eyes and prevent accidental injection of yourself or a colleague when using CFA!

Alternatives to CFA and IFA
Less inflammatory alternatives to CFA and IFA are now available and in use. Examples are the block copolymer adjuvant Titermax®, and the lipid A-derivative adjuvant MPL® by RIBI. Other promising alternative adjuvants are also on the market. Such alternatives can be considered as a means of further reducing inflammation induced by Freund’s adjuvant.

Choosing the Immunization Route
The route of immunization should be chosen to limit pain and inflammation. Regardless of the adjuvant used, the subcutaneous route typically provides a strong immune response, and is recommended. The intravenous route is not appropriate if adjuvant is used because the thick consistency of the adjuvant can result in lethal emboli in the blood stream.

There are several other routes of immunization that are usually discouraged because there is little evidence that they offer any advantage over the subcutaneous route:

• Intradermal (ID): Causes more pain because the skin itself cannot stretch much as body fluids and white blood cells enter the immunization area, resulting in increased pressure and pain.
• Intraperitoneal (IP): Inflammation on surfaces of abdominal organs can result in peritonitis, granulomas, and pain.
• Foot pad: Injections can cause pain and lameness. When allowed by the IACUC, usually only one foot may be injected. Foot pad injections are usually discouraged in rodent species, and deemed inappropriate in larger species. Rabbits will often chew on their own feet after foot pad injections, presumably because of intense pain or irritation.

Spacing Immunizations
Some evidence indicates that immunization injections should be spaced 3-6 weeks apart to elicit an optimal polyclonal antibody response, and the highest possible titer. There may be a temptation to hurry the process and shorten intervals, but a reduction in antibody titer may result. This is because circulating antibody from the previous immunization can remove antigen from circulation and thus limit its ability to induce a strong immune response.

Blood Collection
Blood collection is obviously essential for collecting the immune sera from immunized animals. Often a "pre-bleed" is performed prior to immunization to determine if specific polyclonal antibody is already present in the animal (this could complicate some subsequent antibody studies). Periodic blood collections are needed thereafter to determine when a good antibody response is present. Once a good titer has been produced, serum per protocol objectives will be collected.

Monoclonal Antibody Production

To produce monoclonal antibodies, animals (typically rodents) are immunized with an antigen, then spleen cells or lymph node cells are collected after euthanasia and fused with an immortal cell line. The fused cells are placed in a special medium that allows only hybrid cells to grow.

These hybrid cells, or hybridomas, are expanded in number, and the clones that produce antibody against the antigen of interest are saved.

If adjuvant is used during the immunization process, the same principles apply as described in the polyclonal antibody section.
Two Uses of Animals in Generating Monoclonal Antibodies

The first use of animals in generating monoclonal antibodies is to create the hybridoma cell line.

The second common use of animals in generating monoclonal antibodies is to grow the hybridoma cell line on the peritoneal lining of histocompatible animals, and collect the antibody-rich ascites fluid.

Non-Animal Alternatives

Over the past years, there have been a number of in vitro techniques introduced that can sometimes replace the use of animals for expanding hybridoma cell lines, and for collecting purified monoclonal antibody. Consequently, non-animal alternatives for generating purified monoclonal antibodies must be considered, and found to be unsuitable before the IACUC can approve animal use for that purpose.

Guidelines for Using Animals for Hybridoma Expansion

When requesting approval to use animals for expanding hybridoma cell lines, be prepared to explain why in vitro techniques will not work. In 1999, The Committee on Methods of Producing Monoclonal Antibodies (sponsored by the Institute for Laboratory Animal Research and the National Research Council) suggested the following guidelines for IACUCs to use when evaluating the need for using animals for hybridoma expansion (Recommendation 4):

• 1. When a supernatant of a dense hybridoma culture grown for 7–10 days (stationary batch method) yields a monoclonal antibody concentration of less than 5 mg/ml, or if other systems used yield concentrations less than 500 mg/ml (hollow fiber system) and 300 mg/ml (semi-permeable membrane system).

• 2. When more than 5 mg of monoclonal antibody produced by each of five or more different hybridoma cell lines is needed simultaneously. It is technically difficult to produce this amount of monoclonal antibody since it requires more monitoring and processing capability than the average laboratory can achieve.

• 3. When analysis of monoclonal antibody produced in tissue culture reveals that a desired antibody function is diminished or lost.

• 4. When a hybridoma cell line grows and is productive only in the animal.

• 5. When more than 50 mg of functional monoclonal antibody is needed, and previous poor performance of the cell line indicates that hollow-fiber reactors, small-volume membrane-based fermenters, or other techniques cannot meet this need during optimal growth and production.

These same criteria can help you decide if in vitro methods will suffice. The burden of proof is now on the investigator to show that in vitro methods of obtaining
purified monoclonal antibody do not work, or are not effective in providing the amount of antibody needed.

Guidelines for Using the Ascites Collection Technique

If in vivo methods are needed because in vitro methods cannot replace them, consideration must be given to minimizing the amount of pain and suffering involved. The following parameters should be considered when animals are used to expand hybridomas using the ascites collection technique:

- The amount of pristane used to “prime” the peritoneal cavity and make it better able to support hybridoma growth should be minimized (0.1 to 0.2 ml have been found to be effective).

- The degree of abdominal distension should be monitored at least daily and should distension begin to interfere with breathing, the ascites fluid should be removed.

- The number of peritoneal “taps” used to collect ascites fluid should be minimized. It is customary to limit withdrawals to two taps, unless the investigator provides evidence that the hybridoma is slow growing and additional taps can be accomplished in a humane fashion.

- The needle used should be as small as possible (20 gauge or higher). Because mice with ascites are not good anesthetic risks, ascites fluid is usually collected with a needle and syringe without anesthesia, and smaller bore needles cause less pain.

- Endpoint criteria tailored to collecting ascites should be developed. Typical endpoint criteria include weight loss, extended anorexia, hunched posture, rough hair coat, reduced food consumption, emaciation, inactivity, difficulty in ambulation, or respiratory problems. Additional criteria to consider include a limit on the number of abdominal taps allowed, the presence of dyspnea (difficult breathing) unrelieved by a tap, and the development of solid hybridomas instead of more diffuse neoplasms producing ascites.