Chapter 6. Laboratory Animal Medicine and Toxicology

Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences

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6. Laboratory Animal Medicine and Toxicology

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6.1. Animal Facility Operational Requirements

6.1.1. Regulatory Compliance

- The testing laboratory must comply with the DTT Specifications, the specific regulations listed below, and other applicable federal, state, and local laws, regulations, and policies.
    - U.S. Government Principles[1]
    - Public Health Service Policy on Humane Care and Use of Laboratory Animals[2]
    - Animal Welfare Act Regulations – Title 9: Code of Federal Regulations, Chapter 1, Subchapter A: Animal Welfare[3]
    - Animal Welfare Act[4]
    - Guide for the Care and Use of Laboratory Animals, 8th Edition[5]
- The testing laboratory must have a Public Health Service Assurance from the NIH Office of Laboratory Animal Welfare (OLAW) and must be accredited by AAALAC International.
- The testing laboratory must have a functional Animal Care and Use Committee.
- The laboratory must have a qualified laboratory animal veterinarian (LAV) to supervise the care and health of the animals. Qualifications for the LAV are outlined in Chapter 1.

6.1.2. Disaster Planning and Emergency Preparedness

- Each testing laboratory must maintain an Emergency Notification Procedure that shows who to notify in the event of various types of potential emergency situations. The facility must have an emergency/disaster response plan specifically addressing the animal facility.

[1]https://olaw.nih.gov/policies-laws/phs-policy.htm#USGovPrinciples
[2]https://grants.nih.gov/grants/olaw/references/PHSPolicyLabAnimals.pdf
[3]https://www.ecfr.gov/current/title-9/chapter-I/subchapter-A
[4]https://www.aphis.usda.gov/animal_welfare/downloads/awa/awa.pdf
[5]http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf

- This procedure must be posted in a prominent location and on pertinent equipment (e.g., refrigerators, freezers). Weekend duty personnel, in particular, must be aware of its location.
- All personnel must read and initial, acknowledging they have read and understand the procedures.
- In all cases, the Principal Investigator (or designated alternate, if the principal investigator is absent) must be notified of emergency situations. The LAV must be made aware of any emergency situation, especially those that affect animal welfare.

6.1.3. Pest Control

- An integrated pest management (IPM) program will be in place prior to starting animal studies. This program will be provided by a licensed, commercial pest control company.
- Pesticides and traps may be used as necessary in conjunction with a strict program of sanitary maintenance. However, to prevent toxic effects in research animals and possible interference with experimental procedures, pesticides, including insecticide-impregnated plastic materials, must not be used in animal rooms, feed and bedding storage areas, or any other areas of the facility where animals, cages, racks, feed, bedding, or water may be exposed to either the particulate or vapor form of pesticides (when used).
- Use of nontoxic pest control (e.g., live traps, insect-only sticky traps) is advised.
- The testing laboratory must maintain records that include a schematic of all areas under the IPM with the location of all traps and bait stations clearly marked. The records will include inspection reports by the IPM provider listing the date of the inspection; the number, type, and location of all pests found; and any treatments performed. These records will be available for review by the program contracting officer's representative (COR).

6.1.4. Sanitization Practices

Chemicals used to sanitize must not contain essential oils, perfumes, fragrances, or any other chemicals expected to influence the metabolism of mammalian systems. The contractor must maintain records of all chemicals used for sanitation. These records must be available for review by the program COR.

6.1.5. Movement of Animals between Rooms

- Studies (study animals) must not be moved to a different room during the course of study except for the reasons stated below:
    - Specific statement in the protocol of the study requiring or permitting the move;

  - The physical condition (floor, walls, ceiling, fixtures, etc.) of the room and/or its adjacent supporting area deteriorated to the extent that a safety hazard is judged to exist;
  - The physical plan, ventilation equipment or ventilation, or lighting equipment and fixtures have deteriorated or malfunctioned to cause highly variable environmental conditions in the room;
  - The physical factors in or around the study room have changed causing the procedures employed to control pests, disease, and microbial spread to no longer be effective.
- If a move is necessary, the COR must be notified as soon as possible.
- When a study is moved to a new or different animal room due to reasons listed above, the equipment to control environmental, health, and safety conditions, as well as the procedures to control disease and microbial spread, must be substantially superior in the new room when compared with the previous animal room.
- Except in cases of emergency, approval for the move must be obtained from the program COR in advance. There must be a detailed procedure for the type of move. The study report must include reasons and approvals for the move, date of the move, and detailed procedure of the move.

6.1.6. Required SOPs for Laboratory Animal Medicine and Toxicology

The test facility must have specific standard operating procedures (SOPs) for all laboratory animal medicine and toxicology procedures including, but not limited to, the activities listed below:

- Facility Operations, Management, and Maintenance of Equipment SOPs
  - Emergency/disaster response plan
  - Staff education and training
  - Pest control
  - Routine testing and maintenance of emergency backup system
  - Sanitization (rooms pre- and post-animal arrival; equipment such as racks, cages, feeders, watering systems, enrichment devices, etc.) and monitoring of sanitation practices
  - Quality assurance monitoring of sanitation procedures
  - Routine testing and maintenance of cage and rack washers
  - Environmental monitoring
  - Movement of staff, animals, test articles, supplies, and waste throughout the animal facility
  - Movement of animals in and out of the inhalation exposure rooms
  - Watering system

  - Procedures for disease control and for prevention of microbial spread in the testing facility

- Animal Care, Husbandry, and Procedure Protocols
  - Observation of animals: daily a.m. and p.m. checks, detailed clinical observations
  - Evaluation of feed and/or water consumption
  - Randomization, identification, and weighing of animals
  - Receipt, quarantine, health evaluation, and quarantine release of study animals
  - Handling of dead and moribund animals, and criteria for moribund euthanasia
  - Custody transfer of animals from animal care/toxicology to necropsy/pathology for the interim and final euthanasia of study animals
  - Disposition of escaped animals
  - Care and handling of pregnant and lactating dams
  - Recording signs of parturition
  - Handling, sex determination, weighing, and conducting clinical observations of rodent pups; evaluation of developmental landmarks (anogenital distance, preputial separation, nipple retention, vaginal opening, etc.)
  - Procedures for culling of dams and pups
  - Procedures for examination/staining of the uterus for implantations and resorptions
  - Procedures for weaning pups for rodent toxicology studies
  - Randomization of rodent pups for continuation in study
  - Humane endpoints
  - Environmental enrichment
  - Rack and cage rotation
  - Gavage treatment procedure
  - Dermal treatment procedure
  - Receipt and storage of feed including evaluation of contaminant reports to satisfy standards
  - Receipt and storage of bedding including evaluation of physical quality from a randomly selected bag, and contaminant report to satisfy standard
  - Health monitoring program
  - Rack, cage, and bedding change
  - Feeding and change of feeders

6.2. Animal Husbandry and Facility Management

6.2.1. Ventilation

- The ventilation system must provide a minimum of ten complete changes of room air per hour without drafts.
- There must be no recirculation of room air unless it has been treated to remove all particulates and toxic vapors by effective filters and, where necessary, scrubbers to avoid spread of disease and to eliminate the recirculation of contaminants.
- An automatic recording and alert system must be used to monitor the ambient conditions in each animal room.
    - If a completely automated system is used, the probes to determine room temperature and humidity must be in the exhaust for each room.
    - If a freestanding, portable temperature and humidity recording system is used, the equipment must be located near the room exhaust at a level of 3 to 4 feet from the floor.
- Each month the testing laboratory must record qualitative evidence of the correct direction of airflow in each animal room. Quantitative measurements of flow rate must be made at least twice per year, once in the cooling season and once in the heating season.

6.2.2. Temperature and Humidity

- Temperature of the rodent animal room must be maintained at 72°F ± 3°F. The temperature must not be below 69°F or above 75°F during the course of the study and must be maintained with minimal fluctuations near the middle of the range. There must be an alarm system for warning of temperature fluctuations beyond the 69°F to 75°F ranges. If the temperature is below or above the 69°F to 75°F range, it must be returned to the acceptable limits within 2 hours. Thermometers must be accurate within 2°F or better.
- The relative humidity of the rodent animal room air must be 50% ± 15%. It must not be below 35% or above 65% during the course of the study. If the relative humidity is below or above the 35% to 65% limits, it must be returned to the acceptable limits within 2 hours.
- Accuracy of thermometers and hygrometers must be checked as often as necessary, but not less than quarterly intervals. Animal room temperature and humidity results must be reported as either means ± relative standard deviation or as time-weighted averages.
- The temperature and humidity ranges for rabbit rooms are as follows: 64°F ± 3°F and 50% ± 10% humidity. The temperature and humidity below or above the acceptable range must be returned to the acceptable limits within 2 hours.

6.2.3. Lighting

- The animal rooms must be windowless and uniformly lighted, preferably by diffuse lighting. Windows within doors are acceptable, however, light contamination of the animal room must be avoided.
- The light cycle in the animal rooms must be 12 hours light and 12 hours dark, with the timing of the light/dark cycles varying no more than ±15 minutes from day to day. The light cycle may be adjusted to 14 hours light and 10 hours dark to accommodate breeding.
    - Appropriate means must be taken to prevent light from entering the animal room during the dark cycle.
    - The light cycle must be controlled by automatic equipment. Equipment must be monitored for proper functioning at 2- to 3-day intervals.
- NIEHS requires a uniform light intensity of 30 (±3)-foot-candles at 3.3 feet (1.0 meter) from the floor for normal lighting of the animal rooms. During the observation periods, for convenience of the technicians, the light intensity may be increased up to 45- to 55-foot-candles at 3.3 feet from the floor. To accomplish this lighting, the animal room may be equipped with two-stage lighting, both stages to be automatically turned off by an automatic timer. The second stage will be to facilitate observation of the animals. The second stage must be wired to be turned off manually when not needed for observations. In the event that the second stage lighting is not turned off, the automatic timer must turn off not only the first stage, but also the second stage lighting at the set time.
- LED (light emitting diode) lighting is acceptable for use in animal rooms. Warm white light must be used at a wavelength of 600–680 nm.
- The lights must not be turned off during the light (day) phase or turned on during the dark (night) phase except in case of emergency. With permission from the program COR, exceptions will be made for special circumstances requiring the temporary reversal of lighting, such as retinal evaluations.
- Emergency power must be connected to the light timers/controls and to some lights of the animal room.

6.2.4. Noise and Vibrations

Procedures must be in place to limit and reduce the noise inherent in the day-to-day operations of an animal facility. Procedures that make the most noise, such as cage washing, must be separated from animal rooms used for neurobehavioral testing and reproduction and developmental toxicity studies. Noisy animals, such as dogs, must be separated from reproduction and developmental toxicity studies. Likewise, production of excessive vibrations in animal housing areas must be avoided.

6.2.5. Caging

- Cages for NIEHS studies must be program and test article specific.

- Cages must be returned to the same test agent to avoid possible contamination.
- Each cage containing animals must always be identified with a cage label that includes:
    - Study number
    - Cage number
    - Animal number
- The cage identification card must be attached to the cage and must be transferred along with the animal(s) to a new cage throughout the in-life portion of the study.
- Polycarbonate cages must be used in a suspended cage rack system, unless otherwise specified by NIEHS. The racks must have provisions for placing filter fabric on the shelf above the cages.
- During the quarantine period, study animals, except those on chronic studies, may be caged together according to the weight-space specifications recommended in the Guide for the Care and Use of Laboratory Animals.
- For subchronic and chronic studies, animals must be apportioned to cages at the start of the study as if they were in the upper weight range so that it will not be necessary to redistribute them later to larger cages to remain within the recommended weight-space specifications.
    - Rats and female mice must be group housed, five per cage, except for those used in chronic studies.
        - For chronic studies, male rats must be group housed, up to three per cage, and female rats must be group housed, up to five per cage.
        - Male breeder rats must be individually housed upon separation of breeding pairs.
    - Male mice must be individually housed.
    - Rabbits must be individually housed.
    - For inhalation and dermal studies, all rats and mice must be housed individually.
    - Pregnant dams must be individually housed; there must be no more than one dam with litter per cage.
    - During the mating period, one male rat and one female rat must be housed together, unless otherwise directed by the program COR, depending on study type.

6.2.6. Cage Sizing

- For group-housed rats, pregnant rats, or rats with litters, cages must measure approximately 22” L, 12.5” W, and 8” H.
- For individually housed rats in dermal studies, cages must measure approximately 9” L, 8” W, and 8” H.

- For group-housed female mice, cages must measure approximately 12.5” L, 9.25” W, and 6” H.
- For individually housed male or female mice, polycarbonate, solid-bottom cages must measure approximately 9.25” L, 6” W, and 6.125” H.
- Rabbits must be housed in caging with smooth, slip-proof, perforated flooring. Caging must provide at least 3 sq. ft./animal for rabbits 4.0 kg or less and must be 16” H. Larger rabbits may require more space.

6.2.7. Sanitization

- Group-housed animals must be changed to a sanitized cage twice weekly and individually housed animals must be changed to a sanitized cage once weekly, or as often as necessary to keep the animals clean and dry. Remaining cage groups must not be combined. If cage changing becomes more frequent than the above schedule on a continual basis, the program COR is to be notified as this might indicate a treatment-related effect.
- Dirty cages must not remain in the animal rooms. After changing, cages must be washed promptly in a machine that provides one rinse cycle of at least 180°F water.

6.2.8. Animal Allocation for Chronic Studies

- Animal allocation must be accomplished as follows: Randomly assign animals from weight classes to cages. Randomly assign cages to treatment/dose groups. Rearrange cages in a rack within like treatments/doses in a vertical column. Randomly assign location of treatment/dose columns in racks.
- Cage rotation must be accomplished as follows: Once every 2 weeks, or each time racks are cleaned, rotate each rack of cages vertically within a treatment column. Also, rearrange racks within the original room configuration. Sentinel animals for the animal disease screening program must be included in procedures as if they were another treatment group and must be handled as outlined in the Biosecurity section below.

6.2.9. Racks

- Stainless steel, suspended-type racks must be used, unless otherwise specified.
- Racks must be kept clean while in use and, in particular, the wheel surfaces must be cleaned 360° when the floor is being cleaned.
- Water manifolds on each rack must be flushed daily (for at least 60 seconds) and each watering valve must be checked for proper water flow
- Racks must be capable of being moved to the wash area for periodic machine sanitizing, or, if they are fixed racks, sanitization must be provided for each of these racks.

- It is preferred that racks are run through a rack washer that includes one cycle of 180°F water. If a rack washer is not available, racks must be moved to a wash area, hosed and washed using a suitable detergent, and rinsed under high pressure.
- Racks must be sanitized at least every other week. At the time of rack sanitization, the automatic water manifolds must also be:
    - Sanitized by flushing with hot water at 180°F or higher for a least 1 minute, preferably after flushing with warm detergent solution to remove organic matter; or
    - Sanitized by flushing/exposure to a sanitizing solution (e.g., chlorine) for 30–60 minutes followed by flushing with water for at least 2 minutes.

6.2.10. Filters

- Nonwoven, synthetic fiber filters must be used on the racks.
- A fresh filter sheet must be supplied at least every other week.

6.2.11. Bedding

- For rodents, irradiated, heat-treated hardwood bedding that meets the NIH standards for physical quality and the standards for chemical and microbiological contaminants is available from commercial manufacturers. (See Section 6.6.1 [Table 6-1] for maximum acceptable level of contaminants.)
- The testing laboratories and the NIEHS Laboratory Animal Medicine (LAM) discipline leader must receive the bedding analysis data from a sponsor-approved vendor for each batch of bedding used.
- It is the responsibility of the testing laboratory to make sure that the bedding meets the standards.
- The testing laboratory is not expected to perform additional analyses on the bedding.
- The testing laboratories may be required to ship a sample of the bedding to NIEHS or to a sponsor-designated analytical laboratory for contaminant analysis.
- The bedding must be stored off the floor, away from the wall, and in a fashion that prevents contamination.
- Fresh bedding must be supplied in clean sanitized cages as specified above.
- Bedding is NOT to be used in rabbit studies.

6.2.12. Diet and Water

- Diet
    - Irradiated, certified NIH-07 or NTP-2000 open formula diets must be used for rodents, unless a different diet is specified. NIH-07 and NTP-2000 diets must be certified as outlined in Section 6.6.2.

- The testing laboratory must receive the diet from a sponsor-approved vendor. NIH-07 diet must be used during periods of premating, mating, pregnancy, and lactation. NTP-2000 diet must be used during study phases and for cohorts that do not include mating, pregnancy, or lactation. The diet must be changed from NIH-07 to NTP-2000 at the time of weaning.
- The program COR and LAM discipline leader must approve the use of alternate rodent diets. The feed must be irradiated and analyzed for contaminants prior to use. The LAM discipline leader must approve each lot of diet before use. It is the responsibility of the testing laboratory to ensure that the appropriate documents are provided to NIEHS LAM in a timely manner.
- Feed must be stored at 70°F or lower, 50% relative humidity or lower, and in a well-ventilated area. Feed must be stored off the floor and away from the wall.
- Feed must be used for no more than 180 days post-milling date.
- Rabbits must be fed NIH-09 open formula diet or other approved feed obtained from a sponsor-approved vendor. The diet must be certified and irradiated.
- Rabbits must be fed the sponsor-approved diet at the rabbit vendor for at least 2 weeks prior to mating. The vendor must provide confirmation that rabbits are eating the approved diet prior to shipping.
- Records must be kept on the type of diet used for each study. These records must include the batch number, milling date, date of use, physical form (e.g., wafer or meal), and the supplier or source of the feed. Each batch of NIH-07 or NTP-2000 diet must be analyzed for contaminants, protein, fat, fiber, ash, moisture, and heat-labile nutrients such as vitamin A and thiamine. Lists of contaminants with maximum acceptable levels are provided in Section 6.6.2 (Table 6-2). A copy of the analysis records must be included in the study records and sent to the NTP Archives.
    - It is the responsibility of the testing laboratory to verify that the diet meets the standards prior to use.
    - The testing laboratories may be required to ship a sample of the diet to NIEHS or a sponsor-designated analytical laboratory for nutrient and contaminant analysis.
    - NIEHS anticipates updating the rodent feed analysis guidelines in the near future; labs will be given advanced notice.
- Diet must be provided for the animals in a feeder. Unless otherwise specified, wafer (pelleted) feed must be used for all routes of administration except dosed feed studies. Meal (powdered) feed must be used for dosed feed studies.

  - For rodents, clean feeders with fresh food must be supplied at least once weekly.
  - Rabbits must be fed daily. Sufficient, fresh food must be provided as often as necessary to ensure support of normal growth and maintenance.
  - Feed hoppers for meal (powdered) feed must not be filled to the brim as filling the feeder to <80% capacity will help decrease spillage.
  - Animals must have food ad libitum, unless specified otherwise (e.g., see Section 6.5.1). Feed hoppers must be dumped in the vented enclosure in the dirty cage wash area.
  - Dirty feeders are to be soaked when necessary; after washing, they must be rinsed in at least one cycle of 180°F water.
  - To avoid cross contamination during washing, feeders used for all dosed feed studies must be uniquely marked or labeled to identify the test article being dosed, each dose group, and the control group feeders.
- Water
  - Municipal drinking water must be supplied ad libitum. Water must not be hyperchlorinated or hyperacidified. NIEHS may specify a suitable water treatment procedure for special cases.
  - Laboratories must demonstrate that water provided for animal use meets U.S. EPA National Primary Drinking Water Regulations. Section 6.6.4 contains a list of additional water components and contaminants to be determined and assessed. To satisfy this requirement, the laboratory must provide analyses of water from an animal room or a composite from several animal rooms at least once during the in-life phase of each subchronic study, and at least once a year for chronic studies. For dosed-water studies, water used for such analysis is to be taken from the specific source used to make the dose formulations. Details of any water treatment performed by the laboratory must be provided.
  - For laboratories new to NIEHS, an additional report must be provided to NIEHS within 30 days of contract award. Water analyses must be performed by a laboratory qualified to conduct such analyses on a local, state, or interstate basis. The testing laboratories may also be required to ship a sample of water to NIEHS or an NIEHS-designated analytical laboratory for contaminant analysis once a year.
  - The entire automated watering system must be sanitized at least every 1–3 years to prevent a buildup of biofilms in the system.
  - When an automated watering system is used, the valve end must be located outside the cage, which will require that a stainless steel grommet be affixed around the access port to the watering valve. Care must be taken to ensure that the animals can reach the valves and that the valves are placed such that cages cannot be flooded in the event of a malfunction.

  - If an automated watering system is used, S-shaped stainless steel watering manifolds must be used to facilitate sanitization at the time of rack sanitization. The rack watering manifolds must be flushed or drained at least once a day to prevent matter and bacterial accumulation.
  - Water bottles may be used, although an automated watering system is preferred. Water bottles must be of sufficient capacity so that no more than two bottles/week/cage are routinely needed. Each cage must be supplied with a fresh sanitized water bottle, bottle cap, and sipper tube twice weekly for group-housed animals and weekly for individually housed animals. Dirty (used) bottles must be exchanged for clean bottles, and must not be refilled and reused prior to sanitization. Water bottle caps must be made of an inert material and bottles must be located in a position to prevent the caps from being chewed by the animals.
  - Bottles, bottle caps, and sipper tubes must be soaked and washed promptly. After the wash cycle, water bottles must be rinsed in water of at least 180°F. Water bottles must be:
    - Washed with regular cage washing detergent using a brush apparatus, suitable for the type of bottles being used; or,
    - Washed in an automatic washing system wherein the water outlet for the bottle washing process is well within each bottle being washed.
    - If the bottles are to be washed in a standard tunnel washer, each bottle at each dose (study water bottles only) must be filled with tap water and rinsed twice prior to washing. During washing, control bottles and those from each dose group must be kept separate from each other and not washed with bottles from other dosed-water studies.

- Dosed-water studies
  - For dosed-water studies, water for control groups is to be taken from exactly the same source and at the same time as the water used for the treated group formulations. Depending on the test article, different types of water may be used (e.g., deionized water). The control water and dose formulations are to be stored at 5°C in carboys until it is time to dispense the formulations to water bottles and transport them to the animal rooms.
  - To avoid cross contamination during washing, water bottles used for all dosed-water studies must be marked indelibly, inscribed, or tagged with a permanent color coding so as to identify the test article being dosed (symbol may be used), each dose group, and the control group water bottles.

6.2.13. Environmental Enrichment

Study animals in all dose groups and sentinel animals must be provided with the appropriate enrichment devices upon arrival to the testing laboratory until study termination. Animals on studies that are exposed via dosed-feed, dosed-water, and gavage routes must receive enrichment

items/devices as outlined below. Animals on whole-body/nose-only inhalation studies will only be given enrichment devices when they are in their domiciliary solid-bottom cages, whereas animals on dermal studies will not be provided with enrichment devices during the course of the study.

- For mice, natural crinkled Kraft paper must be used in caging.
- For rats, natural crinkled Kraft paper must be used in cages of rat dams that are pregnant and rat dams with litters. Rectangular shelters must be used for all other rats including weanlings and breeding pairs.
    - The crinkled Kraft paper must be used for pregnant rats during most of the gestation period. On gestation day (GD) 19, all crinkled Kraft paper must be removed from each pregnant dam's cage; on postnatal day (PND) 4, the crinkled Kraft paper must be returned to the cage of each dam with litter. For teratology studies in which the dam is euthanized prior to parturition, the crinkled Kraft paper should remain in the cage for the duration of the pregnancy.
    - For rabbits, contaminant-screened, loose timothy hay will be provided.
    - Additional enrichment devices for rabbits and rodents other than rats and mice will be defined prior to study start by the NIEHS LAM discipline leader.
- All animals on the study must have the same enrichment device for the same amount of time.
- The testing laboratory must provide the COR and the NIEHS LAM discipline leader with a standard operating procedure outlining the use of the enrichment device using NIEHS guidelines.
- Enrichment must be purchased from a sponsor-approved vendor.
- The testing laboratories must receive the enrichment devices and the analysis data from a sponsor-approved vendor for each batch of enrichment used.

6.3. Animals

6.3.1. Humane Care of Rodents and Rabbits in NIEHS Studies

- Animals must be anesthetized to alleviate pain in procedures that may cause momentary or slight pain.
- Animals with the following criteria must be euthanized immediately to avoid further pain and distress (see Section 6.6.5).
    - Large masses or other conditions interfering with their eating and drinking
    - Major injuries or ulcers
    - Debilitating conditions (animal not anticipated to survive until next observation period) or other conditions indicating pain or suffering as judged by the veterinarian or an experienced scientist

- Moribund animals and animals scheduled for interim and final necropsies must be euthanized by personnel trained in methods and techniques established by the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (Leary et al. 2020).
- Approved methods of euthanasia include prolonged exposure to CO_2 gas in cylinders for rodents >10 days old, anesthetic overdose (injection or inhalant), or injection of euthanasia solution. Rodent decapitation with a guillotine or surgical scissors, with or without anesthesia, is acceptable by trained personnel deemed proficient in this technique. Guillotines and scissors must be kept sharp, clean, and in good condition.
- Fetuses GD 15 to birth and neonates from birth to 10 days of age must be euthanized by AVMA-approved methods based on the needs of the study.
- Rabbits must be euthanized by injection with euthanasia solution.

6.3.2. Strains and Source

- The B6C3F1/N (C57BL/6N × C3H/HeN MTV-) hybrid mouse and the Envigo Sprague Dawley (Hsd:Sprague Dawley® SD®) rat must be the study animals used in studies, unless otherwise specified. The B6C3F1/N mouse is maintained by contract under the direction of the NIEHS LAM group and is provided to the testing labs to conduct studies. The CD-1 mouse must be used in studies that use breeding in mice; the BALB/c mouse will be used in hypersensitivity studies. Other rodent models (e.g., NZB mouse, NOD mouse, brown Norway rat) may be used occasionally for certain immunotoxicity studies.
- The New Zealand white rabbit must be used in certain nonrodent studies.
- All animals must be obtained from a sponsor-approved vendor.
- For studies using time-mated SD rats, female rats must be 11–12 weeks of age and 200–220 grams at mating unless specified otherwise by the protocol; male rats should be at least 13 weeks of age at the time of mating.
- Time-mated female rabbits should be 2.5–4.0 kg (4–6 months of age) at either GD 1 or GD 2 upon receipt.
- When animals are shipped by air, they must be transported from the airport to the testing laboratory without delay. All shipments, regardless of route, from sponsor-approved suppliers containing dead, moribund, or otherwise unsatisfactory animals must be reported immediately to the program COR. If a shipment of animals is not received, the NIEHS LAM discipline leader must be notified as early as possible to help trace the shipment.

6.3.3. Animal Receipt and Quarantine

- Shipping cartons/crates and the filter fabric must be examined for damage that occurred during transit. Do not use animals in damaged cartons. Thoroughly wipe the entire outside of the shipping cartons with an appropriate disinfectant. Disinfected

shipping cartons are to be separated from shipping cartons that have not been disinfected. Do not spray directly on or around the shipping cartons.

  - Disinfected, unopened shipping cartons must be taken directly to the door of the specific animal room but must not be taken into the animal room.
  - During unpacking and transfer of animals to cages, neither the animals nor the person removing the animals from the carton must come in contact with the outside surfaces of the shipping cartons.
  - Rats and mice on the same study, received from the same or different shipments from the same supplier, may be maintained together.
  - Rabbits will remain housed separately upon arrival.
  - The testing laboratory's LAV must examine the animals with 24 hours of arrival to assess their health status. Upon receipt of time-mated animals, examinations may need to be conducted sooner, depending on the protocol.

- Animals must be quarantined/acclimated up to a maximum of 14 days under conditions simulating those in the study situation. The maximum quarantine may be increased due to special circumstances, such as receipt of staggered cohorts or multiple orders. Newly received animals may be used in a study after a 3-day acclimation period, at minimum, but must remain under quarantine until released. At the end of the quarantine/acclimation period, the animals must be examined and, if healthy, released from quarantine for study by the LAV.
  - Animals must be housed one to five per cage to simulate the housing conditions that will be used during the testing phase of the study (Section 6.2.5).
  - If automated watering is used in the testing facility, it must be used during the quarantine/acclimation period. Group housing is permitted for up to 7 days at the beginning of the quarantine period for acclimation to automated watering.
  - During quarantine, the animals must receive the same textured feed (wafer or meal) from the same source as they will receive during the study period.
  - Rabbits will be handled daily after arrival to acclimate them to lab conditions, including any procedures that will be performed during dosing and other studies.
  - Animals not eating well, or otherwise unsuitable, will not be placed on study. Rabbits not placed on the study due to inappetence may be placed in the testing laboratory's training colony.
  - Animals must be observed for signs of normal eating, drinking, and behavior, as well as for any untoward signs of health problems, stress, or distress.
- The health of the animals must be assessed during the last few days of quarantine (3–4 days prior to being placed on study). Animals undergoing health assessment must be bled for serology assessment of pathogens, and must be examined for internal and external parasites 1 to 3 days before quarantine is lifted. Abnormal health reports

must be confirmed by necropsy, followed by microscopic examination and/or microbiological culture.

6.3.4. Animal Assignment to Study

- During the last 1–3 days of quarantine/acclimation, or as specified in the protocol for time-mated animals received from the supplier, animals must be assigned to test/control groups following formal randomization routines.
- If sufficient healthy animals are available, these animals must be randomly assigned to weight distribution groups. The weight distribution range of the animals selected for the study should be as narrow as possible and no more than ±20% from the mean body weight (by sex) of all animals available for the study at randomization. If it is necessary to use a few animals outside the ±20% range, approval by the program COR must be obtained. For studies using rabbits, the weight distribution range must be as narrow as possible.
- In all studies the body weight means of the groups within a sex and species must be similar. To achieve this objective, it is required that, before randomization to treatment, the animals must be divided into weight classes and all outliers removed. Animals must be distributed into stratified weight classes using 5-gram intervals for rats and 1-gram intervals for mice and then randomized into treatment groups. Extra animals must be removed from the study room and accounted for in the raw data. There must be no animal substitutions after a study starts. Extra animals may be used as sentinel animals if necessary or for training of technical personnel.

6.3.5. Animal Identification

The method of identification must be approved by NIEHS and must take into consideration pregnancy status to minimize stress, length of time on study, and the need to individually identify animals over that interval. Animals must be uniquely and consecutively numbered. Once a number is used for an animal in a study, it must not be repeated in the same study. A tattoo at the base of the tail with black pigment is the preferred identification method for all studies with albino and pigmented rats and mice. Double-strength black pigment is recommended for tattooing pigmented (B6C3F1/N) mice. Some tattoos of pigmented mice may fade and have to be retouched or re-tattooed once or twice during the course of a 2-year study. Rabbits must be identified with an ear tattoo and implanted transponders. The scheme used for identification must be included in the raw data. The tail, or other body part bearing the identifications marks, must be fixed with the tissues at necropsy.

There must be a method to track parentage of every animal born in a study. Limb or paw tattoos may be used to identify pups.

6.3.6. Weaning

Pregnant rat dams assigned to perinatal studies will be weaned at PND 28 days unless exceptions are indicated at the time of protocol development.

6.4. NIEHS Biosecurity Program

6.4.1. Program Overview

The NIEHS Biosecurity Program is directed by NIEHS. Testing laboratory participation is mandatory. Studies >30 days in duration require additional animals to be used as sentinels (untreated controls). Sentinel animals must be clearly identified as sentinel and must be used only as sentinels, and not as part of the animals used for the study. Rats and female mice must be group housed and male mice housed individually. Rabbits used as sentinels must be individually housed. Sentinel cages must be randomly placed throughout the racks of control and treated animals. Health surveillance samples are to be submitted to a sponsor-approved disease diagnostic laboratory for evaluation of pathogens. If samples are collected from animals used for clinical pathology studies and the NIEHS Biosecurity Program, the anesthetic, the site of blood collection, and the blood collection technique specified for clinical pathology studies must be used. Samples are shipped to a sponsor-approved disease diagnostic laboratory for disease screening (see Section 6.7)

6.4.2. Perinatal Studies

For studies with perinatal exposure using vendor supplied time-mated animals, the testing laboratory must receive 10 age-matched nonmated females to be used for disease screening prior to dosing the time-mated animals.

- For all perinatal studies, sentinel animal testing will start at arrival. These animals must be used progressively through the perinatal range-finding, perinatal subchronic, and perinatal chronic studies until the F_1 control pups are weaned and culled to be subsequently used as sentinels.
- Testing laboratories will continue to use a sponsor-approved testing schematic at each sentinel testing time point, starting at arrival.
    - For perinatal range-finding studies, sentinel testing time points will be at arrival, 4 weeks after arrival, and at study end. For perinatal subchronic studies, sentinel testing time points will be at arrival, 4 weeks after arrival, 7 weeks after arrival, and end of study.
    - For perinatal chronic studies, sentinel testing time points will be at arrival, 4 weeks after arrival, and 7 weeks after arrival. The newly assigned sentinels originating from the F_1 control pups will be used until study end.
    - Blood collection, fur swabs, and fecal collection will be performed at each of the testing time points given above.

Survival procedures will be performed at all testing time points, except for the 7 weeks after arrival and study-end time points. At study end, full necropsies will be performed on all sentinel animals.

6.4.3. Nonperinatal Studies

- Sentinel animal testing (5 age-matched/sex/species) for all nonperinatal studies will start at arrival. These animals will be used progressively through the 3-month and 2-year studies.
- Testing laboratories will continue to use a sponsor-approved testing schematic at each testing time point, starting at arrival.
    - For nonperinatal 3-month subchronic studies, sentinel testing time points will be at arrival, 4 weeks after arrival, and study end.
    - For nonperinatal 2-year chronic studies, sentinel testing time points will be at arrival, 4 weeks after arrival, 6 months after arrival, 12 months after arrival, 18 months after arrival, and study end.
    - Blood collection, fur swabs, and fecal collection will be performed at each of the testing time points given above.
- Survival procedures will be performed at all testing time points, except for the study-end time point. At study end, full necropsies will be performed on all sentinel animals.
- For all other studies outside these parameters, the LAM discipline leader must be consulted.
- If there is indication or suspicion of disease, animals must be necropsied and examined for gross lesions following the collection of blood samples.
- Blood collection for the disease screening specimen must be collected from the orbital sinus or mandibular vein in mice or via a tail nick or jugular vein in rats (survival), or via cardiac puncture or abdominal vessels using CO_2/O_2 (nonsurvival). Rabbit blood samples must be obtained from the marginal ear vein or artery. Alternative methods of blood collection may be considered as deemed necessary depending on needs of the clinical pathology laboratory or other study parameters.

6.4.4. Care of Sentinel Animals

Weighing

It is not necessary to weigh the sentinel animals or measure their food or water consumption at any time during the study. If all animals for a chronic study, including the sentinels, are pooled for randomization purposes, the initial body weights would have been measured.

Moribundity/Mortality Checks

The sentinel animals must be checked at the same time the regular animal observations are made to assure they are alive and healthy. No program notes are necessary, and the animals need not be palpated or otherwise handled unless a moribund or dead animal is found. If a sentinel animal is found dead, the death must be recorded. If a moribund sentinel animal is found, a blood sample and feces must be taken before euthanasia. The samples must then be stored and included with the samples from the next scheduled sampling. The sample, in essence, becomes part of the next scheduled sampling.

6.4.5. Pathology

Any sentinel animals lost to the study must not be replaced. Sentinels that die or are euthanized during the course of a study must receive complete necropsies and must be examined to determine cause of death. Selected histopathology (to include all lesions and grossly abnormal organs) must be conducted on dead and moribund sentinel animals (as well as those necropsied at study end). Results must be reported to NIEHS as soon as they are available, but no later than ten working days after the dead or moribund animal was found.

One copy of the individual animal necropsy record (IANR) for the dead and moribund sentinel animals must be submitted to the program COR. Data from these forms must not be entered into Provantis (see Chapter 11 [Data Collection and Submission]). The slides, blocks, and tissues must be labeled with a non-Provantis label containing the experiment, the animal identified as sentinel, date, tissue, etc. The slides, blocks, and wet tissues of the sentinel animals must be sent to the NTP Archives along with the rest of the tissues from the study.

6.4.6. Collection, Processing, and Shipping of Disease Screening Specimens

- See Section 6.7 for further details.
- Dried blood spot samples
    - A drop of whole blood is required for the serology assays via dried blood spot sampling. The sample area outlined on the blood spot card must be filled with blood and allowed to dry for at least 1 hour before preparing for shipping. The sample must be stored in provided waterproof bags with silica packets until shipment.
    - The testing laboratory must submit dried blood spot samples for screening for the presence of antibodies to pathogens, as indicated in Section 6.4.2 and Section 6.4.3. The screening tests must include but not be limited to the following:

      Mice

        - Ectromelia
        - EDIM
        - Lymphocytic choriomeningitis virus (LCMV)
        - Mouse norovirus (MNV)
        - Mouse hepatitis virus (MHV)
        - Mouse minute virus (MVM)
        - Mouse parvovirus (MPV)
        - *Mycoplasma pulmonis*
        - NS1 (generic parvovirus)
        - Pneumonia virus of mice (PVM)

- Reovirus type 3 (REO3)
- Sendai virus
- Theiler's encephalomyelitis virus (GD VII)

Rats

- Lymphocytic choriomeningitis virus (LCMV)
- *Mycoplasma pulmonis*
- Parvo virus (RPV, RMV, KRV, H-1)
- Pneumocystis carinii
- Pneumonia virus of mice (PVM)
- Rat coronavirus-sialodacryoadenitis virus (RCV-SDA)
- Reovirus type 3 (REO3)
- Sendai virus
- Theiler's murine encephalomyelitis-like virus (rat theilovirus)

Rabbits

- *Filobacterium rodentium* (*Cilia-associated respiratory bacillus* [*CAR bacillus*])
- *Encephalitozoon cuniculi (E Cun)*
- *Clostridium piliforme*
- *Treponema paraluiscuniculi*
- Rotavirus (if rabbits are purchased from a rotavirus-free vendor)

- Blood collection from study animals for disease screening is not required unless requested by the comparative medicine discipline leader and the program COR.

- Feces
  - Fecal samples are collected to test for pinworms and *Helicobacter spp.* via polymerase chain reaction (PCR) in rodents. Fecal samples from rabbits are tested for endoparasite *Passalurus ambiguous* and *Eimeria spp*.
  - Two to three fecal pellets per rodent or rabbit must be collected with clean gloves or sterile forceps and placed in individually labeled sterile containers to test for pinworms and *Helicobacter spp.*
  - Gloves or forceps must be changed between cages. Fecal pellets can be collected directly from the animal or from the cage. Fecal pellets do not need to be chilled or frozen.
  - Fecal samples: 1–5 fecal pellets of the 10 allowed per sample must be from study animals that will be combined with samples of sentinel animals for rodents and rabbits.

- Fur swabs
    - Fur swabs are taken to test for fur mites via PCR. The sterile swab should be run through the hair of the animal, against the direction of the fur growth.
    - Targeting the fur on the face, back, and tail base will ensure the best potential exposure. The dry, fur swab sample collections must be placed in individually labeled sterile tubes.
    - Users can place the swab halfway into the tube and use the cap to hold the swab in place while breaking the swab in half. This method allows the user to store the swab without touching the sample or the inside of the tube.
    - Fur samples must be collected from study animals in addition to sentinel animals.
- Sample handling
    - All dried blood samples, feces, and fur swabs must be submitted to the sponsor-approved disease diagnostic laboratory in containers that must be labeled legibly with a waterproof, indelible permanent marker. Each label must correlate with a corresponding line on the accompanying disease screening specimen form (provided by the program COR). A separate sample form must be included for each species. Ensure vial lids are tight to prevent leakage. Individual wrapping or clustering in groups of three to five with rubber bands, envelopes, or plastic bags can help to prevent moving during shipment.
    - If serum is collected, it must be transported frozen with ice packs in an insulated container.
    - Fecal samples must be transported at ambient temperature.
    - All disease screening specimens must be shipped via an overnight delivery service Monday through Thursday only.

6.5. Special Requirements for Specific Routes of Administration

6.5.1. Inhalation

Typically, inhalation studies will be conducted by whole-body exposure; however, on occasion nose-only or intratracheal exposure may be required. In those cases, specific details will be provided in the individual chemical statement of work. The requirements that follow are provided for whole-body exposure, although some are also appropriate for nose-only exposure.

- Animal housing/exposure room
    - The air entering the chamber room must be filtered and clean. Clean materials (cages, racks, feeders, etc.) must not be stored in the chamber rooms, and dirty materials taken from the chambers must be removed from the chamber rooms as soon as the animals are back in the chambers.

  - Animals used in whole-body and nose-only inhalation studies must be singly housed in polycarbonate cages (as previously described in Section 6.2.5) within the animal room. Quarantine will begin upon arrival and will extend up to 14 days postarrival. Once the study begins, the animals are transported to the exposure room each day prior to the start of exposure and then are placed in the inhalation tube or chamber. After the exposure period is complete for the day, animals are returned to their home cage in the animal room. The animals must be identified by tattoo.
  - Animals must be housed in a room as close as is practical to the chamber room. One room must be allocated for each chemical. The procedures used for transport to the exposure room, caging, feeding, and watering must be described in detail and approved by NIEHS.

- Chambers
  - Inhalation chambers must be of a design that can be demonstrated to provide uniform and reproducible exposure of all animals to the test article. Intake air to the chambers must be filtered through absolute (HEPA), Purafil® and charcoal filters. Intake air is to be analyzed at the conclusion of exposure system development and installation, and at least once during each 90-day and 2-year study. This practice is done to ensure that the quality of the air entering the exposure chambers meets or exceeds human breathing air standard grade E set forth by the Compressed Gas Association "Commodity Specification for Air," G-7.1. Air flow rate, temperature, and relative humidity must be checked and recorded at least every 3 hours or, preferably, continuously. Chamber pressure (negative relative to room pressure) must be checked frequently and recorded at least daily. Flow meters must be calibrated with regard to pressure drop on a routine basis at least once every 2 months or as often as necessary to maintain the required flow.
  - During exposure, all animals must be individually housed in stainless steel wire mesh cages with secure latches during exposure. Additionally, males may need to be housed separately from females, or special study animals housed separately. The cages must be of adequate size such that all animals in a particular dose group can be exposed in a single chamber. The animals themselves must account for not more than 5% of the total volume of a chamber.
  - For chronic inhalation studies: From the start of the study, female rats must be housed in cages with at least 40 sq. in. floor space and male rats must be housed in cages with at least 60 sq. in. floor space. For example, if Hazleton 2000 chambers are used, a combination of R14, R16, R20, and R24 cage batteries will be required to meet space requirements for growing rats throughout the chronic study. From the start of the study, all mice must be housed in cages with at least 24 to 30 sq. in. floor space. Sentinel rats and

mice must be distributed evenly throughout each exposure and control chamber.

- The cage racks must be rotated clockwise daily on exposure days of the repeated dose study and weekly during the subchronic and chronic studies. The cages and the chambers must be washed and sanitized at least once a week and more often if necessary. If cages are mounted on more than one tier in the chamber, pans for collection of excreta must be required between tiers during both the exposure and nonexposure periods. Cage board (pan paper) may be used in the excreta collection pans of the chambers during exposure, provided the test article is not going to react with or be absorbed by the cage board during exposure. The cage board or pan paper must be changed once a day.
- Animals are to be removed from chambers during nonexposure periods and maintained in their home cages. Moribundity/mortality checks are performed twice daily: once when moving animals from their home cage prior to exposure and again when moving animals to their home cage after exposure. Clinical observations are performed during these times, per the study protocol. Only one chamber must be opened at a time. Care must be taken to avoid exposure of animals to pathogens in the chamber room.

- Diet and water
  - Water must be available by automatic watering system during the nonexposure periods as well as the exposure periods. The automatic watering system must be checked daily so that in case of malfunction or air locks, the animals will not be without water for more than a day.
  - The feed must be provided ad libitum in feeders or hoppers during the nonexposure periods while animals are in their home cages. Fresh feed must be provided at least weekly.
- Environmental conditions and animal acclimation
  - The study animals must be acclimated in the chambers for at least 3 days before initiation of chemical exposure. The chamber ventilation system must provide 15 ± 2 air changes per hour and the design of the chamber must afford opportunity for equal exposure to each animal.
  - The temperature of the chamber must be maintained at 75°F ± 3°F. If the temperature is above or below the 75°F ± 3°F limit, it must be returned to the acceptable limits within 2 hours. The temperature must not be below 70°F or above 80°F during the course of the study. There must be an alarm system for warning of temperature fluctuations below 70°F and above 80°F.
  - The relative humidity of the chamber atmosphere must be 40% to 70%, and it must not be below 35% or above 74% at any time during the course of the study. There must be a backup ventilation system in the event of the failure of the primary system supplying conditioned air to the chambers. Chamber

temperature and relative humidity results are to be reported as means ± relative standard deviations or as time-weighted averages.

6.5.2. Dermal Studies

Animal Care

All animals must be individually caged. The standard polycarbonate cages are to be used. Stainless steel wire mesh cages may be employed if the volatility of the vehicle solvent and the resultant inhalation exposure of the animals to the solvent is a significant problem. NIEHS may specify the cages to be used in the chemical specific protocol.

Skin Application

In general, the test material must be applied up to 5 days a week (unless specified for the individual SOW), during a "consistent, specified time" of the morning each treatment day. An entire dose group is to be dosed before moving to the next group. The treatment sequence of control and dose groups for each treatment day must be randomized to avoid a control first and high dose last bias. The dose must be applied uniformly to a fixed standard area of skin in the dorsal (e.g., interscapular) region for both rats and mice. This area is to be the same size and location for each animal of a given species. The application site must not exceed 10% of the animal surface area. An area larger than the application site must be clipped weekly to allow for uniform application of the article and clear observation of the painted area. Electric clippers with the appropriate-sized clipper head must be used. Treated and control animals must be clipped. If needed, CO_2/O_2 anesthesia may be used.

An appropriate vehicle in which the test article is applied must be selected for each chemical and, in general, will be specified in the study SOW for the test article. Ethanol, acetone, and water are common choices. If a vehicle is used, vehicle control animals are required. If the test article is a liquid and no convenient vehicle can be found, it may be applied without a vehicle and clipped untreated controls must be used.

For each dose group, the concentration of the dose formulation will remain constant throughout the study, with the required dose provided by varying the volume administered based on animal body weight. Dosing volume must be 0.5 mL/Kg for rats and 2.0 mL/Kg for mice, or as specified in the individual study SOW.

Documentation that each animal was dosed on each treatment day is to be recorded and submitted with the study files.

If the dose is a free-flowing liquid, it can be applied conveniently using a micropipette or syringe with disposable tip. If necessary, a smooth glass rod or the pipette tip may be used to spread the dose over the application area. When skin tumors occur, a separate disposable rod or pipette tip must be used for each animal to avoid transplanting tumor cells from one animal to another. Nothing that abrades or causes physical damage to the skin must be used.

6.5.3. Gavage Studies

In gavage studies, for each dose group, the concentration of the dose formulation will remain constant throughout the study. The specific dose requirements will be met by varying the volume

administered based on animal body weight. The total volume of material given per animal per treatment must not exceed 5 mL/kg for rats, 10 mL/kg for mice, and 2 mL/kg of corn oil or 6 mL/kg water-based substance for rabbits without consultation with and written approval of the program COR. The volume selected must remain constant throughout all studies for a test article.

All animals of a gavage study must be treated during a "consistent, specified time" of the morning on each treatment day. The program COR must approve the "specified time." An entire dose group is to be dosed before moving to the next group. The treatment sequence of control and dose groups for each treatment day must be randomized to avoid a control-first and high dose-last bias.

Documentation that each animal was dosed on each treatment day is to be recorded and submitted with the study files.

6.5.4. Studies with a Mating Component

- Rodents must be paired 1:1 in the late afternoon (after 3 p.m.). The female must be moved to the male's cage. Sibling matings are to be avoided.
- Vaginal cytology slides must be prepared in a fashion so they may be permanently retained.
- Confirmation of mating is defined as an in situ plug or the presence of sperm in a lavage sample. Cage plugs are not considered to be definitive evidence of mating, but can be used as supportive information (e.g., estimating the gestation day of an apparently pregnant rat).
- Gestation day 0 (GD 0) is defined as the day evidence of mating is noted.
- Calculated reproductive indices
    - Mating index: Number of confirmed mated females/number of cohabiting pairs
    - Fertility index: Number pregnant/number of cohabiting pairs
    - Fecundity: Number females with at least one live pup/number pregnant
    - Littering index: Number of females delivering/number of cohabited pairs

6.6. Laboratory Animal Management

6.6.1. Maximum Levels of Contaminants for Heat-treated Hardwood Bedding

Table 6-1. Heat-treated Hardwood Bedding Maximum Level of Contaminants

Contaminant	Maximum Level
Chemical Contaminants (ppm)	
Pesticide Residues	
Chlorinated hydrocarbons	
Alpha BHC	<0.02
Beta BHC	<0.02
Lindane	<0.02
Aldrin	<0.02
Heptachlor epoxide	<0.02
Dieldrin	<0.02
Endrin	<0.02
DDT	<0.03
DDD	<0.02
DDE	<0.02
Organophosphates	
Diazinon	<0.10
Ethyl parathion	<0.03
Methyl parathion	<0.03
Malathion	<0.05
Ethion	<0.02
Ronnel	<0.03
Triothion	<0.03
Polychlorinated biphenyls	<0.20
Pentachlorophenol	<0.10
Aflatoxins	<10 ppb
Heavy metals	
Lead	<0.5
Mercury	<0.1
Cadmium	<0.1
Arsenic	<0.2
Microbiological Contaminants[a]	
Standard Plate Count	<100
Coliform	<10

Contaminant	Maximum Level
Pseudomonads	Negative
Yeast and Molds	<10
Salmonella/Shigella	Negative

[a]All values in total organisms/g of bedding.

6.6.2. Limits of Contaminants for NIH-07 or NTP-2000 Diet

Table 6-2. Limits of Contaminant Levels – NIH-07 and NTP-2000 Diet

Contaminant	Maximum Level
Aflatoxins (ppb)	
Total	5
B_1	2
Nitrosamines (ppb)	
Total (Volatile)	15
N-Nitrosodimethylamine	10
Heavy Metals (ppm)	
Lead	1.00
Cadmium	0.15
Mercury	0.05
Arsenic	0.50
Selenium	0.50
Chlorinated Hydrocarbons (ppm)	
BHC	
Alpha	0.02
Beta	0.02
Delta	0.02
Lindane	0.02
Heptachlor	0.02
DDE	0.02
DDD	0.02
DDT	0.03
HCB	0.08
Mirex	0.02
Methoxychlor	0.05
Dieldrin	0.02
Endrin	0.02
Telodrin	0.02

Contaminant	Maximum Level
Chlordane	0.05
Toxaphene	0.10
Organophosphates (ppm)	
Chloropyrifos-methyl	0.10
Ronnel	0.03
Ethion	0.02
Trithion	0.05
Diazinon	0.20
Methylparathion	0.03
Ethylparathion	0.03
Malathion	0.50
Endosulfan I	0.02
Endosulfan II	0.02
Endosulfan Sulfate	0.03
PCBs (ppm)	0.20
Miscellaneous (Maximum Limits, ppm)	
Nitrate	20
Nitrite	5
BHA	10
BHT	5
Bacterial Plate Count	
Total (CFU/g)	1,000
Coliform (MPN/g)	10
E. Coli (MPN/g)	10
Salmonella (/g)	Negative

6.6.3. Rating of the Feed for Contaminants

Maximum Points: 100

95–100: Use the feed

91–94: May use it but replace with a new batch within 4 weeks

90 and below: Reject the feed

If all contaminants are at less than the specifications, rating for that batch of feed will be 100.

- Aflatoxin: Deduct 1 point for each ppb above the specification. If the aflatoxin level is more than 10 ppb, the feed must not be used.

- Nitrosamines: Deduct 1 point for each 2 ppb above the specifications.
- Heavy metals:
    - Lead, arsenic, and selenium: Deduct 1 point for each 0.2 ppm above the specifications.
    - Cadmium and mercury: Deduct 1 point for each 0.02 ppm above the specifications.
- PCBs: Deduct 1 point for each 0.02 ppm above the limit.
- Pesticides: Deduct 1 point for each increase equivalent to the maximum allowable level.
- Miscellaneous contaminants: Deduct 1 point for each increase equivalent to the maximum allowable level. If microbiological contaminants are 2X the limit, a new sample is to be tested for possible contamination during sampling. If the repeat sample confirms the original results and the total bacterial count is >5,000 CFU/g, the product was not irradiated properly and cannot be accepted as an irradiated product.

Sample calculation:

Arsenic is reported as 0.675 ppm. Per the limits of contamination, the maximum contaminant level of arsenic is 0.50 ppm. Per the rating, 1 point is deducted for each 0.2 ppm above the specification. Therefore:

$$0.675 - 0.500 = 0.175;\ 0.175/0.200 = 0.875;\ 100 - 0.875 = 99.125$$

Based on this calculation, feed can be used.

NOTE: Calculations are made for contaminants above the limit; calculations are then added together and subtracted from 100 for the feed rating.

6.6.4. Water Analysis

Laboratories must demonstrate that water provided for animal use meets U.S. EPA National Primary Drinking Water Regulations. In addition, the components and contaminants listed below are to be determined and assessed.

Metals (mg/L)

Sodium (Na)

Barium (Ba)

Potassium (K)

Strontium (Sr)

Calcium (Ca)

Boron (B)

Magnesium (Mg)

Phosphorus (P)

Aluminum (Al)

Chromium (Cr)

Iron (Fe)

Copper (Cu)

Manganese (Mn)

Zinc (Zn)

Chlorinated Hydrocarbons (mg/L)

Aldrin

Dieldrin

DDT-related substances

Organophosphates (mg/L)

Phorate

Diazinon

Methyl

Parathion

Malathion

Parathion

Endosulfan

Carbophenothion

6.6.5. Humane Endpoints for Rodents and Rabbits

The final decision for euthanasia of moribund animals must be made by the laboratory animal veterinarian or an experienced scientist and must not be left to the discretion of the technicians. Conditions warranting euthanasia of rodents and rabbits in long-term studies are listed below. These criteria should be supplemented with professional judgment for euthanasia or moribund animals during a study (NIH 2019; Whitehead et al. 2014).

- Loss of 10% to 25% body weight in <1 week
- Gradual but sustained decline in body weight indicating partial and sustained anorexia
- Large masses and other conditions preventing eating and drinking
- Major injuries and lesions, such as nonhealing ulcers

- Diseases, conditions, and behavior indicating severe pain
- Adequate indication that the animal may not survive until the next observation as judged by an experienced laboratory animal specialist
- Prolonged unhealthy appearance such as rough coat, hunched posture, and distended abdomen
- Behavioral indicators of pain such as back arching or grimace scores (Benato et al. 2021; Langford et al. 2010; Sotocinal et al. 2011)
- Prolonged diarrhea leading to emaciation. Testing labs must use a body condition scoring system to determine emaciation per SOP.
- Prolonged or intense diuresis leading to emaciation. Testing labs must use a body condition scoring system to determine emaciation per SOP.
- Persistent coughing, wheezing, and respiratory distress
- Paralysis and other nervous disorders leading to anorexia and continuous decline in body weight
- Conditions severely impeding locomotion (e.g., persistence recumbency, loss of righting reflex, masses)
- Bleeding from natural orifices not due to minor injuries
- Persistent self-induced trauma complicating minor injuries
- Microbial infections interfering with toxic and carcinogenic responses
- Inappetence in rabbits persisting for 7 consecutive days and consuming <15% of total ration
- Loss of ≥10% of weight in rabbits from the initiation of dosing
- Severe wounds/pup mutilation by dam
- Failure to thrive/runting (<50% body weight compared with siblings mean body weight) or signs of maternal neglect
- Prolonged removal of animal from the thermoneutral zone (persistent hypothermia or hyperthermia)

6.7. Guidelines for Collection and Submission of Samples to IDEXX BioAnalytics

IDEXX BioAnalytics (formerly RADIL) will conduct the health monitoring for DTT studies unless otherwise noted. IDEXX BioAnalytics is a service, research, and teaching laboratory animal diagnostic facility located in Columbia, Missouri. The information presented describes the process of submitting samples to IDEXX BioAnalytics.

6.7.1. Advantage Program

The Advantage Program allows the submission of four samples for optimal rodent health monitoring: dried blood spot via the Opti-Spot, feces, fur swab, and oral swabs. All supplies for collection, such as sterile swabs, Opti-Spot sample strips, and sterile tubes must be provided by IDEXX BioAnalytics. DTT recommends the submission of the dried blood spot, feces, and fur swabs for routine health monitoring. The testing laboratory will be directed to collect oral swabs when needed.

All Opti-Spot sample strips must be submitted to IDEXX BioAnalytics. The Opti-Spot sample strips **must be labeled** with the sample ID number. Allow the blood spot to dry completely for at least 1 hour before preparing to ship. Protect the sample strip from moisture after the blood spot is completely dry by storing it in a watertight plastic bag containing the provided silica gel desiccant pack.

Fecal samples are collected to test for pinworms and *Helicobacter spp*. via PCR. The fecal pellets should be collected with clean gloves or sterile forceps and placed into labeled sterile tubes.

Gloves or forceps must be changed between cages. IDEXX BioAnalytics recommends that fecal samples contain no more than 10 fecal pellets per tube (2–3 pellets/animal). The fecal samples can be stored at room temperature if packaged for same-day shipping or placed in the refrigerator if shipping is next day. The fecal samples can be frozen for longer-term storage.

Fur swabs are taken to test for fur mites via PCR. The sterile swab should be run through the hair of the animal against the direction of the fur growth. Targeting the fur on the face, back, and tail base will ensure the best potential exposure. The dry fur swab sample collections must be placed in individually labeled sterile tubes. Users can place the swab halfway into the tube and use the cap to hold the swab in place while breaking the swab in half. This method allows the user to store the swab without touching the sample and the inside of the tube.

Oral swabs are tested via PCR for *Pasteurella pneumotropica*. The animal should be restrained to restrict the head's movement from side to side. The sterile swabs should be inserted at the corner of the mouth and spiraled inside the cheeks. The dry, oral swab samples must be contained in individually labeled sterile tubes. Users can place the swab halfway into the tube and use the cap to hold the swab in place while breaking the swab in half. This method allows the user to store the swab without touching the sample and the inside of the tube. **The DTT COR will direct the testing laboratory to collect samples when oral testing is necessary**.

The fur and oral swab samples can be held at room temperature until shipping.

Opti-Spot screening must be used to detect the presence of antibodies to rodent and rabbit pathogens listed in Table 6-3.

Table 6-3. Lists of Rodent and Rabbit Pathogens for Testing

Mouse Advantage Health Monitoring Profile – Basic Panel	Rat Advantage Health Monitoring Profile – Basic Panel	Rabbit Serology Profile – Comprehensive Panel
Ectromelia	Lymphocytic choriomeningitis virus (LCMV)	Rabbit rotavirus
EDIM	*Mycoplasma pulmonis*	*Encephalitozoon cuniculi*
Lymphocytic choriomeningitis virus (LCMV)	Parvo virus (RPV, RMV, KRV, H-1)	*Clostridium piliforme*
Mouse norovirus (MNV)	*Pneumocystis carinii*	*Filobacterium rodentium (CAR bacillus)*
Mouse hepatitis virus (MHV)	Pneumonia virus of mice (PVM)	*Treponema paraluiscuniculi*
Mouse minute virus (MVM)	Rat coronavirus-sialodacryoadenitis virus (RCV-SDAV)	
Mouse parvovirus (MPV)	Reovirus type 3 (REO3)	
Mycoplasma pulmonis	Sendai virus	
NS1 (Generic Parvovirus)	Theiler's murine encephalomyelitis-like virus (Rat Theilovirus)	
Pneumonia virus of mice (PVM)		
Reovirus type 3 (REO3)		
Sendai virus		
Theiler's Murine Encephalomyelitis Virus (TMEV)		
Ectromelia		

Testing for additional pathogens not listed above requires approval by the COR and the DTT Laboratory Animal Medicine Discipline Leader.

6.7.2. Serology

Dried blood sampling is the preferred diagnostic method for the detection of pathogens. Approval by the COR and the DTT Laboratory Animal Medicine Discipline Leader is needed for the submission of serum samples. Serum samples will be tested for same pathogens as listed above under Opti-Spot screening.

All serum specimens must be submitted to IDEXX BioAnalytics prediluted. IDEXX BioAnalytics recommends the submission of at least 100 µl (0.1 mL) of 1:5 diluted serum (1 part whole blood and 4 parts of room temperature saline). Refrigerate the diluted blood for 6–12 hours, centrifuge at low speed for 5–10 minutes, and recover the 1:5 diluted serum for submission. The serum must not be heat inactivated or treated in any other way and must be stored frozen until shipment. Do not submit whole blood; hemolysis may interfere with the serology assay performance.

6.7.3. Shipping

Opti-Spot, serum, fecal, fur swab, and oral swab samples must be submitted to IDEXX BioAnalytics in containers that must be labeled legibly with a waterproof, indelible permanent marker. Each label must correlate with a corresponding line on the DTT Sample Submission Form. A separate DTT Sample Submission Form must be included for each species and must accompany the shipment. Potential biohazards associated with the sample must be described on the form. Package samples to keep movement at a minimum during shipment. Opti-Spot samples must be protected from moisture by storing them in a water-tight plastic bag containing the provided silica gel desiccant pack. If serum is used instead of Opti-Spot, the samples must be shipped frozen with ice packs in an insulated container. The use of dry ice is not required. The use of Parafilm over tube lids is recommended to prevent leakage. Fecal, fur swab, and oral swab samples must be shipped at ambient temperature.

All samples must be shipped to the IDEXX BioAnalytics prepaid via an overnight delivery service Monday through Thursday. Samples must not be shipped on Friday to avoid weekend delivery. Samples must be shipped to the following address:

IDEXX BioAnalytics
Discovery Ridge Research Park
4011 Discovery Drive
Columbia, MO 65201
e-mail: idexx-radil@idexx.com
Phone: 800-544-5205; 573-499-5700

6.7.4. Results

Results are available online; an e-mail notification will be sent when the results are ready for viewing via the secure online client portal. Final reports will be sent to appropriate individuals via e-mail. IDEXX BioAnalytics also has an email alert system that sends a notification when results indicate that a potential disease problem has been identified.

6.8. References

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6.9. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapter 6 within the draft *Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences* by letter in February 2022 by the experts listed below. Reviewer selection and document review followed established DTT practices. The reviewers were charged to:

1. Peer review the following chapter within the draft Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences.
    - Chapter 6: Laboratory Animal Medicine and Toxicology
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

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