National Institute of Environmental Health Sciences

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

March 2023

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National Institute of Environmental Health Sciences
Public Health Service
U.S. Department of Health and Human Services

Research Triangle Park, North Carolina, USA

Foreword

The Division of Translational Toxicology (DTT) at the National Institute of Environmental Health Sciences ([NIEHS], which is part of the National Institutes of Health), offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base in toxicology, and inform decisions by health regulatory and research agencies to safeguard public health. Many of these studies are conducted in support of the interagency National Toxicology Program.

Test agents (e.g., chemicals, physical agents, and mixtures) are selected for study primarily depending on level of human exposure, amount of commercial production, and chemical structure. Many of these studies are conducted in compliance with laboratory health and safety guidelines and the Food and Drug Administration Good Laboratory Practice Regulations and meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

The *Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences* are used to outline expectations and provide guidance for the conduct of toxicology studies by DTT. Since the 1980s, DTT and its predecessor units within NIEHS have provided contract research organizations with specifications documents to inform the conduct of their toxicology and carcinogenicity studies. In 2011, DTT published its specifications for the conduct of developmental and reproductive toxicology studies, and in 2015, released a specifications document on the conduct of neurobehavioral studies. To facilitate use and increase transparency, DTT has now compiled into a single document its specifications for studies that investigate toxicologic, carcinogenic, developmental, reproductive, or neurobehavioral outcomes. This document defines the expectations and requirements (e.g., facility, personnel, quality assurance, and health and safety) for testing facilities and provides instructions on the conduct of specific study elements (e.g., necropsy, tissue trimming). Previously, DTT's specifications documents also included protocol requirements for its studies; however, they are not included here because over time DTT testing strategies have evolved and protocols to study specific agents are now tailored to address the identified data needs and research hypotheses. Research elements such as data analysis and pathology peer review are also not included because these are outside the scope of the specifications document.

For questions about this document, please email wolfe@niehs.nih.gov or call 984-287-3209.

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1. General Personnel Requirements

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This chapter describes requirements for personnel who conduct laboratory work for the Division of Translational Toxicology, National Institute of Environmental Health Sciences (DTT/NIEHS), including contractors and project staff. Although staff might have other responsibilities within the laboratory's organization, the amount of time devoted explicitly to contract/project activities shall be commensurate with the scale of the contract activities. Specific personnel requirements (i.e., personnel required and any additional requirements) will be defined in the contract. An overview of the requirements for key personnel or critical staff is presented in Table 1-1. Table 1-2 summarizes the general responsibilities of professional or technical support staff.

- Selected personnel categories are identified as key personnel, critical staff, or subject matter experts (SMEs). These categories are defined in the contract.
- The specific personnel fulfilling the roles of key personnel and critical staff will be named in the contract. Critical staff not employed by the prime contractor shall be included in the contract for named subcontractors. As appropriate, additional staff in critical roles shall be considered SMEs on a study-by-study basis.
- If a change is required in key personnel or critical staff, either through the employee no longer being employed by the contractor or because the employee is reassigned to another area within the organization, the contractor shall submit a candidate for replacement to the contracting officer’s representative for approval.
- For key personnel, advance notice of least 30 days is needed before diverting the individual to other programs or contracts (or as soon as feasible, if an individual must be replaced, for example, due to leaving the employ of the contractor). The contractor shall notify the contracting officer in writing and shall submit comprehensive justification for the diversion or replacement request to permit evaluation by the government of the effect on performance under this contract.
- SMEs shall be identified as needed for specific studies.
- Certain professional personnel are required to be employed by the contractor and cannot be consultants or subcontractors (see Table 1-1). The rationale for this requirement is that daily interaction and constant coordination are needed among these personnel throughout the execution phase of studies. This coordination makes it critical that personnel be physically and organizationally in the same location.
- For education/experience requirements, unless otherwise specified, the following combinations are considered equivalent. Table 1-1 expresses the preferred qualifications for a given personnel role; however, a suitable equivalent may be proposed by the contractor as needed.

  - Doctorate (Ph.D.) and 3 years of relevant experience, either through a postdoctoral program or work experience
  - Master's degree and 7 years of relevant experience
  - Bachelor's degree and 10 years of relevant experience
- Relevant discipline areas in the biological/physical sciences include degrees or significant relevant coursework, or both, in: toxicology, pathology, veterinary medicine, biochemistry, chemistry, pharmacology, anatomy, physiology, biology, biochemistry, embryology/developmental biology/endocrinology, neurotoxicology, psychology, and related areas.
- Relevant experience includes performance of a specific role(s), or that which prepares the individual to serve in a specific role(s) (e.g., experience as a study director prepares an individual to serve as a toxicology discipline leader).
- For personnel who have significant experience in a discipline area (e.g., a Ph.D. in an unrelated discipline +3 years of experience or no degree with >10 years of experience), the experience can serve as a substitute for the education requirements.

Table 1-1. Overview of Personnel Requirements for Professional Key Personnel/Critical Staff

Professional Staff	Responsibilities	Personnel Category[a]	Employee of Contractor	Education/Training/ Certification Requirements	Experience
Principal Investigator	Daily management of and point of contact for the contract; up-to-date knowledge of all aspects of the program/contracts to include study activities, cost, and schedule	Key	Required	Doctorate/10 years of experience in the biological/physical sciences or equivalent	Management of large-scale multidisciplinary programs, preferably rodent toxicology programs; demonstrated capability for oral and written communication (e.g., journal publications or lab reports)
General Toxicology Discipline Leader	Establish toxicology scientific guidelines and procedures; train staff; supervise, technically direct, and/or consult with appropriate professional and technical staff on studies/evaluations	Critical	Required	Doctorate/3 years of experience in the biological/physical sciences or equivalent; board certification (DABT) is preferred	Experience in the conduct of discipline-specific in vivo rodent toxicology studies; written communication (e.g., journal publications or lab reports)
Laboratory Animal Medicine Discipline Leader	Closely monitor the health of experimental animals; establish LAM-specific scientific guidelines and procedures; train staff; supervise, technically direct, and/or consult with appropriate professional and technical staff (including other LAM veterinarians as applicable) on studies/evaluations involving issues related to animal care and medicine	Critical	Required	Doctorate (e.g., D.V.M.) from a veterinary medicine program recognized by the AVMA; diplomate (active or eligible) of the ACLAM is preferred	Experience in laboratory animal medicine and in managing large colonies of laboratory animals, in particular, rodents as part of multidisciplinary toxicology studies; if studies with dams/litters required, experience with breeding, pregnant and lactating animals, and fetuses/neonates/pups
Health and Safety Discipline Leader	Monitor worker health and safety conditions; has authority to bring unsafe conditions to the attention of upper management; reports to someone other than the principal investigator/study direction/conduct	Critical	Required	Bachelor's degree/3 years of experience or master's/1 year of experience in industrial hygiene, safety engineering or a related field; ongoing training at intervals not to exceed 1.5 years	Experience and training in occupational health and safety and hazard control, understanding of industrial hygiene; experience working with management and technical staff in implementing a health and safety program, including identification of problem areas and execution of corrective actions

Professional Staff	Responsibilities	Personnel Category[a]	Employee of Contractor	Education/Training/ Certification Requirements	Experience
Quality Assurance Unit Officer/ Discipline Leader	Monitor each study to assure management that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with applicable FDA GLP regulations (citation) and later interpretations; establish QA-specific guidelines and procedures; QA unit staff training; supervise, technically direct, and/or consult with appropriate professional and technical staff on matters related to QA; acts independently from personnel engaged in the principal investigator/study direction/conduct	Critical	Required	Bachelor's degree/10 years of experience in a biological/physical science or equivalent	Formal training/experience with conducting studies according to GLP regulations; experience to provide understanding of the tasks or reports being inspected or audited (e.g., chemistry, inhalation exposure, animal care, toxicology, pathology)
Inhalation Exposure Discipline Leader	Establish inhalation exposure-specific engineering and scientific guidelines and procedures; train staff; supervise, technically direct, and/or consult with appropriate professional and technical staff on the design, development, and fabrication of generation, monitoring, and exposure systems for inhalation studies	Critical	Required	Bachelor's/10 years of experience or master's/7 years of experience or equivalent in engineering or a related physical science or equivalent	Experience in designing and operating generators for maintaining stable inhalation chamber atmospheres for vapors, particulates, and/or liquid aerosols; experience in developing and operating a variety of monitoring systems for determining test article concentration in the test atmosphere; written communication (e.g., journal publications or lab reports)
Chemistry Discipline Leader	Establish chemistry-specific scientific guidelines and procedures; train staff; supervise, technically direct, and/or consult with appropriate professional and technical staff on analytical chemistry, dose formulation preparation/analysis, and (for inhalation studies) test atmospheres	Critical	Required	Master's/7 years of experience or doctorate/3 years of experience or equivalent in chemistry or a related physical science	Experience in dose formulation and analytical chemistry (i.e., identity, purity, and formulation analysis); for inhalation studies, experience in analyses of test agents in atmospheres; written communication (e.g., journal publications or lab reports)
Study Directors	Overall responsibility for the technical conduct of the study, as well as for the interpretation, analysis, documentation, and reporting of results; this shall include the direct observation of activities as required	Subject matter expert	Not required	Doctorate/3 years of experience or in the biological/physical sciences or equivalent; board certification (DABT) is preferred	Formal training/experience with conducting and reporting in vivo studies according to FDA GLP regulations; experience with study designs the individual will be responsible for; written communication (e.g., journal publications or lab reports)

Professional Staff	Responsibilities	Personnel Category[a]	Employee of Contractor	Education/Training/ Certification Requirements	Experience
Anatomic Pathology Discipline Leader	Establish guidelines and procedures for anatomic pathology; train staff; supervise, technically direct, and/or consult with appropriate professional and technical staff on anatomic pathology techniques and evaluations	Critical	Not required	Formal training in a medical specialty (e.g., D.V.M. or M.D.); postdoctoral training and/or board certification in anatomic pathology (DACVP)	Experience in management of laboratory animal rodent pathology, particularly systemic neoplastic/nonneoplastic lesions or those of the reproductive and nervous systems; written communication (e.g., journal publications or lab reports)
Study Pathologists	Responsible for the histopathological evaluation and reporting of a study	Subject matter expert	Not required	Formal training in a medical specialty, (e.g., D.V.M. or M.D.); postdoctoral training and/or board certification in anatomic pathology (DACVP)	Experience in management of laboratory animal rodent pathology projects, particularly systemic neoplastic/nonneoplastic lesions and/or those of the reproductive and nervous systems, as applicable; written communication (e.g., journal publications or lab reports)
Clinical Pathology Discipline Leader	Establish guidelines and procedures for clinical pathology; train staff; supervise, technically direct, and/or consult with appropriate professional and technical staff on clinical pathology techniques and evaluations	Subject matter expert	Not required	Doctorate/3 years of experience or equivalent in the biological sciences; D.V.M. and board certification (ASCP) is preferred	Experience in rodent clinical pathology assays, including hematology, clinical chemistry, urinalysis, and hormone assays
Subject Matter Experts: Inhalation Toxicology, DART, Neurobehavioral, Immunotoxicology, Cardiotoxicology, Others as Applicable	Establish discipline-specific scientific guidelines and procedures; train staff; supervise, technically direct, and/or consult with appropriate professional and technical staff on studies or evaluations involving the specific discipline	Subject matter expert	Not required	Doctorate/3 years of experience in the biological/physical sciences or equivalent; board certification (DABT) is preferred	Experience in the conduct of discipline-specific in vivo rodent toxicological studies; written communication (e.g., journal publications or lab reports)
Subject Matter Experts: Laboratory Animal Veterinarian, Health and Safety Officer, Quality Assurance Officer, Inhalation Exposure, Others as Applicable	For studies conducted at a testing facility other than that of the prime contractor, establish discipline-specific guidelines and procedures; train staff; supervise, technically direct, and/or consult with appropriate professional and technical staff on studies or evaluations involving the specific discipline	Subject matter expert	Not required	Consistent with that described above for the given subject matter	Consistent with that described above for the given subject matter

ACLAM = American College of Laboratory Animal Medicine; AVMA = American Veterinary Medical Association; DACVP = Diplomate of the American College of Veterinary Pathologists; DABT = Diplomate of the American Board of Toxicology; FDA GLP = Food and Drug Administration Good Laboratory Practice; QA = quality assurance.
[a]See text for discussion of key personnel, critical staff, and subject matter experts.

Table 1-2. Overview of Responsibilities for Professional/Technical Support Staff

Professional/Technical Support Staff[a]	Responsibility[b]
Animal Care/Toxicology	Provide animal care/husbandry; administer test articles to rodents (including pregnant dams, neonates/pups, juvenile, and adult animals as required) via required route(s) of exposure; collect in-life data (e.g., body weights, clinical observations, food/water consumption, reproductive evaluations, neurobehavioral evaluations)
Inhalation Exposure	Design, fabricate, develop, and qualify inhalation generation, monitoring, and exposure systems and operate the system during the study
Dose Formulation and Analytical Chemistry	Perform dose formulation, test article identity/purity, and dose formulation analysis (test atmospheres for inhalation studies)
Necropsy and Histology	Conduct dissection and examination of rodents. Specific evaluations will depend on the design of the study and may include reproductive and nervous system examinations; fetal examinations (external, visceral, and skeletal); and assessments of abnormalities/gross lesions; required to be ASCP-registered (HT or MT) technologist to supervise the histology operations
Clinical Pathology	Perform required clinical pathology evaluations
Electronic Data Management (Data Coordinator)	Perform electronic data management activities involving data protocol preparation and data collection, management, reporting, and archiving
Data Management	Perform data management activities, including electronic data capture and archiving aspects
Quality Control	Perform activities to ensure that all information generated by the study conduct staff is accurate, consistent, and complete
Quality Assurance	Perform audits and inspections of study activities

ASCP = American Society for Clinical Pathology; HT = Histotechnician; MT = Medical Technologist.
[a]Professional/technical support staff are not considered key/critical, do not require sponsor approval/notification for reassignment, and are not required to be employed by the contractor.
[b]All technical staff shall have education and training that is appropriate for their responsibilities as deemed by the contractor.

1.1. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapters 1, 2, 3, 4, 11, and 12 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was charged to:

1. Peer review the following chapters 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 1: General Personnel Requirements
    - Chapter 2: Facilities
    - Chapter 3: Health and Safety
    - Chapter 4: Quality Program
    - Chapter 11: Data Collection and Submission

- Chapter 12: Report Formats and Guidance

2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

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2. Facilities

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Facilities to which these specifications apply include those of both the prime contractors and the subcontractors who perform major aspects of the activities required by the contract statement of work.

2.1. Animal Facility Requirements

All facilities for the testing program must be approved by NIEHS and will be evaluated with respect to criteria outlined by the following documents and organizations:

- Guide for the Care and Use of Laboratory Animals[1] (National Research Council) and any other additions and exceptions thereof
- Guidelines for Personal Protective Equipment in Animal Facilities[2] (NIH Office of Animal Care and Use)
- Public Health Service Policy on Humane Care and Use of Laboratory Animals[3] (NIH Office of Laboratory Animal Welfare)

A Public Health Service assurance statement and accreditation of the animal facility by AAALAC International are required in order for a laboratory to conduct in vivo studies.

2.1.1. Entry Requirements

The testing laboratory is responsible for establishing policy and procedures to address entry of approved staff and visitors into the animal facility. However, the following conditions/criteria shall be met:

- Entry shall be prohibited to those individuals who have been in another animal facility within the last 48 hours regardless of the disease status of that facility.
- The animal facilities shall be designed and managed to prevent contamination of animals with pathogenic organisms, contamination of personnel and the environment with test articles, and cross-contamination of animals with other test articles.
- A two-corridor system with intervening animal rooms is the preferred way to fulfill this requirement, assuming that (a) all materials coming in contact with animals are sanitized to a clean state suitable for introduction to the supply (clean) corridor and the animal rooms; (b) after use, these materials are removed from animal rooms by a return (dirty) corridor for disposal, destruction, or reprocessing; (c) the air pressure is

[1]https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf
[2]https://oacu.oir.nih.gov/system/files/media/file/2022-12/d2-Personal_Protective_Equipment.pdf
[3]https://grants.nih.gov/grants/olaw/references/PHSPolicyLabAnimals.pdf

adjusted so that the animal rooms are positive to the return corridor and negative to the clean one. In cases for which animals must be moved from one room to another, such as in multigeneration studies, whole-body inhalation studies, or studies that include neurobehavioral evaluations, precautions must be followed so that the barrier system is not compromised. Animal rooms shall be compatible with direct entry of data into an electronic data capture system.

- If a two-corridor system is not available, detailed measures need to be taken to ensure the health and safety of staff and animals, and these measures shall be reviewed and approved by the contracting officer's representative prior to implementation.

2.1.2. Animal Husbandry

Facilities for sanitization of equipment, including but not limited to cages, racks, inhalation chambers, water bottles, feeders, and environmental enrichment devices, must be available and properly located in relation to the study rooms. Clean, well-ventilated, vermin-free storage space must be provided for clean supplies and equipment, cage filters, and feed/bedding awaiting use. The ability to autoclave feed and bedding is desirable.

2.1.3. Chemistry

Secured, controlled-access storage facilities shall be available for retention/storage of test articles at refrigerated (5°C ± 3°C) or ambient temperature as specified by the protocol. Cold storage at −20°C ± 5°C and −70°C or below must be available for analytical reference standards or biological/biochemical specimens, respectively. Suitable V-blenders with intensifier bars shall be used for diet mixing. The testing laboratory shall contain space and equipment for the performance of selected analytical chemistry activities, including identity and purity evaluations and analysis of dose/exposure formulations or exposure atmospheres. The specific types of equipment required will depend on the test article(s), which can be organic or inorganic. Many evaluations include gas or liquid chromatography, infrared spectrophotometry, UV-visible spectrophotometry, mass spectrometry, nuclear magnetic resonance, and similar procedures.

2.1.4. Pathology

The necropsy facility should be in close proximity to the pathologist(s) office(s). The necropsy facility must be equipped with adequate working surfaces, dissection boards, running water with drains, adequate lighting, ventilation, and exhaust hoods. Necropsy and microscopic photography capabilities are required. Refrigeration shall be available for holding dead animals until necropsy. Dead animals shall not be frozen before necropsy. The histology laboratory should be separated from the necropsy area and equipped with automatic tissue processor(s), microtomes, embedding and staining equipment, and with supplies and appropriate ventilation adequate for the expected volume. Acceptable storage space must be available for storage of residual archival and histological materials that will be retained by the testing laboratory before shipment to the sponsor-designated recipient. These areas must have secured, limited access. Facilities shall be compatible with the direct entry of data into an electronic data capture system.

2.2. Emergency Facility Support

Facilities shall be equipped with a tested back-up power source with automatic change-over equipment that is sufficient to preserve the integrity of the testing experiment. Emergency power must be able to handle those areas critical to the study, including but not limited to animal rooms, inhalation chambers, HVAC, storage freezers/refrigerators, and waste storage. Essential mechanical equipment must be guarded or alarmed. Provisions for prompt maintenance response must be provided. Alternative air handling systems for inhalation studies are required.

2.3. Facility Floor Plans

Floor plans shall document locations where all required study activities will be conducted. Floor plans shall be submitted in an electronic form before the initiation of pre-study and study activities under a contract, every time there are physical changes to the facilities, or if requested by the sponsor.

2.3.1. Types of Floor Plans

Three separate floor plans shall be provided:

(1) The locations of showers, changing areas, and restrooms; quarantine rooms; animal rooms; general storage areas and those for feed and bedding storage; cage and rack washers; and emergency power sources and those areas in which emergency power operates. This floor plan is to indicate traffic flow for personnel, animals, test articles, feed/bedding, supplies, and equipment through the facility.

(2) Room airflow directionality and the location of all safety equipment, including, but not limited to, eyewash stations, safety showers, and fire control equipment.

(3) Ventilation equipment and ductwork, including interior and exterior exhausts. The location of each of the building(s) general air intakes and exhausts and the location of the exhaust for each hood or vented enclosure shall be provided.

2.3.2. Areas to Identify in Floor Plans

- Chemistry: space for test article storage and laboratory space for dose/exposure formulation preparation, test article identity, purity evaluations, analysis of dose/exposure formulations and biological samples analysis, supporting equipment, and exhaust hoods
- Inhalation: laboratory space for the generation and control of vapor and liquid and particulate aerosol atmospheres exposure room(s) for exposure via nose-only and whole-body inhalation
- Clinical pathology: laboratory space for the performance of terminal bleeds and preparation, analysis, and storage of specimens
- Fetal examinations: laboratory space for specimen preparation and storage and the performance of external, visceral, and skeletal exams
- Anatomic pathology: laboratory space for necropsy/gross examinations, histology, and storage of specimens

- Neurobehavioral evaluations: laboratory space for the performance of neurobehavioral tests, including motor activity, motor function, motor-sensory function, and learning and memory
- Additional laboratory spaces: laboratory space for the performance of additional study-related activities, including biochemical, molecular, and in vitro evaluations
- Waste storage: spaces for the storage of hazardous and nonhazardous waste before disposal
- Study data and quality assurance records: secure, limited-access space for the performance of quality assurance audits and storage of quality assurance records, and storage/archival of study data for which Food and Drug Administration Good Laboratory Practices shall be cited

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Peer Reviewer

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3. Health and Safety

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3.1. Administrative Controls

3.1.1. Regulations and Guidelines

The National Institute of Environmental Health Sciences (NIEHS) or its program representatives may inspect, sample, and monitor the laboratory and associated facilities used for its studies at any time to ensure that the requirements and applicable regulations and guidelines (described below) are being followed. Any deviations to these requirements shall be approved by the program.

All work shall conform to applicable local, state, and federal statutes in effect at the time of award and throughout the period of performance. These statutes include the following federal regulations and any updates:

Occupational Safety and Health Administration (OSHA)

- Standards for General Industry, 29 CFR 1910
- Hazard Communication, 29 CFR 1910.1200
- Respiratory Protection, 29 CFR 1910.134
- Occupational Exposure to Hazardous Chemicals in Laboratories, 29 CFR 1910.1450
- Occupational Exposure to Blood-borne Pathogens, 29 CFR 1910.1030
- Formaldehyde, 29 CFR 1910.1048 (applicable to the use of formaldehyde in histology, pathology, and anatomy laboratories)

Department of Justice (DOJ)

- Americans for Disability Act, Accessibility, Design Guidelines, 28 CFR, Title III, Part 36

Environmental Protection Agency (EPA)

- Clean Air Act, 40 CFR 50-80
- Clean Water Act, 40 CFR 100-140 and 400-470
- Resource Conservation and Recovery Act (RCRA) 40 CFR 240-271
- Comprehensive Environmental Response, Compensation and Liability Act (CERCLA, Superfund, SARA), 40 CFR 300

Department of Transportation (DOT)

- General Information, Regulations, and Definitions, 49 CFR 171

- Hazardous Material Table, Special Provisions, Hazardous Materials Communication Requirements and Emergency Response Information Requirements, 49 CFR 172
- Shippers, General Requirements for Shipments and Packaging, 49 CFR 173
- Carriage by Public Highway, 49 CFR 177

Nuclear Regulatory Commission (NRC)

- Standards for Protection against Radiation, 10 CFR 20
- Notices, Instruction, and Reports to Workers; Inspections, 10 CFR 19
- Recommendations described in the most recent version of the National Institutes of Health (NIH) Radiation Safety Guide

Drug Enforcement Administration (DEA)

- Federal Requirements for Controlled Substance, 21 CFR 1300

For contract work involving infectious agents, the Centers for Disease Control guidelines, Biosafety in Microbiological and Biomedical Laboratories (HHS Publication No. (NIH) 93-8395, 2009) and the NIH Guidelines for Research Involving Recombinant DNA Molecules (66 Federal Register 57970, 2001 and updates), shall be followed.

Where not superseded by this document, the American National Standard for Laboratory Ventilation, Z 9.5, published by the American National Standards Institute (ANSI), shall be followed. In many cases, the ANSI Z 9.5 provides requirements and recommendations that describe how to approach defining specific requirements for a given facility or program of work. Thus, the information and requirements presented here may be more specific; however, the overall philosophy presented in the ANSI Z 9.5 shall be applied to the health and safety program of the facility.

Other consensus standards and publications may include: the current edition of the Threshold Limit Values for Chemical Substances and Physical Agents & Biological Exposure Indices published by the American Conference of Governmental Industrial Hygienists (ACGIH), criteria documents for various substances and the recommended exposure limits published by the National Institute for Occupational Safety and Health (NIOSH), and the Workplace Environmental Exposure Levels (WEEL®) published by the American Industrial Hygiene Association. If the acceptable exposure levels conflict with the OSHA permissible exposure limit, the more stringent standard shall be used for workers' protection.

3.1.2. Health and Safety Plan (Chemical Hygiene Plan)

The scope of each health and safety plan shall address the organization's health and safety policies and occupational medical surveillance program, as well as potential chemical, physical, biological, and ergonomic hazards (e.g., acquisition of study materials, storage, and handling through ultimate disposal of contaminated wastes).

No contract laboratory will participate in studies without a health and safety plan that has been approved by the sponsor. An updated plan shall be submitted every 2 years for review. In addition, the contracting officer's representative (COR) shall be informed of any updates to the

plan immediately. If approval of the plan is not granted at the time of award, the laboratory must submit a revised plan for review within 30 days of the receipt of award notification. Revisions to the plan shall be clearly indicated to facilitate reviewer approval.

For all contract laboratories, a chemical hygiene plan as required under the OSHA "Laboratory Standard" may be used in place of a health and safety plan provided it meets or exceeds *all* of the requirements outlined in this chapter.

The health and safety plan shall include procedures for ensuring that subcontractors performing major aspects of work have appropriate health and safety procedures in place to perform required work. These procedures are expected to be commensurate with the nature of the work performed.

Written Policies. In addition to the standard operating procedures (SOPs) outlined below, the health and safety plan shall address (but not be limited to):

- Health and safety responsibilities, policies, and organization
- Record keeping and archiving
- Initial and periodic employee training
- Engineering controls
- Personal and environmental monitoring
- Medical surveillance and biological monitoring
- Respiratory protection program
- Personal protective clothing and equipment
- General housekeeping
- Eating and smoking policies and areas
- Precautionary signs and labels
- Chemical and biological storage
- Fire protection and prevention
- Emergency and evacuation contingencies
- Locations (with schematic diagrams) of fire control equipment, and plumbed eyewash stations and emergency showers
- Laboratory safety inspection
- Waste management and disposal
- Other pertinent personnel, operational, and administrative practices, and engineering controls necessary for the containment and safe handling of chemical, physical, biological, and radiological hazards
- Entry and exit to restricted areas
- Visitors

3.1.3. Standard Operating Procedures

The laboratory shall be required to have written SOPs that have been reviewed and approved by NIEHS for at least the following activities:

- Visitors access to test areas
- Employee training
- Medical surveillance and biological monitoring
- Respiratory protection, mask fit, cleaning/maintenance, and inspection
- Eye and face protection
- Personal protective clothing and equipment
- General housekeeping practices
- Ventilation system maintenance
- Storage, receipt, transport, and shipping of study materials
- Hazardous material handling (e.g., in analytical chemistry labs)
- Dose/exposure formulation preparation (as applicable)
- Entry and exit from the limited access areas (including traffic patterns of formulation preparation facility and room(s) used for housing, handling, or evaluating animals, as applicable)
- Spill cleanup, accident, emergency response and evacuation (including natural disasters), and fires/explosions
- Use of radio-labeled material, infectious agents, or controlled substances (if applicable)
- Hazardous/nonhazardous waste management and disposal

3.1.4. Exposure Evaluation and Control

Permissible Exposure Limits/OSHA-regulated Substances

All laboratories shall ensure that employees' exposures to hazardous substances do not exceed the permissible exposure limits (PELs) specified by OSHA in 29 CFR 1910, subpart Z. In addition, initial monitoring of employees' exposure to any substance regulated by a standard (e.g., 29 CFR 1910.1001-1101), which requires monitoring, shall be conducted if there is reason to believe that exposure levels for that substance routinely exceed the action level (or in the absence of an action level, routinely exceed the PEL). If this initial monitoring reveals that an employee's exposure exceeds the action level or the PEL, the testing laboratory shall comply with the exposure monitoring provisions of the relevant standard.

If a PEL has not yet been established for a study material, alternative acceptable exposure standards (e.g., TLV®, REL, WEEL®) shall be used (refer to Section 3.1.1). In situations for which there is no known exposure standard for a proposed test article, a suitable interim exposure standard that is based on current toxicology and industrial hygiene literature shall be established when feasible.

Formaldehyde Monitoring

Histology, pathology, and anatomy laboratories must comply with the formaldehyde OSHA standard, 29 CFR 1910.1048. The use of formaldehyde in all other laboratories shall be carried out in accordance with the OSHA formaldehyde standard.

Histology, necropsy, tissue storage, and tissue trimming operations shall be conducted in a manner that employs engineering controls to ensure that airborne concentrations of formaldehyde do not exceed 0.75 ppm as an 8-hour time-weighted average (TWA) or 2 ppm as a 15-minute short-term exposure limit (STEL). Monitoring shall be performed to evaluate exposure levels (both TWA and STEL) of workers potentially exposed to formaldehyde hazards. If the results of initial monitoring indicate exposure levels exceeding either 0.5 ppm as an 8-hour TWA (action level) or 2 ppm as a 15-minute STEL, additional monitoring must be performed. If the TWA exceeds the action level, sampling must be repeated every 6 months. If the STEL exceeds 2 ppm, sampling must be repeated annually. The repeat sampling may be discontinued when two consecutive sampling rounds are below the STEL and action level as described in the OSHA formaldehyde regulation, 29 CFR 1910.1048. In addition, the testing laboratory shall adhere to all other provisions of the OSHA formaldehyde standard.

Test Article/Positive Control Monitoring

Exposure monitoring shall be routinely conducted where both test article and controls are handled and when the test article/positive control has an established exposure standard, such as the PEL, TLV®, REL, or WEEL® of 10 ppm or less, or 0.1 mg/m^3 or less. This exposure monitoring shall be performed at least once during initial dose preparation and once during initial dose administration, and at the midpoint of the study (for prechronic studies) or every 6 months (for chronic studies). When there is no known exposure standard for a proposed test article, the testing laboratory shall perform exposure monitoring at the same frequency stated above. Determination of exposure and adoption of controls shall be based on a predetermined interim exposure standard, when feasible.

3.1.5. Occupational Medical Surveillance

An occupational medical surveillance program shall be implemented to cover personnel who will be working with study test articles or animals. The frequency of surveillance as well as the scope of medical examination shall be specified in the laboratory's health and safety plan. Persons who are required to wear respirators must obtain written medical clearance from an occupational health service provider (e.g., an occupational medicine physician, a physician assistant, or nurse practitioner who is supervised by the physician) for use of this equipment.

3.1.6. Injury and Incident Reports

A record shall be kept of all injuries or illnesses, including animal bites. In addition, any record of an OSHA recordable incident shall include a full description of the incident, the test article/positive control involved, the medical attention required, any remedial actions taken, and planned follow-up to minimize the likelihood, or eliminate the potential for, reoccurrence (if pertinent). Copies of such incident reports shall be forwarded to NIEHS. The program COR shall be notified *immediately* if a serious (as defined by OSHA) accident or incident occurs. All occupational injuries and illnesses shall be recorded and reported according to the OSHA recording system.

3.2. Test Article/Positive Control Handling and Safety Policies

3.2.1. Receipt/Handling/Storage

A log shall be maintained that will include the date of test article receipt and a continuous balance of the remaining amount of test article.

Weighing of the test article/positive control shall be done using the smallest quantity needed. An analytical balance shall be used whenever possible to preclude the need for handling large amounts of chemical. At all times, this balance shall be placed in an effective laboratory hood or a vented enclosure exhausted to the outside (see Section 3.3.3). Protocols shall be designed to use the minimum possible quantities of "neat" chemical in preparing solutions.

A nonbreakable, secured secondary container shall be used for transfer of any test article/positive control.

Volatile test articles shall be handled properly (e.g., keeping lids on container when not in use, segregating from unintended contact with heat or high pressure) and stored in an area with adequate ventilation that is directly vented to the outside. All other test articles shall be stored in a secured, designated storage area(s). Flammable liquids must be stored, however, in a nonvented flammable liquid storage cabinet (see Section 3.5.1).

3.2.2. Hazard Communication

Training

Personnel who handle (receive, store, weigh, dilute, transport, package, or administer) hazardous agents shall be provided with written material and trained on the associated hazards of these agents including the contents of the material safety data sheet (MSDS). This training shall be conducted by the health and safety officer (HSO) or a program approved by the HSO and shall be properly documented. Training shall include the recommendations for handling carcinogens and reproductive, developmental, or neurobehavioral toxicants. In addition, training in accordance with the requirements of applicable regulations shall be conducted.

Labeling

Warning signs and labels shall be used wherever test articles are used or stored (e.g., on primary and secondary containers, affixed to entrances to work areas, refrigerators, and on containers holding hazardous waste). These signs and labels shall be conspicuous (especially for containers to minimize handling) and shall indicate the presence of suspected carcinogenic, mutagenic, and other hazards, as required by OSHA.

Health and Safety Documents

The testing laboratory must have health and safety documentation available for each study agent and positive control that includes, but is not limited to, the supplier's MSDS, which includes information on the material's hazards, properties, and appropriate control measures. If a chemical is produced for a user outside of the laboratory, the laboratory is required to develop an MSDS. All employees handling the study material or the positive control, or both, must be trained on the contents of the agent-specific health and safety data document.

The MSDSs shall be accessible at all times at designated locations known by the appropriate employees.

3.3. Engineering Controls

3.3.1. General Facility Requirements

Safety showers, drench hoses, and eyewash stations shall be located throughout the facility as required by local, state, and federal regulations and must be located in close proximity to where potentially hazardous chemicals are stored or used. Only plumbed eyewashes are permitted.

3.3.2. Isolation and Access Restriction

General Requirements

An isolated, posted, restricted access laboratory (or laboratories) separate from other laboratory facilities shall be designated for unpacking, storing, weighing, and diluting of test articles/positive controls and where necropsy, tissue trimming, tissue processing, embedding, microtoming, and staining are performed.

Administration of test articles and positive controls shall be performed in a limited access area that has air supply under negative pressure with respect to connecting laboratories and hallways. This area shall be a separate laboratory from the area described above dedicated to unpacking, storing, weighing, and diluting.

Each laboratory shall have a room inspection program providing monthly checks of the airflow directionality. Relative pressures of laboratory areas shall be checked monthly with smoke tubes to verify that the air flows from relatively clean to relatively dirty areas. Monthly inspections shall be documented.

A record shall be kept of all personnel entering and exiting any limited access area(s).

Barrier Systems

The dose/exposure formulation preparation shall be isolated from general traffic, which can be accomplished by locating this area within the animal facility limited access barrier system, or by establishing a separate limited access area for dose preparation. If the latter approach is used, all areas into which laboratory workers might bring used protective equipment (including gloves, shoes, head covers, and clothing), respirators, or containers of dosed feed or water, etc. shall be behind the barrier. Also, any hallways used by workers for reaching the shower facility shall be considered to be behind the barrier (e.g., limited access area).

Personnel who enter the formulation preparation area, or an area requiring a complete set of clean protective clothing and equipment (e.g., a disposable laboratory suit, safety goggles, disposable gloves with permeation-resistant properties specific to the test article, disposable boots, disposable shoe covers or sneakers or rubber boots, and disposable head covering), must shower out before leaving the barrier facility at the end of the day.

Within the shower facility, the “clean” and “dirty” sides must be physically separated by the shower or by another physical barrier. The facility design and procedures shall be arranged so that it is not necessary to enter the clean side before showering and to prevent returning to the

dirty side after showering (e.g., to store or retrieve items such as shoes, towels, respirators). (See Chapter 2. Facilities.)

Facility Design for Barrier Systems

Air exhausted from formulation preparation areas involving the particulate form of the test materials shall be passed through HEPA filters. If volatile chemicals are handled, charcoal filters shall also be used. These filtration systems shall be periodically monitored and maintained and personnel performing maintenance shall wear the protective clothing described for neat test article handling (see Section 3.4.1).

The relative location of external air intakes and exhausts for both local and general ventilation systems must be arranged to minimize the risk of re-entrainment of exhaust air. Documentation (e.g., schematic diagram) shall be provided that indicate the location of intakes and exhausts, stack height, discharge velocities, and the direction of prevailing winds. The use of weather caps shall be approved by the COR before implementation. No other obstructions shall be in the path of vertical discharge.

Within the barrier facility, walls, floors, and ceilings shall be sealed around all incoming and outgoing pipes, conduits, and other utilities to prevent release of contaminated material to surrounding areas. Animal rooms and formulation rooms shall be constructed of wall, floor, and ceiling materials that form chemical-tight surfaces. Animal room doors shall include windows to permit observation of workers within each room.

3.3.3. Hoods and Vented Enclosures

Where not superseded by requirements in this section, all work shall conform to the current edition of the Laboratory Ventilation Standard, Z 9.5, published jointly by ANSI and the American Industrial Hygiene Association. Effluent exhaust concentrations shall not exceed federal, state, and local air pollution emission requirements.

Operations and Requirements

The following operations, unless otherwise noted below, shall be performed in a laboratory hood or other enclosure:

- All dose preparation operations (e.g., weighing, premix, micro encapsulation, mixing of dosing solutions), as well as diluting or administering (gavage, dermal, intra-peritoneal injection, inhalation chamber administration) of study materials/positive controls
- Test article weighing in laboratories (e.g., analytical laboratories)
- Transfer/filling of dosed-feed containers
- Unpacking, analysis, and other handling operations involving test article/positive control or other hazardous agents
- Necropsy, tissue trimming, tissue processing, and staining
- Handling tissues, fluids, and exhaled air collected from animals for evaluation
- Cage and feed container dumping

- Plastic-backed absorbent matting shall be secured inside of any hood wherever the test articles/positive controls (including dilutions) are being handled. After each working session in the hood, or sooner if there is known contamination, this matting shall be disposed of as hazardous waste.

NOTE: Operations that cannot be performed within a laboratory hood or other enclosure due to the size of the containers or equipment will be conducted using other engineering controls (e.g., local exhaust, enclosed systems), administrative controls (e.g., restricted access during operations), additional personal protective equipment (PPE), or a combination of controls that will provide equivalent protection of employees. The HSO shall make the determination of appropriate controls.

Weighing, Diluting, or Administering Test Articles/Positive Controls

Laboratory hoods for diluting and administering test articles/positive controls (including gavage, dermal, intra-peritoneal injection and dosed-feed hoods) shall provide sufficient contaminant and containment capture velocities (an average air flow velocity of 100 ± 20 fpm at the operating sash height with no individual point <80 fpm or >120 fpm unless it can be demonstrated by testing—e.g., yearly use of smoke candles—that values >120 fpm provide adequate capture and do not cause turbulence). In addition, face velocities of balance enclosures shall be at least 50 fpm.

Biological safety cabinets used for dilution or administration of toxic agents shall recirculate no more than 30% of their air.

Automatic Tissue Processing/Staining

An effective exhausted enclosure or hood for automatic tissue processing or staining machines with exposed solvent systems shall supply sufficient capture velocities (e.g., 50 fpm minimum), as evaluated by a combination of velometer and smoke tube tests. Exhausted enclosures for automatic processors having exposed solvent systems shall be provided with a fire protection system or emergency power backup, or both.

Necropsy, Tissue Trimming, Manual Tissue Processing, and Manual Staining

An effective exhausted enclosure or hood for necropsy, tissue trimming, manual tissue processing, and manual staining as well as for all handling operations involving tissues, fluids, and exhaled air collected from animals considered to be contaminated with test article/positive control shall provide capture velocities of 80 ± 10 fpm (with no individual point <70 fpm or >90 fpm unless it can be demonstrated by testing [e.g., yearly use of smoke candles] that values >90 fpm provide adequate capture and do not cause turbulence).

Venting

Hoods and glove boxes used for weighing, diluting, or administering test articles/positive controls shall be exhausted to the outside.

Effluent exhaust vapor from sample oxidizers and analytical instruments (e.g., gas chromatograph, atomic absorption spectrophotometer) shall be vented to the outside.

Motors for hoods and enclosures exhausted to the outside shall be mounted outside the building such that all ductwork shall be under negative pressure.

Recirculation of air from local exhaust systems into occupied spaces shall not be permitted. The only exception to this requirement will be for dosed-feed container-filling hoods, cage-dumping hoods, or vented enclosures for studies involving nonvolatile, solid test articles. If recirculation is desired in this case, the air discharged from hoods or vented enclosures must be equipped with HEPA filtration to clean air before its discharge to the study room. The HEPA filter shall be disposed of as hazardous waste (see Section 3.7.2).

Monitoring

Exhaust enclosures shall be smoke tested using smoke tubes to demonstrate no leakage of smoke out of the enclosure during normal operating procedures.

All ventilation systems shall be routinely monitored. During chronic studies, laboratory hoods and all other local ventilation enclosures shall be quantitatively monitored on a quarterly basis. For studies of 90 days or fewer duration, each hood or vented enclosure shall be verified within 45 days before the beginning of the study unless monitoring data indicate a different frequency.

The sash height at which the face velocity has been measured shall be marked on each hood along with the date of the last measurement, the measured flow, and name of the person performing the monitoring.

The HSO shall maintain records of ventilation system checks. The records shall indicate for each hood, room, and area, at a minimum, when air was tested, what was found, who conducted the test, and what equipment was used.

3.4. Personal Protection Equipment Selection

3.4.1. Selection

Handling Neat Test Article/Positive Control and Activities in Animal Rooms

Where the neat test article/positive control (as neat material or in formulated doses) is stored and weighed in dose-formulation rooms and in animal-study rooms, or areas into which personnel directly exit when leaving animal study rooms (e.g., dirty side of the barrier), the following minimum personal protective clothing shall be worn at all times:

- Disposable full-body Tyvek® (or equivalent) suit and disposable head covering, unless Tyvek® suit includes a hood
- Gloves: If chemical-specific gloves cannot be identified, two pairs of dissimilar, disposable gloves (e.g., N-Dex® or equivalent, PVC, latex, natural rubber) will be worn when handling test article/positive control (as neat material or in formulated doses); both pairs of the two dissimilar gloves shall be changed after any known chemical contact and after every 2 hours of handling test article/positive controls or dose formulations
- Respirator: Appropriate NIOSH-approved respirators
- Eye protection: Splash-proof safety glasses, goggles, or other eye protection specified by OSHA and ANSI

- Footwear: Disposable shoe covers, disposable boots, or facility-dedicated rubber boots

Operations Not Involving Neat Chemical/Positive Control

For laboratory operations not involving the handling of neat test article/positive control (e.g., chemical analysis, histology, tissue trimming, and necropsy on the clean side of the barrier), the following shall be worn:

- Single pair of disposable gloves
- Laboratory coat
- Splash-proof safety glasses, goggles, or other eye protection specified by OSHA and ANSI

Animal Barrier "Clean" Corridor

All staff entering the clean corridor with the intention of entering animal rooms must follow PPE requirements as defined above for activities in animal rooms.

All staff entering the clean corridor for purposes other than entering animal rooms must wear disposable suits, scrubs, lab coats, or other launderable clothing dedicated to the facility, and disposable head and shoe covers.

3.4.2. Respiratory Protection

Where specific engineering controls (e.g., vented enclosure for test article/positive control weighing) have been demonstrated to be effective in controlling exposure levels, the need for respiratory protection shall be determined by the HSO.

The HSO in accordance with OSHA regulations and NIOSH Respirator Decision Logic recommendations shall select suitable, NIOSH-approved, task-specific respirators. Where air-purifying respirators (APRs) are used (e.g., with gas/vapor and particulate combination cartridges), written provisions shall describe when cartridges are to be changed and the logic used to make this determination. The date and time of installation shall be marked on all cartridges. Where air supplied devices are used, breathing air is to be analyzed periodically to ensure that the quality of air meets human breathable air standards. Personnel who are required to wear respirators shall be medically cleared, trained, and mask-fitted before they are allowed to wear the respirator.

A respirator program that meets the requirements of OSHA 29 CFR 1910.134 shall be implemented for routine and emergency use of respirators.

Any respirator cartridge used during a cleanup of spilled chemical shall be disposed of as hazardous waste.

3.4.3. Usage and Storage Practices

All protective equipment used in a particular laboratory shall be stored in accessible and convenient locations as dictated by the barrier design or procedures.

Disposable protective clothing shall not be worn out of the laboratory/test work area where neat chemical is handled.

Work clothing shall be removed upon exit from the laboratory on a daily basis. Disposable clothing shall not be reused.

Nondisposable items are to be stored in covered containers until washed. If laboratory personnel do the washing, they shall wear gloves and disposable suits while handling contaminated items. If washing is done by an outside service, they shall be notified in writing that they are handling items with potential contamination.

3.5. Fire Safety

NOTE: Fire safety requirements for inhalation studies are described in Section 3.8 below.

The facility and operations shall comply with applicable federal, state, and local fire and building codes.

3.5.1. Storage and Handling

Flammable liquids shall be stored and handled in a manner that will reduce the risk of fire and explosion. This conduct includes:

- All nonworking quantities of flammable liquids shall be stored in storage cabinets approved by Underwriters Laboratories or Factory Mutual, or in a designated flammable liquids storage room with suitable fire protection, ventilation, spill containment trays, and with equipment meeting the requirements of OSHA. In either storage arrangement, the flammable liquids shall be segregated from other hazardous materials such as acids, bases, oxidizers, etc.
- Flammable storage cabinets shall not be vented unless required by a chemical-specific OSHA regulation or by local authorities. Metal bung caps shall be used in place of flash arrestor screens. If it is necessary that venting be provided, the following instructions shall be followed: (1) Remove both metal bungs and replace with flash arrestor screens. The top opening shall serve as the fresh air inlet. (2) Connect the bottom opening to an exhaust fan by a substantial metal tubing having an inside diameter no smaller than the vent. The tubing shall be rigid steel. (3) Ensure that the fan has a nonsparking fan blade and nonsparking shroud. It shall exhaust directly to the outside where possible. (4) The total run of exhaust duct shall not exceed 25 feet.
- Class I flammable liquids shall not be stored in conventional refrigerators/freezers. If flammable liquids must be kept at low temperatures, they shall be stored in Underwriters Laboratory (UL)-listed/Factory Mutual (FM) Global-approved refrigerators/freezers designed for flammable storage. In a potentially flammable or explosive atmospheric environment, only those explosion-proof refrigerators/freezers listed for Class I. Division 1, Group C and D, and listed by UL as a “Special Purpose Refrigerator and/or Freezer” shall be used. All explosion-proof refrigerators shall be labeled as such.

- Whenever flammable liquids are stored or handled, ignition sources shall be eliminated. Smoking is prohibited.
- Flammable liquid transfer shall be done in the designated storage room or over a tray within an effective laboratory hood. In the former location, all transfer drums shall be grounded and bonded and shall be equipped with pressure relief devices and dead man valves.
- Safety cans shall be used when handling small (e.g., no more than 2 gallons) quantities of flammable liquids, unless chemical purity requirements require otherwise (e.g., distilled-in-glass grade).

3.5.2. Fire Safety Equipment

Fire extinguishers: Fire extinguishers shall be conspicuously located where they will be readily accessible and immediately available in the event of fire as required by local, state, and federal regulations. Placement of portable fire extinguishers shall conform to OSHA 1910.157. The specific type and size of extinguisher shall be selected with consideration for the hazards to be protected and the strength of the personnel who might use the extinguishers. For most laboratory applications, water and aqueous film forming foam (AFFF) extinguishers shall have a capacity of 2.5 gallons. Dry chemical, carbon dioxide, and foam extinguishes shall have 20–30-pound capacity.

Safety showers: Safety showers shall be located in the immediate vicinity of every laboratory where flammable liquids are stored/used. Fire blankets may be used if available.

3.5.3. Training

All personnel shall receive training in fire safety. Course material shall include hazard awareness, proper techniques for the handling and storage of flammable liquids, and a briefing on the alarm system and emergency evacuation preplanning. In addition, “hands-on” training for appropriate personnel on fire extinguishers is encouraged.

3.6. Emergency Procedures

The written set of general safety policies shall include actions to be taken in case of fire or explosion. They will address personnel assignments, evacuation routes, and notification procedures. The National Fire Protection Association Life Safety Code, Number 101, and existing manual pull-box locations shall be considered when establishing means of egress.

A written set of emergency/evacuation procedures to be followed by all project personnel in the event of a spill or leak involving the test article/positive control shall be developed and posted in each laboratory. Personnel shall be instructed to call for appropriate help (e.g., inhouse emergency group or poison control center) in case of an emergency. This plan shall address the storage, use, and maintenance of emergency protective equipment.

The location and phone number of the nearest poison control center and any other emergency phone numbers shall be prominently posted in each laboratory.

Emergency protective equipment shall not be stored in the laboratory where test articles are stored and handled.

3.7. Waste Disposal/Test Article Shipment

3.7.1. Disposition/Shipment of Surplus/Residual Test Article

The practices described below shall be adhered to concerning the disposition of surplus/residual test article.

Thirty days before shipment, the testing laboratory shall notify the program COR of its intention to ship surplus or residual test article, including the amount to be shipped, and complete details of the shipping procedures, including the contractor that will be used.

Upon completion of testing and after receiving approval from the program COR, the testing laboratory shall immediately ship excess quantities of test article after the final bulk chemical analysis has been completed. In addition, a 100-gram aliquot of each batch of the test article is to be reserved and shipped separately to the sponsor-designated chemistry support contractor after the final bulk chemical analysis has been completed. For reactive chemicals gases, or other test articles that may be difficult to store long term, the program COR shall be contacted to determine if any test article is to be reserved and shipped.

The following requirements for packaging these test articles are made to minimize the possibility of exposure to personnel involved in the packaging, transportation, and receipt of these test articles. The requirements shall be consistent with the Department of Transportation (DOT) regulations (or International Air Transport Association [IATA] regulation for contractors outside the USA) as outlined in 49 CFR, parts 100 to 199.

Test articles shall be shipped in primary containers compatible with the physical and chemical properties of the substances that prevent contamination of the study material. Each primary container must be securely sealed to prevent leakage during transport. After being sealed, the exteriors of each primary container must be decontaminated and labeled with all pertinent information (including chemical name, lot number, amount, date, and source). Test articles that are gases or liquefied gases in cylinders shall be shipped without additional packing and according to appropriate transportation procedures.

All primary containers shall be sealed in double plastic bags to prevent leakage and exposure if broken, surrounded by absorbent material, and placed in secondary containers. Larger amounts of liquids may be shipped in 5-gallon metal drums, which shall be individually packaged and which shall meet all DOT regulations. These 5-gallon drums must be overpacked in larger drums with absorbent material, securely sealed, and fully labeled. All overpacked drums shall be fully filled, securely sealed, and completely labeled on the outside.

Outside containers must be free from extraneous and ambiguous labels. Labeling must include a directional label to indicate the top of the container, appropriate warning labels (e.g., SUSPECT CANCER AGENT, FLAMMABLE), and all required DOT labels and identification. All shipments shall be made in compliance with DOT regulations (or IATA regulations where applicable) and accompanied by a completed Shipper Certification Form for Hazardous Materials. A detailed packaging list must be placed on the outside of the shipping container identifying each chemical fully by name, amounts shipped, and lot numbers of each chemical. The sponsor shall be consulted if the quantity or type of substance to be shipped renders these requirements inappropriate.

3.7.2. Potentially Contaminated Material

All potentially contaminated material (e.g., dose formulations, bedding, used personal protective clothing and equipment, absorbent materials for handling test materials, disposable cages, lab ware, filters, respirator cartridges) shall be incinerated or disposed of in a licensed hazardous waste landfill, in a manner consistent with federal, state, and local regulations. Animal carcasses, blood samples, animal tissues, or any other materials that are grossly contaminated with blood, including sharps and syringes, shall be collected and disposed of by incineration. The laboratory shall indicate whether it plans to fulfill this requirement with its own incinerator, or by use of a licensed waste disposal firm. If the laboratory's incinerator is to be used, specifications (e.g., temperatures and residence times), operating procedures, and information on licensing by local regulatory authorities shall be provided for evaluation. If a contract disposer is to be used, complete information on the firm’s licensing and hazardous waste transporter shall be provided.

Computer terminals used to enter laboratory animal data into an electronic data capture system shall be decontaminated after each use and when removed from an animal room, using a chemical-specific solution. Terminals must be disconnected from any electrical power sources before decontamination, and care will be taken to ensure that any solvents used do not damage the plastic parts of the computer terminal.

Vacuum lines, including water aspirators, used when working with test article/positive control shall be protected with an absorbent or liquid trap and a HEPA filter.

3.8. Inhalation Studies

The following requirements apply to inhalation studies and supplement the requirements described in other parts of this chapter.

3.8.1. General Requirements

The test atmosphere generation apparatus and flow meters through which test atmospheres pass shall be contained in enclosures exhausted to the outside (see Section 3.3.3). All connections in the piping and ducting between the test atmosphere generator and the exhaust air filters shall be either compression-fitted, threaded, welded, or enclosed and vented and leak-tested before use. All equipment through which test article flows shall be electrically grounded and bonded according to the provisions of the National Electrical Code and the National Fire Protection Association Standard 77, “Recommended Practice on Static Electricity.” Use of plastics, such as PVC, is not permitted. All piping, ducting, and other materials must be compatible with the test article.

A full description of all safeguards, safety procedures, alarms, shutdowns, emergency plans, cleanup procedures and disposal methods must be reported to the program COR and be in place before the start of all studies.

At least one sampling port connected to the test article concentration monitoring system shall be located in each animal room involved in the study. Test article monitoring strategy must be submitted and be based on the physical properties of the test article for the exposure room. Exposure rooms do not have to be monitored during nonexposure periods.

All exhaust air from the inhalation chamber must be cleaned with HEPA or charcoal filters (depending on physical form) or other air cleaning devices (e.g., scrubbers, incinerators, electrostatic precipitators), unless the laboratory provides written documentation that local and state air pollution regulatory agencies have been informed of both the laboratory's operating practices and the potential hazards of the test articles in use. Compliance with all federal, state, and local air pollution laws and regulations is required.

Trained inhouse staff or emergency response HAZMAT personnel shall don appropriate self-contained breathing apparatus if emergency entry into a study room following a leak is required. If available inhouse, these units shall be maintained and inspected as required under 29 CFR 1910.134 of the OSHA respiratory protection standard.

The personal protection requirements for inhalation studies as specified in Section 3.4. shall apply except as follows:

When the test article is a gas or vapor and the ambient sampling port indicates that the air in the study room is not contaminated, personnel entering the study room need not wear respirators and disposable overgarments. However, when the exposure chambers are open, personnel entering the exposure rooms must wear appropriate respirators, gloves, eye protection, disposable overgarments, and head and foot coverings.

When the test article is a particulate, personnel transporting animals and necropsy personnel shall wear the same APRs equipped with P-100 filter cartridges and disposable overgarments, which are required at all times in the exposure facility unless all animals are bagged or otherwise enclosed and the containers are only opened under a vented enclosure. Necropsy shall be performed in an enclosure vented to the outside.

3.8.2. Combustible/Flammable Test Articles

When a test article is flammable or explosive, NIEHS requires that the test system minimize the probability of, and the consequences associated with, fire or explosion. The laboratory shall provide data on its test system that includes equipment and techniques for reducing the fire or explosion hazard. As NIEHS recognizes that there could be alternative approaches for minimizing the risks due to fire and explosion, the program COR may grant approval to test system configurations that differ from the following provisions on a site-specific basis.

If the exposure concentration is below 25% of the lower flammable limit or the minimum explosive concentration, the following provisions shall be made:

- Flow monitoring equipment shall be used to determine variations in the flows of the test article and carrier air. In the event that there is a 10% change in flow, system shutdown shall occur with an audible alarm signaling such action at a manned location.
- The instrumentation used to continuously monitor the inhalation chamber and chamber room for the test article shall be equipped with an audible alarm that signals a manned location when a concentration equal to 25% of the lower flammable limit or the minimum explosive concentration is detected.

- A flame arrestor shall be installed on the gas or vapor supply line. If the test article is a combustible dust, an optical flame detector must be located in the supply line and connected to trip a fast acting shut-off valve upstream.
- An alarm shall be in place to indicate when the air flow through the vented enclosure, which surrounds the test article generation devices(s), falls below 85% of its nominal value.
- All equipment through which the test article flows shall be electrically grounded and bonded according to the provisions of the National Electrical Code and the National Fire Protection Association Standard 77, "Recommended Practice on Static Electricity."

If the exposure concentration is ≥25% of the lower flammable limit or the minimum explosive concentration, the following provisions shall be made in addition to those stated above:

- The inhalation chamber study room shall be isolated from the other operations by walls with a fire resistance of 1 hour such that it is solely dedicated to the testing of the flammable or explosive study material.
- Explosion venting shall be installed on each inhalation chamber. The recommended vent ratio is 1 ft^2/10 ft^3.
- All electrical equipment shall be suitable for a Class I, Division II (flammable gas or vapor) or Class II, Division II (combustible dust) location as defined by the National Electrical Code.

If the exposure concentration is ≥100% of the lower flammable limit or the minimum explosive concentration, the following provisions shall be made in addition to those stated above:

- The inhalation chamber study room shall be located such that one wall of the room is common to an area outside of the building that is typically unoccupied. Explosion venting shall be installed in that wall. National Fire Protection Association 68, Guide for Explosion Venting, shall be used for reference when designing the installation.

3.8.3. Reporting Requirements for Inhalation Studies

The following information shall be included in the prestart inhalation report:

Effluent Exhaust Monitoring

- Description of the method(s) to be used for effluent exhaust treatment during generation runs at the protocol-required concentrations, in all chambers under actual animal exposure conditions.
- Data demonstrating the effectiveness of the effluent exhaust treatment unit immediately after the effluent treatment unit or at the point of exhaust from the building.
- Percent efficiency of the exhaust treatment—the effluent exhaust treatment must be effective in removing the test article to an acceptable concentration (e.g., >90% efficiency of removal by the treatment system and <50% of the TLV, if a TLV exists), or written documentation for a waiver from appropriate air regulatory agencies must be provided.

- Determination of the lifetime expectancy of any proposed filtration/treatment units and the amount of treatment media that will be required.
- Confirmation that none of the exhausted test article is re-entrained.

Room Air Monitoring

- Description of the test method(s) for room air monitoring during generation runs at protocol-required concentrations in all chambers under actual animal exposure conditions.
- Definition of the lower limit of detection of the monitoring method(s).
- Documentation that this level provides an adequate safety margin for personnel.

Special Requirements for Aerosol Studies

When a test article is an aerosol (e.g., particulate or liquid), NIEHS requires that the test system minimize the probability of, and the consequences associated with, fire or explosion. The laboratory shall provide data on its test system that includes equipment and techniques for reducing the fire or explosion hazard. The laboratory shall also provide complete information on the test article that includes determination of the following: the minimum explosion concentration, minimum spark ignition energy, explosion severity, minimum ignition temperature of a layer, and the volume resistivity. As NIEHS recognizes that there may be alternative approaches for minimizing the risks due to fire and explosion, the program COR may grant approval to test system configurations that differ from the following provisions on a site-specific basis.

Demonstration of containment of particulate study material during generation of protocol-required concentrations in all chambers under actual animal exposure conditions, description of the monitoring strategy including methodology, frequency of sampling, and results are to be provided.

Standard Operating Procedures

All SOPs that are specific to the study are to be attached to the prestart inhalation report.

3.9. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapters 1, 2, 3, 4, 11, and 12 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was charged to:

1. Peer review the following chapters 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 1: General Personnel Requirements
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    - Chapter 3: Health and Safety

- Chapter 4: Quality Program
- Chapter 11: Data Collection and Submission
- Chapter 12: Report Formats and Guidance

2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

Peer Reviewer

Catherine Spong, M.D.
Professor and Chair, Department of Obstetrics and Gynecology
Chief of Maternal Fetal Medicine
UT-Southwestern Medical Center
Dallas, Texas, USA

4. Quality Program

M.D. Stout[1], G.K. Roberts[1] (Editors)

[1]Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Last Updated: March 2023

4.1. Quality Management Plan

The contractor shall establish and maintain a quality management plan (QMP). A QMP governs quality aspects of all work performed. The QMP shall follow the requirements set forth in the U.S. Environmental Protection Agency (EPA) Requirements for Quality Management Plans (EPA QA/R-2).[1] QMPs shall be submitted before performance of work under the contract and updated versions submitted at regular intervals (e.g., biannually), or if the plan has been updated substantively.

4.2. Quality Assurance Unit

The contractor shall establish an effective and independent quality assurance unit (QAU).

The purpose of quality assurance (QA) is to provide assurance that studies are conducted in compliance with Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations. National Institute of Environmental Health Sciences (NIEHS) toxicology studies shall be conducted in compliance with FDA GLP regulations as specified in Part 58 of "Good Laboratory Practices for Nonclinical Laboratory Studies" (21 CFR Part 58[2]), in addition to these DTT specifications, unless otherwise specified in the work assignment and the resulting laboratory study protocol. On occasion, NIEHS might require that a study be conducted in compliance with EPA regulations ("Toxic Substances Control; Good Laboratory Practice Standards; Final Rule," Federal Register, Tuesday, November 29, 1983, Part III) in addition to FDA GLP regulations, or, conversely, not in compliance with either FDA or EPA regulations (i.e., non-GLP). The quality standard for a particular project will be clearly communicated in writing upon assignment of the project. For non-GLP studies, all quality aspects of the studies shall occur with the exception of those specifically related to the QAU.

For testing facilities that conduct studies in compliance with FDA GLP regulations, the facility shall have a QAU, which shall be responsible for monitoring each study to provide assurance that the facilities, equipment, personnel, methods, practices, records, and controls are in compliance with the regulations. For any given study, the QAU shall be entirely separate from and independent of the personnel engaged in the direction and conduct of that study (FDA GLP 58.35).

The conduct of QA audits and inspections is the responsibility of the testing facility/test site laboratory QAU. Aspects of the studies are to be inspected and resulting data audited according to the frequency of the data collected, the extent of quality control (QC) review, and the potential

[1]https://www.epa.gov/quality/epa-qar-2-epa-requirements-quality-management-plans
[2]https://www.ecfr.gov/current/title-21/chapter-I/subchapter-A/part-58

relevance of errors. These factors and the monitoring frequency are to be considered and established in concert with the study director/test site principal investigator (PI), discipline leaders, contract PI, and the laboratory's management. The schedule of inspections and audits reflects a dynamic process and will be influenced by a variety of factors such as previous findings, follow-up activities, workload and quality issues expressed by management, and inspectional findings from outside reviewers.

Audits and inspections by the QAU shall encompass all aspects of the studies performed under the purview of the laboratory's QAU, from prestart chemistry and inhalation through the final report and archiving of data/specimens (where applicable). The study protocol and amendments shall be reviewed by the testing facility's QAU for compliance with GLP regulations, with signed and dated documentation of this review before the initiation of the study, or before the amended change effective date. Those phases of a study that occur only once and include procedures or conditions that can be directly observed shall be inspected at the time they occur and resulting data audited. If a once-only phase undergoes a formal and documented QC review by technical staff, the QAU might limit its responsibility to inspecting the procedures of the QC review for that phase. Repetitive or routine procedures that affect the generation, collection, and handling of study data need to be subjected to inspection and audit on a periodic basis for each study. All data and statements of fact included in study reports submitted shall be audited by the testing facility's QAU. In addition, the QAU shall prepare and sign a statement that will be included with the final study report that specifies dates of inspections and dates of reports provided to management, the contract PI, and the study director (FDA GLPs). If a laboratory or testing facility has one or more test sites, each test site QAU shall be responsible for the critical phase inspections and data and report audits occurring at its site and shall prepare and sign the QAU statement. Ultimately, it is the study director's responsibility to determine the overall effect of the audit findings on the study.

4.3. Quality Control

The contractor shall establish policies and procedures that ensure QC at all levels, including development of a QC program. The purpose of QC is to ensure that all information generated by the study-conduct staff (e.g., data, records, and reports) are accurate, consistent, and complete. The laboratory shall have a robust and comprehensive QC program in place to accomplish this. This program shall be in operation for all projects, regardless of whether they are to be performed under FDA GLP regulations. This program shall be separate from and subject to periodic assessment by the QAU. Ensuring that the procedures, studies, data, and reports reflect contractual standards, including these specifications, is a QC function and shall be the responsibility of study management.

NIEHS's independent quality assessment support contractor performs retrospective assessments of NIEHS studies, data, and reports. The project officer will provide the results of the assessments to the study laboratory. It is expected that the study laboratory will review these reports and, if required, use the findings to inform improvements to the quality program. On occasion, NIEHS might determine that a finding(s) is sufficient to require a revision to a report. In this case, the study laboratory shall submit a revised report and respond to the assessment.

4.4. On-site Inspection

At its discretion, NIEHS may perform on-site inspection with prior notification, by the contracting officer's representative (COR) or his/her designee, of equipment, facilities, records, and procedures, including those of the QAU periodic site visits to the laboratory. These visits may include technical discussions, reviews of schedules, audits and inspections of studies, procedures, data, records, and reports for projects in progress. In addition, the visits may include evaluation of the organization and function of the laboratory's QAU, with evaluations to include the organization and function of the laboratory's QAU as well as audit/inspection of various aspects of ongoing studies.

The laboratory shall maintain a file of QAU reports (inspections, audits, master schedule entries) and responses to them in connection with NIEHS studies. As the sponsor for the studies, NIEHS management, QA personnel, and the program COR shall have access to the file for review purposes. The confidential, proprietary, and predecisional information contained in this file shall not be divulged by NIEHS reviewers. The file shall not be revealed to any outside parties, including QA support contractors for the NIEHS or GLP compliance inspectors for the FDA or EPA. When a study is completed, the QAU reports file shall not be part of the study record and shall not be submitted to the NTP Archives.

4.5. Standard Operating Procedures

The contractor shall develop and maintain standard operating procedures (SOPs) for all contract operations, procedures, assignments, and tasks that describe how each activity is to be performed. In addition to the study protocol, laboratory SOPs are considered essential to the successful conduct, documentation, inspection, and auditing of a study. The review and revision of existing SOPs, the creation of new SOPs, or the retirement of outdated SOPs shall be a continuing process. For this reason, all new SOPs shall be reviewed by the laboratory's QAU at a frequency documented in the laboratory's QMP (e.g., biannually). New or revised SOPs shall be prepared, reviewed, and approved by appropriate study facility personnel, including the QAU, before implementation. The decision to develop and implement program-specific SOPs shall be that of study laboratory management. All SOPs used in connection with each study shall be maintained as records in the study file and submitted to the NTP Archives. These SOPs are subject to review by NIEHS personnel upon request, as they document how work is performed.

4.6. Subcontractors

The contractor shall establish procedures to ensure that work performed by subcontractors meets the same quality requirements as work performed by the prime contractor. Testing facility management, the contract PI, and the study director shall assume responsibility for all the work assigned to the testing facility. For multisite studies, in which aspects of the studies conducted under GLPs are delegated to independent subcontractors (test sites), the delegated phases of the study performed by an independent subcontractor are audited by the test site QAU according to its SOPs. Before the initiation of this subcontract work, the testing facility's QAU shall assess the subcontractor's quality system. The appropriate staff, including discipline leaders or the study director, the contract PI, and testing facility management, as appropriate, shall review data and reports submitted by a subcontractor and determine their acceptability.

4.7. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapters 1, 2, 3, 4, 11, and 12 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was charged to:

1. Peer review the following chapters within the draft 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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    - Chapter 11: Data Collection and Submission
    - Chapter 12: Report Formats and Guidance
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

Peer Reviewer

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5. Chemistry

S. Waidyanatha[1], B.J. Collins[1], M.D. Stout[1] (Editors)

[1]Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Last Updated: March 2023

5.1. General Requirements

- The National Institute of Environmental Health Sciences (NIEHS) will typically supply the test article.
- NIEHS will typically provide procedures for test article purity analysis, dose formulation preparation, and dose formulation analysis. Modest modifications may be made to suit existing instrumentation per NIEHS approval.
- For inhalation studies, the development of methods for generation, monitoring, and characterization of the chamber atmosphere shall be the responsibility of the testing laboratory.
- In some cases, the testing laboratory may be required to conduct one or more of the following activities or part of an activity per direction by the contracting officer's representative (COR), including but not limited to procuring and handling the test article, characterizing the test article, including bulk stability; developing a formulation method and developing and/or validating analysis methods; developing and validating biological sample analysis methods; and conducting toxicokinetic studies.
- Toxicokinetic study may be conducted as a part of the toxicology study or as a separate study. The testing laboratory's responsibility is typically limited only to the collection and shipment of biological matrices to a designated analytical chemistry contractor. However, in some cases the testing laboratory shall be required to develop and/or validate analysis methods for quantifying the analyte concentration in biological matrices, analyze the samples, model the data, and provide an interpretation of the results.
- Standard operating procedures (SOPs) shall be prepared for performance of all chemistry activities, including but are not limited to purity analyses, dose formulation preparation, dose formulation analysis, inhalation technology operations, biological sample method development, validation and/or analysis, and toxicokinetic studies.
- The results of all analyses shall be reported to three significant figures unless directed otherwise by the COR.
- The contractor shall refer to DTT Chemistry Specifications[1] for details when applicable.

[1]https://ntp.niehs.nih.gov/howwework/research/chemistry/index.html

5.2. Bulk Test Article

5.2.1. Receipt, Handling, and Storage

The test article(s) and reference standard(s), as well as corresponding storage conditions, will be supplied by NIEHS. Whenever feasible, a sufficient quantity of the test article will be provided so that only one lot of the material will be needed to complete all of the contracted study phases. Testing laboratories shall plan to have adequate storage at the specified condition(s) for bulk test article(s). Upon receipt, the amount received shall be document in a use log. A use log of the bulk test article shall be kept and submitted as part of the raw data at the end of the study.

Upon receipt of each test article, the testing laboratory shall remove samples (typically 15 × 1 gram) to be used as reference samples. The COR will notify the testing laboratory when a smaller amount shall be archived (e.g., in the case of limited test material availability). All reference samples shall be placed in a freezer and maintained at −20°C ± 5°C. In some cases, the storage temperatures of the reference sample may be different depending on the nature of the test article.

The bulk test article shall be stored per directions provided by NIEHS. If appropriate storage conditions for the bulk test article are not provided by NIEHS, it may be possible to use the manufacturer's data to establish the stability and thus the storage conditions for the bulk test article without the need for additional studies by the testing laboratory. If stability studies of the bulk test article are required by the COR, refer to the DTT Chemistry Specifications for details.

5.2.2. Initial Identity and Purity

Methods to confirm the initial identity and purity of the bulk test article upon receipt at the testing laboratory shall be provided by NIEHS and will generally involve up to two identity analyses and one purity determination.

The testing laboratory shall confirm the identity and purity of the test article upon receipt using the method provided by NIEHS. The testing laboratory shall remove one 5-g aliquot of the test article and analyze it with the NIEHS-supplied frozen reference standard. The purity of the bulk test article shall be determined relative to the frozen reference standard.

5.2.3. Chemical Reanalysis

The testing laboratory shall determine the relative purity of the bulk test article against a frozen reference sample taken at the time of receipt. For all studies, this shall be conducted within 30 days prior to start of the study. For studies with an exposure duration >30 days, relative purity of the bulk test article shall be repeated within 30 days after removal of the last animal. For studies with an exposure duration >6 months, relative purity shall also be conducted at 24 ± 2-week intervals throughout the duration of the study. Each frozen reference sample shall only be used for one purity analysis.

The principal investigator shall immediately notify the program COR via telephone or email and in the next monthly progress report of any significant change in purity (e.g., a difference not explained by variability within the analytical procedure used) or appearance of the test article during the study.

5.3. Noninhalation Studies

A summary of the required activities for noninhalation is provided in Table 5-1. Additional information for each activity is provided in the following sections. In some cases, it will be appropriate to refer to the DTT Chemistry Specifications.

Table 5-1. Summary of Chemistry Activities for Noninhalation Studies[a]

Category	Type	Anticipated Frequency	Reporting Requirement
Initial Purity[b]	Identity and purity	Once upon receipt	Prestart chemistry report
	Stability study	Not always required; to be conducted at the direction of the COR	Prestart chemistry report
Chemical Reanalysis	Purity analysis	Within 30 days prior to exposure for all studies; within 30 days of end of exposure for studies with exposure durations longer than 30 days; every 24 ± 2 weeks for studies with durations longer than 6 months	Study report
Vehicle Analyses[b]	Identity and purity analyses	Once upon receipt	Prestart chemistry report
	Purity analysis	Every 24 ± 2 weeks	Study report
	Corn oil analysis, peroxide level determination	Bimonthly	Study report
Method Performance Evaluation for Dose Formulation Analysis[b]	Dose analysis	Once[c]	Prestart chemistry report
Dose Formulation Analyses (Exposure Duration <30 days)	Preadministration dose analyses	Once, prior to study initiation, all batches and each dose	Study report
	Animal room dose analyses	Once, after dose administration, each dose for each sex/species	Study report
	Homogeneity study[d]	Once, prior to study initiation	Prestart chemistry report
Dose Formulation Analyses (Exposure Duration >30 and <90 days)	Preadministration dose analyses	Initial, middle, and final; all batches and each dose for each sex/species	Study report
	Animal room dose analyses	Initial, middle, and final; each dose for each sex/species	Study report
	Homogeneity study[d]	Once, prior to study initiation	Prestart chemistry report
Dose Formulation Analyses (Exposure Duration >90 days)	Preadministration dose analyses	Initial and every 10 ± 2 weeks; all batches and each dose	Study report

Category	Type	Anticipated Frequency	Reporting Requirement
	Animal room dose analyses	Initial and every 3rd scheduled formulation room analysis; each dose for each sex/species	Study report
	Homogeneity study[d]	Once, prior to study initiation	Prestart chemistry report

[a]These activities apply when NIEHS provides the bulk test article to the testing laboratory.
[b]Methods provided by NIEHS.
[c]If concentrations change in subsequent studies, an additional method performance evaluation will be required.
[d]Prior to preparation of formulation designated for dosing; required only for feed and suspension dose formulations; total of three samples from each of three locations.

5.3.1. Dose Formulation

Methods for formulating the test article shall typically be provided by NIEHS.

- Each time an aliquot of the bulk chemical is weighed and formulated with the vehicle, that formulation is defined as a BATCH. For each dose prepared on each formulation day two or more batches might be required.
- An archival sample and an analysis sample of each batch shall be taken at the time of preparation and stored in individually labeled, sealed containers under the same storage conditions as the bulk formulations.
    - The quantities of the archival samples are approximately 50 mL for gavage and drinking water studies, 100 g for feed studies, and 25 mL for dermal studies. The COR will notify the testing laboratory when a smaller amount shall be archived (e.g., in the case of limited test material availability).
    - When a dose analysis of a formulation is planned, an analysis sample shall be used to determine the formulation concentration. Archival sample can be used if a reanalysis is required.
    - Archival and analysis samples that are not used shall be discarded as hazardous waste in accordance with federal, state, and local regulations 90 days or more after preparation.
- To prevent improper dosing of study animals:
    - The containers shall be labeled with the test article name, batch number, storage conditions, other identifying data, and intended species. The inclusive dates that a formulation can be used must appear on the containers.
    - The labels on the containers shall be color coded for different dose groups, species, and sex (if different concentration used for species/sex).
    - Control and dosed formulations shall be stored separately from the bulk test article.
- Formulation shall be aliquoted into dosing bottles (e.g., daily, weekly) as appropriate for study.
- An inventory of each dose formulation shall be maintained. A record shall be kept of the formulation date and the dates/duration the formulation can be used. This record, which shall be signed by the dose formulation supervisor or designee, shall contain

information on the quantities of dose formulation prepared and identifying numbers (e.g., lot and/or batch numbers) for both the test article and dosing vehicle.

- If suspensions are to be formulated, the resuspendability and syringeability of the highest concentration formulation shall also be determined after preparation and following storage for at least 24 hours under the recommended storage conditions.
- The dose formulation storage condition and duration of stability/use period typically shall be established by NIEHS. The dose formulations shall not be used beyond their stability period. In some cases, the testing laboratory might need to determine the stability and conditions to mimic dosing and stability prior to study start. In such cases, refer to the DTT Chemistry Specifications for requirements.
- For dosed water studies, water for control groups is to be taken from the identical source and at the same time as the water used for the treated group formulations. The control water and dose formulations are to be stored as specified by NIEHS until it is time to dispense the formulations to water bottles and transport them to the animal rooms. For dosed feed studies, feed for the control groups is to be taken from the identical source and at the same time as the feed used for treated group formulations.
- If the batch sizes required for studies are twofold higher than those evaluated by NIEHS (for feed and gavage suspensions only), or homogeneity is not provided by NIEHS, per COR direction, the testing laboratory shall assess homogeneity of the batch size equivalent to the batch size anticipated for the study at the lowest and highest formulation concentration prior to study initiation. Refer to the DTT Chemistry Specifications for requirements.
- To ensure that homogeneous dosed feed or suspensions are prepared, homogeneity shall be checked prior to initiation of each study or study phase (e.g., 14-day study, 90-day study and 2-year study) at the highest and lowest formulation concentrations, unless blend parameters (size and concentration) have not changed from the previous study.

5.3.2. Formulation Analysis

Formulation analysis methods shall typically be provided by NIEHS. The testing laboratory shall evaluate the method performance prior to study start. If concentrations required for analysis during study phases are outside the qualified/validated range, the testing laboratory shall qualify or validate the method over the proposed range of dose concentrations per the DTT Chemistry Specifications prior to study initiation.

As a quality control check, formulations shall be analyzed periodically by the testing laboratory. The results of analysis for formulations shall be reported using the units specified in the study protocol.

When formulation analyses are required, analyses shall be completed prior to administering the dose formulations per requirements in the DTT Chemistry Specifications.

Determined formulation concentration should be within 10% of target concentration. Values that deviate from the target concentration will be considered out of tolerance/specifications. There may be cases where a 10% tolerance limit cannot be attained; these will be addressed on an

individual basis and must be approved by NIEHS. The cause of any deviation from the approved tolerance limit shall be discussed in the monthly progress report. If the dose formulation is out of tolerance, the dose formulation shall not be given to the animals without NIEHS approval. When NIEHS determines that a re-mix is necessary, it shall be analyzed according to the original procedure. Re-mixes shall be shown to be within 10% of target before they are used for dosing.

The frequency of formulation analysis shall be based on the study duration as given below and in Table 5-1. Each formulation sample shall be analyzed in triplicate.

If NIEHS has directed that the formulations be analyzed by another laboratory, the samples shall be labeled according to the example below, and a sample submittal form (example shown in Figure 5-1) shall be prepared and included with the shipment. The laboratory shall be notified (by email or phone) at least 24 hours prior to arrival of the shipment. Information regarding the carrier and tracking number shall be provided when available. In addition, appropriate return address information shall be included on the package. If the shipment will arrive during nonworking hours or requires special handling or storage conditions, the laboratory shall be contacted at least 48 hours prior to arrival so that arrangements can be made to receive and handle the shipment properly.

Studies with Exposure Durations of Less Than 30 Days

All batches for the initial dose formulations of each dose group shall be analyzed to demonstrate the accuracy of the formulation procedure.

Samples of the formulations shall be taken from the animal room according to the following scheme and analyzed:

- The samples shall only be taken from formulations for which dose formulation samples have already been analyzed.
- The sample shall be taken on the last dosing day prior to the expiration date of the batch.
- The sample shall be taken at the end of the dosing day.
- The sample submitted for analysis shall be the residual formulation in the original dosing vessel. For drinking water studies, the sipper tube assemblies are to be removed and the bottles capped. For dosed feed studies, the contents of the feeders are to be emptied into clear, interferent-free containers. In addition, for dosed feed and drinking water studies, samples of the unused formulation from which feeders or bottles are filled shall be collected and analyzed along with animal room samples in determining animal room sample stability.
- Samples shall be taken from one sex of each species and each dose group (if dose groups for each sex are different, samples must be taken from each sex).
- The quantities of the samples are approximately 50 mL for gavage and drinking water studies, 100 g for feed studies, and 25 mL for dermal studies.

The results of these analyses shall be compared with the results of the original analyses.

Studies with Exposure Durations of Greater Than 30 Days and Equal to or Less Than 90 Days

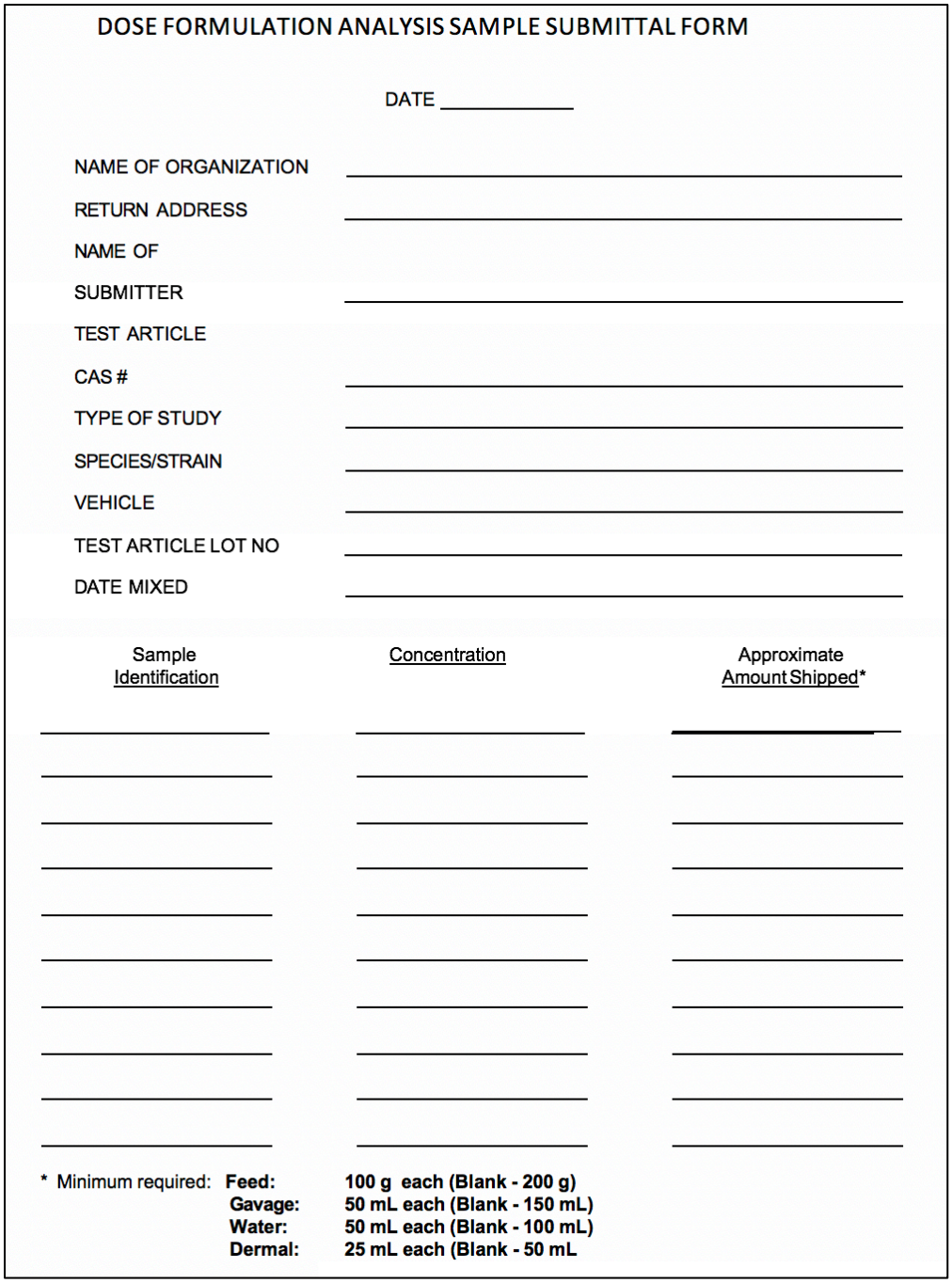
All batches prepared for the initial, midway, and final dose formulations for each dose group shall be analyzed to demonstrate the accuracy of the preparation procedures and analytical methods.

Samples of these same preparations shall be taken and analyzed from the animal room (upon completion of dosing) as stated above.

Studies Greater Than 90 Days in Duration

All batches prepared for the initial set of dose formulations shall be analyzed. Thereafter, these analyses shall be carried out every 10 ± 2 weeks.

Samples of all initial dose formulations shall be taken from the animal room for analysis as described above. Thereafter, similar animal room samples shall be taken during every third scheduled analysis period.



DOSE FORMULATION ANALYSIS SAMPLE SUBMITTAL FORM

DATE _____

NAME OF ORGANIZATION _____

RETURN ADDRESS _____

NAME OF SUBMITTER _____

TEST ARTICLE

CAS # _____

TYPE OF STUDY _____

SPECIES/STRAIN _____

VEHICLE _____

TEST ARTICLE LOT NO _____

DATE MIXED _____

Sample Identification	Concentration	Approximate Amount Shipped*

* Minimum required: **Feed:** 100 g each (Blank - 200 g)
Gavage: 50 mL each (Blank - 150 mL)
Water: 50 mL each (Blank - 100 mL)
Dermal: 25 mL each (Blank - 50 mL

Figure 5-1. Sample Dose Formulation Analysis Submittal Form

5.3.3. Analysis of Dosing Vehicles

The testing laboratory shall perform analyses of dosing vehicles to confirm the identity and purity of the test article. Refer to the DTT Chemistry Specifications for details.

5.4. Inhalation Studies

A summary of activities for inhalation studies is provided in Table 5-2.

Table 5-2. Summary of Activities for Inhalation Studies

Activity	Anticipated Frequency	Phase[a]
Test Material and Analysis		
Receive or procure test material	Once	Prestart
Bulk Chemical Analysis		
Method development	Once	Prestart
Identity and purity analysis	Once[b]	Prestart
Reanalysis	Within 30 days prior to exposure for all studies; within 30 days of end of exposure for studies with exposure durations longer than 30 days; every 24 ± 2 weeks for studies with durations longer than 6 months	Study
Exposure System Design and Characterization		
Exposure system design and schematics	Once[b]	Prestart
Monitor development/qualification	Once	Prestart
Stability in reservoir, generator, and chamber/carousel		
Without animals	Once	Prestart
With animals	At the beginning of study[c]	Study
Identity of test article in reservoir, generator, and high and low concentration chamber/carousel (particulate aerosol)	Once during study (exposure duration <3 months) or annually (exposure duration >1 year)	Study
Evaluation of Aerosol (Vapor Generation from a Liquid)		
Without animals	Once	Prestart
With animals	At the beginning of study	Study
Exposure Concentration/Environment Stability		
Without animals	Once (3-day test generation)	Prestart
With animals	Daily during exposure period	Study
Chamber/Carousel Uniformity		
Without animals	Once	Prestart
With animals	At the beginning of study; every 3 months (studies >3 months)	Study
Chamber Concentration vs. Time Plots		
Without animals	Once	Prestart
With animals	At the beginning of study	Study

Activity	Anticipated Frequency	Phase[a]
Chamber Post-exposure Monitoring		
Without animals	Once	Prestart
With animals	At the beginning of study	Study
Oxygen Determination When Inert Gas Is Used		
Without animals	Once	Prestart
Determine Particle Size (Aerosols)		
Without animals	Once	Prestart
With animals	At the beginning of study, then monthly	Study
Room Air Monitoring	Once	Prestart
Effluent Exhaust Treatment	Once	Prestart

[a]Methods and data shall be presented in the report corresponding to the phase listed.
[b]Preliminary results shall be reported to NIEHS for approval as soon as results are available.
[c]If the test article is to be generated as a particulate aerosol, test article identity shall be confirmed in the generator, distribution line, and high and low chamber/carousel during the prestart effort, once for studies with exposure durations of 1–3 months and annually during the chronic studies.

5.4.1. Test Material and Analysis

The testing laboratory shall either procure or receive the test material from NIEHS. Color photos of the test material compared with a color spectrum shall be generated.

The testing laboratory shall generally be responsible for analytical chemistry, including method development and analysis for the determination of the identity, purity, and as required, physical properties of the bulk test material. If specified by NIEHS, specific assays shall be included (see DTT Chemistry Specifications). In some cases, methods and/or analyses shall be provided by NIEHS.

5.4.2. Exposure System Design and Characterization

Design

Chambers for whole-body inhalation studies or carousels for nose-only inhalation studies can be any design that can be demonstrated to provide uniform and reproducible exposure of all animals to the test article and allows exposure under appropriate environmental conditions. However, the design shall be such that the chamber air supply and the incoming test material will be thoroughly mixed prior to entering the chamber. Schematics of the entire system, including generation, distribution, chambers/carousels, and monitoring system shall be generated.

The testing laboratory shall develop methodology for exposure generation and monitoring exposure concentrations, including calibration of the online monitoring system. The specificity, precision, linearity, absolute recovery, measurement limits, and relative error shall be established for the online monitoring method, along with appropriate methods of calibration.

The concentration of test article in the treated and control chambers/carousels must be monitored and recorded once per hour at minimum, and preferably continuously using a monitoring method from a single representative port.

If an aerosol is being generated, then the vapor (if present) as well as aerosol concentration shall be determined. Once the ratio of aerosol to vapor concentration has been calculated for each exposure concentration, only the aerosol concentration needs to be monitored on a daily basis. (The vapor concentration monitoring methodology developed by the testing laboratory shall be able to quantitate vapor at a level of 1% of the targeted aerosol concentration.)

Characterization

All evaluations with animals shall occur at the beginning of the studies, with a maximum load of animals in the chambers/carousels.

Aerosol Particle Size

If the test atmosphere is an aerosol, the particle size distribution shall be controlled and monitored. The measurement method must provide the mass median aerodynamic diameter (MMAD) and the geometric standard deviation for the distribution. The initial particle size distribution determination shall be done by impactor and shall have a MMAD of <3 microns and a sigma g of <3.

Evaluation for Potential Degradation Products

The testing laboratory shall develop methods for analysis of known or suspected degradation products or impurities. The studies shall be carried out at the lowest and highest exposure concentrations during developmental work without animals in chambers/carousels and at the beginning of each study with animals in chambers/carousels. Methodology developed by the testing laboratory must be sensitive enough to detect the degradation products down to a level of 0.1% of the chamber/carousel concentration target. Stability/degradation studies to confirm the integrity of the generated chemical atmosphere shall be conducted by

(1) Establishing there is no degradation of test article in the reservoir during the expected residence time in the reservoir. The reservoir sample shall be taken at the end of an exposure day. If the reservoir is not refilled daily, the sample shall be taken immediately before a refill. This sample shall be compared with a sample collected from the container of test article used to fill the reservoir or an analytical standard collected after receiving the test material, if it is necessary to avoid repeated opening of the vessel.

(2) Establishing the generation system does not cause degradation of the test article prior to introduction into the inhalation chamber/carousel. The distribution line sample shall be taken at a point after generation but prior to final dilution of the chemical stream before introduction into the exposure chamber/carousel.

(3) Determining to what extent, if any, the test article is degraded after introduction into the chamber/carousel atmosphere. The chamber/carousel sample to be tested shall be taken during the first and last hours of the exposure. The testing laboratory shall establish a generator/reservoir change-out schedule.

The testing laboratory shall establish a generator/reservoir change-out schedule and develop operating procedures covering the maintenance of the generator/reservoir based on these stability studies.

Evaluation for Aerosols

When the test atmosphere of a liquid (at standard temperature and pressure) test article is to be generated as a molecular vapor of that test article rather than as an aerosol, the testing laboratory shall demonstrate by photometric (or other appropriate means) that the test atmosphere does not contain aerosolized test article.

Exposure Concentration/Environment Stability

The method of generation of the test atmosphere shall be reproducible so that the daily average chamber/carousel concentration does not vary by more than ±10% from the target concentration from exposure period to exposure period. The daily relative standard deviation (RSD; standard deviation divided by the mean, expressed as %) of chamber concentration shall not vary by more than ±10%. Daily average chamber/carousel concentrations, relative error (RE), and RSD shall be reported. For certain test materials, low exposure concentrations, nose-only exposures, and others, it may be necessary to broaden the RE/RSD limits, which will be determined on a case-by-case basis.

The stability of environmental parameters (temperature, relative humidity, air flow, and vacuum) and of exposure concentrations must be established for a period of the daily exposure duration to be used in the studies, plus T_{90} (for whole-body studies) for 3 days. All the data shall be obtained in the same exposure room and under identical conditions that will be used during the animal exposures.

Exposure Uniformity

The uniformity (homogeneity) of the exposure concentrations in the chambers/carousels shall be demonstrated to confirm that the challenge to each animal is the same. Total port variability consists of within port and between port variability (WPV and BPV, respectively). WPV and BPV shall not exceed 5% RSD.

Build-up and Decay

Chamber concentration versus time plots shall be developed for each chamber. These data will be used to evaluate: the time necessary to reach the target concentration; the ability of the generation system to maintain a stable concentration over a full exposure period; the length of time necessary to clear the chamber of test article; the times necessary to reach 90% of the target concentration at exposure initiation (T_{90}) and to reach 10% of the target concentration (T_{10}) after exposure termination shall be estimated from chamber concentration versus time plots and compared with the theoretical T_{90} and T_{10}, respectively. The T_{90} shall be added to the nominal daily exposure duration (e.g., 3 or 6 hours).

Post-exposure Monitoring

The residual concentration of test article in the high chamber atmosphere shall be monitored overnight or until the concentration for two consecutive hours is <1% of the target concentration. The overnight monitoring period shall begin immediately after exposures are completed. If possible, opening and servicing of chambers shall be delayed until the concentration has decayed to <1%.

Oxygen Determination

If an inert gas (e.g., nitrogen) is used to generate or transport the test article to the inhalation chamber/carousel, the oxygen content in each exposure chamber/carousel shall be measured. A minimum oxygen concentration of 19% is required.

Room Air Monitoring

Method(s) of room air monitoring shall be developed and evaluated at exposure concentrations used in the studies in all chambers/carousels under exposure conditions. The lower limit of detection of the monitoring method(s) shall be defined. It shall be documented that this level provides an adequate safety margin for personnel.

Effluent Exhaust Treatment

Method(s) for effluent treatment shall be developed and tested at protocol-required concentrations in all chambers/carousels under exposure conditions. The effectiveness of the exhaust treatment unit immediately after the effluent treatment unit and at the point of exhaust from the building shall be demonstrated. The percent efficiency of the exhaust treatment shall be determined. The effluent exhaust treatment must be effective in removing the test material to an acceptable concentration, that is, >90% efficiency of removal by the treatment system and <50% of the threshold limit value (TLV), if a TLV exists; otherwise, written documentation for a waiver from the appropriate air regulatory agencies must be provided. The lifetime expectancy of any proposed filtration/treatment units and amount of treatment media required shall be determined. It shall be confirmed that none of the exhausted test material is re-entrained.

5.5. Completion of Test Article Program

5.5.1. Exposure System Closeout

The exposure system for a specific test article may be used for one or more studies. Following completion of the testing program for a given test article, the exposure system shall be decommissioned. All consumable/disposable materials shall be removed and disposed of according to the appropriate guidelines (see Chapter 3 [Health and Safety], Section 3.7 Waste Disposal/Test Article Shipment). If reusable, the materials or equipment shall be sent through a cage wash if appropriate (e.g., will not cause damage to equipment based on water temperature, detergents).

Test article requirements could stipulate that some studies have additional provisions, such as for wipe samples or air sampling, aimed at ensuring proper cleaning has occurred so that no additional hazards are present on the materials or equipment or in the study rooms.

5.5.2. Disposition of Surplus/Residual Test Article

Thirty days prior to the shipment of the test article, the testing laboratory shall notify NIEHS of its intention to ship surplus or residual chemical, including the amount to be shipped. Shipment is to be made within 30 days after the terminal sacrifice for the last study for that test agent. (See Section 3.7 for complete details.)

A completed surplus test article aliquot transmittal form (example in Figure 5-2) shall accompany shipments of aliquots and surplus test article. In addition to the surplus chemical, a

100 g aliquot of each batch of chemical shall be reserved and shipped. The chemistry support contractor shall be notified (by email or phone) at least 24 hours prior to arrival of the shipment. Information regarding the carrier and tracking number shall be provided when available. In addition, appropriate return address information shall be included on the package. If the shipment will arrive during nonworking hours or requires special handling or storage conditions, the laboratory shall be contacted at least 48 hours prior to arrival so that arrangements can be made to receive and handle the shipment properly.

SURPLUS TEST ARTICLE TRANSMITTAL FORM

DATE _____

NAME OF ORGANIZATION _____

RETURN ADDRESS _____

NAME OF SUBMITTER _____

TEST ARTICLE* _____

CAS# _____

Provide information below for each lot used.

Lot #	Date lot received	Temperature lot stored	Amount of Test Article Returned	List study types for which lot used

* Use full NTP test article name.

Figure 5-2. Example Surplus Test Article Submittal Form

5.6. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapter 5 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was 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 5: Chemistry
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

Peer Reviewer

Erin Baker, Ph.D.
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6. Laboratory Animal Medicine and Toxicology

A.P. King-Herbert[1], T.L. Blankenship[1], S.R. Churchill[2], D. Kurtz[1] (Editors)

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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.

Peer Reviewers

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7. Clinical Pathology

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At a minimum, the laboratory must be capable of satisfactorily performing the clinical pathology assessments/measurements listed in this chapter.

7.1. Hematology

The laboratory must be capable of performing the following required hematology measurements using automated or semi-automated systems (impedance or laser-optic instruments) optimized and validated for rodent species:

- Erythrocyte count
- Hemoglobin concentration
- Hematocrit (automated)
- Mean corpuscular volume
- Mean corpuscular hemoglobin
- Mean corpuscular hemoglobin concentration
- Leukocyte count
- Leukocyte differential count
- Reticulocyte count
- Platelet count
- Cell hemoglobin concentration mean (CHCM)
- Cellular hemoglobin (CH)

If the laboratory lacks a hematology analyzer that measures CHCM and CH, a spun (manual method) hematocrit (packed cell volume) shall be performed.

Blood smears shall be made for each animal. A morphological assessment (microscopic evaluation) of erythrocytes, leukocytes, and platelets shall be performed and documented. Nucleated erythrocyte (nRBC) counts (nRBC/100 leukocytes) shall be reported. The requirement for blood smear evaluation and nRBC counts may be waived **ONLY** if the laboratory has approved blood smear review guidelines in an applicable hematology standard operating procedure (SOP).

Instead of an automated leukocyte differential count, a leukocyte differential count—determined by microscopic examination of a Wright's-type stained blood smear and identification of at least 100 leukocytes (manual method)—is acceptable. A manual differential count will be performed if the automated leukocyte count or leukocyte differential count generates instrument errors or

abnormal cell counts/distributions/findings—or as outlined in an approved applicable hematology SOP.

Laboratories shall possess the capability to perform an automated reticulocyte count. If an automated reticulocyte count cannot be performed, a reticulocyte count determined by microscopic examination (manual method) of a supravitally stained blood smear (e.g., new methylene blue) is acceptable. The manual reticulocyte count must be reported as an absolute number based on the proportion of reticulocytes in 1,000 erythrocytes or by use of a Miller disc.

Platelet, reticulocyte, and leukocyte cell counts will be expressed as absolute counts. The raw data will be determined by electronic or laser-optic methods.

When a manual leukocyte differential count is required (see above), absolute leukocyte cell counts may be derived by calculation using the instrument-derived total leukocyte count and the microscopically derived percentages obtained for the individual cell types. The reporting of data based on percentages, estimates, and manual (i.e., hemocytometer) counts is not acceptable.

7.2. Clinical Chemistry

The laboratory must be capable of performing the following required serum clinical chemistry measurements using automated or semi-automated systems optimized and validated for rodent species:

- Total protein concentration
- Albumin concentration
- Globulin concentration (total protein minus albumin)
- Albumin/globulin ratio
- Urea nitrogen concentration
- Creatinine concentration
- Alanine aminotransferase activity
- Sorbitol dehydrogenase activity
- Alkaline phosphatase activity
- Total bile acid concentration
- Aspartate aminotransferase activity
- Total bilirubin concentration
- Direct bilirubin concentration
- Indirect bilirubin concentration (total bilirubin minus direct bilirubin)
- Glucose concentration
- Creatine kinase activity
- Cholesterol concentration
- Triglyceride concentration

7.3. Urinalysis

The laboratory must be capable of performing the following required urinalysis/urine chemistry measurements using manual, automated, or semi-automated systems optimized and validated for rodent species:

- Urine appearance
- Urine volume
- Urine specific gravity or osmolarity
- Microscopic assessment of urine sediment
- Urine protein concentration
- Urine glucose concentration
- Urine creatinine concentration
- Urine enzyme activities as specified for individual studies (e.g., N-acetyl-β-glucosaminidase, lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase, and gamma glutamyl transferase)

7.4. Laboratory Requirements

The clinical laboratory scientist is responsible for the oversight of analysis and storage of clinical pathology samples and shall be available for consultation or questions. The training or approval of personnel to assume these tasks is also the responsibility of the clinical laboratory scientist, who shall review and sign off on data at the end of each day (or designate qualified personnel to perform this task).

The laboratory shall have in place all equipment necessary to perform (at a minimum) the aforementioned tests.

The laboratory shall have routine capability to collect and analyze blood, serum, and urine samples from rats and mice at a capacity of at least 60 samples of each sample type for each study day.

SOPs for performing the aforementioned clinical tests shall be made available for review and approval and submitted with the study file to the NTP Archives. The SOPs shall be accompanied by documented performance of the laboratory's ability to interpret results. Each laboratory shall maintain clinical pathology historical control data for each sex and species used, and those data shall be made available for review upon request.

Each laboratory performing clinical laboratory tests for NIEHS shall have written quality control procedures (i.e., SOPs) that are routinely followed and shall subscribe to a proficiency-testing program. In-house quality control procedures include scheduled equipment maintenance and calibration and cumulative records of performance utilizing normal and abnormal control reference materials or samples. Cumulative records of proficiency program testing results shall be maintained. Prior to approval to conduct clinical laboratory tests, 6-month cumulative data shall be submitted for evaluation; the data shall be in graph or tabular form.

The laboratory shall follow the criteria for rejecting sample runs based on the Westgard Rules (Westgard et al. 1981). The laboratory shall have written procedures for the application of the Westgard Rules and shall describe the criteria used for the determination of sample run acceptability. For quality control data, the source of control materials, the mean, and a measure of variability used in testing control materials to determine acceptability of sample runs must be maintained. If the mean and the measure of variability of commercially obtained controls were established in the testing laboratory, both in-house and manufacturer's values shall be maintained. The laboratory shall have written procedures for documenting incidents and deviations from the study protocol and SOPs. The degree to which deviations and incidents may influence the clinical laboratory measurements shall be identified. Examples of such deviations and incidents include: (1) technician error, (2) variation in reagent lots, (3) equipment drift or failure, and (4) the rejection of data of sample runs based on the concurrent analysis of control samples. Regardless of incident type, documentation of rejection and repeat of individual samples or sample runs must be made along with corrective action.

Protocol-required blood samples for hematology, clinical chemistry, etc. shall be obtained and analyzed in random order (not by dose group) for a given sex and species. The laboratory shall have written procedures for collecting and processing specimens in a randomized order. This requires an appropriate scheme for identifying and tracking specimens throughout all procedures.

Blood collection procedures shall be clearly identified. Blood collection sites, procedures, and anesthetics used shall be defined for both interim and terminal sampling. Unless otherwise specified by the study protocol, all terminal blood samples from rats and mice shall be collected from the retro-orbital sinus/plexus using a carbon dioxide/oxygen mixture as the anesthetic. In rats, survival (interim) blood collection shall be collected from the jugular vein without the use of anesthetic unless otherwise specified by the study protocol. All blood samples from the same study shall be collected from the same site. Sample volumes taken at interim bleeds shall not exceed 2.0% body weight for rats and 2.5% body weight for mice. Animals shall not be fasted prior to sample collection. For dermal, gavage, and inhalation studies, at each collection time point, animals shall be treated for a minimum of 2 consecutive days (within 24 hours) prior to sample collection. Animals are not to be treated on the morning of collection for these routes unless the protocol requires it. Blood samples for analysis in the clinical pathology laboratory shall be collected from the appropriate animals the morning of sample collection during a 3-hour period, including samples for analysis of routine hematology, clinical chemistry, urinalysis, and hormone variables. Methods for harvesting serum and plasma shall be described in the written procedures. All males and females of a given species shall be treated the same number of days before collection of samples, and all animals of a sex shall be bled on the same day. Perinatal and DART studies may require that blood collection occurs on a specific perinatal day (i.e., pups need to be exactly the same age) or gestation day (i.e., collection of blood from dams). Thus, blood collection may need to occur over several days instead of 1 day; in these studies, an appropriate scheme for the blood collection and analysis must be reviewed and approved by the program contracting officer's representative (COR). Overnight urine collection procedures must be clearly explained. As part of the demonstration of capability to collect blood and harvest serum, each laboratory must submit a listing of the total volumes per animal of whole blood, serum, and plasma that can be routinely obtained from rats and mice of 6 weeks, 17 weeks, and 6 months of age, using the retro-orbital bleeding technique. Both interim and terminal sacrifice volumes shall be listed.

If unsuitable blood samples are obtained from individual animals, those animals shall not be re-bled on subsequent days to fill the data gap. If the data gaps are significant, the program COR will determine if and when it may be necessary to re-bleed all animals.

Automated hematology measurements and blood smear preparations shall be made within 6 and 2 hours of sample collection, respectively. Constituents in serum (or plasma, if specified) shall be assayed the same day of sample collection. Samples collected for routine hematology assays (EDTA) shall not be stored on ice before analysis. During the period when the samples are not being assayed, they shall be kept tightly sealed at 4°C. With study-specific approval, samples (e.g., serum) may be frozen at −20°C or colder for subsequent analysis. The freezing of samples for storage prior to the performance of routine assays is not acceptable unless otherwise specified by the study protocol.

The laboratory shall have sufficient facilities for frozen storage of biological samples at −60°C or below. Such samples may be retained for up to 6 months following receipt of the relevant study report(s). Stained and cover-slipped peripheral blood, reticulocyte, and bone marrow smears shall be appropriately identified (as is the case with histology slide identification) and packaged for delivery to the NTP Archives at the conclusion of each phase of the study, as specified in Section 8.13.

7.5. Reporting Requirements

7.5.1. Submission of Unaudited Data

Summary tables—as well as individual animal data for all sample collections of routine chemistry assays, automated hematology analyses, and urine chemistry determinations—shall be submitted electronically to the program COR and pathology coordinator within 21 calendar days of sample collection. White blood cell differentials, reticulocyte counts, and morphologic evaluations of blood smears are to be included in the final report.

Data to be retained in the study file include original and repeat sample assays for individual animals. It is not necessary for these data to be subjected to internal quality assurance prior to submission.

7.5.2. Final Study Report

Summary clinical pathology data considered to be related to treatment shall be included in the "Results" section of the final report. All summary and individual animal data shall be organized by species, sex, and treatment group and included in the report appendices. Notations of any observation and/or action taken to confirm or explain atypical data points are to be included (e.g., dilution and reanalysis to confirm or establish values that exceed linearity of assay or reanalysis to confirm low values). Relevant comments concerning sample quantity (QNS) and quality (e.g., lipemia, hemolysis, icterus, etc.) are to be included. The methodology used to obtain samples and measure analytes must be described in the "Materials and Methods" section of the study report. Interpretation of the biological significance of the results shall be presented in the "Discussion" section of the study report and shall include correlations between clinical laboratory findings and anatomic pathological changes and/or clinical signs exhibited by the study animals.

7.6. References

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7.7. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapters 7 and 8 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was charged to:

1. Peer review the following chapters 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 7: Clinical Pathology
    - Chapter 8: Anatomic Pathology
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

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8. Anatomic Pathology

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This chapter specifies information on pathology requirements, organ weights, clinical observations, necropsy instructions, and gross and microscopic approaches for histopathological evaluation of the pathology of rodent species from perinatal, chronic (general toxicity and carcinogenicity) and subchronic studies, developmental and reproductive toxicology (DART) studies, immunotoxicity studies, and other study types such as the subchronic cohort of modified one-generation studies. This chapter also provides information regarding necropsy and tissue collection, fixation, trimming, slide preparation, written and photo documentation, and specimen handling for all studies.

8.1. Necropsy Capabilities and General Requirements

Unless otherwise noted in the study protocol outline, a complete necropsy shall be performed on all animals from all control and treatment groups that either die or are euthanized due to moribundity or scheduled sacrifice. All organs/tissues shall be examined in situ, then dissected from the carcass in the manner specified below, re-examined (including cut surfaces), and fixed in 10% neutral buffered formalin (NBF) immediately after examination and/or weighing, unless otherwise directed (such as fixation in Davidson's solution for the eyes/optic nerves and modified Davidson's solution for the testis/epididymides or samples of tissues that may be directly frozen in liquid nitrogen and stored at −80°C for molecular studies). All tissues shall be immersed in at least a 10:1 ratio of fixative to tissue by volume. Additional fixation instructions are provided below in their respective chapter sections.

The study pathologist at the testing laboratory assigned to conduct the study shall be responsible for monitoring the in-life phase of the study, as well as for assuring that the protocol-required tissues (PRTs) from all study animals of a given species (including early death animals) are collected and trimmed for microscopic evaluation at study termination. The pathologist assigned to the in-life phase shall also perform the histopathological evaluations on all interim removal animals (if an interim time point is required), animals euthanized when moribund, and early deaths.

Necropsy procedures shall follow those described below. All animals shall be euthanized according to standard operating procedures (SOP) at individual institutions, always adhering to the American Veterinary Medical Association (AVMA) guidelines as described in Chapter 6 of this document.

Scheduled necropsies shall be performed in the presence of and under the supervision of the assigned study pathologist. Animals shall be treated for at least 2 consecutive days prior to being euthanized and shall be euthanized within 1 day after the last dose or exposure to prevent repair

of damage to some organs or recovery in body weight effects. Necropsy shall be initiated immediately after an animal is euthanized (or found dead) to minimize postmortem autolysis.

It is preferable to dose animals for additional days and schedule necropsies in a timely manner rather than to have the exact number of prescribed study days with delayed necropsies following the last dose. For some studies, as specified by the protocol, it is required that animals be necropsied on a specific postnatal day. For logistical reasons, the start of studies shall be staggered so that scheduled necropsies can be performed without delay. The following requirements are detailed to assure that necropsy occurs promptly after euthanasia.

The order in which animals are necropsied (within a species/group/sex) shall be randomized. When feasible, a minimum of one sex per species shall be necropsied per day so that all animals of both sexes of one species are completed within 2 consecutive days. If necessary, based on protocol-required endpoints, subchronic study necropsies for each species and sex shall be completed within 4 consecutive days, while for chronic studies, all necropsies at the scheduled terminal sacrifice for a species shall be completed within 7 consecutive days. Animals may be dosed for an additional week to allow necropsy of all animals of both sexes and species within 1 day after the last dose. When necropsies for a given sex/species are conducted over more than 1 day, a randomized block design shall be used so that animals from all dose groups are equally represented across all scheduled necropsy days.

Sentinel animals that die or are euthanized during the course of a study shall be undergo a necropsy. (See Chapter 6 [Laboratory Animal Medicine and Toxicology] for sentinel necropsy requirements.) Animals removed from the study with removal reasons of “other” (such as accidental deaths) shall also be necropsied. Animals used in special studies shall be euthanized according to that specific protocol. All gross (macroscopic) lesions observed at necropsy are to be recorded in the raw data.

Unscheduled early death and found-dead animals shall be necropsied within 8 hours after death or discovery and, if necessary, these animals shall be refrigerated (not frozen) for no longer than 8 hours prior to necropsy. Unscheduled necropsies shall be performed in the presence of a pathologist when such deaths occur during normal working hours. An effort shall be made at necropsy to establish the probable cause of death (e.g., gavage-related, other accidental death, infectious disease, developmental abnormality, treatment-related toxicity or neoplasia).

The date and time of the last dosing period are to be recorded for each animal. Feed and water, including dosed feed or dosed water, if appropriate, shall be provided until the actual time of euthanasia.

8.2. Organ Weights

Terminal body weights shall be collected prior to necropsy. Organs shall be weighed to the nearest 0.1 mg. Organ-to-body-weight ratios shall be reported, if required in the protocol outline. The organs typically weighed in subchronic studies are liver, thymus, kidney, testes, epididymides, ovaries, heart, and lung. If indicated, bilateral organs shall be weighed and recorded separately. Organ weights are not collected in chronic studies unless specified in the study protocol.

8.3. Observations at Necropsy

Prior to euthanasia, clinical observations related to body condition, behavior, and movement of the animal shall be assessed. All clinical signs including coat abnormalities, state of emaciation or dehydration, breathing patterns (e.g., rapid, shallow, labored) as well as ambulatory ability and gait (e.g., limping, circling, tremors) shall be observed and recorded. A complete necropsy shall include external examination of the animal including body orifices (ears, eyes, nose, anus, genital openings, and oral cavity) and examination, collection, and fixation of all organs/tissues from animals in all treatment groups for histopathological examination (see Table 8-1).

8.4. Gross Evaluations and Tissue Collection

All gross lesions shall be described by the designated/supervising pathologist (or designee) and recorded in the NIEHS-leased Laboratory Information Management System (LIMS; currently Provantis) using the terminology/nomenclature listed in the Division of Translational Toxicology (DTT) computerized data management system (DTT Provantis Glossaries[1]) inclusive of anatomic site (topography); lesion (morphology), when possible; distribution; size (in three dimensions, largest to smallest, in units of centimeters [cm] or millimeters [mm], or volume in milliliters [mL]); number; shape; color; and consistency as appropriate. If necessary, any paper records shall be transcribed into the electronic data capture system as soon as is feasible. Each gross lesion shall be given a Traceable Gross Lesion (TGL) number. Each TGL shall be correlated with a microscopic diagnosis.

For studies with perinatal exposure, in addition to the standard necropsy procedure, the following data shall also be recorded during necropsy in age-appropriate pups, unless otherwise indicated: patency of vagina, enumeration of corpora albicans, and gubernacular length.

Table 8-1. Tissues to Be Collected during Necropsy

Organ/Tissue
Adrenal glands
Brain with olfactory bulbs
Clitoral glands
Cowper's (bulbourethral) gland[a]
Esophagus
Eyes w/optic nerve
Femur
Gallbladder (mouse)
Gross lesions (including tissue masses)
Harderian glands
Heart and aorta
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Kidneys

[1] https://cebs.niehs.nih.gov/cebs/paper/14901

Organ/Tissue
Levator ani bulbocavernosus (LABC) muscle complex[a]
Larynx[b]
Liver
Lungs and mainstem bronchi
Lymph nodes
Mandibular
Mediastinal
Bronchial
Mesenteric
Mammary gland with adjacent skin (unless requesting mammary gland whole mount)
Muscle, thigh
Nerve
Sciatic
Tibial[c]
Trigeminal and ganglion
Nose (three sections including nasal passages and nasal turbinates)
Oral cavity and pharynx
Ovaries
Pancreas
Parathyroid glands
Pituitary gland
Preputial glands
Prostate gland (ventral and dorsolateral lobes)
Salivary glands
Seminal vesicles (with coagulating glands)
Skin (collected with the mammary gland) Skin, Site of Application (to be collected for dermal studies only)
Spinal cord
Spleen
Stomach (forestomach and glandular stomach)
Sternum
Testes, including epididymides and vaginal tunic
Thymus
Thyroid gland
Tissue masses
Tongue
Trachea
Urinary bladder
Uterus, including cervix
Vagina

Organ/Tissue
Zymbal's glands

[a]To be collected in studies with perinatal exposure.
[b]To be collected in studies with inhalation exposure.
[c]Rats only.

8.4.1. Tongue, Oral Cavity, Pharynx, Trachea, Lung, Heart, Aorta, Thyroid Gland, Parathyroid Glands, Esophagus, Thymus, and Mediastinal Tissues

The mandible shall be removed to allow visualization of the tongue and posterior pharynx and examination for gross lesions. If there are no gross lesions, continue with removal of thoracic tissues by severing the ribs, so that the thoracic pluck, consisting of the tongue, trachea (with larynx, thyroid/parathyroid glands and esophagus attached), lungs, heart, thymus, and mediastinal tissue, is removed as a unit for examination. When gross lesions (masses/nodules) are present in the lung, the five largest shall be recorded and examined. If additional masses/nodules exist after the largest five have been recorded and collected, the Individual Animal Necropsy Record (IANR) shall specify under "Notes" that the number of masses/nodules was "greater than five" for that lung.

The thymus and the mediastinal tissue containing the mediastinal lymph nodes shall be dissected free of the lungs and heart. The thymus shall be dissected free from the mediastinal tissue, weighed (if required by the study protocol), placed flat on an index card, dorsal surface down, and placed in a labeled cassette to avoid loss during fixation.

The mediastinal tissue containing the mediastinal lymph nodes shall be placed in a labeled cassette to avoid loss during fixation.

If the lung and/or heart are to be weighed, the heart shall be carefully removed with the cranial vessels. Prior to weighing, the heart shall be removed from the lung/mediastinum tissue at its base with cranial aorta and be free of the pericardial sac. Additionally, the major vessels at the base of the heart can be blotted on fresh gauze or a paper towel to absorb excess blood remaining in the heart before weighing.

After the lung is weighed, it shall be infused by introducing fixative (approximately 1–2 mL for mice and 4–8 mL for rats or using a Mariotte bottle at 25 cm water pressure or until fixative flow stopped due to pressure equalization) into the distal trachea until the lungs are fully expanded to normal inspiratory volume. Care shall be taken to prevent over- or underinflation. The distal trachea shall be ligated to prevent leakage (backflow) of fixative as the lungs and trachea are immersed in fixative. For studies in which inhalation is the route of exposure, the mainstem bronchus to the accessory lobe (rats) or the apical lobe (mice) shall be ligated and removed prior to intratracheal infusion of fixative and subsequently frozen for molecular studies.

The thoracic aorta shall be removed and placed on an index card inside a cassette to prevent curling and loss during fixation.

For studies not requiring thyroid gland weights, the remaining proximal trachea with the thyroid gland (including parathyroid glands) and esophagus attached shall be immersed in fixative as a unit. For studies that require prefixation thyroid gland weights, both lobes of the thyroid gland

(with parathyroid glands) shall be dissected from the trachea, weighed together, placed in a labeled cassette, and immersed in fixative to avoid loss during fixation.

The entire remaining portions of the esophagus and proximal trachea shall be opened with dissecting scissors and carefully examined. If gross lesions are observed, including masses and/or other abnormalities, they shall be collected and fixed and recorded as TGLs for microscopic examination.

8.4.2. Head, Brain, Pituitary Gland, and Nose

The calvarium shall be removed for examination of the brain and pituitary gland, after which the brain (with olfactory bulbs) shall be removed, weighed (if required by the protocol), and immediately immersed in NBF. The brain shall be gently removed without excessive pressure in a manner that minimizes artifacts and damage to the brain, auditory canal, and caudal nasal cavity.

The pituitary gland and both trigeminal nerves (with ganglia) shall be left in situ for fixation to avoid excess handling and reduce the potential for creating artifacts (Figure 8-1). The nasal bones shall not be removed as the head is to be fixed and then decalcified to facilitate trimming of the nasal bones at the anatomical landmarks specified in Section 8.7.11.

Prior to immersion fixation, the nasal cavity shall be flushed with fixative by gently inserting a blunt needle attached to a syringe into the nasopharyngeal duct and slowly instilling fixative until drops of the liquid appear at the external nares. The entire head with the nasal portion shall then be immersed in the fixative with the pituitary and trigeminal nerves (with ganglia) in situ. Note that the pituitary gland and trigeminal nerves (with ganglia) are to be removed after fixation, but before decalcification (see Sections 8.7.10 and 8.7.13, respectively). The decalcification solution used shall be one that preserves immunogenicity in the nasal epithelium should future immunohistochemical and/or molecular analyses be directed (i.e., decalcification in Immunocal™ solution).

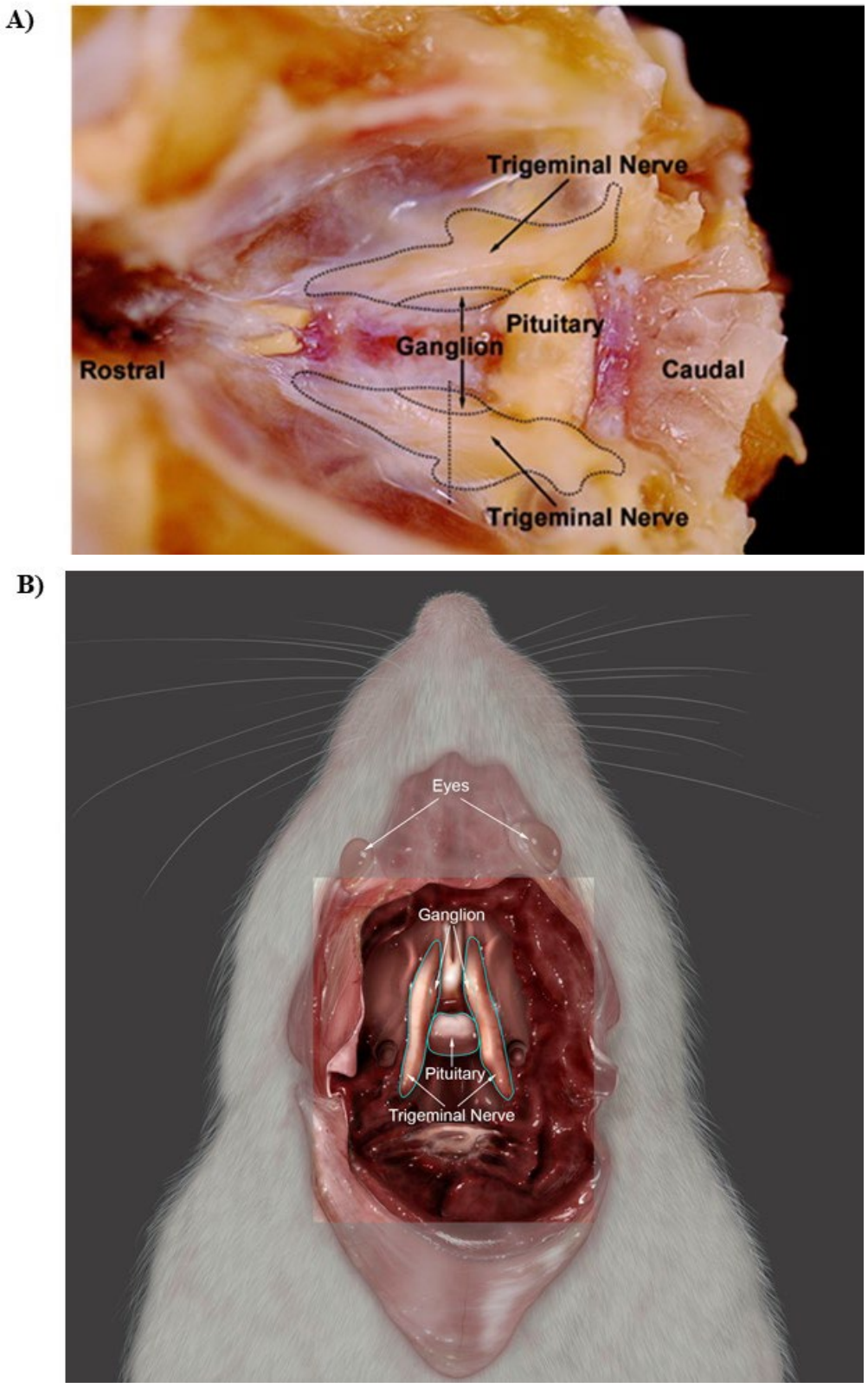


Figure 8-1. (A) Pituitary Gland and (B) Trigeminal Nerves with Ganglia

8.4.3. Spinal Cord

The spinal cord shall be exposed for examination at necropsy by removing some of the dorsal processes of the vertebral bodies over the cervical, mid-thoracic, and mid-lumbar regions before the vertebral column with the entire spinal cord is immersed in situ in fixative.

8.4.4. Liver and Gallbladder

The liver shall be free of adjacent tissues (diaphragm, ligaments, and other attachments) before weighing and fixation. In mice, the gallbladder shall be incised to allow drainage of bile prior to weighing.

The liver, including the gallbladder (mice), shall be removed as a unit for examination at necropsy. When multiple gross lesions (nodules/masses) are observed in the liver, the five largest shall be recorded and sampled. If more than five are observed, it shall be recorded on the IANR under “Notes” that the number was “greater than five” for that liver. The liver lobes shall be separated, the surface of the larger two lobes (median and left) shall be slightly incised to facilitate fixation, and the lobes shall be immersed in fixative.

8.4.5. Spleen

The spleen shall be removed for examination at necropsy by severing its attachments to the stomach and pancreas and immersed in the fixative. The spleen shall be free of adjacent tissues (pancreas, mesenteric fat, splenic ligament) before weighing (if required by the protocol) and fixation.

8.4.6. Pancreas

The pancreas shall be gently dissected from the duodenum for examination at necropsy taking care not to compress or tear the tissue, laid flat on an index card, and immersed in fixative. Note: A small segment of the pancreas shall remain attached to the duodenum for inclusion with the transverse section of the duodenum.

8.4.7. Kidneys

Both kidneys shall be removed at necropsy, examined, and weighed (if required by the protocol). Prior to weighing, each kidney shall be dissected from the perirenal fat and the adrenal gland. After weighing, the capsular surface of each kidney shall be slightly incised (but not completely removed) to facilitate fixation. For specific identification of each kidney at tissue trimming, the left kidney shall be incised along the longitudinal axis and slightly off-center. The right kidney incision shall be transverse and slightly off-center of the median plane. Both kidneys shall be immersed in fixative.

8.4.8. Adrenal Glands

The adrenal glands shall be dissected free from each kidney and surrounding adipose tissue for examination at necropsy and placed in labeled cassettes to avoid being lost during fixation. Both adrenal glands shall be immersed in fixative.

8.4.9. Gastrointestinal Tract

The pelvis shall be split longitudinally along the pelvic symphysis and the entire gastrointestinal tract removed from the stomach to the anus for examination at necropsy.

The stomach shall be transected at the junction of the pylorus and opened along the greater curvature for examination (Figure 8-2A). The opened stomach shall be pinned flat on an index card, serosal side down (Figure 8-2B), and immersed in fixative.

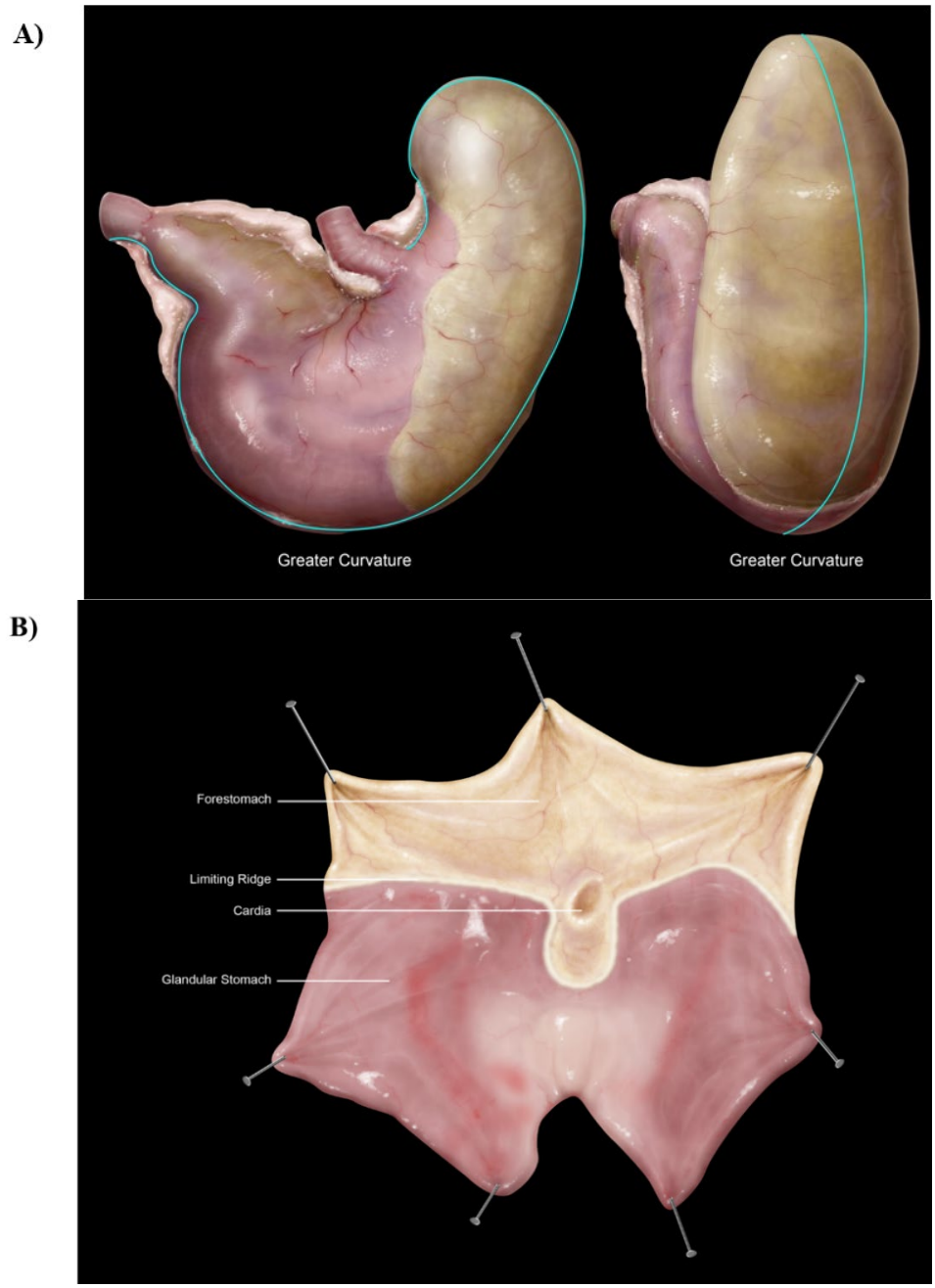


Figure 8-2. (A) Stomach Transection and (B) Pinning to Index Card for Fixation of the Glandular and Forestomach Regions

Fixative shall be gently injected into the remaining small and large intestinal tracts at several locations approximating the different anatomic segments (taking care not to overinflate), and the entire length of the small and large intestines shall be laid stretched out as a unit on an index card and immersed in fixative.

8.4.10. Hind Legs (Femur), Skeletal Muscle, and Peripheral (Sciatic and Tibial) Nerves

The right and left sciatic and tibial nerves shall be exposed by gently separating the muscles of the hind limb (Figure 8-3A). The tibial and sciatic nerves (Figure 8-3B) shall be trimmed for histopathological examination if required by an individual study protocol or if neurological signs were observed in the study. When collected, the nerves shall be placed on an index card to minimize curling during fixation, labeled appropriately, and immersed in fixative.

For rats, the distal portion of one femur with the knee joint and proximal portion of the tibia attached shall be removed for examination at necropsy, immersed in fixative, and then decalcified after fixation. Care shall be taken not to damage the head of the femur during dissection. The legs shall be placed in fixative with the other organs. The rat carcass shall be immersed in a separate container of fixative after the necropsy is completed.

For the mouse, only muscles surrounding the right and left sciatic nerves shall be removed and placed in fixative with the other organs, leaving the hind legs with nerves and muscles attached to the carcass and immersed in fixative.

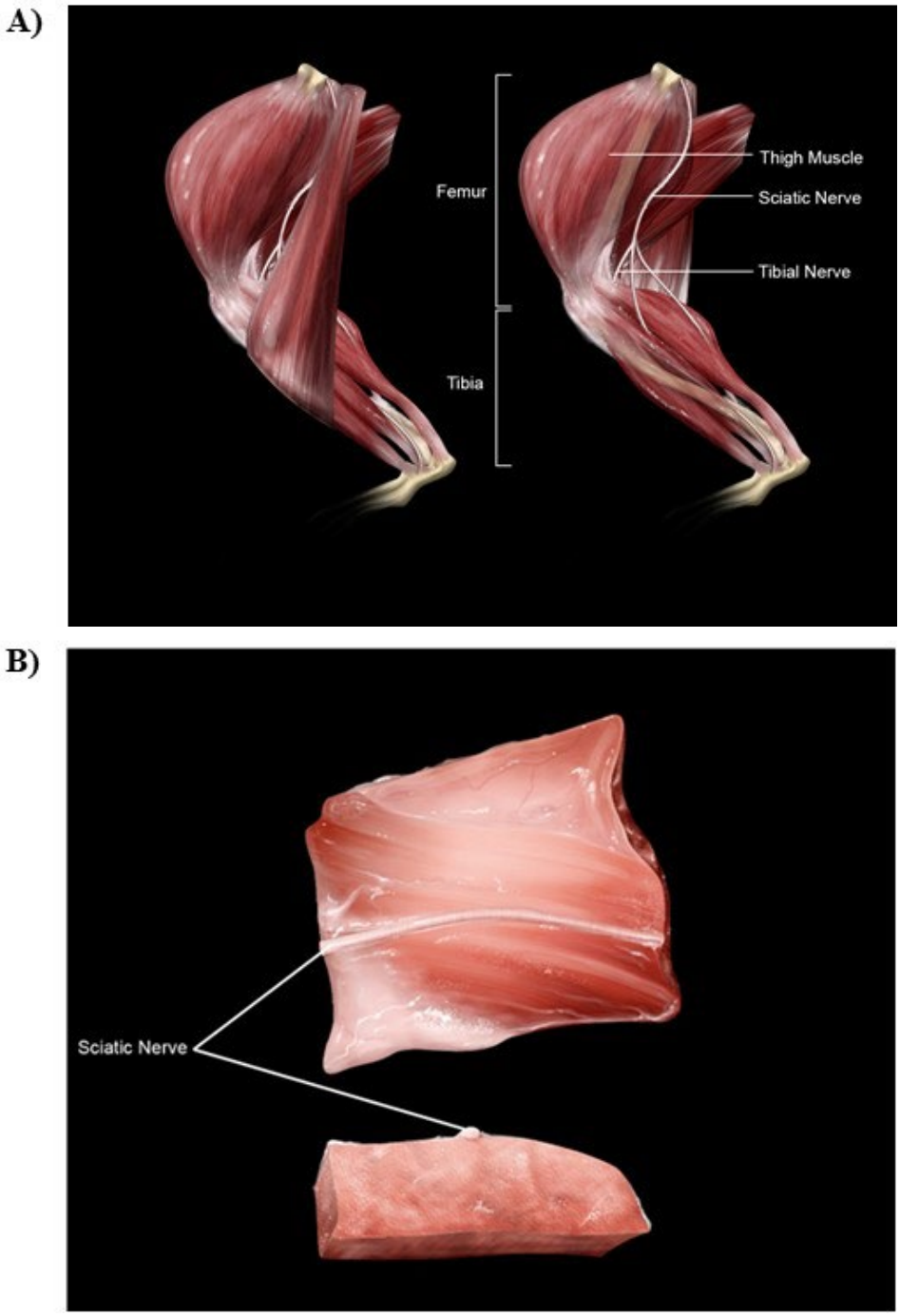


Figure 8-3. (A) Muscles of the Hind Limb Dissected Out from Lateral Surface to (B) Demonstrate the Sciatic and Tibial Nerves

8.4.11. Testes and Epididymides

The testes and epididymides shall be removed en masse at necropsy for examination by severing the vas deferens and the attachments to the scrotum, and excess adipose tissue shall be removed. The epididymides shall then be separated from the testes prior to weighing and fixation and laid flat on an index card to minimize twisting or curling during fixation. The testis and epididymis

shall be fixed in modified Davidson's fixative for 48 to 72 hours, after which they can either be immediately processed and embedded or transferred to 10% NBF to be processed and embedded within 90 days.

Collection for Sperm Motility and Counts

In studies with less than chronic exposure, the left testis with epididymis is used for sperm motility and counts, and the right testis is used for histopathological evaluation. In these instances, the left testis and epididymis shall be removed as soon as possible following euthanasia. The left epididymis shall then be immediately dissected from the left testis and excess adipose tissue removed. An incision shall be made through the epididymis to isolate the cauda from the remainder of the epididymis. The left testis and cauda epididymis shall be individually weighed and the fluid from the cauda immediately assessed for sperm number and motility (note, the left cauda epididymis and the fluid must be kept at 37°C for accurate assessment of sperm motility). The cauda and the residual sample of caudal sperm (after assessment of sperm) shall be placed in individually labeled tubes, capped, and stored at −70°C to −80°C until assessment of sperm concentration. The left testis shall also be frozen and stored at −70°C to −80°C until later assessment of spermatid concentration. The remainder of the left epididymis (corpus and caput together) shall be weighed and placed in modified Davidson's fixative. Sperm motility and counts shall be evaluated using a method validated by the study lab and approved by the contracting officer's representative (COR). Additional information about conduct of motility and counts are available in the literature (Chapin et al. 1992; Chapin et al. 1997; Seed et al. 1996; Stockard and Papanicolaou 1917).

The right testis and epididymis shall be removed at necropsy for examination taking care not to apply pressure to the testis. The right epididymis shall be dissected from the right testis and excess adipose tissue removed. The right testis and right epididymis shall be weighed separately and immersed in modified Davidson's fixative as described above.

Note: If the left testis has not descended (i.e., is cryptorchid) or if there are any gross lesions in either the left testis or epididymis, the right testis and epididymis shall be collected for sperm assessment. If both testes are cryptorchid or if there are gross lesions in both testes or epididymides, both testes/epididymides shall be fixed in modified Davidson's fixative and processed for histopathological evaluation as described below (i.e., andrology evaluations will not be performed). If only one testis/epididymis is present (i.e., there is unilateral testicular/epididymal agenesis), the testis/epididymis shall be immersed in modified Davidson's fixative and processed for histopathological evaluation as described below (i.e., andrology evaluations will not be performed).

8.4.12. Male Accessory Sex Glands

The prostate gland, seminal vesicle, and coagulating gland shall be removed with the urinary bladder en masse for examination at necropsy and laid flat on an index card to prevent or minimize twisting and curling during fixation. Adipose tissue surrounding these tissues shall be removed when collecting. If the prostate gland and/or seminal vesicles are to be weighed, the ventral and dorsolateral prostate and seminal vesicles with coagulating glands shall be dissected from the urinary bladder. The ventral and dorsolateral prostate lobes shall be weighed as described in the protocol and separated prior to or following weighing as appropriate. The secretory content of

the prostate and seminal vesicles represents a large proportion of the weight of the glands. Therefore, it is critical that the organ weight of these tissues includes all the secretory content.

For DART studies, as specified in the study protocol, the Cowper's (bulbourethral) glands, preputial glands, and levator ani bulbocavernosus (LABC) muscle complex shall be grossly examined in all necropsied males. If gross lesions, malformations, and/or abnormalities consistent with anti-androgenic activity are present, these tissues shall be collected, fixed, and examined microscopically.

8.4.13. Urinary Bladder

A urinary bladder distended with urine shall be fixed as is. Contracted, empty bladders shall be partially distended by slowly injecting a small amount of fixative (approximately 0.2 mL for mouse) into the lumen using a tuberculin syringe. Care shall be taken to insert the needle into the lumen rather than in the wall of the bladder (which may result in artifact) and to avoid overdistension. Insertion of the needle into the bladder wall may result in artifact. Urinary bladders shall be opened and examined after fixation at trimming.

8.4.14. Ovaries, Uterus, Cervix, and Vagina

The ovaries, uterus, cervix, and vagina shall be removed en masse for examination at necropsy. Prior to weighing, adipose tissue shall be removed from each ovary. The ovaries with oviducts shall be removed from each uterine horn and placed in a labeled cassette to prevent loss during fixation, and then immersed in 10% NBF.

In some studies, corpora lutea counts may be required (refer to study protocol). If required, these counts shall be performed prior to placing the ovaries in fixative and shall be performed as quickly as possible to minimize autolysis. For a description of corpora lutea of ovulation and corpora lutea of pregnancy, consult an appropriate textbook, such as *Practical Teratology* (Taylor 1986).

For pregnant dams assigned to natural delivery, the uterus shall be examined for nidation scars (implantation sites). If no nidation scars are observed, the uterus shall be stained with potassium ferricyanide solution to reveal implantation sites. Staining and implantation site enumeration shall be performed, and the uterus, cervix, and vagina immersed in fixative as quickly as possible to minimize autolysis. The uterus, cervix, vagina, and urinary bladder shall be laid flat on an index card prior to immersion in fixative to minimize curling and to facilitate sectioning in the longitudinal plane. The urinary bladder shall be dissected from the uterus, cervix, and vagina after fixation.

8.4.15. Eyes and Harderian Gland

The eyes, with the optic nerve and Harderian gland, shall be dissected free from the orbital socket as a unit for examination at necropsy. The Harderian glands shall be dissected free and placed in a labeled cassette to avoid being lost during fixation in 10% NBF. The eyes, with a segment of the optic nerve still attached, shall be immersed in Davidson's fixative (not modified Davidson's fixative). Eyes shall be fixed for 24 to 48 hours, after which they may either be immediately processed and embedded or transferred to 10% NBF and processed and embedded

within 90 days. After removal from Davidson's fixative, tissues must be thoroughly rinsed in physiological saline before further processing.

8.4.16. Salivary Glands

The submandibular, parotid, and major sublingual salivary glands are closely associated with the mandibular lymph node in the ventral cervical region. These shall be removed en masse for examination at necropsy, laid flat on an index card, and immersed in fixative.

8.4.17. Zymbal's Glands

The Zymbal's glands shall be dissected free of the surrounding tissue for examination at necropsy, placed in a labeled cassette to prevent loss during fixation, and immersed in fixative.

8.4.18. Preputial and Clitoral Glands

The preputial and clitoral glands shall be dissected free of the surrounding tissue for examination at necropsy, placed in labeled cassettes to prevent loss during fixation, and immersed in fixative.

8.4.19. Skin and Mammary Gland

A section of skin, approximately 2.5 cm wide × 3.5 cm long, with subcutaneous tissue and mammary gland attached, shall be collected from the inguinal region with the longest portion oriented along the longitudinal axis of the body. The section of skin shall be sampled so that the orientation of the anterior and posterior edges is well defined. For example, the anterior edge of the sample can be marked with indelible ink (India) or trimmed in the shape of an arrow.

Prior to immersion in fixative, the skin shall be placed, subcutaneous side down, on an index card to prevent curling during fixation. These procedures also apply to sampling at the site of application in dermal studies. The sections of skin sampled from the inguinal region and the site of application shall be placed in separately labeled cassettes to avoid confusion when the blocks and slides are prepared.

On select studies, as directed by the study protocol, the skin and mammary gland shall be collected separately. The entire fourth and fifth mammary glands shall be collected and laid flat on an index card prior to immersion in 10% NBF. The skin shall be collected as above, but without the mammary gland attached.

8.4.20. Tissue Masses

Multiple representative portions of gross lesions that represent large or heterogeneous tissue nodules/masses shall be recorded and collected with surrounding unaffected tissues, assigned a TGL number, and immersed in fixative. Masses <0.5 cm diameter shall be fixed in their entirety. When there are multiple masses in an organ, up to five of the largest masses per organ shall be sampled. If more than five are observed, they shall be recorded as a gross finding, or by using an observation comment, that the number was "greater than five" for that tissue.

8.5. Storage

All organs and tissues (with possible exceptions of skin, mammary glands, bone, and muscle) shall be saved and fixed in their entirety; no part of the organ or tissue shall be discarded. Eosin

shall be added to the stock NBF solution in sufficient quantity to impart a pink tinge to the fixative solution. Tails (or other body parts) that have been used in any way for animal identification during the in-life phase of the studies shall be saved in 10% NBF along with the animal tissues. If an ear tag or other identifying methods are used for any reason, these too shall be saved in 10% NBF with the animal tissues. For archiving purposes, all residual wet tissues shall be stored in 10% NBF in labeled, heat-sealed bags (double bagged) with sufficient 10% NBF to completely cover the tissue. Each bag label shall include the contract number, chemical name and CASRN, data management system reference number, strain, sex, treatment group, animal number, generation, and other important identifying information as appropriate.

Following necropsy, the carcass of each rat shall be immersed in properly labeled containers of fixative. Carcasses of rats shall be discarded only after the sponsor-driven pathology peer review has been completed and it has been determined that no gross lesions were missed from necropsy tissue collection and tissue trimming. Carcasses of mice shall not be discarded but retained in the container of fixative (with all other collected tissues) and shall be submitted to the NTP Archives at the end of the study. Disposal of the rat carcasses shall require the approval of the principal investigator, study director, quality assurance officer, and program CORs (including consultation with the COR for the NTP Archives).

8.6. Photodocumentation

Publication quality (per *Toxicologic Pathology* publication standards; see "Instructions for Authors" on *Toxicologic Pathology* website) color images (TIFF files) of selected representative gross lesions in target tissues or those considered to be unusual or rare (as determined by the study pathologist) shall be prepared. During photography, tissue surfaces of fixed gross specimens must be kept moist to prevent drying. All images are the property of NIEHS. Each image shall be identified with contract number, chemical name and CASRN, data management system reference number, sex, treatment group, animal number, generation, and description/diagnosis.

8.7. Tissue Trimming, Processing, and Embedding

Specific instructions for tissue trimming, processing, and embedding are provided in this section. For additional reference information, please refer to Kittel et al. (2004), Morawietz et al. (2004), and Ruehl-Fehlert et al. (2003).

To facilitate possible use of tissues for immunohistochemical or molecular analyses, fixed tissues from all animals, including early death, euthanized moribund, and scheduled removals, shall be trimmed, processed, and embedded within a period of not <48 hours but not >3 months from the day of necropsy.

The eyes and testes shall be fixed and stored in Davidson's fixative and modified Davidson's fixative, respectively.

Tissues shall be embedded in a consistent manner so that the same tissues with similar trimming/orientation are in the same numbered blocks for all animals.

If protocol requires molecular analyses, the program COR, NIEHS PI, and NIEHS study pathologist shall discuss the details and develop a strategy to collect tissues optimally for molecular analysis. A general approach and caveats are presented in Section 8.11 below.

Tissue trimming shall be supervised by the assigned pathologist and manager of the histology laboratory, although their continued presence is not required during trimming. An electronic copy of an IANR shall be used for each animal and shall be available for the technician at the time of tissue trimming. A hard (paper) copy of the IANR shall only be used in the event of computer failure or prior approval from the COR. Any additional gross observations identified during the trimming procedure shall be recorded and assigned a TGL number.

Parenchymal organs shall be free of attached extraneous tissues and trimmed to allow the largest cross-sectional surface area possible for microscopic examination. Tissues shall be trimmed to a thickness of not <0.4 cm and placed in labeled cassettes for processing. Small (<0.4 cm) endocrine organs, lymph nodes, and tissue masses may be submitted intact for histological processing, paraffin embedding, and microtomy. Large tissue masses that cannot fit in standard cassettes shall be trimmed to obtain one representative section of the mass.

All residual tissues from all animals shall be stored in fixative in heat-sealed bags (double bagged) following trimming (see Section 8.5 Storage). The animal identification label shall be included with the tissues for all study animals.

8.7.1. Gross Lesions (Masses, Nodules, Abnormalities)

Multiple portions of each gross lesion described as a mass/nodule shall be trimmed and submitted if large or variable in appearance. Surrounding, normal tissue shall be included if possible. Careful documentation of the number of samples taken per mass shall be maintained on the electronic IANR form so that multiple masses (potential neoplasms) per animal can be assessed.

Parenchymal organs shall be free of attached extraneous tissues and trimmed to allow the largest cross-sectional surface area possible for microscopic examination. For liver and lung, one section of each mass/nodule shall be prepared (up to five for each organ). If there are more than five masses/nodules, the five largest nodules/masses shall be trimmed. Adjacent normal tissue shall be included with gross lesions (mass/nodule or other abnormality) whenever possible.

8.7.2. Liver

Two standard sections of normal liver, one each from the left and median lobes, shall be prepared. These sections shall be transverse sections taken midway along the greatest dimension of these lobes. The median liver lobe section shall be trimmed across both the left and right median lobes to include the fissure. In the mouse, the section of median lobe shall include the gallbladder. If the sections are >2.5 cm in length, one end shall be trimmed slightly so that the sections fit into the cassette. The anatomic location and orientation for trimming the liver are shown in Figure 8-4A and Figure 8-4B for the rat and mouse, respectively.

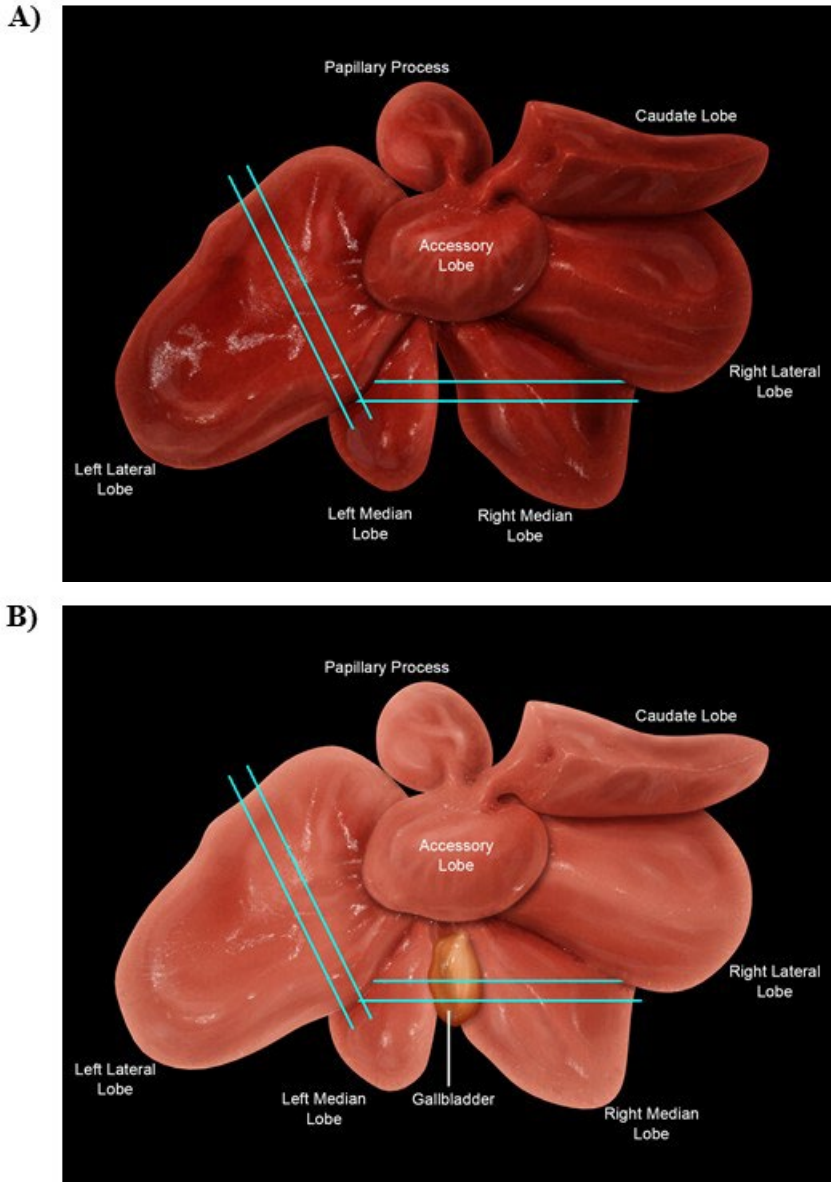


Figure 8-4. Ventral Surface of (A) Rat and (B) Mouse Liver Showing Left, Median, Caudate, Right Anterior, and Right Posterior Lobes

Lines represent anatomic location and orientation for trimming the (A) rat and (B) mouse liver.

8.7.3. Kidney

The fixed kidneys shall be bisected through the entire cortex, medulla, renal pelvis, and tip of the papilla and the cut surfaces examined for potential gross lesions. A representative section shall be taken from the fixed left and right kidneys and submitted for processing and embedding. The left kidney shall be bisected longitudinally slightly off-center of median plane (paramedian) pole to pole (Figure 8-5A). The right kidney shall be bisected transversely slightly off-center of the medium plane (paramedium) through the hilus to include the entire renal pelvis and renal papilla (Figure 8-5B). For rats, the trimmed kidneys shall be submitted in separate cassettes; for mice,

they shall be submitted in the same cassette. For enlarged kidneys, one pole of the trimmed section may be removed so that the section fits into the cassette.

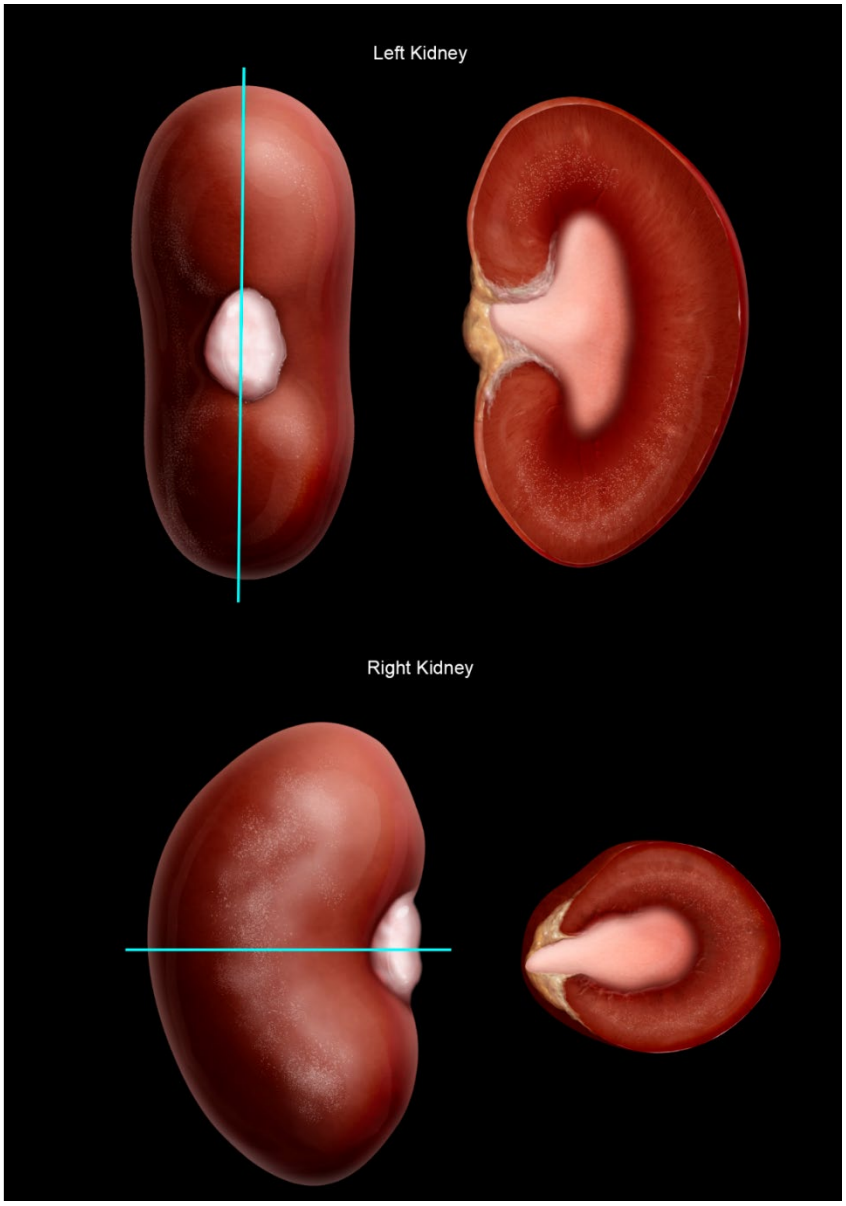


Figure 8-5. Bisection of the (A) Left and (B) Right Kidneys

8.7.4. Lung

For rats, the left and right lobes, including the mainstem bronchi, shall be submitted in separate cassettes, ventral surface down. Alternatively, if the right and left lobes are too large to submit as a unit in a single cassette, then the respective lobes may be hemisectioned and submitted in separate cassettes.

For mice, the entire lung shall be submitted ventral surface down in a cassette. The trimming methods for the rat and mouse lung are shown in Figure 8-6A–E.

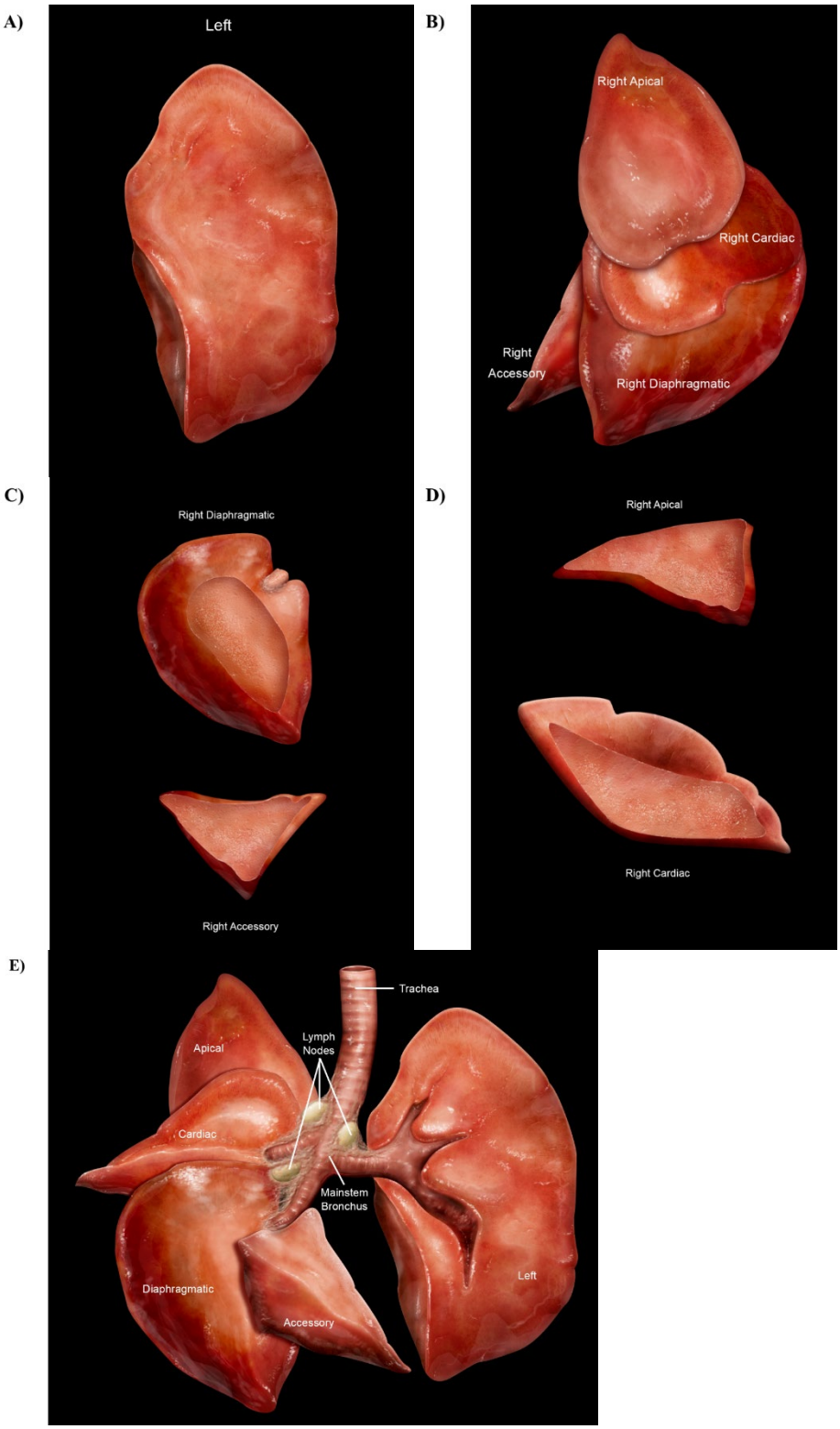


Figure 8-6. Lung Lobes of the (A–D) Rat and (E) Mouse

For the mouse, entire lung embedded ventral surface down.

8.7.5. Trachea, Thyroid Gland, and Larynx

If postfixation thyroid weights are not required, the thyroid gland shall be trimmed with the trachea and esophagus. A 4-mm transverse section shall be taken through the trachea that includes both thyroid/parathyroid glands and the adjacent segment of the esophagus attached (Figure 8-7). Following fixation, the remaining trachea shall be opened and examined. Any gross abnormalities shall be recorded, trimmed, and submitted for processing and embedding for histopathological examination.

If the thyroid gland is to be weighed after fixation, both lobes shall be submitted for processing and embedded flat to obtain longitudinal sections that also include the parathyroid glands.

The larynx shall be removed from the trachea by transection at its distal end (immediately proximal to the thyroid glands) and submitted in a labeled cassette for processing. The distal surface may be inked to facilitate recognition during embedding. The larynx shall be embedded distal surface down so that serial transverse sections are taken from the distal cut surface, sectioned cranially through to the base of the epiglottis, taking a section when the required level is observed. Level 3 shall be the first level observed during microtomy:

- Level 1. Through the base of the epiglottis
- Level 2. Through the ventral pouch and adjacent vocal processes of the arytenoid cartilages
- Level 3. Through the ventral pouch with the caudoventral extensions of the vocal processes and the vocal folds

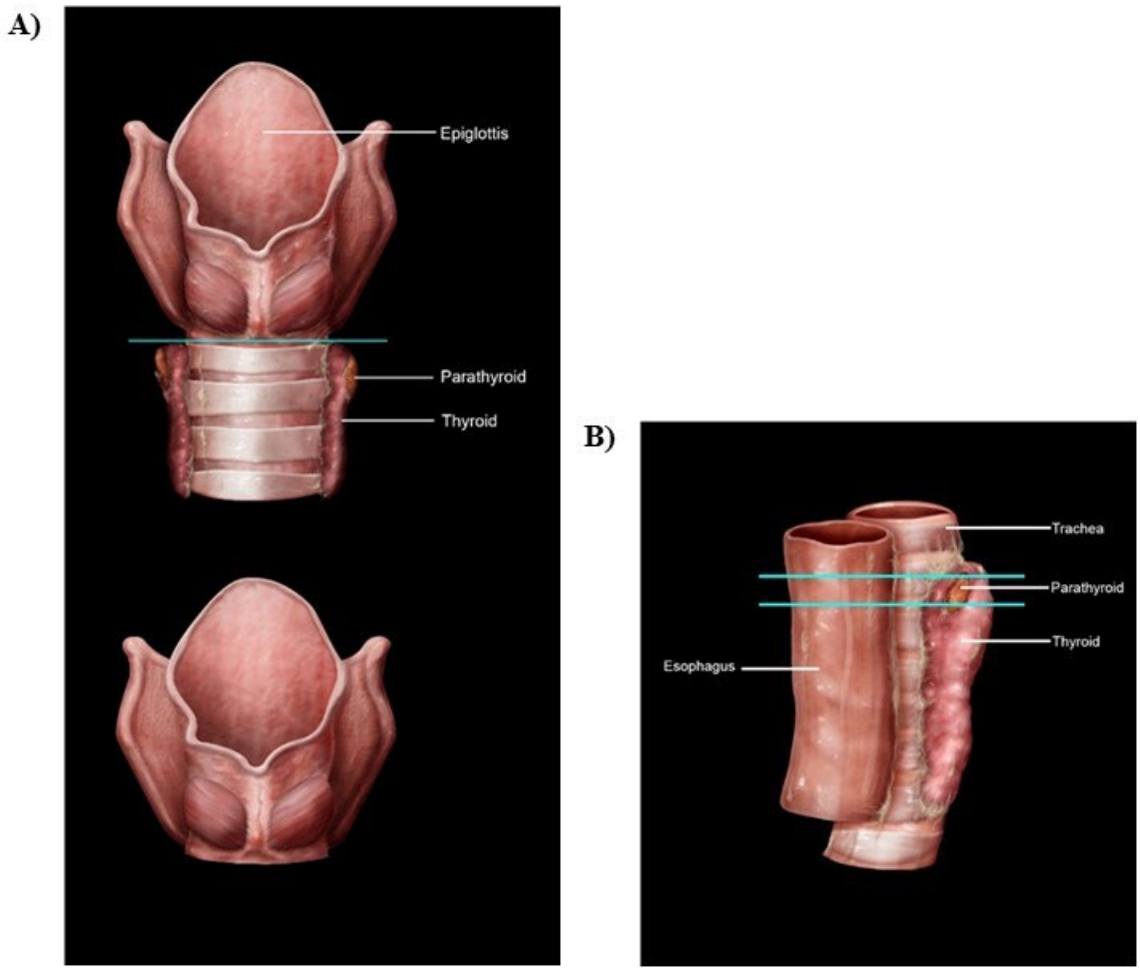


Figure 8-7. (A, B) Trachea, Thyroid Gland, and Larynx

8.7.6. Heart and Aorta

After fixation, the heart shall be sliced longitudinally from the base through the apex so that all four heart chambers (left and right ventricles and left and right atria) are visible (Figure 8-8). The left ventricle and the aorta shall be used for orientation during trimming. The largest, most visible structures of the heart are the ventricles. The position of the aorta emerging near the left ventricle from the base of the heart and making an arching curve back to the right shall be noted. After the ventricles and the aorta are located, the heart is held with the base and the left ventricle facing upward and toward the prosector. Thus, the base of the heart is seen when the prosector views it from above (Figure 8-8, left image). The trimming blade is placed on the base of the heart so that the blade slices through both atria, the aorta, and both ventricles and extends to the apex of the heart. The cut surface shall be examined for gross lesions (e.g., thrombi), and if present, recorded and left in place. All visible valves shall be examined and any abnormalities recorded. A 4-mm transverse section of the aorta shall be taken and placed in a separate cassette.

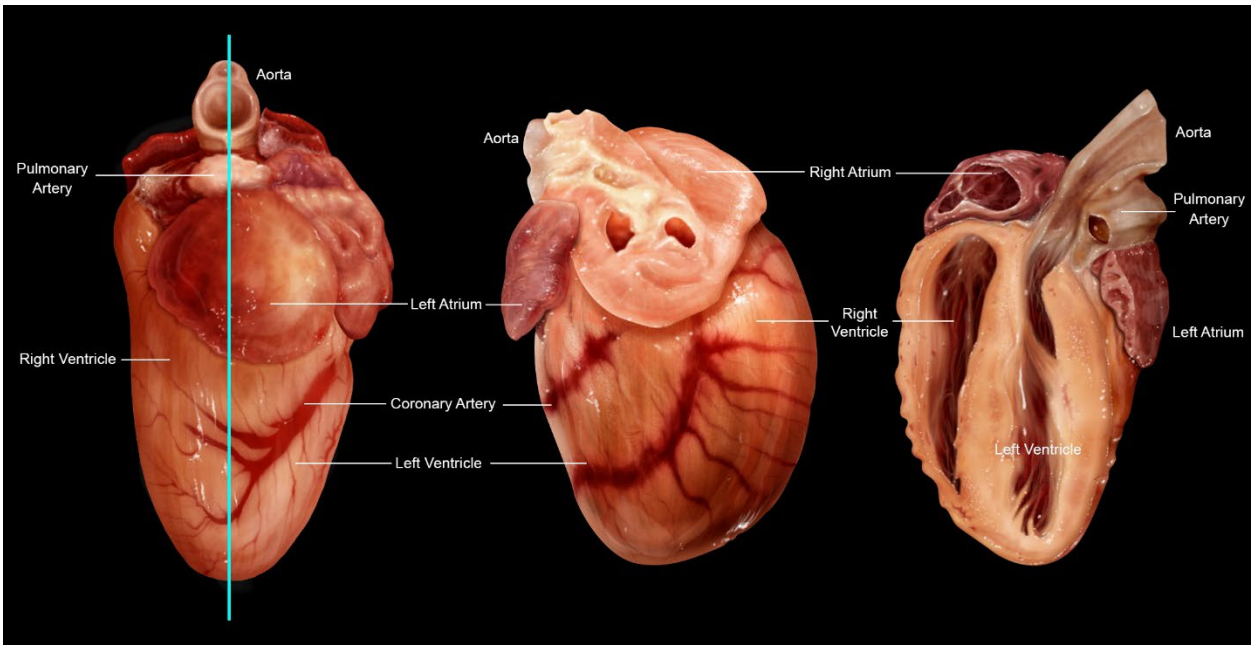


Figure 8-8. Anatomy and Positioning of the Heart and Great Blood Vessels to Demonstrate the Desired Plane of Section

8.7.7. Small and Large Intestine

The intestines shall be carefully separated from the mesentery (Figure 8-9). For all studies, segments will be consistently sampled from the same area for each region (include Peyer's patches with the ileum and jejunum when possible) and placed in labeled cassettes. One 4-mm transverse section from each segment (specific locations described below) of the fixed, unopened small and large intestines shall be taken for processing and histology. For consistency, the following guide shall be used for sampling:

Rat

- Duodenum: 1 cm distal to the pyloric sphincter that includes a portion of the adjacent pancreas (leave a small portion of the pancreas attached)
- Jejunum: section from the midportion containing Peyer’s patch if visible
- Ileum: 1 cm proximal to cecum to include Peyer’s patch
- Cecum: central section (due to larger diameter, it is advisable to open the section and then take a transverse section)
- Colon: transverse segment of the proximal colon 0.5 cm distal to the cecum and a second transverse segment of the distal colon 5 cm proximal to the anus
- Rectum: 1 cm proximal to the anus

Mouse

- Duodenum: 0.5 cm distal to the pyloric sphincter that includes a portion of the adjacent pancreas (leave a small portion of the pancreas attached)
- Jejunum: section from the midportion containing Peyer’s patch if visible

- Ileum: 0.5 cm proximal to cecum to include Peyer's patch
- Cecum: central section (due to larger diameter, it is advisable to open the section and then take a transverse section)
- Colon: transverse segment of the proximal colon 0.2 cm distal to the cecum; and a second transverse of the distal colon 2 cm proximal to the anus
- Rectum: 0.5 cm proximal to the anus

The entire chain of mesenteric lymph nodes shall be dissected free from the mesentery and placed in a labeled cassette to prevent loss during fixation.

For all studies, the residual segments of the intestinal tract shall then be opened and examined. All gross lesions in these segments shall be trimmed from the adjacent intestine, pinned flat on an index card, labeled as to location, and recorded on the IANR. TGLs shall be assigned to all gross lesions identified.

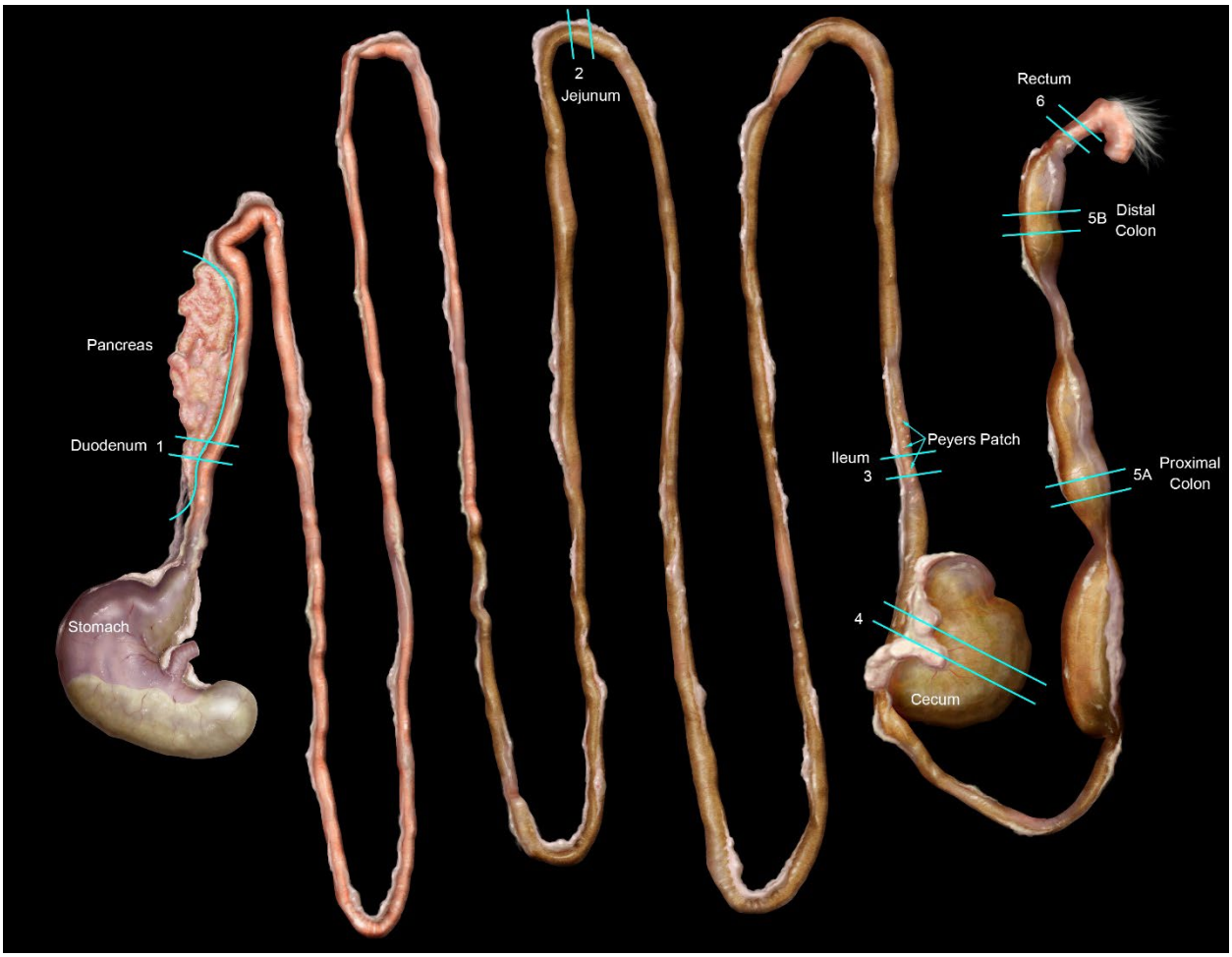


Figure 8-9. Small and Large Intestines

8.7.8. Tongue

If gross lesions are present, a 4-mm transverse section shall be trimmed through the entire tongue that includes the lesion(s) and adjacent tissue. Each lesion shall be placed in a separate cassette and immersed in 10% NBF.

8.7.9. Stomach

Three sections of the stomach shall be taken for histopathology (Figure 8-10):

- Section 1. From the cardia through the fundus and pyloric sphincter to the duodenum (if section is too large to fit in the standard cassette, bisect in half)
- Section 2. From the forestomach across the limiting ridge into the fundus
- Section 3. Through the fundus

Note: The location/orientation of this trimming shall be consistent.

All gross lesions shall be recorded, assigned a TGL, and trimmed for processing and histology.

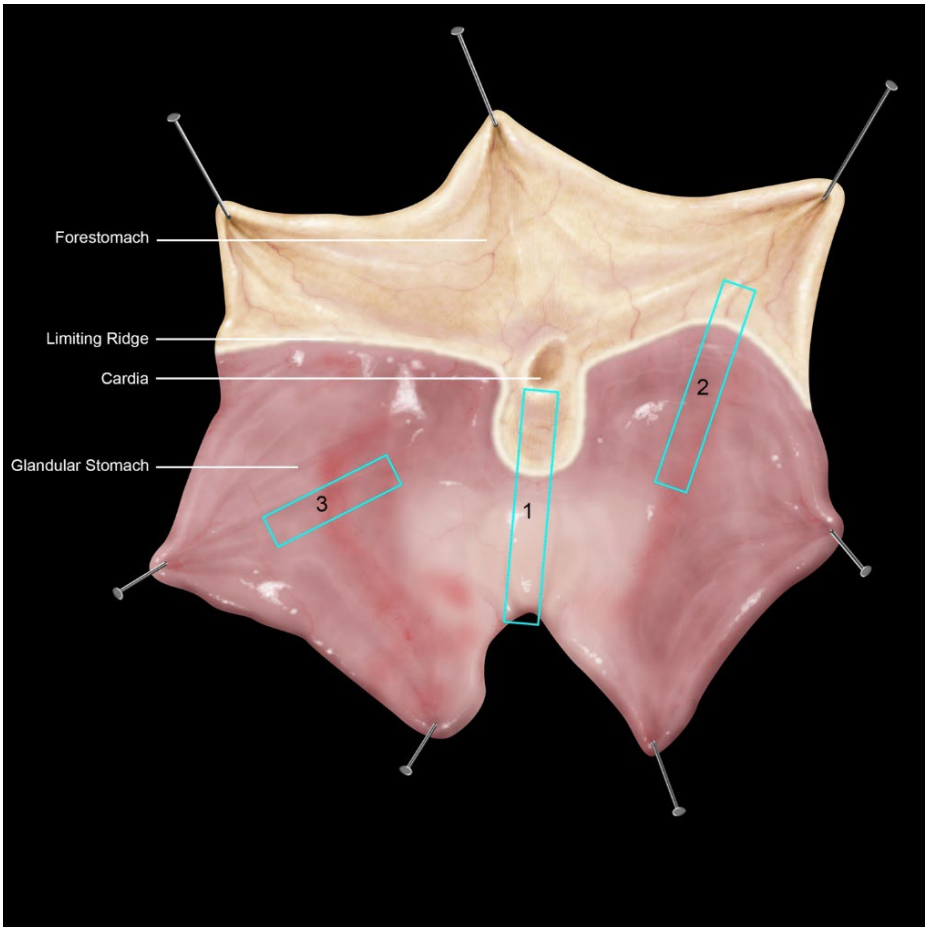


Figure 8-10. Trimming of the Stomach for Histology

8.7.10. Pituitary Gland

Following in situ fixation, the pituitary gland shall be carefully removed from its in situ location in the sella turcica of the sphenoid bone at the base of the skull and embedded whole with the caudodorsal surface down so that the histologic section will include all three anatomic regions (pars distalis, pars nervosa, pars intermedia) of the gland. If the pituitary gland is enlarged (noted as a TGL), the gland shall be bisected along the largest diameter with half of the tissue placed in a labeled cassette to be processed and embedded.

8.7.11. Nasal Cavity

After fixation, all muscle and extraneous tissue shall be dissected from the head, and the head shall be decalcified in a mild/gentle decalcification solution, such as Immunocal™, according to product specifications. After decalcification of the head, three separate transverse slices of the nasal portion of the head shall be taken through the following anatomic landmarks/levels (Figure 8-11):

- Level I. Immediately posterior to the upper incisor teeth
- Level II. Through the level of the incisor papilla midway between incisors and first molar teeth
- Level III. Through the middle of second molar teeth (olfactory region)

The nasal turbinates of the required trimmed sections and the remaining nasal cavity shall be carefully examined for gross lesions. All gross lesions shall be recorded on the IANR (Figure 8-11), placed in cassettes for fixation, and assigned a TGL (Maronpot et al. (1999), Nose, Larynx and Trachea in Pathology of the Mouse [pg. 261]).

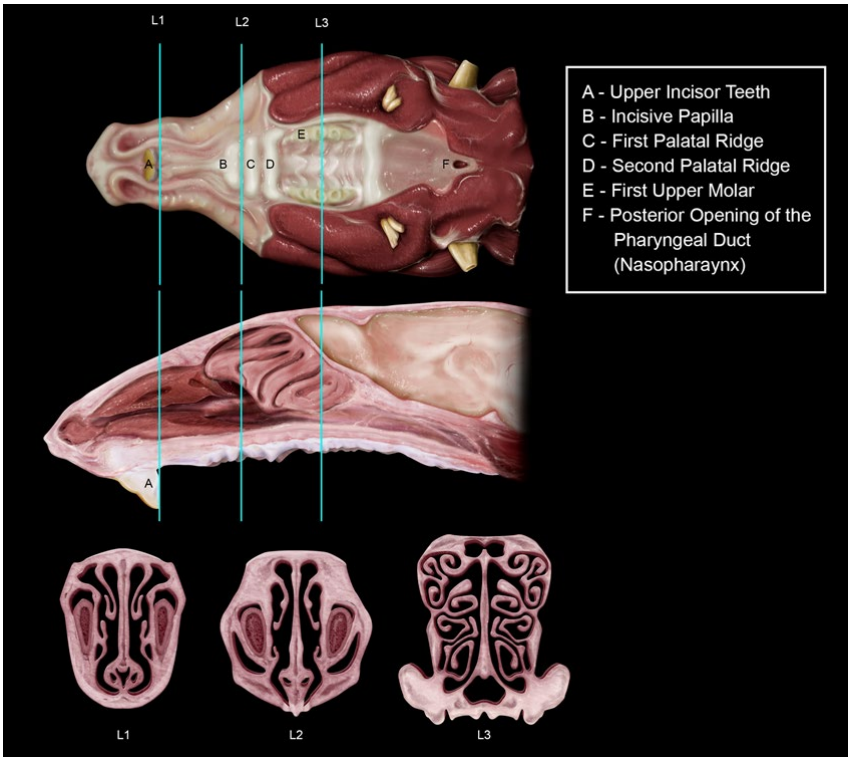


Figure 8-11. Nasal Cavity Sections

8.7.12. Brain

Seven transverse slices of the brain shall be taken at the anatomic landmarks/levels as shown from the ventral surface of the brain (Figure 8-12) (Rao et al. 2011; Rao et al. 2014). Brain matrix molds may be used to facilitate consistent section trimming. These sections shall include:

(1) Olfactory bulb (mid-level)

(2) Fronto-parietal cortex, including basal ganglia (1–2 mm cranial to the optic chiasma)

(3) Midpoint of the infundibulum (mid-parietal cortex and thalamus)

(4) Caudal half of the cerebral peduncles and interpeduncular nucleus (midbrain with substantia nigra and red nucleus)

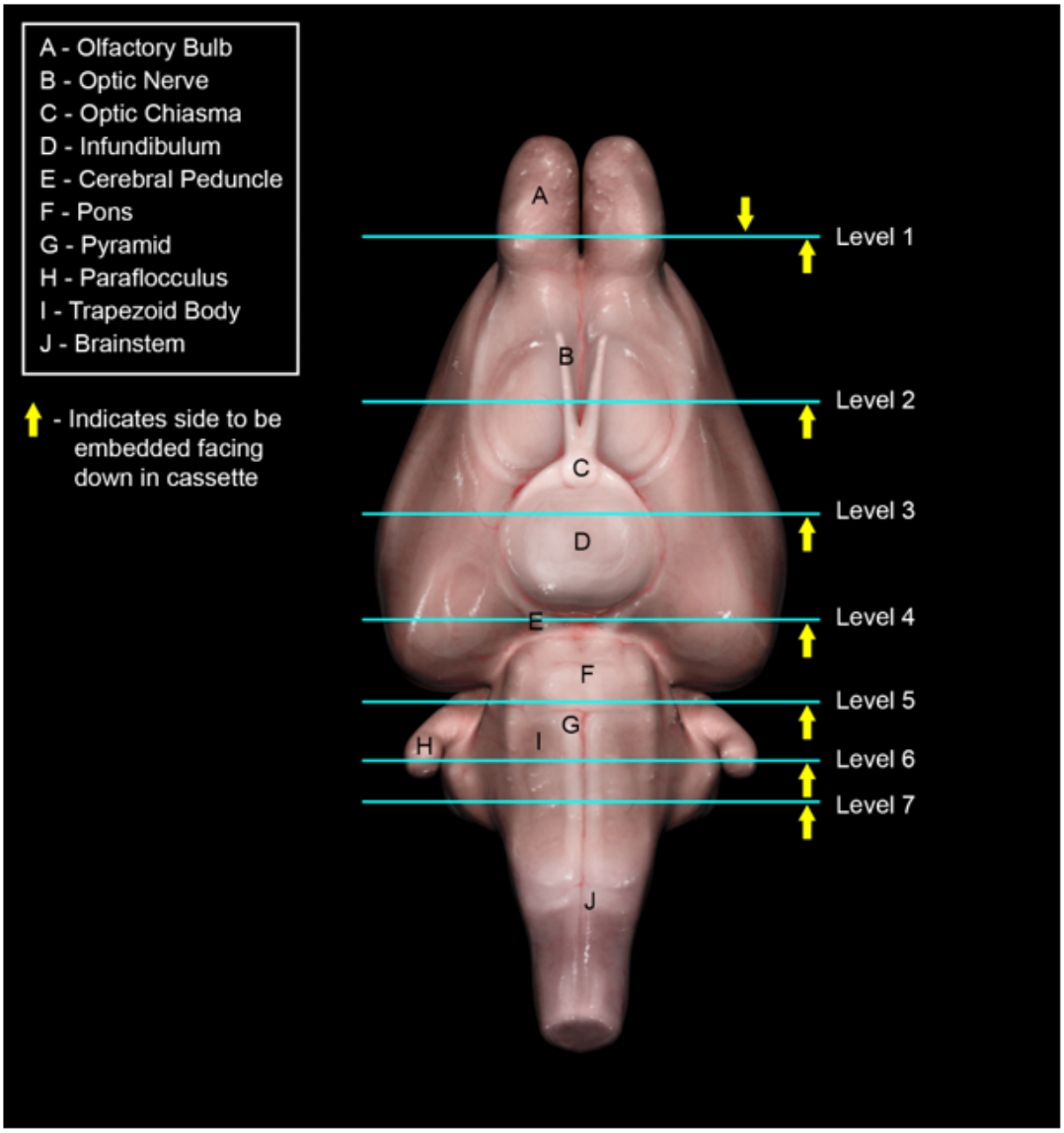
(5) Midpoint of posterior colliculus

(6) Mid-cerebellum at the level of the VIII cranial nerve

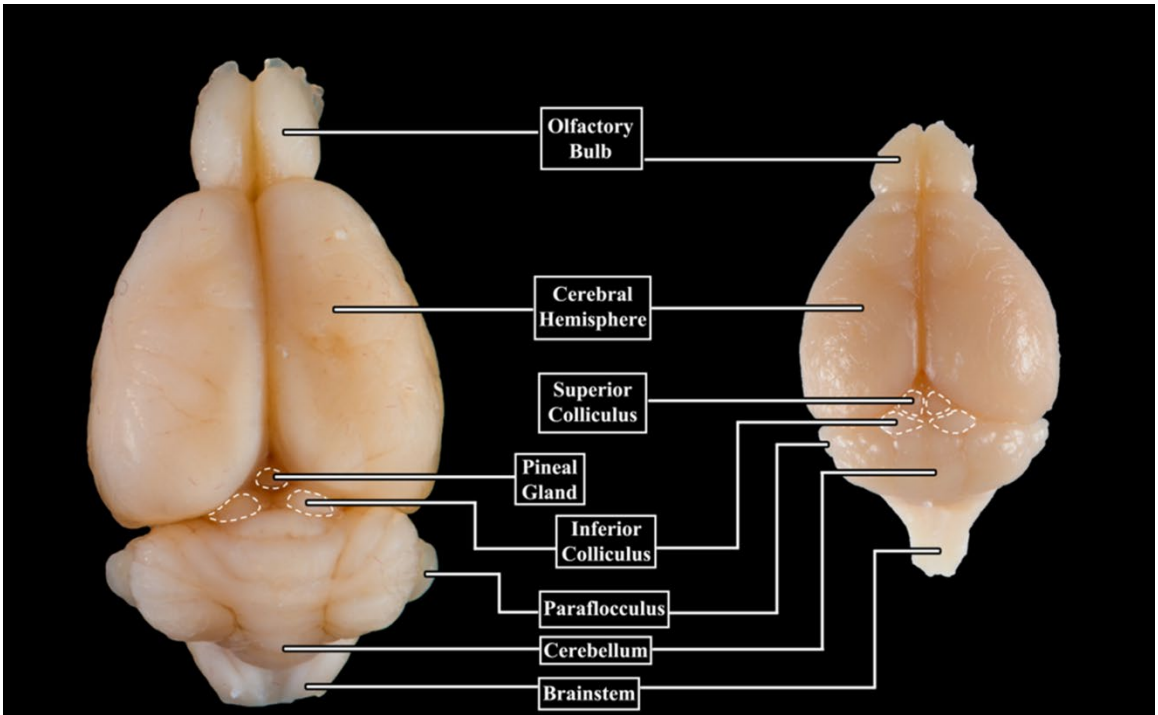
(7) 2–3 mm anterior to caudal termination of the cerebellum (posterior medulla through the area postrema)

If small brains preclude obtaining seven quality sections, a minimum of five slices shall be obtained to include sections 1–4 and 6 as identified above. These shall be placed in the cassettes with the rostral cut surface placed down for embedding and sectioning. If gross lesions are observed during trimming, they shall be noted on the IANR.

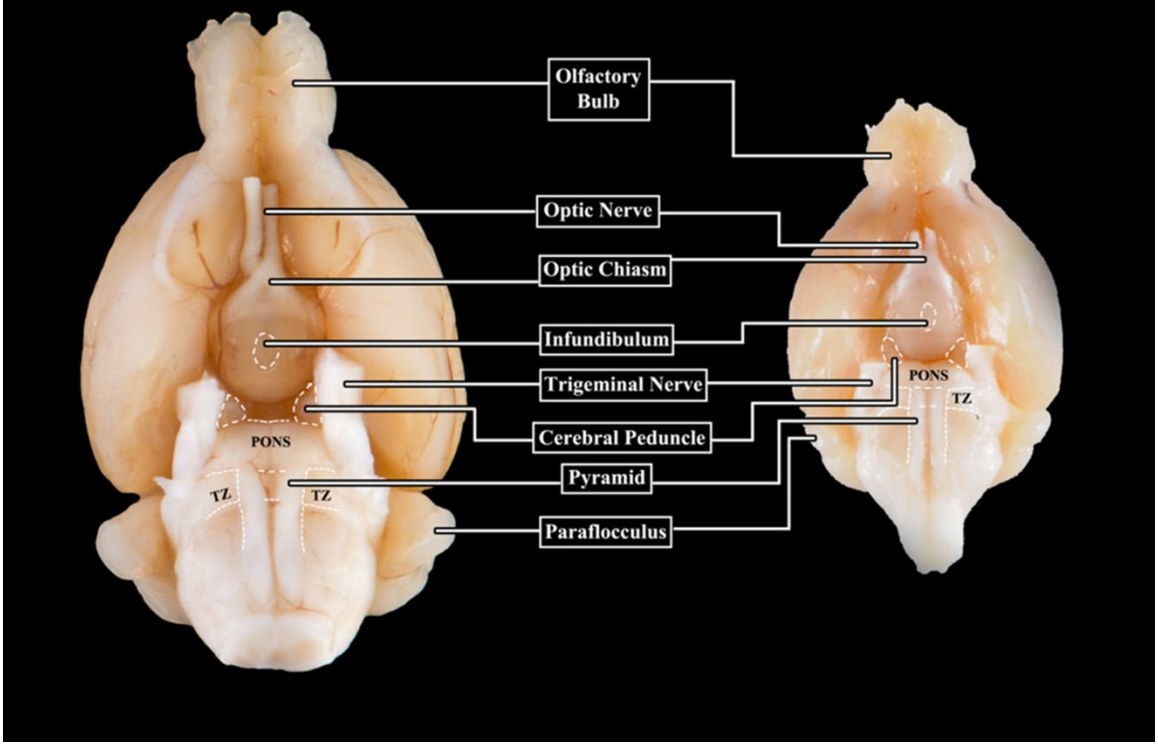
A)


A - Olfactory Bulb
B - Optic Nerve
C - Optic Chiasma
D - Infundibulum
E - Cerebral Peduncle
F - Pons
G - Pyramid
H - Paraflocculus
I - Trapezoid Body
J - Brainstem
- Indicates side to be embedded facing down in cassette
A
B
C
D
E
F
G
H
I
J
Level 1
Level 2
Level 3
Level 4
Level 5
Level 6
Level 7

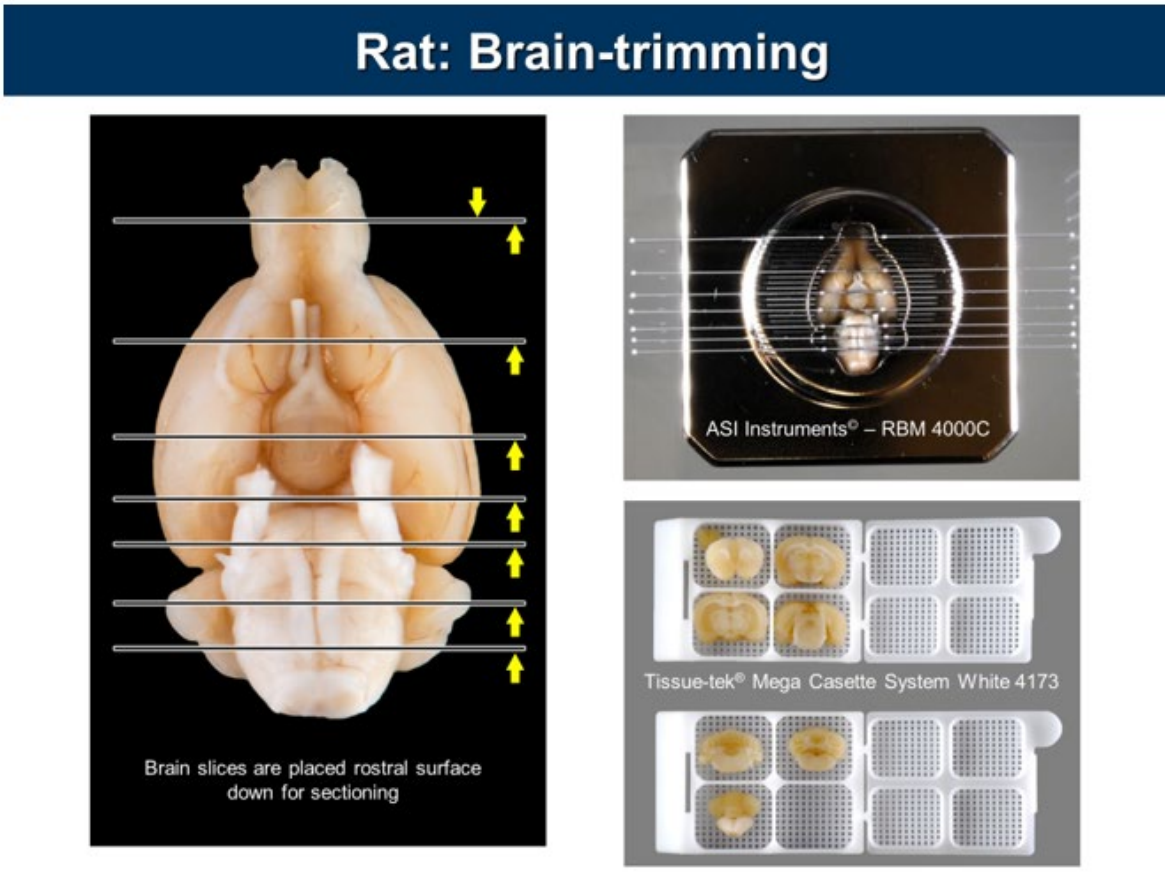
B)


Olfactory Bulb
Cerebral Hemisphere
Superior Colliculus
Pineal Gland
Inferior Colliculus
Paraflocculus
Cerebellum
Brainstem

C)


Olfactory Bulb
Optic Nerve
Optic Chiasm
Infundibulum
Trigeminal Nerve
Cerebral Peduncle
Pyramid
Paraflocculus
PONS
TZ
PONS
TZ
TZ

D)


Rat: Brain-trimming
Brain slices are placed rostral surface down for sectioning
ASI Instruments© – RBM 4000C
Tissue-tek® Mega Casette System White 4173

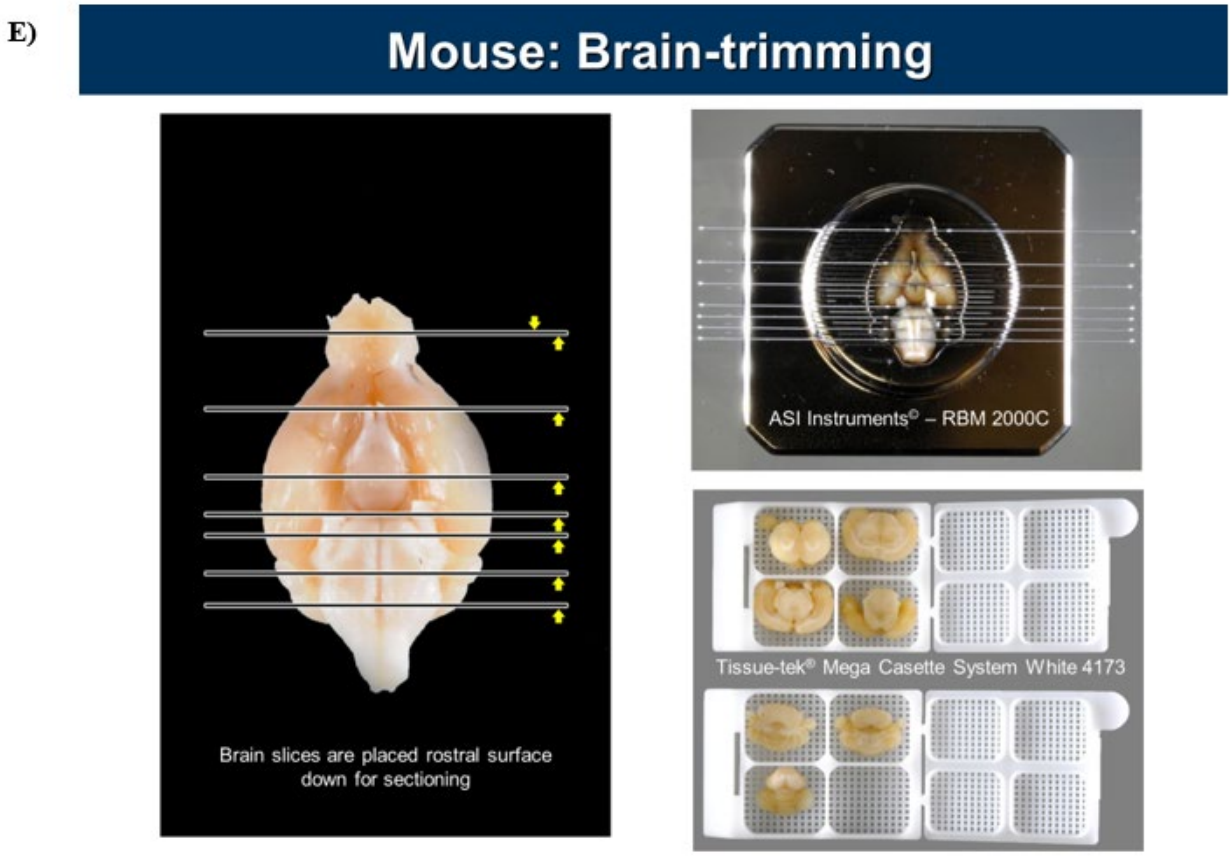


Figure 8-12. (A–F) Trimming and Sectioning Protocol for the Rat and Mouse Brain

8.7.13. Trigeminal Nerve/Ganglion

If a study protocol requires histopathological evaluation of the trigeminal nerve or if neurological signs were observed during the study, the right trigeminal nerve and ganglion shall be dissected from the cranium after fixation and embedded to obtain a cross-section that includes the ganglion (Figure 8-13).

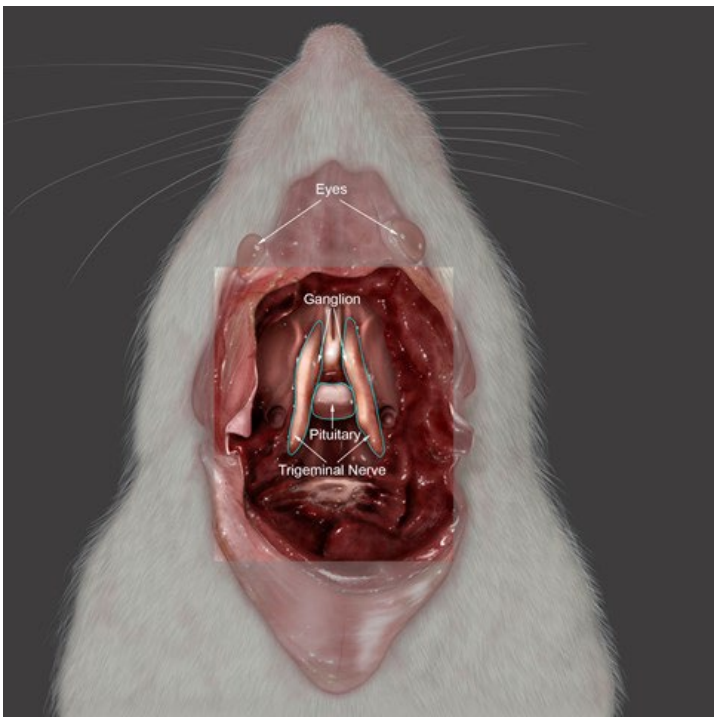


Figure 8-13. Trigeminal Nerve and Ganglion

8.7.14. Spinal Cord

Should a study protocol require histopathological examination of the spinal cord or if neurological signs were observed during the study, the vertebral column shall be decalcified and trimmed to obtain transverse and longitudinal slices (Figure 8-14) through the following:

(1) Anterior cervical segment (C1–C2)

(2) Mid-thoracic segment (T7–T9)

(3) Mid-lumbar (at intumescence) segment (at level of vertebrae L2–L3)

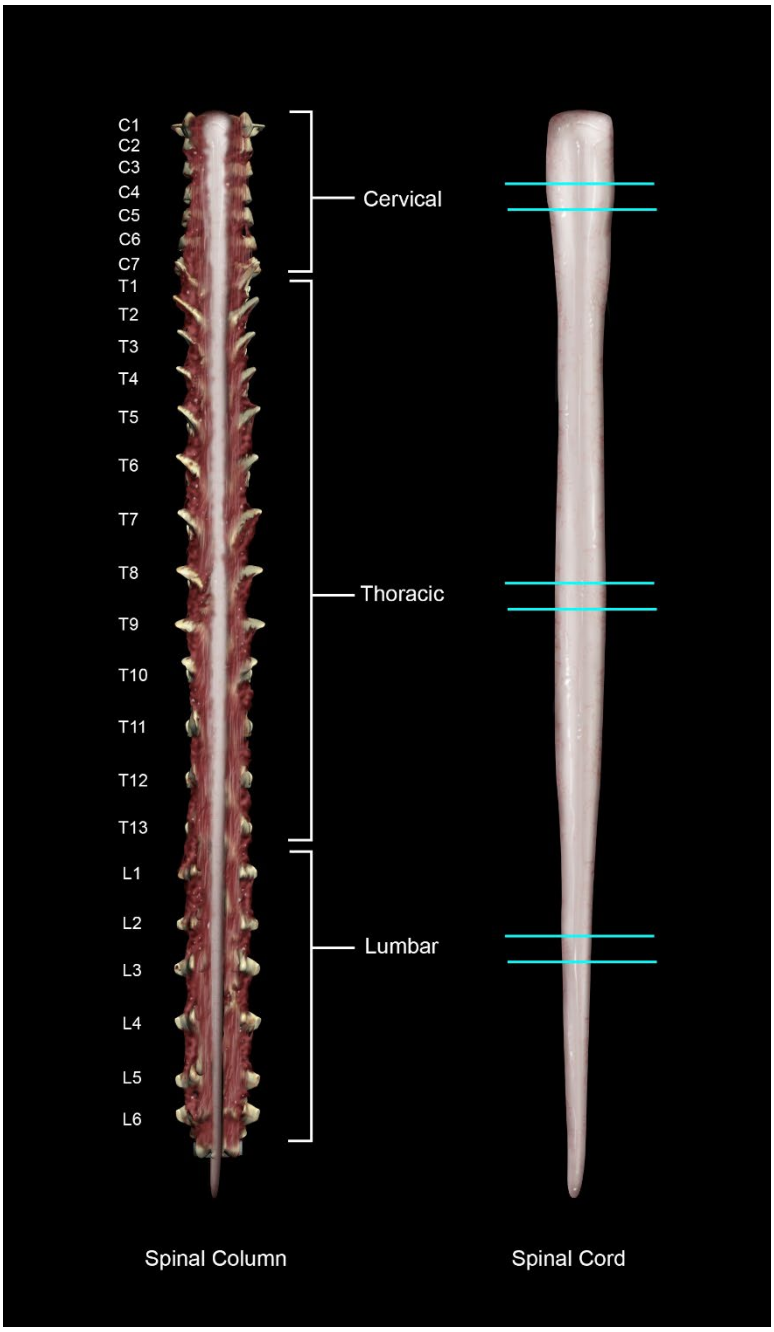


Figure 8-14. Spinal Cord

8.7.15. Skeletal Muscle, Peripheral (Sciatic and Tibial) Nerve

The biceps femoris muscle shall be trimmed to obtain longitudinal and transverse sections.

In standard subchronic and chronic studies, the sciatic and tibial nerves shall be collected and fixed in situ with the muscle. Should an individual SOW or study protocol require histopathological examination of the tibial and sciatic nerves or if neurological signs were observed during the study, the right sciatic (rat and mouse) and right tibial (rat only) nerves shall each be trimmed to obtain one transverse and one longitudinal section at least 0.5 to 0.7 cm (rat)

and 0.4 to 0.5 cm (mouse) in length. Transverse sections shall include associated skeletal muscle to stabilize the transverse plane and to obtain quality sections of the nerves.

8.7.16. Femur and Tibia

After fixation, all muscle and extraneous tissue shall be dissected from the femur and tibia, and both bones shall be decalcified in a mild/gentle decalcification solution, such as Immunocal™, according to product specifications. After decalcification, the shafts (diaphyses) of the femur and the tibia shall be bisected mid-shaft. The distal portion of the femur with the knee joint and proximal portion of the tibia (attached) shall be embedded as a unit to obtain a longitudinal section that includes the distal end of the femur, the knee joint (the articular cartilage and articular surface), and the proximal end of the tibia with the marrow cavity (Figure 8-15).

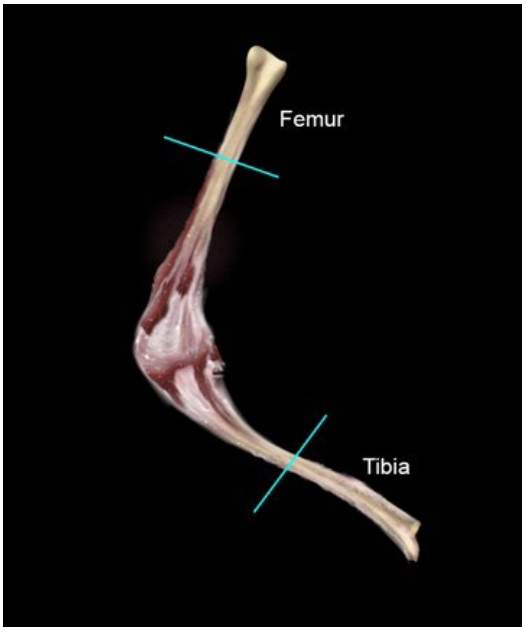


Figure 8-15. Femur and Tibia

8.7.17. Eyes and Harderian Gland

After fixation (with Davidson's fixative), the eyes, with the optic nerve attached, shall be embedded whole and oriented in manner that allows step-sectioning to obtain a longitudinal section through the anterior pole of the globe, the lens, and optic nerve together in one section (Figure 8-16). A small, shallow incision shall be placed in the sclera to allow infiltration of paraffin during processing.

The Harderian glands shall be embedded flat to obtain a longitudinal section through each gland.

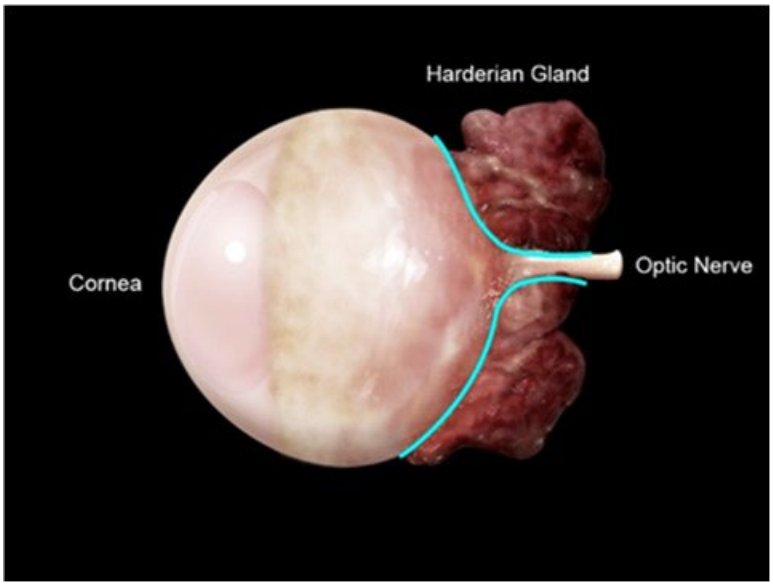


Figure 8-16. Eye and Harderian Gland

8.7.18. Pancreas

The pancreas shall be trimmed transversely through a region that results in the largest area possible for examination. The remaining pancreas shall be embedded flat to provide a longitudinal section.

8.7.19. Salivary Glands

The submandibular and major sublingual salivary glands are closely associated and together constitute an oval dorsoventrally compressed structure in the ventral cervical region. The mandibular lymph node(s) and parotid salivary gland are located at the cranial border of these salivary glands. The left mandibular and left major sublingual salivary gland and the mandibular lymph node shall be embedded flat as a single unit so that all three salivary glands and the mandibular lymph nodes are included in the histological section (Figure 8-17).

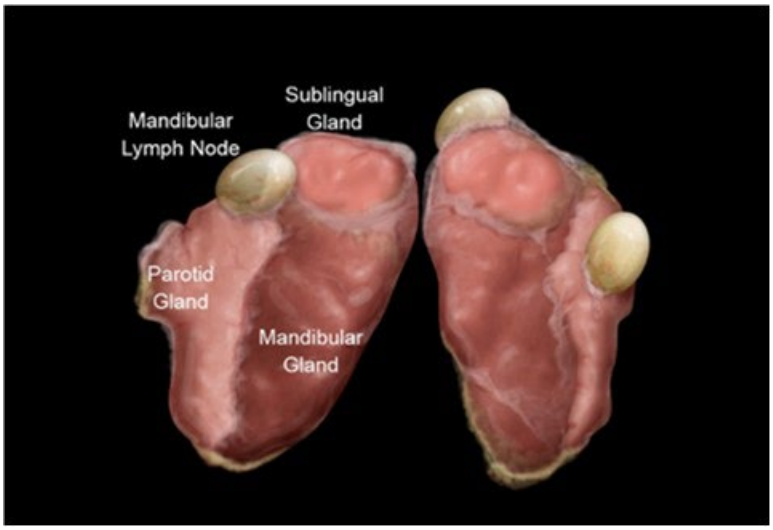


Figure 8-17. Profile of Required Sections of Salivary Glands and Mandibular Lymph Node

8.7.20. Lymph Nodes

Unless grossly enlarged, the protocol requires lymph nodes be embedded whole. The mesenteric lymph nodes shall be embedded as a block with the mesenteric tissue. A section shall be taken through the middle of the longitudinal axis of the lymph node at the area of greatest thickness to obtain the greatest amount of tissue for histopathological evaluation of all major areas (i.e., cortex, paracortex, and medulla). If necessary, large samples can be trimmed on one end to fit in the cassette and to allow for placement on the slide.

8.7.21. Adrenal Glands

Both adrenal glands shall be processed and embedded intact. Histological sections of the adrenal glands shall include the cortex and medulla.

8.7.22. Spleen

A single transverse section of the spleen shall be taken at the largest diameter and thickest area of the organ with the cut surface placed down in the cassette. The remaining (longer) section shall be bisected longitudinally and the cut surface of one half placed down in the cassette (Figure 8-18). If the spleen is diffusely enlarged, for example due to leukemia or lymphoma, either section can be trimmed on one side to allow placement in the cassette and on the slide.

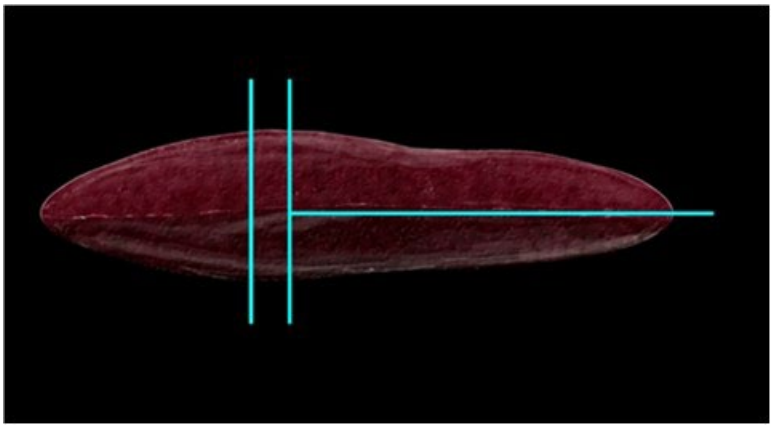


Figure 8-18. Spleen

8.7.23. Thymus

The entire thymus shall be placed in a cassette with the dorsal aspect down to facilitate sectioning. Sectioning shall be done along the longitudinal axis at the **thickest area** of both lobes. This method yields a standardized longitudinal section showing all anatomical structures of this organ.

8.7.24. Urinary Bladder

The urinary bladder shall be trimmed transversely through the body and embedded cut surface down (Figure 8-19).

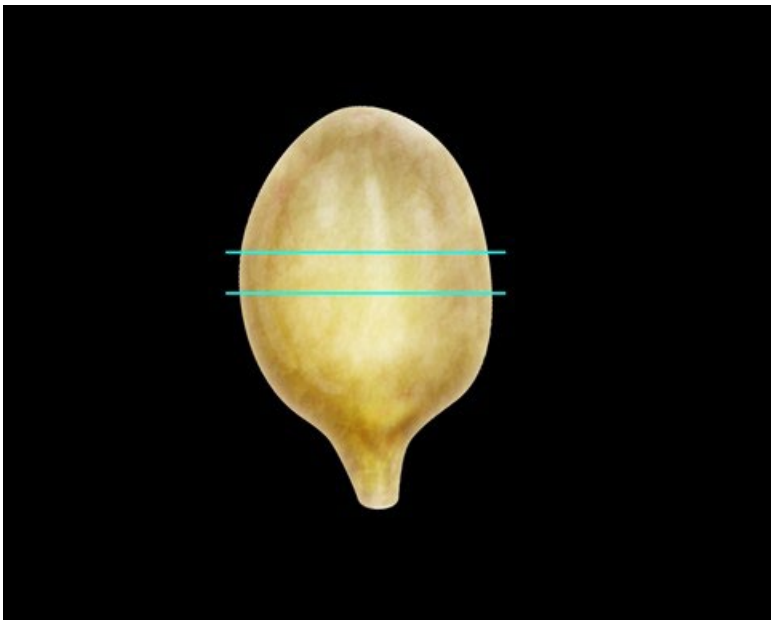


Figure 8-19. Urinary Bladder

8.7.25. Male Reproductive System

Testis

For studies with adult-only exposure, a single transverse cut shall be made through the rete testis approximately one-third distal from the proximal pole, and a second transverse cut shall be made 4–5 mm distal to Cut 1 (Figure 8-20). The resulting slice shall be placed in a cassette with the cut proximal surface down in the cassette for embedding and sectioning (Figure 8-20A, Cut 1 and Cut 2). The remaining slices shall be retained in the wet tissue bags. Additional information can be found in Lanning et al. (2002) and Foley (2001).

For perinatal exposure studies, the testis shall be trimmed to obtain three single transverse slices (Figure 8-20) as follows:

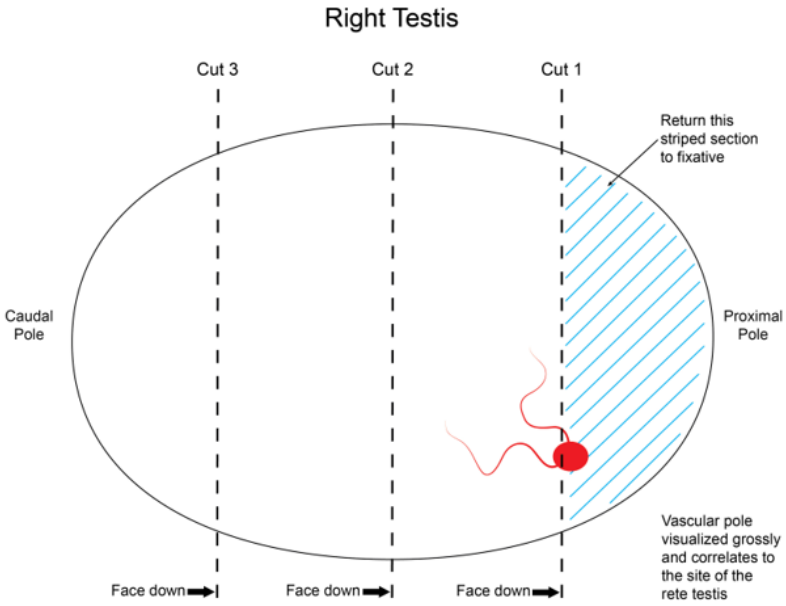
(1) At a point one-third distal from the proximal pole, to include the rete testis (same section as for adult-only exposure)

(2) At the midpoint

(3) At a point two-thirds distal from the proximal pole

All slices shall be placed in a single cassette with the proximal cut surfaces down for embedding and sectioning.

A)



B)

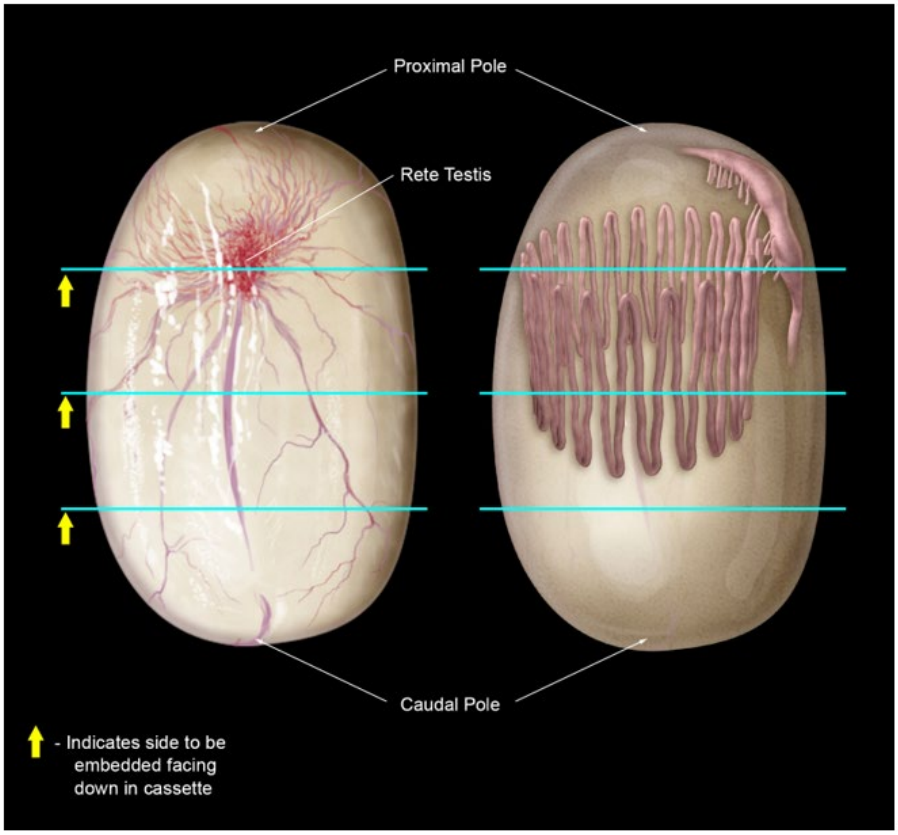


Figure 8-20. (A, B) Profile of Required Sections of Testis

As shown in panel A, trim the testis at the dotted line (Cut 1), approximately one-third distal from the proximal pole to obtain a section that includes the rete testis. Dotted lines (Cuts 2 and 3) provide sections from midpoint and caudal pole.

Epididymis

For the rat, the epididymides (left and right or right only [based on use for other endpoints and as specified by the protocol outline]) shall be bisected through the length of the epididymis (from head to tail through the body) and one ipsilateral half placed flat in the cassette adjacent to the respective left or right testis, such that the histological sections of each epididymis shall include the head, body, and tail.

For the mouse, the intact ipsilateral left and right epididymides shall be placed flat in the cassettes adjacent to the respective left and right testis.

Note: Maintaining the epididymal section with the corresponding testis is of utmost importance to corroborate pathologic findings since abnormal findings in the epididymis typically reflect abnormal findings or events in the ipsilateral testis.

Accessory Sex Glands

Seminal Vesicles and Coagulating Glands

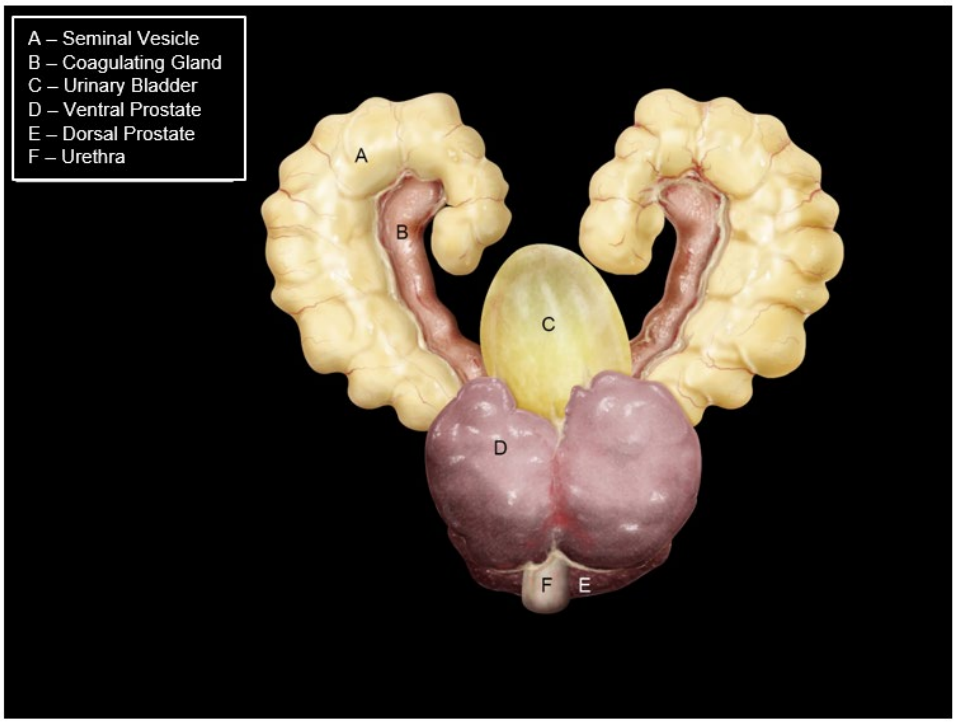
The seminal vesicles with the coagulating glands in situ shall be separated from the prostate glands, placed intact ventral surface down in a cassette for embedding, and sectioned along the longitudinal plane (Figure 8-21A, B) such that both lobes of the seminal vesicle with the coagulating glands attached are visible in the section. If too large to be placed in a single cassette, each seminal vesicle/coagulating gland shall be placed in separates cassettes.

Prostate Gland

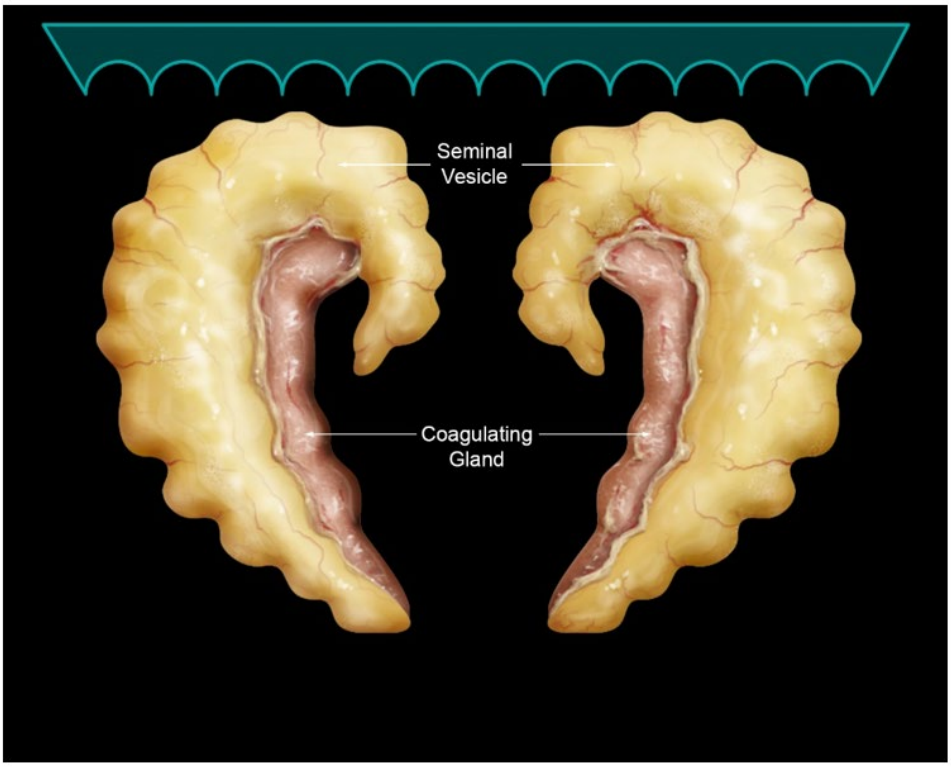
For standard studies, the lobes of the prostate gland shall be gently separated, spread horizontally (in butterfly fashion), placed flat with the ventral aspect down in a cassette for embedding, and sectioned along the longitudinal plane (Figure 8-21A, C) such that all lobes are visible in the histological section. If enlarged, the lobes shall be separated and placed in separate cassettes.

For DART studies, make a mid-transverse section (approximately 4 mm in thickness) of the dorsolateral lobe and ventral lobe of the prostate gland (these lobes were separated for weighing prior to fixation) and embed on the cut surface. Tissues <5 mm in their largest dimension may be embedded whole.

A)


A – Seminal Vesicle
B – Coagulating Gland
C – Urinary Bladder
D – Ventral Prostate
E – Dorsal Prostate
F – Urethra
A
B
C
D
F
E

B)


Seminal Vesicle
Coagulating Gland

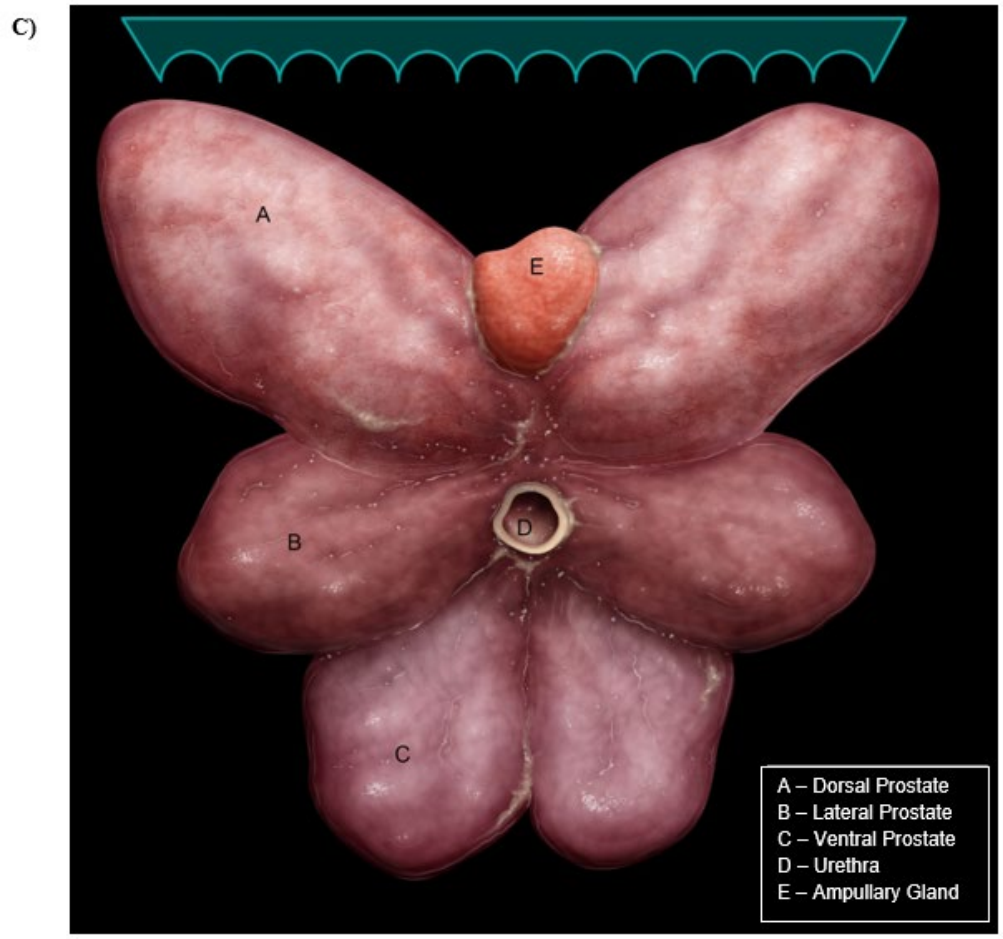


Figure 8-21 (A) Accessory Sex Glands, (B) Seminal Vesicles and Coagulating Glands, and (C) Prostate Gland

Blue outline indicates the plane of sectioning.

Levator Ani Bulbocavernosus Muscle Group

For DART studies, make a mid-transverse section (approx. 5 mm thickness) of the levator ani bulbocavernosus muscle group. If these tissues are <5 mm in their largest dimension, they may be embedded intact.

Preputial Glands

The preputial glands shall be embedded flat so that a longitudinal section is obtained through each gland.

8.7.26. Female Reproductive Organs

Ovaries

The ovaries (with oviducts attached) shall be removed from the uterine horns. The ovaries shall be embedded whole such that they may be sectioned parallel to the long axis. During sectioning, the first full-face section shall be taken at approximately one-third into the ovary to leave enough residual ovarian tissue for ovarian follicle counts should they be required per the study protocol.

Uterus, Cervix, Vagina

Transect the uterine body to separate the uterine cervix and vagina from the uterine body and horns. Place the uterine cervix and vagina in a cassette as a single unit. The uterine horns shall be transected at their midpoint, taking one 4-mm-thick transverse section from each horn and placing both sections in a second cassette. The uterine body with attached portions of uterine horn and the two free portions of uterine horn shall be placed in a third cassette (Figure 8-22).

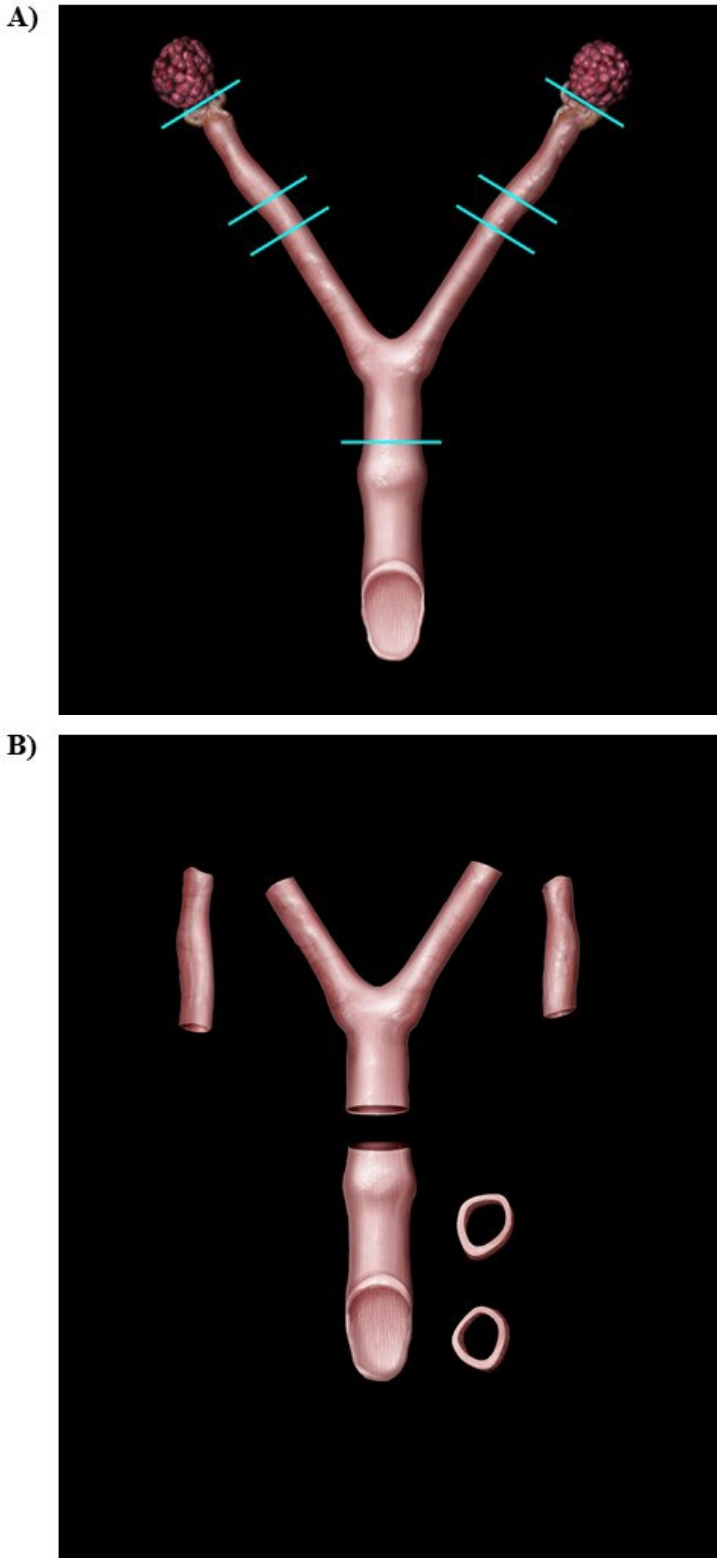


Figure 8-22 (A, B). Uterus, Cervix, and Vagina

Clitoral Glands

The clitoral glands shall be placed in a single cassette and embedded flat so that a longitudinal section is obtained through each gland.

8.7.27. Skin and Mammary Gland

The section of control skin with the mammary gland attached (taken from the inguinal region) shall be kept in a separate labeled cassette to avoid confusion with the skin sampled at the site of application. The section (strip) to be embedded shall be approximately 0.3–0.4 cm × 1.5 cm and shall be embedded cut surface down (on end) in the cassette.

For dermal studies, the strip of control skin is taken in the inguinal area to include the mammary gland. The section of skin taken from the site of application shall be trimmed so that the orientation of the examined section is parallel to the longitudinal axis of the body and embedded cut surface down (Figure 8-23). As specified in the study protocol, a third section of skin (control), at a site distant from the inguinal region and site of application may be requested.

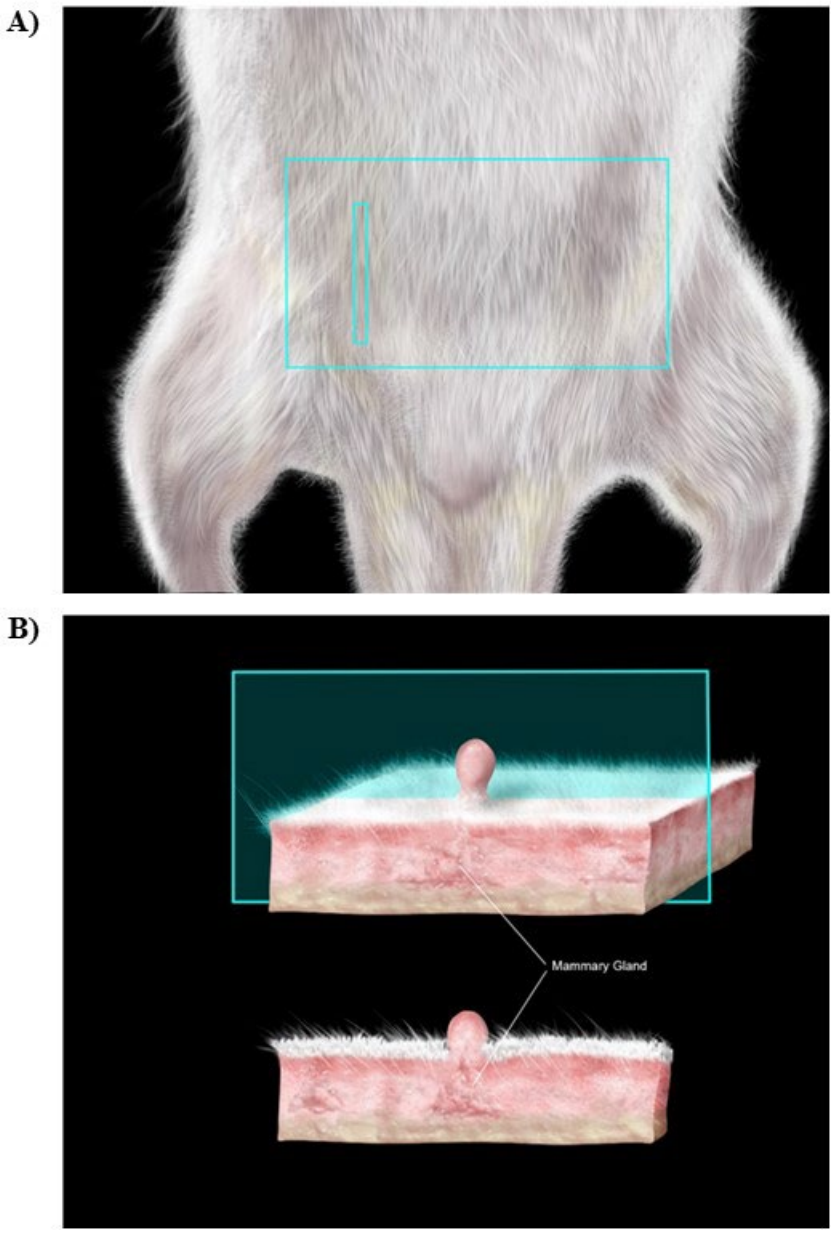


Figure 8-23 (A, B). Skin and Mammary Gland

For mammary glands collected for histopathological examination (right fourth and fifth glands prior to postnatal day 90, fourth only in postnatal day 90 rats), the trimmed sections shall include the region deep to the fourth nipple and the lymph node. These glands shall be sectioned in the dorsoventral plane (i.e., parallel to the animal's body).

For storage, all residual tissues from all animals shall be double bagged and immersed in sufficient 10% NBF to completely cover the tissues. The animal identification label shall be affixed to the inner bag containing the tissues for subchronic and chronic study animals.

8.8. Histology

8.8.1. Tissue Sectioning and Processing

A unique histology number shall be assigned to each animal to be evaluated histopathologically. At the time of assignment, this number shall be entered in a permanent log and cross-referenced with the unique animal identifier.

- This histology number shall appear on the labels placed on the tissue block, on the slides, and affixed to the outer and the inner bags containing the wet tissues. The label on the paraffin tissue block and the labels on the tissue bags shall have the group and animal number, and the name of the testing laboratory. Alternatively, the designated letter code (acronym) for the testing laboratory may precede the histology number. In addition, for each animal, each block shall be linked to the corresponding slide by the same designated sub-number from 1 to n, on each label.
- All slides (including tissue slides, mammary gland whole mounts, cover-slipped vaginal cytology slides, cover-slipped semen evaluation slides, cover-slipped blood smear slides, reticulocyte preparations, and bone marrow preparations, if required by the protocol) shall be labeled using the slide label format presented at the end of this section.

All trimmed tissues shall be processed (dehydrated and infiltrated with paraffin) in an automatic tissue processor (loaded with the appropriate solutions and reagents using a standardized protocol), sectioned, and stained as described below.

After tissues are processed, they shall be embedded in paraffin blocks. The blocking scheme shall be consistent within and across all studies.

All blocks shall be subject to quality control/assessment.

Tissue blocks shall be sectioned at 4–6 microns in thickness. After sectioning, each block shall be resealed using a warm spatula to melt the surface paraffin, or by dipping the block surface in melted paraffin wax.

Tissue slides shall be stained routinely with hematoxylin and eosin (H&E) or other special stains, such as Periodic Acid-Schiff/Hematoxylin (PAS/H) as required by the study protocol or specifications for specific study types (refer to Table 8-2, Table 8-3). All slides shall be stained on the same day to prevent staining variation, after which they shall be covered with glass cover slips. Each slide shall be permanently paper labeled or labeled using an approved alternate method such as an automated slide printer.

All slides, including stained and cover-slipped smears, when required, shall be subject to quality control/assessment before submitted for microscopic evaluation.

Slides shall be compared with the blocks (slide-block match-up) to ensure that all embedded tissues are represented on the slide and that the slide number matches the block number.

A histology processing record shall be completed for each animal for which histology slides are prepared and shall be submitted to the NTP Archives with the IANR. The histology processing record shall include, but is not limited to, the following information:

- Header information to include test article, histology accession number, species, dose group, generation (when applicable), and sex
- List of tissues trimmed, number of cassettes prepared, and verification by trimming technician with initials and date
- List of tissues embedded, number of paraffin blocks prepared, and verification by embedding technician with initials and date
- Number of blocks sectioned, number of slides prepared, and verification by microtomy technician with initials and date
- Number of slides stained and cover-slipped with verification by technician with initials and date
- Number of blocks prepared correspond with the number slides prepared with verification by technician with initials and date
- Blocks and slides match with verification by technician with initials and date
- Number of slides checked out during quality control procedure and verification by technician with initials and date
- Number of block re-cuts and/or wet tissue re-cuts and verification by technician with initials and date
- Notes documenting deviations from protocol, missing tissues, missing gross lesions, problems, and/or comments
- Signature of histology laboratory supervisor indicating review and approval of histology processing record

8.8.2. Format for Slide Labels

- Line 1: Laboratory acronym/pathology subcontractor acronym [if appropriate]/NIEHS [acronyms will be supplied by the program COR]
- Line 2: Study number – test number [supplied by the program COR]
- Line 3: Treatment/treatment group designation and individual animal number – generation and litter designations
- Line 4: Histology number – slide number
- Line 5: Treatment/treatment group, sex, generation and litter designations (where applicable)

Sample Slide Label

BC/NIEHS or BC/PI/NIEHS

05921-01

UF048

881750-9

The treatment/dose group designation (line 5) will consist of a single letter and will be immediately followed by M for male or F for female, which will be immediately followed by the animal number. The letter designations for treatment/dose group are as follows:

Prechronic Studies/Transgenic Studies

V = Vehicle or Chamber Control

X = Untreated Control

P = Positive Control

A = Low Dose/Exposure Concentration

B = Low Medium Dose/Exposure Concentration

C = Medium Dose/Exposure Concentration

D = Medium High Dose/Exposure Concentration

E = High Dose/Exposure Concentration

F–L = High Dose/Exposure Concentration (for studies with up to 12 treated groups)

Chronic Studies

V = Vehicle or Chamber Control

L = Low Dose/Exposure Concentration

M = Medium Dose/Exposure Concentration

H = High Dose/Exposure Concentration

For multigenerational studies, the generation and litter designations shall be separated from the treatment/dose group and sex designations by a dash. The generation shall be identified by the letter F followed by the generation number: 0 = original parental generation, 1 = offspring of F_0 generation, 2 = offspring of F_1 generation, 3 = offspring of F_2 generation, etc. The litter will be identified by a lower-case letter following the generation designation: a = first litter, b = second litter, c = third litter, etc.

Examples (Line 5)

Subchronic Study: CM035 (Medium dose male #035)

Transgenic Study: PF139 (Positive control female #139)

Chronic Study: HF389 (High dose female #389)

Chronic Study: NM235 (High intermediate dose male #235)

MOG Study: BM273-F1b (Male #273 from the second litter of the low medium dose F_1 generation)

8.9. Histopathological Evaluations

One pathologist shall perform histopathological evaluation on all PRTs, including controls for one species for a given test article. It is preferable that the same pathologist conduct the histopathology on all phases of the studies and for both species when possible. If necessary, the histopathological evaluations for a study may be conducted on the females by one pathologist and on the males by another, or on mice and rats by separate pathologist, but the two pathologists shall use similar evaluation criteria (e.g., thresholds or grading schemes) and should be in close communication during their evaluation.

Routinely, complete histopathological evaluation shall be performed for most subchronic toxicology and chronic toxicology/carcinogenicity studies. Complete histopathological evaluation for these studies is generally defined as histological evaluation of the tissues listed below in Table 8-2. For DART studies, routine histopathological evaluation is defined as histological evaluation of the tissues listed below in Table 8-3. In some studies, fewer tissues will be examined at the discretion of NIEHS (refer to the study protocol). In rare cases, the tissue collection protocol shall be customized to the study to answer specific questions (see study protocol).

Table 8-2. Tissues for Complete Histopathological Evaluation (General Toxicity Studies)

Organ/Tissue
Adrenal glands
Brain (seven sections)
Clitoral glands
Esophagus
Eyes w/optic nerve
Femur
Gallbladder (mouse)
Gross lesions
Harderian glands
Heart and aorta
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Kidney
Larynx (inhalation studies)
Liver (two sections including left lobe and median lobe)
Lungs and mainstem bronchi
Lymph nodes
Mandibular and mesenteric (all studies)
Bronchial and mediastinal (inhalation studies)
Mammary gland and adjacent skin

Organ/Tissue
Muscle, thigh (if neuromuscular signs present)
Nasal cavity and nasal turbinates (three sections)
Nerve (if neurological signs present or required by SOW)
Sciatic
Tibial (rat only)
Trigeminal (with ganglion)
Ovaries
Pancreas
Parathyroid glands
Pituitary gland
Preputial glands
Prostate gland
Salivary glands
Seminal vesicles
Skin (collected with the mammary gland) Skin, Site of Application (for dermal studies only)
Spinal cord (three sections, if neurological signs are present or required by SOW)
Spleen
Stomach (forestomach and glandular)
Testis
Epididymis
Thymus
Thyroid gland
Trachea
Urinary bladder
Uterus, including cervix (longitudinal and cross-sections [uterine horns])
Vagina (longitudinal)

Table 8-3. Tissues for Complete Histopathological Evaluation for Reproductive Studies[a]

Organ/Tissue
Adrenal glands
Liver (left and median lobes)[b]
Kidneys[b]
Pituitary gland
Thyroid gland
Right testis[c]

Organ/Tissue
Right epididymis
Dorsolateral prostate
Ventral prostate
Seminal vesicles[d]
Coagulating glands[d]
Paired Cowper's (bulbourethral) gland[e]
Preputial glands[e]
Levator ani bulbocavernosus (LABC) muscle complex[e]
Ovaries
Uterus, including cervix (longitudinal and cross-sections [uterine horns])
Vagina (longitudinal)
Clitoral glands
Retained nipples (if collected)
Mammary gland (tissue section, if collected)
Mammary gland whole mount (if collected)
Gross lesions

[a]The side of each paired reproductive organ shall be recorded for the reproductive organs.
[b]These organs are routinely collected, weighed, and fixed. They are examined histologically on a case-by-case basis if they are known target organs or have gross lesions. Refer to study protocol for specific instructions.
[c]The testes shall be evaluated in a "stage-aware" manner. However, "staging" of the testes should not be performed unless directed by the COTR.
[d]Although the seminal vesicles and coagulating glands are collected together, kept together throughout histological processing, and adjacent to each other on the H&E slides, they are to be treated as separate organs during histopathological evaluation and data entry.
[e]The Cowper's (bulbourethral) glands, preputial glands, and levator ani bulbocavernosus (LABC) muscle complex shall be grossly examined in all necropsied animals. However, they shall be collected, fixed, and examined histologically only if there are other lesions, malformations, or abnormalities consistent with anti-androgenic activity.

8.9.1. Guidance for Histopathological Evaluation

Severity Grades

Severity grades shall generally be applied according to the generic scheme adopted by NIEHS unless a compelling reason suggests otherwise.

Most nonneoplastic lesions should be assigned severity grade. NIEHS uses an ascending four-level numerical scheme for gradable nonneoplastic lesions: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. For a given study, the histological criteria for each grade for each lesion shall be informed by the NTP Nonneoplastic Lesion Atlas[2] (NNLA) and can be established by the study pathologist. The criteria for treatment-related findings and severity grading shall be described in the pathology narrative.

In NIEHS studies, some morphological tissue changes (e.g., cysts) shall not be graded but are routinely recorded as "present" (refer to the NNLA to determine whether a lesion should be

[2]https://ntp.niehs.nih.gov/nnl/index.htm

graded). However, such changes sometimes exhibit treatment-related, toxicologically relevant differences in extent or magnitude, and in these instances, they shall be assigned severity grades rather than being listed as "present."

In some studies, the test article is detectable by light microscopy in target tissues. Test articles (diagnosed as foreign material) can be recorded as "present" when the accumulations appear similar from animal to animal. However, when there are toxicologically relevant, dose-related differences in the degree of test article accumulation, these differences shall be documented with severity grades. Conversely, organisms (bacteria, fungi, protozoan, and metazoan parasites) in tissues shall not be graded for severity regardless of their numbers.

Thresholds

Diagnostic thresholds shall be applied at the discretion of the pathologist(s) evaluating the studies. Spontaneous "background" and/or age-related lesions, such as extramedullary hematopoiesis in the spleen or mononuclear cell infiltrates in the Harderian gland or liver, are common in rats and mice. In many studies, such incidental lesions have similar incidences in control and treated groups and lack toxicological relevance. In such instances, diagnostic "thresholds" can be established, in which only occurrences with severity above a predetermined threshold are diagnosed and recorded. Thresholds are implemented and encouraged for lesions such as inflammation, extramedullary hematopoiesis, thymic cysts, and other lesions that are not treatment related. In some studies, common, background, age-related lesions can exhibit treatment-related, toxicologically significant differences in incidence and/or severity. In these situations, the lesions should be diagnosed and graded without a threshold.

The following lesions should always be evaluated without a threshold to provide historical data for the following lesions:

(1) All neoplasms
(2) Preneoplastic lesions, with or without a corresponding neoplastic response
(3) Treatment-related increases or decreases in severity, i.e.,
    a. Islet cell hyperplasia
    b. Pancreas, acinar cell, hyperplasia
    c. Glial hyperplasia
    d. Gliosis
    e. Focus of altered hepatocytes
    f. Thyroid, follicle, epithelium, hyperplasia
    g. Thyroid, follicular cell, hypertrophy
    h. Thyroid C-cell hyperplasia
    i. Cardiomyopathy
    j. Schwann cell hyperplasia, endocardial/myocardial
    k. Chronic progressive nephropathy
    l. Liver, hepatocyte hypertrophy
    m. Liver, hepatocyte hyperplasia

n. Liver, hepatocyte necrosis
o. Alveolar/bronchial hyperplasia
p. Squamous metaplasia
q. Endometrial stromal polyp
r. Atypical hyperplasia, endometrium
s. Inflammatory polyp
t. Oncocytic hyperplasia, kidney
u. Meningeal hyperplasia, brain

Other lesions may be added to this list as determined by NIEHS.

These parameters are determined under the judgment of the study pathologist. Determination of lesions that might be related to treatment can be discussed with the NIEHS pathologist.

Read Downs

A read-down approach is often used in short-term studies. When this approach is utilized, histopathological evaluation of the PRTs shall be performed on all control animals, all animals in the highest exposure group with at least 60% survivors at study termination, plus all animals in the higher exposure groups. Exposure-related lesions (target organs) shall be identified and examined in lower exposure groups to a no-effect level.

For studies with chronic exposure, or as specified in the study protocol for shorter studies, histopathological evaluation of protocol-required tissues shall be conducted in all animals from control and exposure groups, unless otherwise directed by the study protocol.

In all studies, whether or not a read-down approach is used, gross lesions observed at necropsy or during trimming and all tissues from all early death animals (i.e., those found dead or subjected to moribund sacrifice) shall be evaluated microscopically.

Informed Evaluation (Non-blinded)

For the initial histopathological evaluation, NIEHS recommends using an informed analysis (non-blinded) approach. The rationale and benefits of this approach have been summarized by Sills et al. (2019).

In some instances, a blinded approach is appropriate for histopathological evaluation, particularly during the review of subtle lesions or for common background lesions in which only an exposure-related change in severity is observed. In these cases, a select sample of slides can be reviewed in a blinded fashion to verify diagnoses and define a no-effect level.

8.10. Recording of Results

All pathological findings for each animal shall be entered into a computerized data management system, such as Provantis, to record raw data generated during studies.

The electronic IANR shall be used to record necropsy and trimming observations. Descriptive narratives at necropsy shall be provided for all animals. The number and description of tissue lesions shall be included. This information shall be recorded electronically for all animals. In the

event of a computer failure, the information may be recorded on paper and transcribed into an electronic record. At completion of the necropsy, the IANRs shall be signed and dated by the necropsy prosector and the attending pathologist.

The IANR shall record the following information:

- Test facility, study number, personnel information: all personnel (prosector, trim technician, pathologist, etc.) shall be identified as providing the applicable information below for each animal, and the time and date for each activity shall be recorded.
- Exposure: test article, dose group, route, days on test, death date.
- Animal: species/strain, sex, individual animal number, unique histology number, body weight at death/removal.
- Death: disposition (scheduled removal, moribund, natural death, dosing accident, etc.) and condition (fresh, autolyzed, cannibalized, etc.).
- Organs (as specified in the protocol): organ weights and appearance; if appearance of an organ at necropsy or trim is considered to be abnormal a TGL shall be recorded.
- TGLs shall be numbered sequentially for a given animal and the following information shall be provided: organ, site, morphology, size, distribution, color; after processing to slides, the corresponding slide number for each TGL shall be recorded for correlation during histopathological evaluation.
- Observations: the probable cause of death (PCOD) based on histopathology review and other applicable clinical observations shall be recorded.

The pathologist or data clerk shall use the computer terminal to record all microscopic findings.

- Histopathological diagnosis of all lesions shall be entered under Organ and Diagnosis. Indicate primary versus metastatic neoplasms (e.g., (1) liver hepatocellular carcinoma; (2) lung, hepatocellular carcinoma, metastatic).
- Using terminology primarily from the NNLA, International Harmonization of Nomenclature and Diagnostic Criteria[3] (INHAND), standardized vocabulary and nomenclature based on the two main references of pathology of the rat and mouse (Maronpot et al. (1999) and Suttie et al. (2018) textbooks endorsed by NIEHS), and the NIEHS-leased Provantis Pathology Code Table (PCT).
- Designated nonneoplastic lesions shall be graded using a four-grade system of minimal, mild, moderate, and marked. Sponsor-approved nomenclature shall be used in the Provantis lexicon.
- All gross abnormalities shall be correlated with a microscopic evaluation where applicable.

[3] https://www.toxpath.org/inhand.asp

8.11. Collection and Storing Tissues for Molecular Pathology

8.11.1. Frozen Tissue Collection

The following information is supplied for sampling, freezing, and storage of tissues for later analysis of RNA, DNA, and protein analysis, if required by the study protocol. (Analyses shall be conducted at a sponsor-specified laboratory or at laboratories at DTT/NIEHS)

When frozen tissue samples are collected for RNA, DNA, and protein assays, it is important to recognize that the speed of tissue collection is critical because sample integrity decreases rapidly following euthanasia. Therefore, all samples shall be collected and processed within a maximum of 5 minutes after euthanasia. When several tissues need to be collected from each animal, the tissue collection should be prioritized based on the levels of endogenous RNases in various tissues. The order of tissue prioritization for collection based on the level of endogenous RNases (from highest to lowest) should be pancreas, gastrointestinal tract, spleen, lung, liver, thymus, kidney, heart, and brain. It may be necessary to have specific designations for necropsy staff (such as prosector, recorder, frozen tissue processor) to streamline and expedite sample collecting and processing/freezing for each animal/tissue/necropsy station. Testing laboratories shall configure the necropsy and sample-collecting process in a manner that optimizes collection of these samples within the 5-minute timeframe especially for the first 4 organs listed above. The total time should not exceed 10 minutes for the frozen collection of remaining organs. Staggering of necropsy is strongly recommended to ensure proper collection of frozen tissue from each animal in a study. The testing laboratory shall document the time taken to collect frozen samples.

The frozen tissues should be collected without cross-contamination with other tissues. Clean disposable scalpels and forceps should be used when cutting different tissue types from the same animal or from different animals. Contact should be avoided with absorbent materials that may contaminate dissected tissues or capillary action that may draw fluid from tissue samples. Thorough cleaning of all instruments using RNaseZap, 70% ethanol, and water in that sequence twice and then wiping with a clean gauze is recommended to prevent cross-contamination due to nondisposable surgical instruments.

Tissue samples shall be quickly removed from the animal. Small organs such as ovary, adrenal gland, and pituitary shall be placed directly into cryotubes. Larger organs shall be minced into approximate 5 × 5 × 5 mm cubes while kept cold and frozen as quickly as possible after collection. Frozen tissue shall be placed in RNase-free, DNase-free, pyrogen-free 5-ml screwcap containers (with external threads and lip seal) that shall be permanently labeled with the appropriate information (see Section 8.5). The cryovials (filled to no more than 80% capacity to allow for tissue expansion during freezing) are then weighed to the nearest milligram and quick frozen (not longer than 5–10 minutes after the time of euthanasia) in liquid nitrogen. These cryovials may be placed in a CoolRack® that is located in a Coolbox or Styrofoam box and partially filled with LN2 to submerge half of the CoolRack. In about 30 seconds, the tissues within the cryovials will be frozen. Filled cryovials that are to be stored >1 year should be transferred to a liquid nitrogen container. For samples to be stored ≤1 year, samples may be kept in a −80°C freezer.

Filled containers shall be placed immediately in liquid nitrogen and then transferred to a −120°C freezer if samples are to be stored for longer than 1 year. Samples to be stored for <1 year shall be stored in a −80°C freezer.

In some cases, NIEHS may request that harvested tissue specimens shall be immediately immersed in a tissue reagent (such as "Allprotect Tissue Reagent" or another similar product) for stabilization of RNA, DNA, and protein. Use of such agents eliminates the need for dry ice or liquid nitrogen.

8.11.2. Frozen Tissue Requirements

Study-specific requirements will be included in the study protocol. General requirements are provided here as guidance.

In general, for all studies less than a chronic exposure, after the collection of tissues for routine pathology, the additional remaining left lateral lobe of the liver (and left kidney or right caudal lobe lung, if they are target organs) from all animals from all dose groups shall be frozen. In rare instances of tumor incidences in short-term studies, the sample collection shall be similar to the frozen tissue collection as described for the chronic studies.

For chronic studies, masses must be sufficiently large enough to permit tissue to be fixed for routine histopathological evaluation with enough remaining tissue for molecular biology studies. All spontaneous and chemical-induced tumors larger than 5 mm in diameter shall be collected for genomic analysis. For each organ bearing tumors, adjacent nontumor tissues shall also be collected. In addition, tissue from other organs without tumors, such as the tail and ear, shall also be collected to serve as genomic controls as outlined in the instructions below.

For the five largest tumors in each organ, up to five cryovials shall be collected, one tumor per vial. Representative normal tissue (if available) shall be collected using the same number of vials as tumor vials.

If the tumors are <5 mm and are diffusely distributed (i.e., miliary) throughout the organ, then representative tumors with adjacent normal tissue shall be collected for histology and up to five tumors with adjacent tissue shall be collected for freezing.

If there is a solitary tumor <5 mm in any organ, then the entire tumor shall be collected for histology only.

For all frozen samples collected above, representative histological sections must be made. Each tissue with a tumor >5 mm should be cut in half with one half frozen and the other half processed for histology.

Two histological sections immediately adjacent to all the collected frozen tissues (with the exception of tail and ear) shall be prepared to document the microscopic pathology of the frozen tissue (one slide accompanies the frozen tissue and the other slide accompanies the paraffin block). These instructions apply to all animals sacrificed due to moribund condition or during the scheduled interim or final study termination.

8.11.3. Cryovial Labeling and Packaging

The cryovials shall be labeled with permanent ink to ensure that the labeling will not be lost during storage in liquid nitrogen, during storage at −70°C, or during shipping on dry ice. The labeling shall be unique to the animal and to the specific sample (e.g., "tumor-only" tissue, adjacent nontumor, distant normal tissues as well as tail snip and ear punches to serve as genomic controls). If a given animal has two or more masses, each mass and nontumor tissue shall be uniquely identified. The unique number for each sample, which will include a letter code designating the laboratory (BC = Battelle Columbus; SO = Southern Research Institute, etc.) and a sequential number, shall be included on the cryovial. On subsequent days, numbering shall be resumed where numbers stopped on the previous day. This system ensures each sample/number will be unique for the study. Each cryovial shall be placed in a 5 in. × 5 in. box with a grid in sequential order using the unique number. The outside of each box shall be labeled with unique numbers.

8.11.4. Frozen Tissue Documentation

Each study laboratory shall assign a pathologist to be responsible for ensuring that the frozen tissues are collected from all masses (treated and control), adjacent nontumor tissue, and normal tissue. In the notes section of the IANR, the collection of each frozen tissue sample, as well as the sacrifice time and freezing time, shall be recorded. A photocopy of the IANR from each animal shall be sent to the NTP Archives and Frozen Tissue Bank with the frozen and histological samples. The NTP Archives Frozen Tissue Sample Collection Form shall be used to record all tissue collection information. Once sample collection has been completed and the study has been terminated, an Excel spreadsheet shall be prepared for each species/strain. This Excel spreadsheet shall contain information on the sample collection, such as date and time of necropsy, time from necropsy to sample freezing, sample/specimen weight, unique sample IDs, and other relevant study details. The comments section of the spreadsheet shall be used to provide additional information clarifying sample collection issues, such as explanations for time to freezing >5 minutes, why adjacent nontumor or normal tissue was not collected, identifying the source of the normal tissue if taken from another lobe or bilateral organ, etc. Each page of the spreadsheet shall be signed indicating that the spreadsheet is an accurate representation of the samples collected for the study. A hard copy of the Excel spreadsheet and an electronic copy (CD) shall accompany the frozen samples when shipped to the NTP Archives. A hard copy of the spreadsheet shall be retained in the study file.

In cases when the laboratory was unable to meet the requirements of the frozen tissue collection protocol, the study laboratory shall document why it was unable to do so. For example, *"the tumor effaced the adjacent nontumor tissue and therefore nontumor tissue was not obtained from a specific animal."*

8.11.5. Preparation of Histological Material

A tissue sample shall be collected immediately adjacent to the collected frozen tissue samples (with the exception of tail and ear) to determine the histopathology of the frozen tissue samples. These tissues shall be fixed for 18–24 hours in 10% NBF and then transferred to 70% ethanol and processed into paraffin blocks within 2 weeks. Two H&E-stained slides shall be prepared for each "tumor-only" tissue, nontumor sample, and normal tissue. One of the H&E-stained slides shall be shipped to the NTP Archives and the second slide plus the paraffin blocks shall be

retained by the laboratory and included with the remainder of the study histologic material for shipment to the NTP Archives.

8.11.6. Shipping Frozen Tissues and H&E Slides

Frozen samples shall be shipped to the NTP Frozen Tissue Bank (FTB). The cryotubes shall be placed in a 5 in. × 5 in. moisture-repellant, cryo/freezer boxes with cardboard grid dividers and placed in sequential order using the sample number. The outside of the box shall be labeled with permanent ink to show the phase of the study, the treatment and group, the generation, group numbers, animal numbers and the name of the testing laboratory. Frozen samples shall be shipped on dry ice in boxed Styrofoam containers. A sample inventory list must accompany the shipment. The inventory list must be approved by the COR prior to shipment. One to 2 days prior to shipment of the frozen samples and associated paper records, the FTB coordinator shall be notified by phone and by email to establish the time of shipment and expected arrival at the FTB. Samples shall be shipped by overnight delivery on Monday, Tuesday, or Wednesday to ensure that FTB personnel are on hand to receive the specimens.

8.12. Quality Control of Pathology Activities and Data

Quality control of pathology activities and data must include, but is not limited to, the following procedures.

8.12.1. Histology and Histotechnique

Before slides are given to the pathologist for evaluation, all slides must be examined to ensure that full-face sections of the required tissues are present on each slide, that staining of the tissue is optimum, and that the tissue sections have a minimum of artifacts, such as folds, knife marks, air bubbles, chatter, and shrinkage. Histology records for all animals shall be audited to ensure that all PRTs and gross lesions have been sectioned or otherwise accounted for, and that sections and slides have been prepared according to provided guidelines.

8.12.2. Residual Wet Tissues

After all slides have been prepared (e.g., all study PRTs including gross lesions have been trimmed, embedded, sectioned, and stained), the residual wet tissues must be reviewed for the presence of untrimmed lesions and for animal/carcass identification. A 10% random sample of animals of each treatment group shall be examined according to the following guidelines.

- All residual tissues from animals in the random samples shall be examined by a pathologist or a histology technician experienced in tissue trimming for untrimmed masses/nodules that are potential neoplasms. If any are found, they shall be confirmed by a pathologist and then residual tissues from all animals of that sex and species must be examined for untrimmed lesions.
- Note: In some organs, such as the liver of rats with mononuclear cell leukemia, determining whether small nodules are neoplasms requires considerable scientific judgment and experience. It is imperative that over-sampling and biased sampling of organs does not occur as a result of poor judgment in making these determinations, which is why a pathologist must confirm the presence of untrimmed potential neoplasms before animals are sampled or additional histological sections are

performed. All potential neoplasms found as a result of this quality control review shall be trimmed and sections prepared for microscopic examination by the designated study pathologist.

- The residual tissues from all animals in the random sample shall be examined for verification of animal/carcass identification. The identifying markers (tails, or other) shall be compared with the bag identification label. If any discrepancy exists, all animals of all treatment groups including controls of that particular sex and species are to be examined. Discrepancies shall be reported to the program COR and an attempt shall be made to resolve the discrepancies, but bags shall not be relabeled.

The completed IANR forms (paper or electronic equivalents) for all animals shall be reviewed for thoroughness of completion, documentation consistency, conformance to NIEHS specifications, and for correlation of gross observations with histopathological diagnoses and agreement with preferred terminology. If any discrepancies exist, the IANR forms shall be returned to the study pathologist or other appropriate personnel for correction of problems prior to auditing by the testing laboratory quality assurance unit and subsequent submission to NTP Archives.

In rare circumstances, if the computer entry of histopathology data is performed from a written worksheet, the worksheet shall be retained within the study records and confirmation of its accurate transcription shall be documented on the worksheet and in the computerized data management system. If computer entry of histopathology data is performed from an audio recording, all entries must be confirmed by a second person (who can be the study pathologist). A record of the primary entry shall be retained, and confirmation of its accurate transcription shall be documented with the IANR in the computerized data management system.

The histopathology reports must be reviewed within the testing laboratory to confirm the correct header information, the correct selection of PRTs, and the pathology data entry.

After the study pathologist completes the first evaluation of the slides from all animals in all treatment groups, including controls, the pathologist shall examine the pathology summary tables for positive or negative trends in the incidences of neoplastic or non-neoplastic lesions, and for redundant terminology or inappropriately formatted diagnoses. The pathologist shall reexamine the tissues for which there is a significant positive or negative trend from all animals (by sex/species) to confirm the initial findings. If redundant terminology is present in the pathology tables, diagnoses must be changed to consolidate the data in an appropriate manner.

The study pathologist shall consult DTT pathologists for guidance on the use of preferred terminology for diagnoses and diagnostic terminology.

8.13. Submission of Pathology Data (Slides, Blocks, and Wet Tissues)

The pathology submission to the NTP Archives shall consist of all materials and records generated during the conduct of the study. These may include tissue slides, blocks, wet tissues, cytology slides, semen evaluation slides, hematology slides (if blood smears or bone marrow smears are prepared), slide inventory, histology processing records, IANRs if paper forms were used, and notification that the pathology is complete.

All slides (including tissue slides, mammary gland whole mounts, cover-slipped vaginal cytology slides, cover-slipped semen evaluation slides, cover-slipped blood smear slides, reticulocyte preparations, and bone marrow preparations, if required by the protocol), blocks, wet tissues from all animals shall be retained unless otherwise specified until completion of the prechronic or chronic study and submission of the final reports. Histopathology materials shall be organized, packed, marked, and shipped prepaid to the NTP Archives as directed below.

- Prior to shipping materials, the inventory of residual material must be completed. A separate inventory shall be submitted for the prechronic and chronic studies. The number of slides and blocks, as well as the condition of wet tissues, shall be recorded on this form.
- In addition to the separate inventory of residual histopathology materials provided for each prechronic and chronic study, the scheme or SOP used to identify the animals in each study (including an appropriate figure or diagram) shall be submitted at the time that wet tissues are sent to the NTP Archives.
- Blocks and slides shall not be shipped on the same day. The preferred procedure would be to ship the blocks first followed by the slides.
- A letter of intent to ship showing how many boxes of each type of pathology samples/material(s) and shipment date(s) shall be directed to the NTP Archives with a copy to the program COR and Pathology Coordinator at least 7 calendar days in advance of shipment. This letter is required to aid in tracing lost or misdirected shipments.

8.13.1. Wet Tissues

- All residual, fixed animal tissues shall be double bagged at the trimming station, packed in animal number and treatment group order by sex, and shipped to the Archives after completion of the study.
- Wet tissues (residual from harvested tissues) from each animal shall be stored in a clear, heat-sealable plastic bag appropriate for storing biological tissue samples; heat sealed inside another similar bag to prevent leakage; and organized by sex, species, group, and animal number. A permanent ink label (not ballpoint pen) showing the study number, testing laboratory, group number, and animal number shall be affixed to both bags. A similar label shall be placed on the external surface of the outer bag. All wet tissues, including mouse carcasses, shall be shipped to the NTP Archives. Once the bags are organized, they shall be packed in two layers, separated by a cardboard divider, in double-wall cardboard boxes (350 lb.-test/51ECT) approximately 15 in. × 18 in. × 7.5 in. with a plastic liner in each box. The boxes shall be marked on one end to show:
    - Per the OSHA Formaldehyde Standard and the OSHA Hazard Communication Standard, labels are required for materials containing formalin. A label containing appropriate hazard warnings is to be placed inside the box on each container with formalin. A material safety data sheet (MSDS) for formalin is required to be sent to the receiver for the initial shipment and does not have to be included in the box with the wet tissues but can be sent under separate cover. The MSDS is not required to be sent with

each subsequent shipment from a testing laboratory, only when there is a change in the information in the MSDS.

- These boxes shall be sealed shut and bound with filament tape and shipped promptly to the NTP Archives following submission of the final report or when otherwise specified. Special handling procedures may be required in extreme weather conditions.

8.13.2. Blocks

- Blocks shall be resealed with a warm spatula or the surface dipped in warm paraffin and organized by histology number. Blocks shall be labeled or permanently marked with a testing laboratory's letter code and the histology number.
- When histopathology is complete, the residual material, including blocks and slides prepared during prechronic and chronic evaluations, shall be prepared for shipment to the NTP Archives. Blocks shall be placed in animal order by treatment group into single-wall cardboard boxes the size of approximately 80 blocks. Rows of blocks shall be separated by cardboard dividers; in case of partial boxes, spacers will be used to maintain the order of the blocks, and then these smaller boxes (7.5 in. × 9 in. × 1.75 in.) shall be taped and placed into double-wall cardboard containers (350 lb.-test/51ECT) approximately 15 in. × 18 in. × 7.5 in. All boxes shall be marked on one end to show the same information as indicated for wet tissues.
- Shipping cartons shall be sealed and bound with filament tape for shipment. Special handling procedures may be required in extreme weather conditions.

8.13.3. Slides

- All tissue slides, stained and cover-slipped blood smears, cytology slides, etc. from all phases of the studies shall be organized by species, treatment group, and animal number, and sent to the NTP Archives when specified or upon completion of the study and submission of the final report. Unstained blood smears those that are not cover-slipped shall not be shipped to the NTP Archives.
- For shipment, the slides shall be placed in plastic slide boxes with "bubble pack" and taped shut. These plastic slide boxes shall be placed in double-walled cardboard boxes (350 lb.-test/51ECT) 15 in. × 18 in. × 7.5 in., separated by abundant packing material, for shipment to the NTP Archives. An inventory listing shall accompany the shipment. Slides sent separately in the slide set will be counted as present in the inventory. A copy of the slide set inventory shall accompany the major inventory document.
- Each plastic shipping box shall be marked to show the phase of the study, the treatment, treatment group/animal numbers, and the name of the testing laboratory.
- Each cardboard box shall contain a packing list identifying the name of the testing laboratory, the number of slide boxes, and the cross-reference information (e.g., animal identification numbers, histology numbers, and study numbers), which will allow complete identification of the contents.

- Each testing laboratory shall be responsible for purchasing all supplies for the shipment of residual material to the Archives.

8.14. Release of Slides

Histological slides prepared routinely to support these studies shall not leave the testing laboratory's facility without the specific permission of the program COR. If it is necessary to remove slides to obtain assistance in their interpretation, an inventory sheet shall be prepared and placed in the suspense file until these slides are returned to the testing laboratory's slide file.

If it is desired to use sample tissues from these studies for workshops or other purposes for which the slides would have to leave the facility, the testing laboratory shall first obtain permission from the program COR. After permission is obtained, the testing laboratory shall prepare a separate set of slides and label them in the prescribed manner adding the words "Study Set." Because slides such as these are not used to make diagnoses, the slides shall not be shipped to the NTP Archives upon completion of the study. The program COR and the head of the NIEHS Pathology Group shall determine what will be done with these slides.

8.15. References

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8.16. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapters 7 and 8 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was charged to:

1. Peer review the following chapters 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 7: Clinical Pathology
    - Chapter 8: Anatomic Pathology
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

Peer Reviewer

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9. Fetal Examinations and Vaginal Cytology

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9.1. Prenatal Study Necropsy

9.1.1. Caesarian Section

Dams or does shall be euthanized before expected parturition (the morning of gestation day [GD] 21 for rats, GD 18 for mice, and GD 29 for rabbits) or as defined in the test article-specific Protocol Outline. Rats, mice, and rabbits shall be euthanized in a manner consistent with guidance provided in Chapter 6 (Laboratory Animal Medicine and Toxicology). Dams or does shall be examined and any abnormalities in the maternal thoracic or abdominal viscera recorded. Collection of tissues related to gross observations shall be conducted as described in the study protocol.

The uterus and ovaries shall be excised and weighed, and the number of corpora lutea in each ovary counted and recorded. The uterus of animals that do not appear pregnant shall be examined for nidations (implantation sites) by staining with 10% ammonium sulfide (or another suitable agent, such as Prussian blue).

The fetuses shall be removed from the uterus and their membranes as soon as possible after the dam is euthanized. The number and location of each implantation shall be recorded. The status of each implant site (live or dead fetus, early or late resorption) shall be recorded. The number of implantations shall be compared with the number of corpora lutea to determine pre-implantation loss. Corpora lutea shall be enumerated grossly at necropsy using a dissecting microscope.

- An early resorption is a conceptus where organogenesis has not been completed and is characterized by a grossly necrotic mass that has no recognizable fetal form; nidation sites (“pregnant by stain”) are also considered early resorptions.
- A late resorption is defined as one in which organogenesis has been completed and is characterized by a grossly necrotic but recognizable fetal form with visible placental remnants.
- A live fetus is pink in color and responds to stimulation.
- A dead fetus is a term fetus, often pale to tan in color and is not responsive to stimulation but does not demonstrate marked autolysis. Fetuses with marked autolysis are considered late resorptions.

Fetuses shall be separated from the placenta by cutting the umbilical cord, and any membranes shall be removed. The fetuses shall be blotted dry, individually identified, and individually weighed. Placentas shall be examined for abnormalities in appearance. Collection of tissue related to gross observations shall be conducted as described in the study protocol.

Dams euthanized moribund, delivered early, or found dead shall receive a gross necropsy, including an examination of the thoracic and abdominal viscera for evidence of dosing trauma, toxicity, or gross lesions. The uterus shall be examined and stained, if necessary, to determine pregnancy status. The animal shall be discarded without further examination.

9.1.2. Fetal Exams

Euthanize fetuses prior to visceral examination (e.g., oral administration of sodium pentobarbital). Confirm death by the absence of respiratory movement, absence of response to external stimuli, and/or body color change. Each fetus shall receive an examination as described below. Examination findings shall be recorded in accordance with the computer data system library (or other COR-approved process).

For rodent and rabbit dose range-finding studies:

- Fetal weights (live fetuses only) shall be obtained, and external examinations shall be conducted.
- The external examination shall include all body surfaces (including orifices) and appendages, and the mouth shall be opened and examined for cleft palate.
- The sex of each fetus shall be determined by inspection of the anogenital area for rodents and internally for rabbits (internal confirmation for rodents when it is in doubt).

For definitive rodent and rabbit studies:

- All fetuses shall be subjected to external, visceral, and skeletal examinations.
- Visceral examinations shall be conducted under a dissecting microscope.
- The sex of each fetus shall be confirmed by internal examination of the gonads.
- Approximately 50% of each litter shall have the head removed just below the exoccipitals, leaving the cervical region intact. The isolated head should be fixed in Bouin's solution (rodents) and subjected to examination by serial razor blade sectioning. Alternative methods for fixation may be required by the sponsor or proposed by the testing laboratory for review and approval by the contracting officer's representative (COR). Heads from all rabbit fetuses will be examined by sectioning the skull and brain at the level of the frontal-parietal suture and examining the brain in situ.
- The remaining carcass shall be processed for skeletal examination (along with the other half of the litter with skulls attached), and fetuses shall be individually identified in a manner (e.g., tag, container coding) so they can be identified throughout processing, reading, and archiving.
- All skeletons shall be double-stained with Alcian blue and alizarin red (Ovchinnikov 2009) and stored in an appropriate manner (the staining procedure shall be optimized for a respective lab and the methodology approved by the COR).
- Fetal skeletal exams shall be conducted under a dissecting microscope.

The external examination shall include fetal weight (live fetuses only) and all body surfaces (including orifices) and appendages, and the mouth shall be opened and examined for cleft palate.

The visceral examination and evaluation shall include an assessment of color, size, shape, and position of:

- The abdominal viscera, including the organs of the digestive system (intestines, stomach, pancreas, spleen, liver [and gall bladder, except rats]), urinary system (kidneys, ureters, urinary bladder), adrenals, and reproductive system (for the female: ovaries and uterus; for the male: testes and epididymides) (kidneys shall be sectioned at the hilus and the renal papilla examined)
- The following thoracic viscera: thyroid, trachea, esophagus, thymus, lungs, diaphragm, and greater vessels of the heart (right and left subclavians and carotids, innominate, pulmonary arch, pulmonary artery, vena cava, aortic arch, aorta, and the ductus arteriosus)
- The following aspects of the heart and vessels:
    - the shape and position (with any abnormalities recorded)
    - the aorta, pulmonary artery, descending aorta, innominate (brachiocephalic), subclavian, common carotid arteries, and the ductus arteriosus
    - the internal anatomy of the heart, including the aortic, tricuspid, mitral, and pulmonic valves, as well as the interventricular septum

The skeletal examination shall include:

- The bones of the skull from approximately 50% of the fetuses: premaxillae, maxillae, nasals, frontals, parietals, interparietal, supraoccipital, exoccipitals, zygomatics, squamosals, and mandibles
- The axial skeleton: vertebrae (centra and arches), sternebrae, and ribs
- The pectoral girdle and pelvic girdle:
    - dorsal scapulae and clavicles
    - ilia, ischia, and pubis
- The forelimbs and hindlimbs:
    - humerus, radius, ulna, carpals, metacarpals, phalanges
    - femur, tibia, fibula, tarsals, metatarsals, phalanges

For fetal head examinations from approximately 50% of the fetuses (Thompson 1967):

- A ventrodorsal section (mouth and bisecting both ears) shall be made and the following tissues examined: tongue, palate, upper lips, and lower jaw.
- A frontal section anterior to the eyes shall be made and the following tissues examined: nasal septum, nasal sinuses, and palate.
- A frontal section that bisects the eyes shall be made and the following tissues examined: olfactory lobes of the brain, posterior areas of the nasal septum, nasal sinus

(nasopharyngeal cavity), palate, and fetal eye (including optic cup, retina, vitreous chamber, lens, and cornea).

- A frontal section posterior to the eyes (middle of the parietals) shall be made and the cerebral hemispheres (including the third and lateral ventricles) examined.
- Sections shall be retained in a manner in which each fetal head may be reassessed.

During the fetal examinations, all unusual (notable) observations shall be recorded using a sponsor-approved data collection system. The observations subsequently shall be deemed "malformations" or "variations," as per established criteria and designations [consistent with Makris et al. (2009)]. Historical control data are essential (along with concurrent controls) to determine the designation and occurrence of the findings in the context of normal background. In general, malformations are considered incompatible with—or severely detrimental to—postnatal survival (e.g., ventricular septal defect, exencephaly, diaphragmatic hernia). Variations are nonlethal and not considered detrimental to postnatal survival (e.g., reduced ossification of fore- and hind paws).

9.2. Vaginal Cytology Evaluation

9.2.1. Background

Many studies performed serve to identify target organs for toxicants. An important part of this strategy is evaluating the reproductive system. Microscopic evaluation of the types of cells present in vaginal smears has long been used to document the stages of the estrous cycle in laboratory rats and mice and as an index of the functional status of the hypothalamic-pituitary-ovarian axis. As such, assessment of the estrous cycle has been used both as a principal measure in determinations of reproductive cyclicity and as an ancillary test in reproductive toxicological studies (Goldman et al. 2007). Estrous cyclicity is a proven measure of female fecundity; prolonged cycles correlate with reduced litter size (Chapin et al. 1997).

Other female reproductive endpoints, such as reproductive organ weights, fluctuate with the estrous cycle stage; thus, weights are a less-than-useful endpoint for study-day-driven necropsies as females will be scattered through the cycle and weight variances will be high. Additionally, ovarian follicle counts, while specific, are a poor use of resources when exposure occurs in adulthood because (a) a small proportion of compounds has been shown to affect this parameter and (b) the data are exceedingly tedious and laborious to collect.

This relationship between estrous cycle stage and vaginal luminal cells (in guinea pigs) was first reported by Stockard and Papanicolaou (1917). Later, Long and Evans (1922) published on the vaginal cytology of rats and Allen (1922) on the vaginal cytology of mice. Much of the information regarding the evaluation of vaginal cytologies in rats and mice can also be found in the recent publication by Cora et al. (2015).

A detailed description of techniques to be used by the sponsor-designated laboratories to conduct vaginal cytology evaluations in toxicity studies properly and uniformly is provided in this document. The program COR for the toxicology testing laboratory will designate the dose levels for these studies. For vaginal cytology, 10 female rats or mice in each of three of the five dose groups, plus the control group(s), will be used. Specific selection of the three dose groups will be

based on the results obtained during the first 70 days of the subchronic studies. The objective is to select doses that are not causing overt toxic effects (e.g., mortality, depressed weight gain).

9.2.2. Evaluation in Mice and Rats

The following section is adapted from Cora et al. (2015).

9.2.2.1. The Estrous Cycle

The typical estrous cycle in rats and mice lasts 4 to 5 days, but 6-day cycles are encountered occasionally. Many environmental factors influence cycle length and include the light:dark cycle, age, noise, stress, social interactions (e.g., group housing), and whether a male rat or mouse are in the room. The estrous cycle consists of the four consecutive stages called proestrus (P), estrus (E), metestrus (M), and diestrus (D).

9.2.2.2. Cells of the Rat and Mouse Estrous Cycle

The stages of the estrous cycle are identified by the absence, presence, or proportion of the four cell types (Figure 9-1) described below. The relative changes in the cells present directly reflect the circulating levels of estrogen and progesterone secreted by the ovaries. Cell density and the arrangement of the cells in the smear also might aid in identifying the stages. The following descriptions of the cell types relate to dry-fixed smear preparations stained with a Romanowsky-type stain (e.g., Wright-Giemsa, modified Wright's) or Toluidine blue.

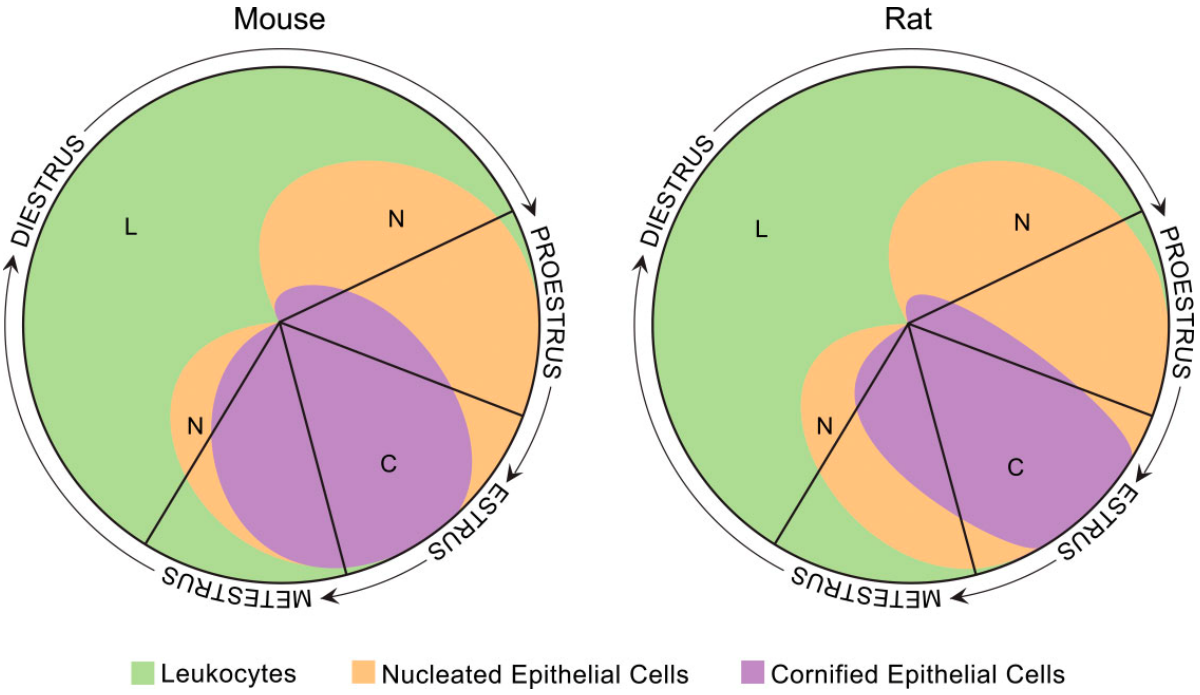

Figure 9-1. Estrous Cycle Wheels

These diagrams are visual representations of the cell types and relative proportion of each cell type present during the four stages of the estrous cycle in the mouse and rat. The size of each quadrant does not directly correlate to the length of each stage. From Cora et al. (2015); adapted from Byers et al. (2012)

Neutrophils (Figure 9-2). Neutrophils are also known as leukocytes or polymorphonuclear cells. These cells are very small and round and possess a multi-lobulated nucleus. During processing,

they can sometimes condense or "ball up," and have the appearance of a dark round dot. On higher power and with scanning of the smear, condensed neutrophils can usually be identified as such. Neutrophils are also relatively delicate and can partially rupture during collection or processing. The evaluator should be familiar with the appearance of condensed or ruptured neutrophils for accurate interpretation of vaginal smears.

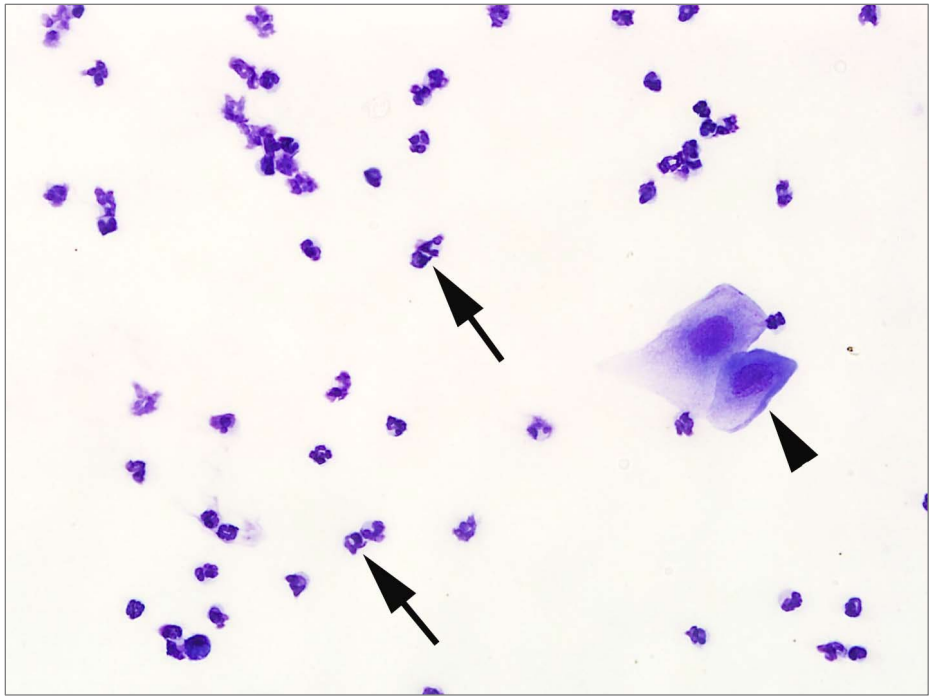

Figure 9-2. Appearance of Neutrophils in Vaginal Smears

Numerous neutrophils (arrows) are present. Note their small size in comparison with the large nucleated epithelial cells (arrowhead).

Small Nucleated Epithelial Cells (Figure 9-3). These cells are small and round to oval and have a round nucleus and blue cytoplasm. They can stain very darkly, precluding visualization of the nucleus. Small epithelial cells of proestrus may contain small cytoplasmic vacuoles.

Large Nucleated Epithelial Cells (Figure 9-2, Figure 9-4). These cells are round to polygonal and have smooth, jagged, or irregular borders. They possess moderate or abundant amounts of blue cytoplasm. Their nuclei could be intact, degenerate, or pyknotic. Large epithelial cells can have some degree of keratinization.

Anucleated Keratinized Epithelial Cells (Figure 9-4). Anucleated epithelial cells are aged cells with abundant blue to sky-blue cytoplasm and jagged or angular edges. As the name infers, they lack a nucleus but can sometimes contain a pale, round area where a nucleus once existed (a ghost nucleus).

9.2.2.3. Cytology of the Estrous Cycle

The cellular composition of the four basic stages of the estrous cycle is described below. Most smears can be evaluated with a 10X objective, but a 20X or 40X objective could be needed (for example, to verify the presence of neutrophils). Sole use of higher objectives is not

recommended because they prevent one from appreciating the overall impression of the smear. It is important to evaluate the whole smear because cell types and numbers can vary throughout the sample.

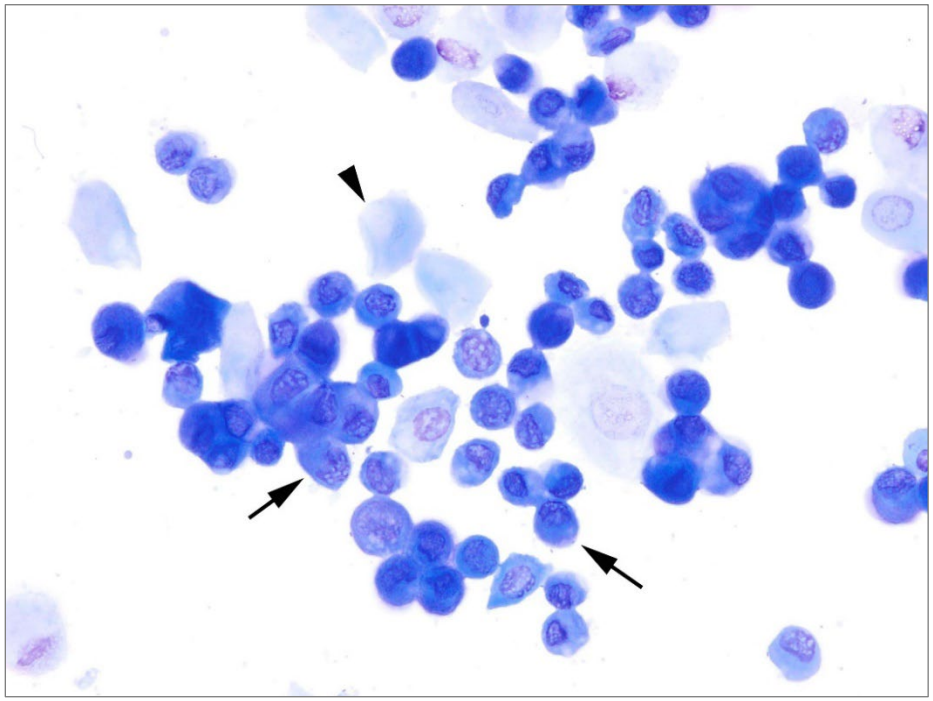

Figure 9-3. Appearance of Various Epithelial Cells in Vaginal Smears, Proestrus

Small nucleated epithelial cells (arrows) with a few anucleated epithelial cells (arrowhead) from a proestrus vaginal smear of a rat.

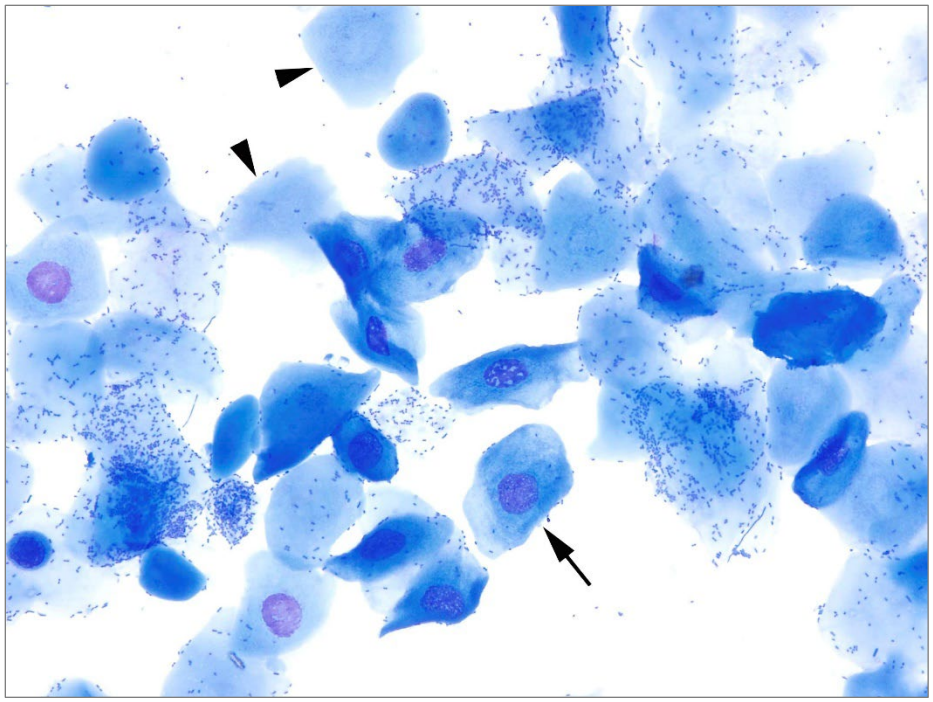

Figure 9-4. Appearance of Various Epithelial Cells in Vaginal Smears, Late Estrus

Large nucleated epithelial cells (arrow) and nucleated epithelial cells (arrowheads) from a vaginal smear of a rat in late estrus.

Evaluating all the collected days of an individual before making a diagnosis for each individual day can be important because it gives one a basic impression of how the rat or mouse is cycling and helps put each day into context. Care should be taken not to introduce bias and misidentify an abnormal stage as normal.

Although **relative** cell density can aid in defining a stage, it should **not** be used in isolation because it can vary independent of the actual stage of the estrous cycle. Collection technique, compliance of the rat or mouse during collection, and preparing more than one smear for each collection day can all affect the cell density of the smear.

The estrous cycle is a dynamic process with stages that are not all the same length—some lasting <24 hours. As such, some stages might be "missed" when sample collection occurs at the typical interval of every 24 hours.

One general approach to staging vaginal smears is to first assess the presence of neutrophils. Are there a good number of them or just an occasional one? In general, if neutrophils are a dominant feature or consistently observed, the stage is either metestrus or diestrus; if neutrophils are rare to absent, the stage is proestrus or estrus.

Proestrus (Figure 9-3, Figure 9-5, Figure 9-6). Proestrus is a relatively short stage that averages 14 hours in rats and <24 hours in mice. It is characterized by the presence of small nucleated epithelial cells of relatively uniform size and appearance. These cells can sometimes stain deeply basophilic. Other times, the small epithelial cells can take on a delicate or wispy appearance, especially in low-cellularity smears. The small epithelial cells can be observed in cohesive clusters (so-called "grape" clusters), sheets, or strands. **However, the observation of cohesive**

clusters, sheets, or strands should not be considered a prerequisite in the determination of proestrus as they might not always be visible, especially in low-cellularity samples. Neutrophils are usually not observed but can be found in low numbers in early proestrus as the rodent would have recently transitioned out of diestrus. Low numbers of large nucleated and anucleated epithelial cells might also be visible throughout proestrus, and, as the cycle approaches estrus, anucleated keratinized cells will become more abundant. The presence of low numbers of neutrophils, large epithelial cells, or anucleated epithelial cells does not preclude the recording of a proestrus stage when the predominating feature of the smear is the observation of high numbers of small epithelial cells.

Estrus. Estrus averages between 24 and 48 hours in rats and 12 and 48 hours in mice. Keratinized anucleated epithelial cells heavily predominate during estrus. Numerous bacteria might be adhered to the cells or scattered throughout the background of the smear. Low numbers of nucleated epithelial cells might be observed throughout estrus. Neutrophils are absent, although rare-to-occasional neutrophils might be seen toward the end of estrus as the rodent begins to transition to metestrus; once neutrophils are consistently visible throughout, the smear should be interpreted as metestrus.

Although consisting mostly of anucleated epithelial cells, the first and second “phases” of estrus differ in their appearance and also differ between the two species.

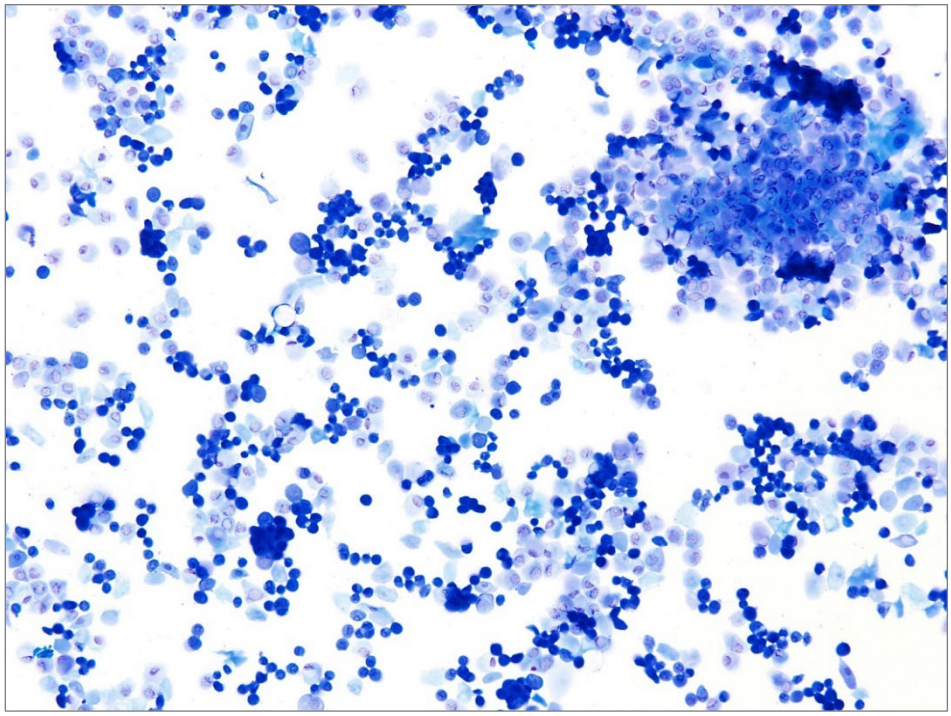

Figure 9-5. Proestrus: Example 1

Small, round nucleated epithelial cells, sometimes in clumps, predominate. No neutrophils are present.

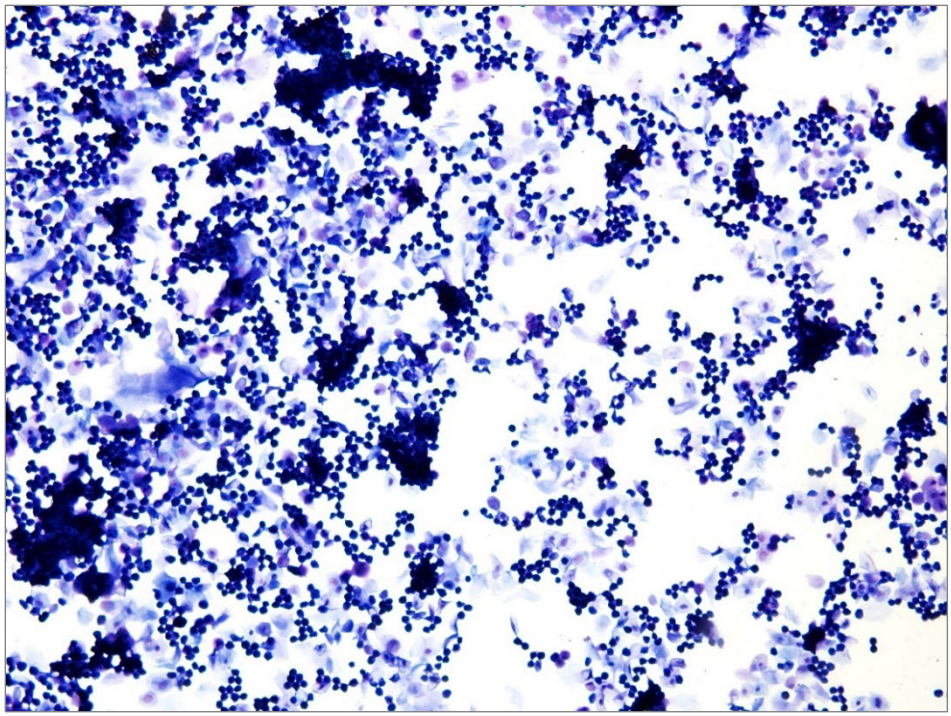

Figure 9-6. Proestrus: Example 2

Clumps of darkly stained, round, small epithelial cells predominate.

Rats (Figure 9-7, Figure 9-8, Figure 9-9, Figure 9-10). In rats, the so-called late estrous "phase" is distinctly different in appearance from the rest of the stage; however, it is a short phase, so it might not always be observed. Late estrus is characterized by the emergence of high numbers of small and large nucleated epithelial cells interspersed among the anucleated cells. The nucleated cells are smooth to irregular and might be round, oval, or spindle-shaped and sometimes stain deeply basophilic. The phase of late estrus should not be mistaken for proestrus. Evaluating the previous day's smear should help avoid confusion. Additionally, the nucleated cells of proestrus are generally more uniform in appearance.

Mice (Figure 9-11, Figure 9-12). When 2 days of estrus are observed in mice, the two different "phases" of estrus are usually appreciated. At the start of estrus in mice, the anucleated epithelial cells are smaller and usually arranged in loose clusters or sheets reminiscent of proestrus. As estrus progresses, the anucleated epithelial cells become larger and are usually more evenly distributed. The cells might be in stacks or layers.

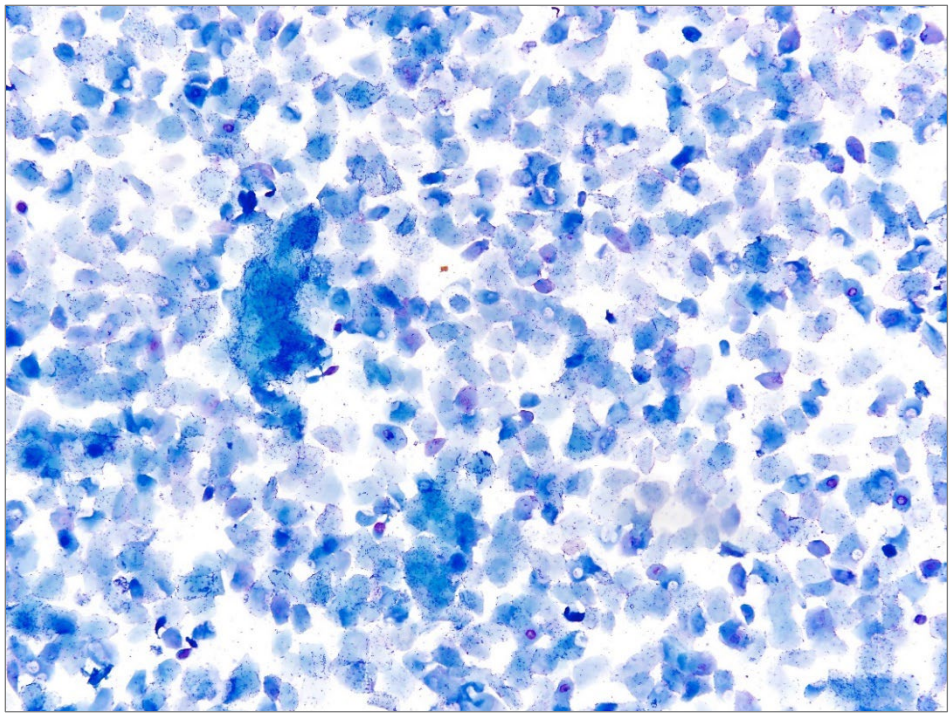

Figure 9-7. Estrus, Rat

Anucleated keratinized epithelial cells predominate.

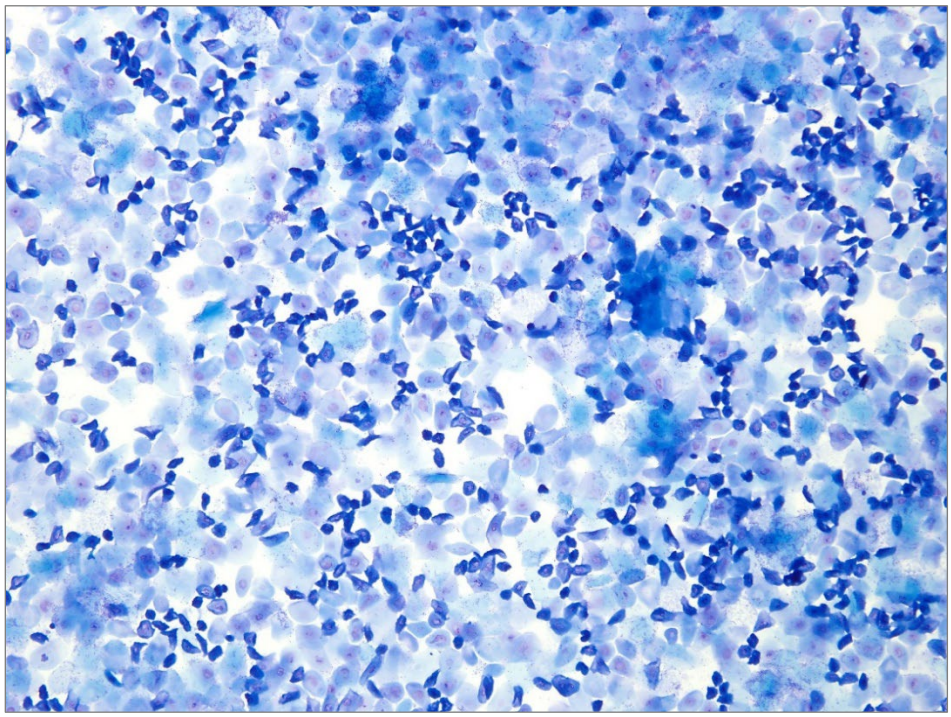

Figure 9-8. Late Estrus, Rat: Example 1

Darkly stained, oval to spindle-shaped nucleated epithelial cells are interspersed among the anucleated cells.

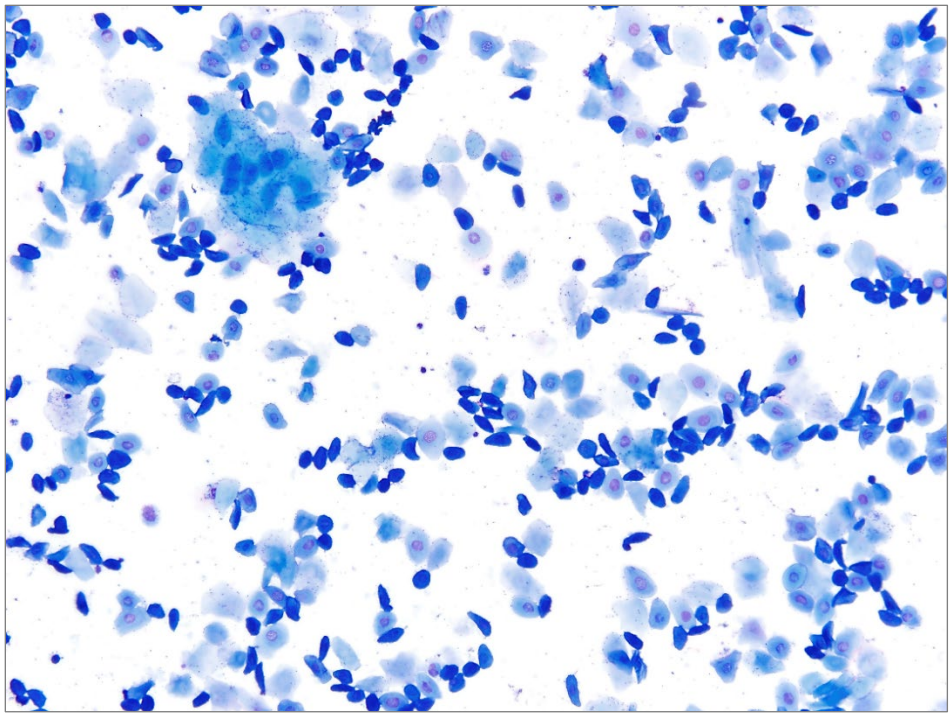

Figure 9-9. Late Estrus, Rat: Example 2

This image is another example of late estrus characterized by anucleated epithelial cells mixed with oval and spindle-shaped nucleated cells, some of which have stained deeply basophilic. Late estrus should not be mistaken for proestrus.

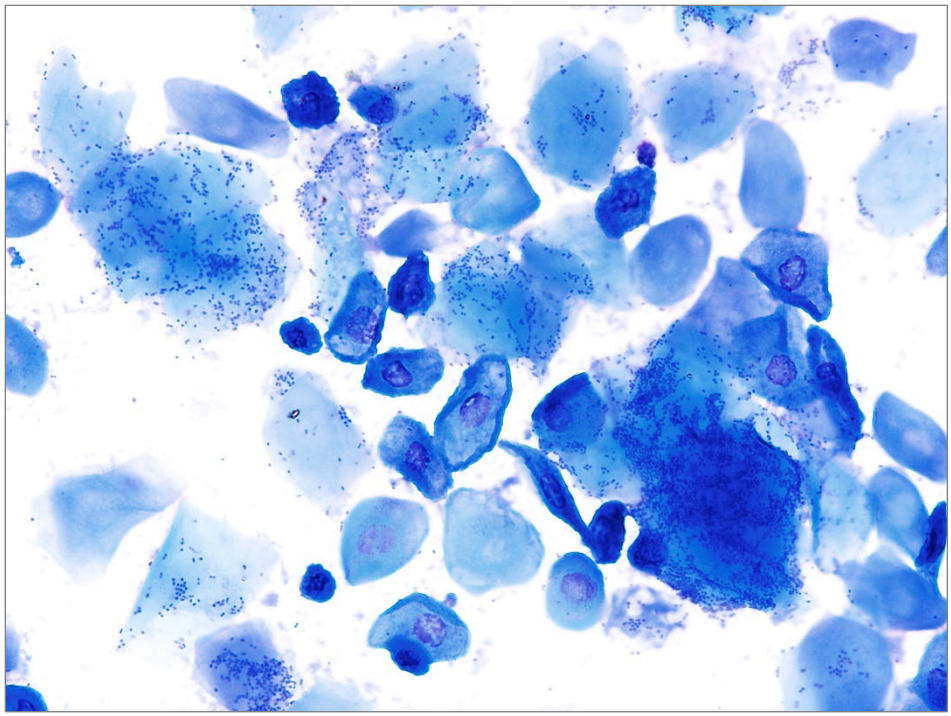

Figure 9-10. Late Estrus, Rat: Example 3

This image is a higher magnification of late estrus in a rat, showing the varying shapes of the nucleated cells. Also note the bacteria adhered to the cells and in the background, which is a normal finding in estrus.

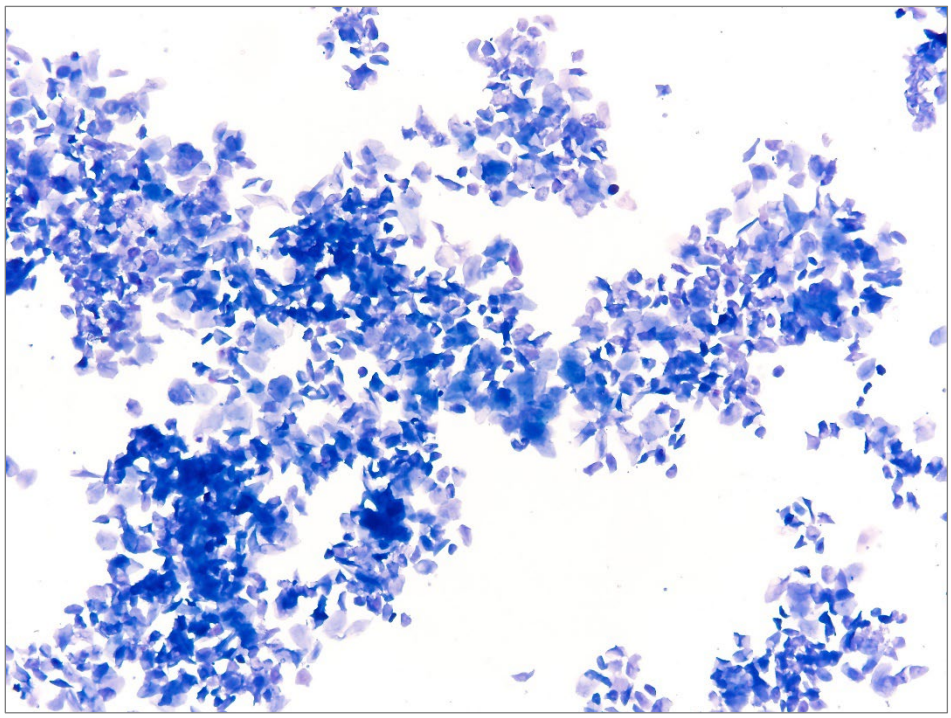

Figure 9-11. Estrus, Mouse: Example 1

In earlier estrus of the mouse, the anucleated cells are smaller and usually arranged in loose clusters reminiscent of proestrus.

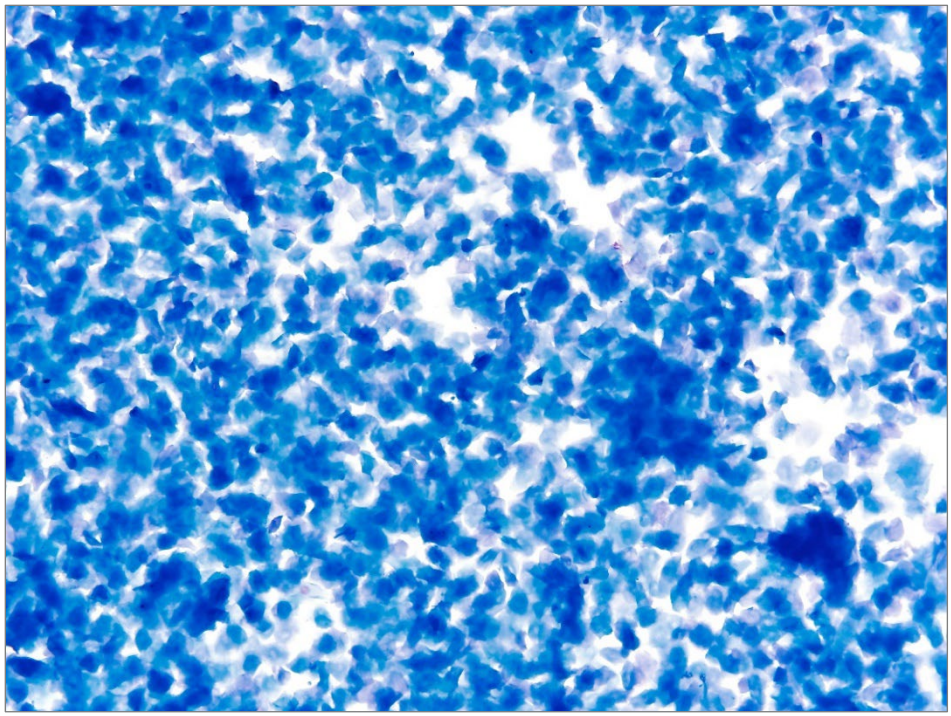

Figure 9-12. Estrus, Mouse: Example 2

As estrus progresses, the anucleated cells generally become larger, more evenly dispersed, and higher in numbers. Figure 9-11 and Figure 9-12 are 2 consecutive days of estrus from the same mouse.

Metestrus (Figure 9-13, Figure 9-14, Figure 9-15, Figure 9-16, Figure 9-17). Metestrus is a short stage in rats, lasting 6–8 hours. In mice, it can last as long as 24 hours, and, although infrequent, might be observed on two consecutive smears in mice. Metestrus is characterized by a combination of anucleated epithelial cells and neutrophils. In mice, rare-to-occasional nucleated epithelial cells might be observed. In rats, the nucleated epithelial cells of late estrus are present in moderate numbers throughout the stage.

In early metestrus, neutrophils are scattered among the epithelial cells and are sometimes found in clumps or tightly packed around the epithelial cells. At this point, the epithelial cell numbers predominate or are equal to the neutrophil numbers. As metestrus progresses, the neutrophil numbers greatly increase, resulting in a smear that is highly cellular; neutrophils can outnumber the epithelial cells by as much as 10-fold. Neutrophil and epithelial cell numbers decrease as the rodent transitions to diestrus.

The point at which metestrus ends and diestrus begins is not always obvious because they are defined by the same cell types and can have a similar appearance. Early and mid-metestrus are easily identified, however. If it is not clear whether a smear is late metestrus or early diestrus, it is best to be as consistent as possible and “err” on the side of diestrus. (There is minimal value in overly scrutinizing a late metestrus/early diestrus smear.)

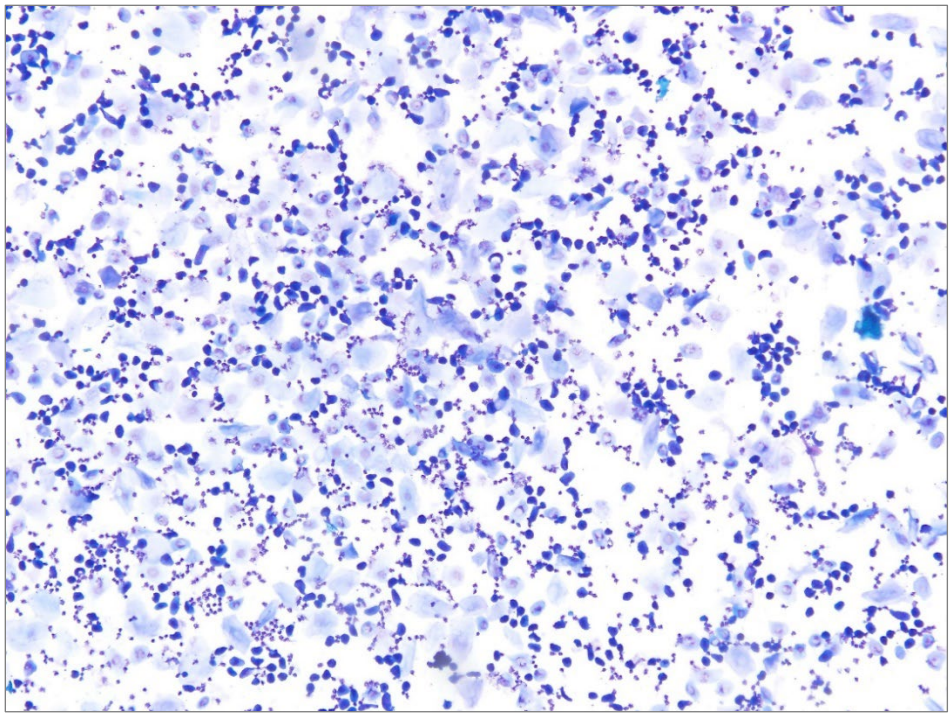

Figure 9-13. Metestrus, Rat: Example 1

Metestrus begins with the emergence of neutrophils interspersed or clumped among the nucleated and anucleated epithelial cells that are visible in late estrus.

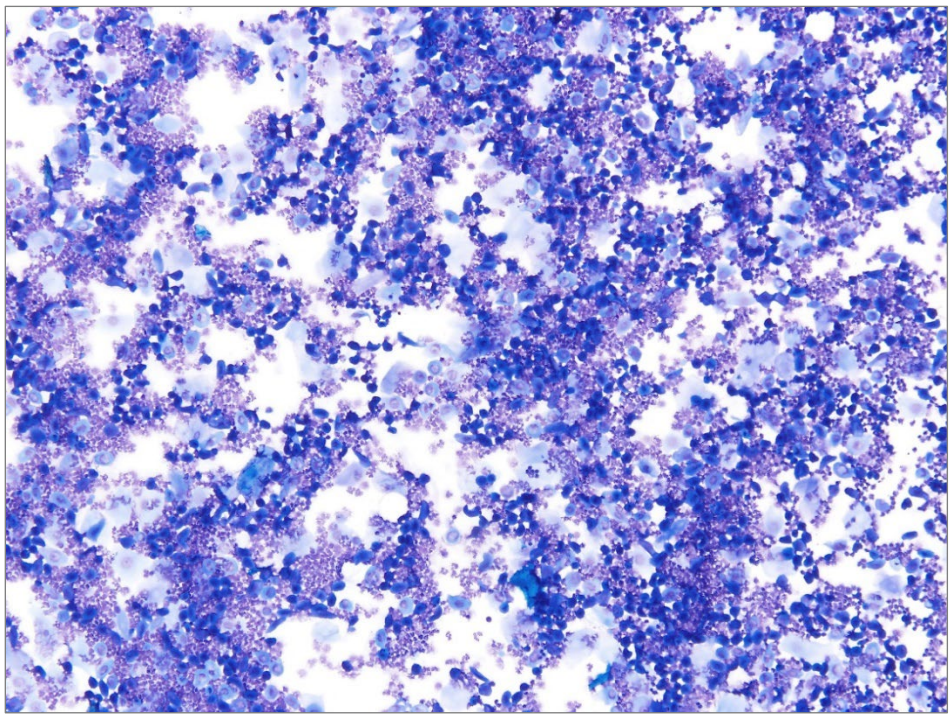

Figure 9-14. Metestrus, Rat: Example 2

As metestrus progresses, the neutrophil numbers increase substantially, resulting in a highly cellular smear.

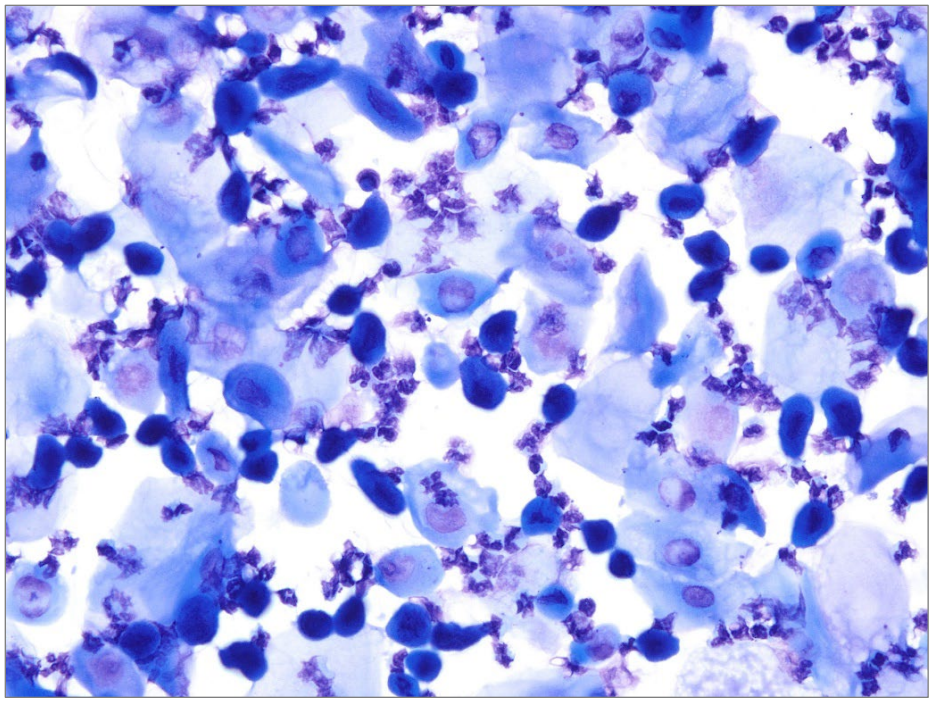

Figure 9-15. Metestrus, Rat: Example 3

This image is a higher magnification of rat metestrus, showing the mixture of anucleated epithelial cells, nucleated epithelial cells, and neutrophils.

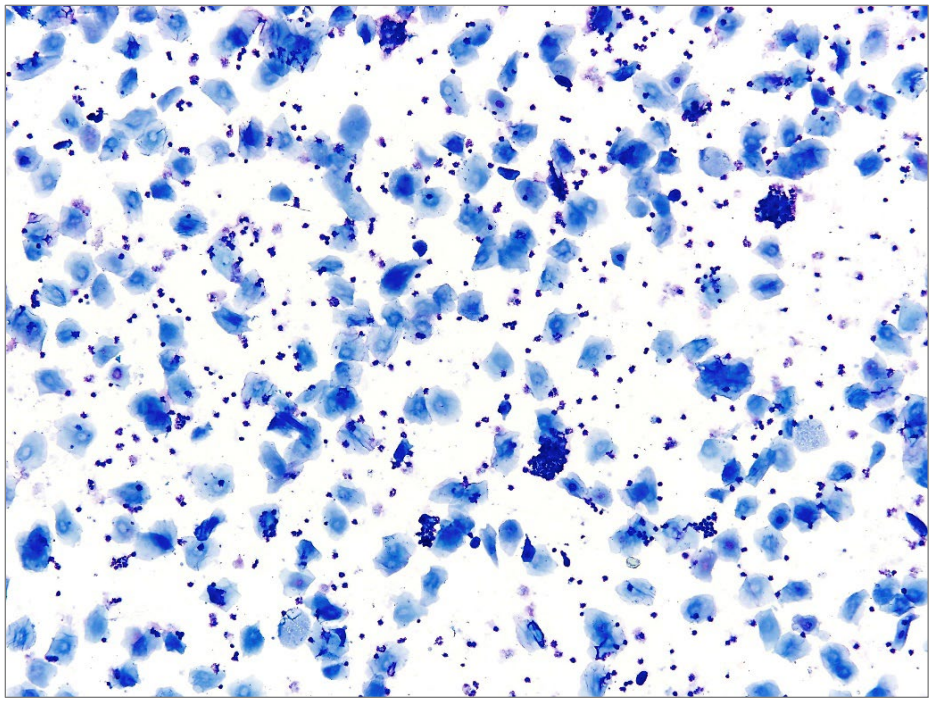

Figure 9-16. Metestrus, Mouse: Example 1

At the start of metestrus, neutrophils are interspersed or clumped around the anucleated epithelial cells. Note that metestrus differs between the mouse and rat in that rats have much higher numbers of nucleated cells mixed among the neutrophils; nucleated cells are in very low numbers in mouse metestrus.

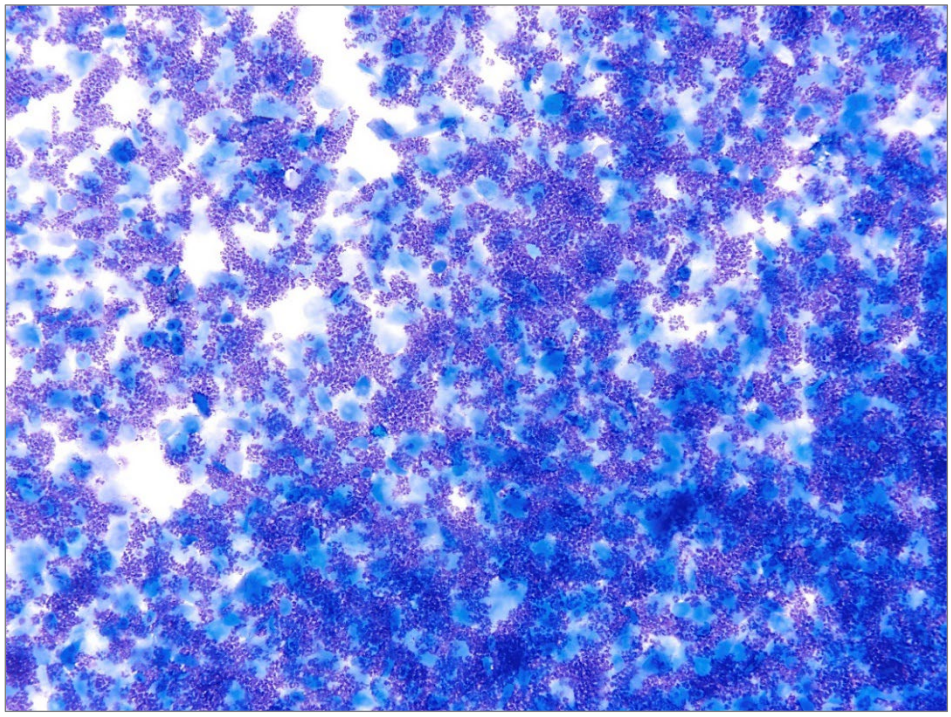

Figure 9-17. Metestrus, Mouse: Example 2

As metestrus progresses, the neutrophils become very high in number, resulting in a high-cellularity sample.

Diestrus (Figure 9-18, Figure 9-19, Figure 9-20). Diestrus is the longest stage, averaging 48–72 hours in rats and mice. There is a substantial decrease in the numbers (although not necessarily an absence) of anucleated epithelial cells. The cellularity is moderate to low, with a combination of small and large epithelial cells, neutrophils, and low numbers of anucleated epithelial cells. Neutrophil numbers are usually higher than epithelial cell numbers, with some smears being solely neutrophilic. In early diestrus, neutrophils might be visible in small clumps.

It is not uncommon for diestrus smears to have a very low cellularity with just a scattering of cells. This is especially true for days 2 and 3. Toward the end of diestrus, epithelial cells might also be observed in small clumps, indicating proestrus the next day, but neutrophils will still be a predominant feature of the smear.

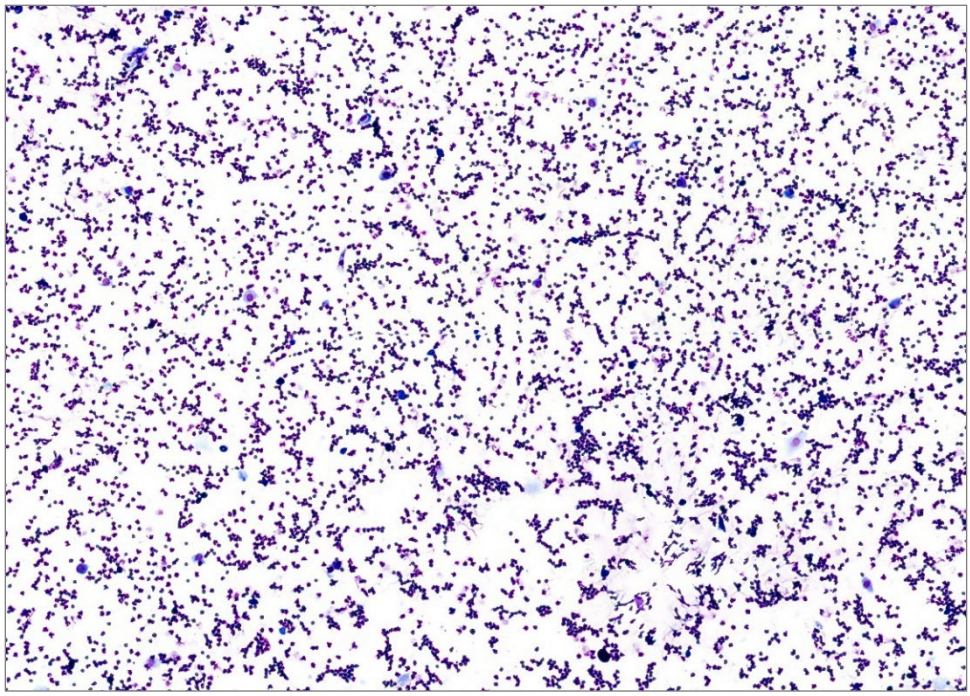

Figure 9-18. Diestrus: Example 1

Neutrophils predominate in this particular diestrus smear.

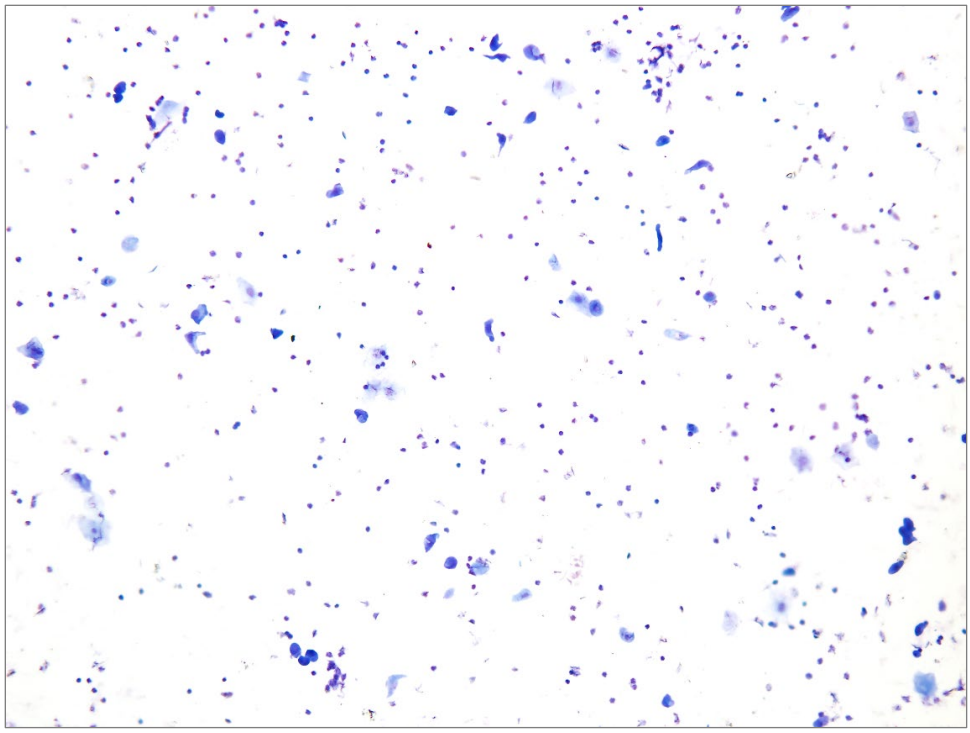

Figure 9-19. Diestrus: Example 2

Epithelial cells are interspersed among the neutrophils.

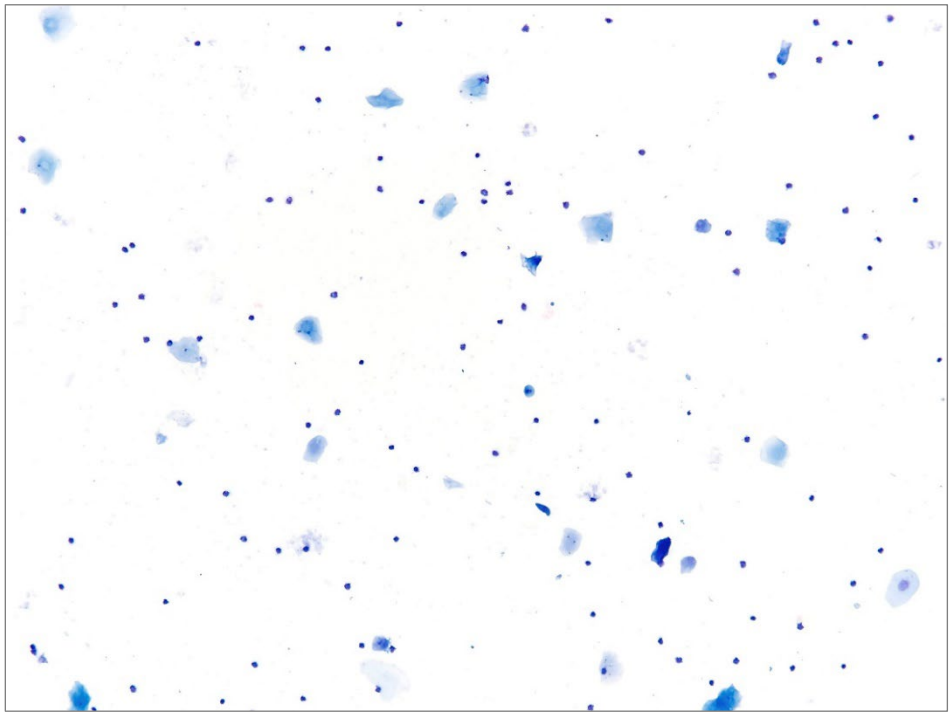

Figure 9-20. Diestrus: Example 3

Low-cellularity smear with a mixture of neutrophils and epithelial cells.

Transitional Stages (Figure 9-21). In general, classification of the individual stages, based on the criteria, is easily performed. It should be appreciated, however, that the estrous cycle is a process of constant change. As such, some samples may be collected at a time of transition (e.g., proestrus transitioning into estrus) and possess characteristics of both stages. In these situations, the recorded stage should be consistent with the most predominant feature(s) of that particular smear. For example, if the majority of cells in a smear transitioning from proestrus to estrus are small nucleated epithelial cells, the stage should be recorded as "P" for proestrus.

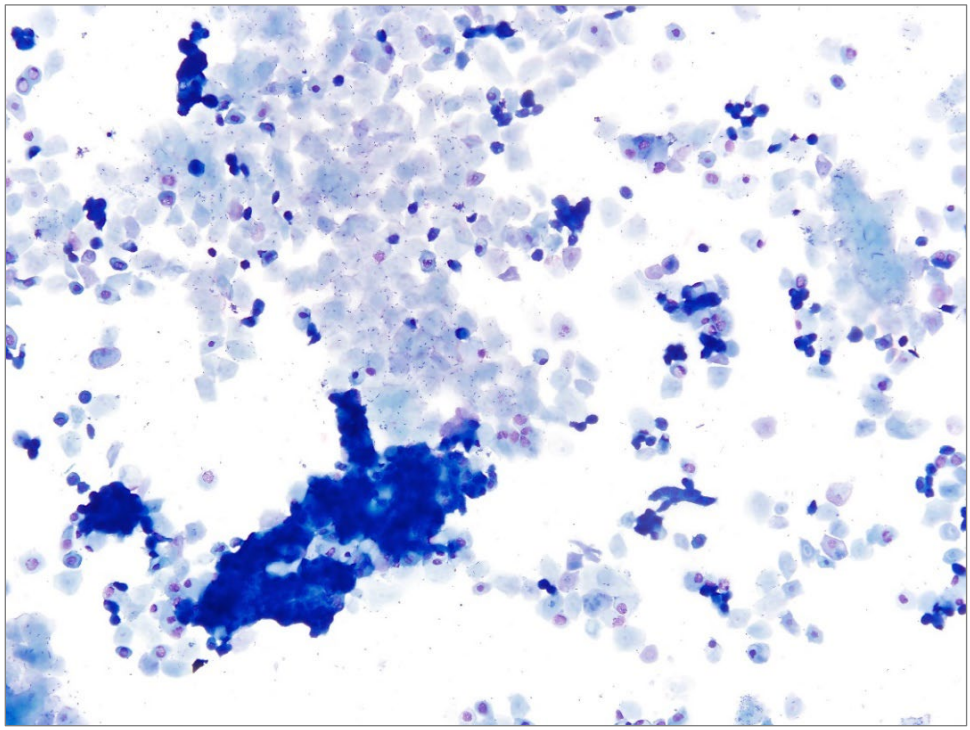

Figure 9-21. Proestrus to Estrous Transition, Rat

Many individual and clumps of small epithelial cells are interspersed with lesser numbers of lighter blue large nucleated and anucleated epithelial cells. A full evaluation suggests this smear was ultimately recorded as proestrus, as the small epithelial cells outnumbered the anucleated cells.

9.2.3. List of Suggested Materials for Vaginal Cytology Assays

These materials will be supplied by the toxicology testing laboratory:

- 0.9% saline solution or phosphate-buffered saline
- 100-slot slide boxes
- Beakers
- Coverslips (24 × 60 mm)
- Medicine droppers
- Microscope slide labels
- Parafilm
- Permount mounting medium and xylene

- Precleaned clear microscope slides (3 × 1 in.) with a frosted end
- Solvent-resistant permanent marker
- Trays

9.2.4. Procedure for Collecting and Preparing Vaginal Smears

Two vaginal smears shall be prepared from each animal between 8:00 and 10:00 a.m. each day during the final 16 consecutive days of the study. Duplicate smears may be used interchangeably during slide evaluation. The dose levels at which these are prepared shall be designated by the program COR for the testing laboratory.

Clear slides with a frosted end shall be used to make vaginal smears. All smears are made on the clear portion of the slide.

Using a solvent-resistant permanent marker, microscope slides are marked with a grid consisting of six squares per slide on the clear portion. The frosted end is marked with a permanent marker or pencil to indicate the species/animal number/slide (e.g., "01-M-001B" is sufficient at this stage because a label with complete information shall be applied later). The squares are labeled 1 through 6, 7 through 12, and 13 through 16 in the upper right corner. This technique is convenient as it allows the incorporation of 6 days of smears on 1 slide.

A 3–4 in. medicine dropper is moistened by aspirating a 0.9% saline or phosphate-buffered saline solution. A very small amount of the solution (up to the shoulder of the medicine dropper, approximately 0.2 mL) is left in the medicine dropper and then placed in the vagina (depending upon the strain, this depth should be approximately one-quarter to one-half of an inch for rats and less for mice) and the vaginal fluids are aspirated back and forth several times.

The contents of the medicine dropper are transferred onto the slide within the appropriate square for that animal-day. The use of excessive saline should be avoided to prevent flow onto surrounding squares. The slide should be checked for sufficient cell numbers (approximately 10–20/40× field) using a microscope. The slides are to be stored in slide folders in a dust-free atmosphere between collection dates.

After the 16th smear is taken and allowed to completely air-dry overnight, the slides can be stained with a Romanowsky-type stain (e.g., modified Wright's, Wright-Giemsa, or equivalent) by way of an automated slide stainer and according to manufacturer's instructions; this is the most consistent and efficient method.

Alternatively, slides may be stained manually with Diff-Quik, according to manufacturer's instructions, or with Toluidine blue as outlined in the following protocol:

- Preparation of 0.5% Toluidine blue stain: Mix 2.5 g of Toluidine blue with 500 mL of 20% ethanol and allow to sit for 1 hour before filtering through Whatman grade filter paper.
- Slides are loaded in glass racks and dipped in the following solutions:
    - 95% ethanol for 30 minutes
    - 80% ethanol for 1 minute with gentle shaking

  - 70% ethanol for 1 minute with gentle shaking
  - 50% ethanol for 1 minute with gentle shaking
  - 20% ethanol for 1 minute with gentle shaking
  - 0.5% Toluidine blue in 20% ethanol for 30–45 seconds (personal judgment should be used because Toluidine blue improves with age)
- Slides are rinsed with running tap water until the tap water runs clear. (At this point, if the slides are not dark enough when checked under a microscope, dip them in 20% ethanol and put the stain back in; cornified cells should be stained blue with a purple nucleus visible.)
- The stained slides are blotted gently under bibulous paper.
- The wet papers are thrown away because material on a slide can be transferred to other slides.

The slides shall be allowed to dry overnight and then cover-slipped using Permount™ or another suitable mounting medium. If the slides cannot be cover-slipped the following day, store them in a slide folder and coverslip as soon as possible.

9.2.4.1. Slide Labeling and Coding

Each laboratory participating in a study that requires vaginal cytology collection will be assigned an identification number by the sponsor-designated laboratory. (Contact that laboratory to find out your assigned identification number.)

Each slide shall include four lines with the following information (see Figure 9-22 for an example):

(1) C number

(2) Lab code, species, animal number with slide (e.g., 01-M-001A)
  - "01" designates the lab code
  - "M" indicates mouse ("R" would indicate rat)
  - "001A" designates the number randomly assigned to an animal, plus the slide ("A" indicates the first of three slides for that animal, whereas "B" and "C" would indicate the second and third slides, respectively)

(3) The date the first smear was made

(4) The date the last smear was made

C62408 01-M-001A 04-06-06 04-21-06	1	2	3
	4	5	6

C62408 01-M- 001B 04-06-06 04-21-06	7	8	9
	10	11	12
C62408 01-M-001C 04-06-06 04-21-06	13	14	15
	16		

Figure 9-22. Slide Labeling and Coding Example

All decoding information is to remain with the individual testing laboratory and a copy sent to the SMCVC laboratory to be opened only after all evaluations have been completed and in the presence of the Quality Assurance Officer, who will date and sign when opened. Decoding information to be provided is shown in Figure 9-23. All slides will be shipped according to instructions provided in Section 9.2.5.

LABORATORY: XYZ
TEST ARTICLE: Emodin
CAS # 518-82-1 C # C62408
DATES SMEARS TAKEN: 4/6/06–4/21/06

LAB # 01
ROUTE/VEHICLE: Dosed Feed/NTP2000
STRAIN: B6C3F1 Mice
TSAC DATE: 4/22/06

SLIDE CODE	TREATMENT GROUP	ANIMAL NUMBER	BODY WEIGHT @ TSAC (g)
01-M-001	8000 ppm	113	29.2
01-M-002	Control	61	30.6
01-M-003	1000 ppm	85	29.7
01-M-004	2000 ppm	97	28.9
Etc.			

Figure 9-23. Slide Decoding Example

9.2.5. Instructions for Shipping Slides to the SMCVC Laboratory

If required by the study protocol, SMCVC slides shall be shipped to the COR-designated laboratory for evaluation. If slides are to be analyzed by the study laboratory, SMCVC slides shall be submitted to the NTP Archives with the remaining study materials.

- Shipping and packaging shall be incurred by the testing laboratory conducting the toxicity studies.
- Stained and cover-slipped slides shall be randomized by animal and shipped to the sponsor-designated SMCVC laboratory within 2 weeks of necropsy. When randomizing by animal number, keep all slides from each animal together.
- A copy of the code for all slides shall be placed in a sealed envelope and sent to the SMCVC laboratory along with the coded slides.

- All vaginal cytology slides shall be placed in plastic slide boxes, keeping slides from mice and rats separate.
- These slide boxes shall be placed in 350 lb. test cardboard boxes, separated by abundant packaging material for shipment.
- An appropriate listing of slides shall be packed in the slide boxes.
- Slide boxes shall be packed to avoid any breakage.
- Shipping cartons shall be sealed and bound with filament tape prior to shipment.
- Slides sent separately in slide set shall be counted as present in the inventory; a copy of the slide set inventory shall accompany the major inventory document.
- Each plastic shipping box shall be marked with the name and address of the laboratory, the test article, CASRN, NIEHS study number (i.e., C #), strain, and the range of animal numbers included in that box.
- Shipping cartons containing slides and the appropriate information shall be directly mailed to the sponsor-designated SMCVC laboratory.

9.2.6. Suggestions for Improving Vaginal Cytology Quality

General instructions to make vaginal smears are described in detail in Section 9.2.4. A few simple precautions, however, can significantly increase the quality of the slides. Below are suggestions and explanations for correcting potential problems that could be encountered during the preparation of a vaginal cytology smear. A list of vaginal cytology quality codes used by laboratories to evaluate slides is provided in Table 9-1.

Debris. Presence of debris in vaginal smears will interfere during the evaluation. Debris usually comes from dirty slides, tissue fragments, or dust in the air. Therefore, all slides should be precleaned by wiping them with a clean gauze or Kimwipe™. Most of the tissue fragments can be eliminated by carefully aspirating vaginal fluids. It is also important to place all smears that do not have a coverslip in a dust-free environment so dust settling on slides is minimized. Coverslips, if dusty, should be cleaned before use.

Staining. The major problem encountered in this category is nonuniformity of the stain, which can result from the different densities of cells in each smear; however, with optimized automated stainers, smear stain quality is usually sufficient for smear evaluation. With manual stains, one can scan the slide to determine whether all the smears are adequately stained. If the stain quality is poor, place the slides back in the Toluidine blue/Diff-Quik to restain them. Be careful not to stain cells too dark because doing so severely interferes with evaluation.

Clumping and Cell Density. The clumping of cells is mainly due to an excess number of cells on the slide, which can be remedied by not placing too many cells on the slide. If the smear is too dense, it will stain heavily and clump. If too much saline is used in making the smear, the cell density will be very low, not allowing for accurate evaluation. Some stages have inherently high cell density, whereas other stages have very low cell density, thus contributing to this challenge of attaining the ideal number of cells on a slide.

Air Bubbles. Air bubbles are a major problem, especially when they occupy a large portion of the slide. They are mainly caused by faulty technique and/or carelessness. More mounting

medium might be needed to cover the entire slide, or better pressing of the coverslip to squeeze out air bubbles could be required.

9.2.6.1. Vaginal Cytology Quality Codes

Table 9-1. Codes Used by the Laboratory for the Evaluation of Slides

Code	Code Description	Subcode[a]	Subcode Description
00	Excellent	-1	Refers to day 1
01	Good	-2	Refers to day 2
02	Moderate crystallization	-3	Refers to day 3
03	Heavy crystallization	-4	Refers to day 4
04	Moderate debris	-5	Refers to day 5
05	Heavy debris	-6	Refers to day 6
06	Debris surrounding cells	-7	Refers to day 7
07	Staining too light	-8	Refers to day 8
08	Cornified cells stained lightly	-9	Refers to day 9
09	Staining too dark	-10	Refers to day 10
10	Nonuniform staining	-11	Refers to day 11
11	Cells heavily clumped	-12	Refers to day 12
12	Few cells	-13	Refers to day 13
13	No cells	-14	Refers to day 14
14	Air bubbles (>5% and <20% of the slide)	-15	Refers to day 15
15	Air bubbles (>20% of the slide)	-16	Refers to day 16
16	Cells incorrectly applied to the slide (i.e., applied on the side of the slide lacking the frosted end or not in the appropriate grid on the clear portion of the side of the slide with the frosted end)		

[a]A subcode is used in conjunction with a code when that remark applies to a particular day (e.g., "13-2" means "No cells on day 2").

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9.4. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapter 9 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was 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 9: Fetal Examinations and Vaginal Cytology
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

Peer Reviewer

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10. Neurobehavioral Testing

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10.1. General Guidelines for Conducting Neurobehavioral Studies

Behavioral testing is a critical tool in assessing nervous system disorders. There are many well-established tests for evaluating various behavioral test results; however, details of how these tests are conducted and how environmental and experimental design factors are controlled are critical to providing reproducible data (Graham et al. 2018; Mandillo et al. 2008; Wahlsten 2011).

It is also known that different outcomes can arise from laboratory conditions extraneous to the test protocol (Crabbe et al. 1999; Harry et al. 2022; Kafkafi et al. 2005; Kafkafi et al. 2018; Lewejohann et al. 2006; Mandillo et al. 2008). These factors have been discussed in various publications and provide the basis for many of the details of the current guidelines (Genzel 2021; Hånell and Marklund 2014; Saré et al. 2021). To address the issue of test quality and consistency, a number of guidance documents, protocol publications, and data analysis papers have been published over the years (Bailey et al. 2006; Hånell and Marklund 2014; IPCS 1986; Moser 2011; NAFTA-TWG 2016; Saré et al. 2021; Slikker et al. 2005; Vorhees and Williams 2021).

Specific aspects of the handling of rodents during mating, pregnancy, and lactation are provided in Chapter 6 of the Specifications (Laboratory Animal Medicine and Toxicology). Specific timing for clinical observation assessments will be provided in a protocol outline directly to the contractor. Neuropathology requirements are described in Chapter 8 of the Specifications (Anatomic Pathology).

10.1.1. Housing

- During periods of gestation and lactation, crinkled Kraft paper shall be used in cages of rat dams. At each cage change occurring between time of birth to time of weaning, new cage bedding will be enhanced with a sampling (approximately one-fourth cup) of the original litter bedding to provide home-cage olfactory cues to minimize stress associated with cage change. This practice is specific to neurobehavioral studies and differs from standard Laboratory Animal Medicine and Toxicology practices of removing enrichment between GD 19 and PND 4 (Section 6.2.13, Environmental Enrichment).
- All animals at the age of weaning and older shall be group housed (by sex and dose group) per cage size guidelines and study needs. Male and female animals shall be housed similarly. Individual housing of mice shall be considered in the presence of aggressive behavior. If an animal is single housed, new cage bedding shall be enhanced with a sampling (approximately one-fourth cup) of the original litter, and a form of cage enrichment shall be used to minimize the stress of isolation. Scientific

justification for deviation from group housing shall be provided by the Contracting Officer's Representative (COR).

10.1.2. Animal Identification and Selection

- If a study design requires individual identification of preweanling pups, a temporary marking (e.g., paw tattoo, tail marking) shall be implemented in a manner to not cause damage that can compromise behavior (e.g., toe/paw damage that may compromise strength or motor-dependent behaviors). Temporary markings shall be replaced by a more permanent marking system upon weaning. If damage occurs (as confirmed by veterinary staff), the animal should be excluded from behavioral assessments due to possible effect on performance.
- For developmental exposure, litters shall be standardized on postnatal day (PND) 4 to 8–10 pups with specific sex distribution determined by the study protocol.
- Unless specifically noted for identified endpoints, for gestational and/or lactational exposure, one animal/sex/litter shall be randomly selected for any specific cohort for a specific behavioral test or sequence of behavioral testing.
- One animal can undergo more than one type of assessment according to a testing schedule ensuring no confounding across tests (e.g., tests are spaced at adequate intervals [days]; tests involving aversive stimuli [e.g., shock] shall be conducted at the end of the testing sequence).
- As determined by the study protocol, more than one cohort of animals can be used for behavioral testing if the testing history is consistent within a cohort (e.g., all animals assessed for any one endpoint will have had identical testing history in that all will have experienced the same test sequence).
- If, for any reason, an animal is removed from study due to health issues, it may be replaced with a matched animal from that same litter and dose group, per agreement of the COR.
- A process shall be put in place to ensure experimenter blinding of animal exposure status while running studies.

10.1.3. Randomization and Counterbalancing

- Animals shall be randomly selected for assignment to the behavioral testing cohort(s).
- All testing shall be counterbalanced for dose across testing apparatus and order of testing. If the same animal is to be tested multiple times for an activity, it shall be placed within the same apparatus for each test session.
- Males and females shall be tested at separate times within any single day. If the study design requires counterbalancing for sex, that information will be provided by the COR.
- If it is necessary for multiple technicians to perform any one task, the assignment of technicians shall be counterbalanced across dose groups to ensure an equal distribution of dose groups across technicians. For any observational endpoints, this requires technicians with a priori demonstrated >80% interrater reliability as

statistically determined from the ratings of two or more technicians on a specific test. For endpoints that can be significantly influenced by the technician (e.g., grip strength, rotarod), consistency of >80% inter-experimenter reliability across the actual technicians to conduct the study shall be demonstrated prior to the study (≤1 year).

- If an animal is terminated prior to testing, a secondary animal may be identified as a replacement and noted as such. The decision shall be made in consultation with the COR.

10.1.4. Animal Clinical Observations

- Body weights shall be recorded as specified in the study protocol.
- Formal (out-of-cage) clinical observations (characterized as "routine" or "clinical observations" in NTP Provantis) for clinical signs of toxicity will be recorded as specified in the study protocol. Clinical observations considered related to chemical exposure and observed at times other than scheduled observations will also be recorded in NTP Provantis.
- Additional nonexposure-related observations, outside of clinical observations, which may affect behavioral testing, shall be recorded in NTP Provantis. Examples include, but are not limited to, loss of toenail, injury to limb and/or tail, dropped to floor, or seizure during testing. Observations should be recorded in such a manner as to easily reflect testing days during review. Animals may continue in behavioral testing if their injury does not affect their overall well-being. The COR shall be notified of such conditions as they may influence study outcomes.

10.1.5. Selection of Testing Equipment and Procedures

- Commercially available equipment that is based on well-established methods for assessing the various neurobehavioral endpoints as demonstrated by published literature shall be used.
- Documentation shall be provided indicating that each test, as conducted by the testing facility (animal handling, environment, testing procedures and conditions), generates data reflecting a normal expected (per published literature) pattern of behavior of naive age-, sex-, and species-specific animals relevant to the study. This documentation can be provided within the form of historical control data captured on the equipment within the last 5 years. If data are not available, then prior to initiating testing of any study animals, a pilot study shall be run to confirm assay setup and laboratory proficiency. A cohort of five naive males (and five females, if females are to be assessed in the study) shall undergo each behavioral test at the defined ages.
- If there is a requirement to test for an acute (not developmental) effect on a specific behavior, a study using a positive control may be required to be performed prior to approval of the test paradigm. The specific experiments required prior to study initiation will be at the direction of the COR.
- Expected results shall have been demonstrated in control animals (species, strain, and sex) within approximately 1 year from the start of the study.

- Historical data, basic response, and positive control data for the most recent 5 years shall be available to the COR.
- All equipment and testing paradigms shall be approved by the COR prior to the start of the study.
- Detailed physical descriptions of the test equipment shall be reported (and can be provided via the commercial source manual). Details shall be supplied from each commercial supplier regarding definition of terms, recommended calculations of data (e.g., ambulatory activity), and calibration. Methods of calibration of each testing apparatus and identification of when and how often calibration is conducted shall be provided.

10.1.6. Handling for Specific Behavioral Tests

- Before behavioral testing, animals shall be acclimated with handling to ensure they do not undergo undue stress. Proper handling of rats consists of scooping up the animal with one hand under the chest and supporting the bottom with the other hand (UNC Basic Rat Handling and Technique Guide[1]). Mouse handling is improved by either nonaversive tunnel or cupping methods (Gouveia and Hurst 2017; Marcotte et al. 2021; Sensini et al. 2020). If tail handling is required, it must occur at the base of the tail. Tail handling is stressful to animals, so it should be used minimally.
- Further acclimation to any specific type of handling required for a behavioral test (e.g., grip strength, startle restraint) shall occur prior to testing to minimize handling stress at time of testing. This handling can be accomplished by acclimation to unique handling required for placing the animal in the restrainer used in the startle apparatus or on strain gauges for grip strength analysis.
- Consistency shall be maintained across animals within any specific test in terms of technicians' handling of the animal, placing of the animal within a test apparatus, removal of the animal from an apparatus, and returning the animal to its home cage.

10.1.7. Minimizing Olfactory Stimuli

- To minimize the influence of olfactory cues, the test environment shall be devoid of all specific odors to the extent possible, including, but not limited to, odors from chamber cleaning solutions and from the experimenter (e.g., perfumes, tobacco smoke, hand sanitizer, food).
- Animal-specific odors as they relate to stress, urine trail, or sex require minimization. Between animals (of the same sex) test arenas or restraints shall be wiped clean using a mild fragrant-free detergent with a disinfectant (e.g., Nolvasan [chlorhexidine diacetate] followed by a distilled water rinse). At completion of all testing for the day, any portion of the test apparatus that was in contact with the animal shall be cleaned with an excess of a mild fragrant-free detergent with a disinfectant, allowing the solution to remain on the apparatus for approximately 1 minute before wiping with distilled water, and wiped dry. For the Morris water maze (MWM), disturbance of the water between animals will minimize any urine trail.

[1] https://research.unc.edu/wp-content/uploads/sites/61/2020/12/rat-handling-and-techniques.pdf

- The influence of sex-specific odors (e.g., urine) on behavior shall be minimized. Unless otherwise requested that the experimental design include full counterbalancing for sex, testing each sex on either separate days or separate times of day with enhanced cleaning between sexes can minimize the influence of sex-related odors. Before handling animals of different sexes for behavioral testing, the experimenter shall change gloves and change or wipe down protective body coveralls to remove any traces of influencing odors.

10.1.8. Behavioral Testing Environment

- Outside of the home-cage room, lack of access to drinking water and food during the daytime shall not be >6 hours. Maintaining dosing via food or drinking water over the period outside of the home cage shall be determined by the study protocol.
- Ambient noise level shall be minimized in the testing facility. Testing rooms shall be maintained with white noise. A random examination shall confirm that the white noise (62–70 dB) is consistent across test units closest to and furthest from the white-noise speaker. Any occurrence of a loud noise during testing shall be recorded.
- If animals are tested in a sound-attenuating chamber with an individual background noise generator (e.g., startle apparatus) and the testing room is within a quiet area of the laboratory, considerations of not having full white noise for the room will be made by the COR.
- Conversation between experimenters within testing rooms shall be kept at a minimum.
- Quiet shall be maintained when placing animals into a test apparatus and during a test session.
- The testing units shall be stabilized to minimize any vibration, as applicable. Assessment of vibration of the testing room due to mechanical activity shall be conducted and confirmed absent or within a specific time of day/week for adjustment.
- Only the test cohort undergoing assessment shall be in the test room at any one time (e.g., animals not within the startle apparatus during a test session shall not be within the test room; animals not being tested for motor activity on that specific day shall not be within the test room). An exemption can be made for long-term learning and memory tests, such as the MWM.

10.1.9. Time of Testing

- All scheduled behavioral testing shall be conducted at an interval within 1 hour of a standard 12-hour light/dark cycle. Thus, testing will not commence until 1 hour after lights are turned on and will end by 1 hour before lights are turned off. The time of day of testing for each animal will be recorded. This time restriction does not apply to transport of animals to/from the testing room.
- Any shorter time interval required due to study design requires approval by the COR.
- Time of testing shall be counterbalanced across exposure group (for each sex per study design). Any alternative specific order for each test shall require COR approval.

- Any endpoints of repeated measures shall be conducted at approximately the same time of day for each animal.
- The effects of a direct dosing of a compound on the behavioral performance shall be considered, and the schedule of dosing to testing shall be coordinated to minimize confounding of such effects.
- Determination of the time of testing relative to the time of last dose will be made by the COR.
- When possible, behavioral testing shall be conducted before any direct dosing (e.g., gavage, injection, dermal application, inhalation) for that day unless study design is targeted to examine acute effects of chemical delivery or examine the peak time of effect.
- The timing of cage changes shall be controlled to ensure animals have a minimum of 24 hours post-cage change prior to testing.

10.1.10. Transport and Location of Animals for Pretest Holding

- Animals shall be transferred from their home-cage room and placed in a quiet holding area close to the testing room and outside of any high-traffic areas. Before the first behavioral test (open field), the animals will have become familiar with cage rack movement (down the hall or in the home room).
- Animals shall be transported and held in the holding area for a minimum of 30 minutes prior to initiating the first test of the day and for a minimum of 10 minutes if sequentially transported to a separate room for testing. Transport of animals is not restricted by the 1-hour lights-on/lights-off time interval required for behavioral assessments.
- Animals shall not be held within the testing room for tests that include a stimulus (e.g., startle), as nonspecific exposure would occur to animals outside of the test chamber.
- Animals shall not be held within the testing room for tests in which light level is a factor (i.e., open-field activity).
- Animals can be held within the testing room for the MWM, per conditions stated in the MWM description.

10.1.11. Retrieval of Animals after Test

- Animals shall remain in the test apparatus (e.g., activity chamber, startle apparatus, rotarod) until the test session times out for all animals to minimize disturbance and distraction.
- For the MWM, animals shall be gently wiped off upon removal from the water tank. Care shall be taken to not subject wet animals to air drafts and to minimize the potential for hypothermia.
- Animals shall be removed from the test apparatus and placed in the transport cage prior to returning to the home cage. If animals are transported in the home cage, they can be returned to that cage (without any requirement of a specific transport cage).

- The “tested” animals shall not be returned to a cage containing animals that have not been tested but shall be placed in a holding cage until all animals in the cage have been tested for the day.

10.1.12. Order of Behavioral Assessments

The initial assessments will acclimate the animal to handling, and assessments in the open field for motor activity will allow the animal to experience a novel environment and allow for detection of any severe motor deficits that may compromise subsequent behavioral assessments. Grip strength and rotarod training assess motor strength and coordination as well as learning. They are noninvasive and can be conducted in sequence prior to or after the startle response, depending on study design. The startle response is primarily a reflex response; however, there are different types of habituation (intrasession and across sessions) that require consideration if more than one testing session is conducted. Different tests for learning and memory can be integrated at different ages and is often the final test of the sequence. The inclusion of additional tests will require consideration of testing history and possible interference across tests.

- In general, excluding clinical observations, only one specific test will occur in any single day.
- Tests should be performed within a short time window to minimize variability associated with age or time from dosing.
- Behavioral assessments shall be performed in a sequential manner that shall not interfere with performance on subsequent tests (e.g., any test involving aversive stimuli, such as shock, shall be conducted at the end of the testing sequence or in a separate set of animals).
- Time intervals between tests shall be consistent across animals.

10.1.13. Age of Behavioral Assessments

Comprehensive evaluation for potential neurotoxicity includes assessments performed prior to weaning, and during the adolescent and adult life phases. A critical determination regarding the actual age of testing for a young animal is the time of weaning. All recommendations are to refrain from behavioral testing within 24 hours postweaning. The below age ranges for juvenile animals are based on the Division of Translational Toxicology practice of weaning at PND 28.

- Assessment of animals <60 days of age requires a very small window of age for testing to account and control for the developmental process of neural circuitry controlling such behavior. Assessment of animals as adults allows for a broader range in age but should be within 65–80 days of age, or 80–90 days of age and counterbalanced across groups.
- Assessment of preweaning behaviors, such as motor activity, requires age-appropriate testing apparatus to provide sufficient sampling of the behavior.
- Assessment of startle and prepulse startle inhibition (PPI) shall adhere to the developmental ontogeny of the associated neural circuitry and use equipment appropriate for the size and weight of the animal.

- Motor activity shall be measured in juveniles at PND 31 (±2) (rats)/PND 24 (±2) (mice) and adults PND 60 (±3) (rats/mice).
- Startle response and PPI shall be conducted at PND 32 (±3) (rats)/PND 25 (±3) (mice), and during adulthood PND 62 (±3) (rats/mice).
- MWM shall be conducted at PND 68 (±4) (rats/mice).
- *Additional behavioral testing, if required by the study protocol:*
    - Forelimb/hindlimb (FL/HL) grip strength shall be conducted in juveniles at PND 32 (±2) (rats)/PND 25 (±3) (mice) and/or adults at PND 60 (±3) (rats/mice).
    - Rotarod shall be conducted in juveniles at PND 32 (±3) (rats)/PND 25 (±3) (mice) and/or adults at PND 60 (±3) (rats/mice).
- It is recommended that the additional observational endpoints listed be included in the standard observational assessments taken over the course of the study.
    - Note: The above time frames are assuming animals are weaned at PND 28 (rats)/PND 21 (mice); relative adjustments shall be made if weaning occurs at a different time point.

10.2. Data Collection and Transmittal

- Data sheets shall include individual animal identifiers: litter (dam) number, sire number, pup number, dose group, sex, age, test date, apparatus identifier, and experimenter identifier. Dose group shall be coded in a manner to maintain experimenter blinding.
- For all computer-assisted tests, software-generated files describing the configuration of the test will be provided (these will include all parameters and units of measures and any time intervals and intensities used).
- For all computer-assisted tests, individual animal raw data files of all endpoints shall be submitted. These include arena maps, endpoints, and pathway tracking data.
- Video-capture images and/or pathway tracking and associated software files shall be provided in a format accessible by NIEHS.
- For each endpoint, all original raw data shall be provided in addition to the Excel or CSV files of specific endpoints.

10.3. Data and Statistical Requirements

- All information related to data processing, analysis, and outputs described in this section shall be provided in an electronic format; however, the specific disposition can be discussed with the COR on a study-specific basis. Examples of specific files include, but are not limited to, outlier analyses and justification for removed data, as well as graphical approaches for assessing normality (e.g., model residual plots, boxplots).
- Procedures for outlier identification/removal should be clearly described.
- The final statistical procedures used for all analyses should be clearly described.

10.3.1. One Measurement per Animal

- Account for censoring using time-to-event/survival models such as Cox proportional hazards modeling. Time-to-event modeling should be used for the rotarod and MWM analyses. (Do not remove censored values from the analysis.)
- Account for deviations from normality and heteroscedasticity across groups using the proper modeling approach (nonparametric Kruskal Wallis analysis of variance [ANOVA], data transformation and parametric ANOVA, or generalized linear modeling).
    - If the data are approximately normally distributed and the variances are similar between different groups, parametric ANOVA is recommended.
    - If parametric assumptions are not reasonable, use a nonparametric approach. Since the nonparametric Kruskal Wallis ANOVA assumes that the distributions of the different groups have a similar form, this assumption should be investigated by plotting the raw data distributions.
    - For count data, a Kruskal Wallis test may be sufficient, but a generalized linear model may be needed if the distributions from different groups are very different from one another. See below for more details on assessing distributional assumptions.
- Use the Shapiro-Wilk (SW) test to test for departures from normality and use Levene's test to test for heteroscedasticity. Graphical approaches using raw data should also be used to help inform whether to use a nonparametric statistical analysis approach. There is no need to rely solely on the SW test or Levene's test to assess parametric assumptions. Expert judgment may be used about the distributional assumptions based on the plots and the SW test and Levene's test results (Holson et al. 2008). Some examples related to determination of normality:
    - Graphical methods should be used to check for substantial deviations from normality and heteroscedasticity (e.g., model residual plots, normal quantile-quantile plots, and boxplots) because the SW test and Levene's test can be restrictively conservative or liberal for determining suitability of the data for ANOVA.
    - It is not necessary to account for unequal variance between groups in modeling if variance in the group with the smallest variance and variance in the group with the largest variance is within a factor of four of each other. A fourfold difference between variances is a general rule, not an absolute criterion, and should be considered part of expert judgment (Moore and McCabe 2001).
    - If there are very large differences in variance between treatment groups and the distributions seem to be very different from one another based on visual plots, then statistical methods should also account for heteroscedasticity as well as departures from normality (e.g., generalized linear model).
- Display data as mean/standard error of the mean (SEM), as well as plots of raw data with boxplots. The boxplots should help determine whether the distributions have a

similar form, find large deviations from normality (e.g., censoring or heavy skew), or indicate whether there is very large heteroscedasticity.

10.3.2. Multiple Measurements per Animal

- Similar to the above, account for censoring by using time-to-event modeling.
- All study designs should be appropriately counterbalanced during conduct, which should be either stated or described in the statistical analysis methods summary.
- Account for deviations from normality or heteroscedasticity. If the data distributions are very different from normal or if there is a large amount of heteroscedasticity, log transformation of the data should be considered before applying repeated measures ANOVA (RMANOVA). Assuming the study design is appropriately counterbalanced, the nonparametric Friedman test for continuous data can be used for nonnormal data. If the Friedman test is not appropriate, then generalized linear modeling can be used for count data.
- Use the SW test, Levene's test, ***and*** graphical methods to look at data distributions and help make decisions about whether to use RMANOVA or nonparametric Friedman (or another approach). It is acceptable to emphasize the plots for assessing the distributional assumptions and not rely solely on the SW test or Levene's test results.
- Display data as mean/SEM ***and*** boxplots to help determine whether the distributions are dissimilar between groups, are not normal, or have substantial heteroscedasticity. These plots can be placed in an appendix or delivered using another electronic format (see above).

10.3.3. Analyses for Sex Effects

- The study design must be appropriately counterbalanced during conduct. Counterbalance time of testing across dose levels and sex to avoid confounding. Counterbalancing is needed to include separate factors in statistical modeling.
- Present the data separately for each sex in data plots. To test for sex effects, use the two approaches described below. Each question compares the larger model to the smaller model using the likelihood ratio test. For each likelihood ratio test result (for each question), the value of the test statistic, the degrees of freedom for the test, and the p value should be presented. No additional statistical analysis or investigation is needed, even if the full model is shown to be more appropriate.

Question 1

Effect of SEX: Compare the Full to Simple Model (get a p value for likelihood ratio test).

Full Model: R = dose + time + sex + dose:time + time:sex + dose:sex + dose:time:sex + ~litter

Simple Model: R = dose + time + dose:time + ~litter

Question 2

Effect of INTERACTIONS: Compare the Full to Simple Model (get a p value for likelihood ratio test).

Full Model: R = dose + time + sex + dose:time + time:sex + dose:sex + dose:time:sex + ~litter

Simple Model: R = dose + time + sex + ~litter

10.4. Observational Assessments

10.4.1. Cage-side Observations in Juvenile Pups

Rodent juvenile activity accounts for many critical features of nervous system integration, including motor function, strength, and social behavior (Vanderschuren et al. 2016). Rats begin to play toward the end of the third week of life, peaking during the fourth and fifth weeks and then decreasing with sexual maturity (Pellis and Pellis 1990). Play by juvenile rats is often considered a potential indictor of good animal welfare (Oliveira et al. 2010) and has been demonstrated to be associated with the development of sociocognitive skills (Baarendse et al. 2013; Stark and Pellis 2020). It has been used as a model for analyzing neurodevelopmental disorders (Burke et al. 2017; Pellis et al. 2022). Play behavior can be scored as it occurs naturally in a litter (Lampe et al. 2019); however, this limits the level of detail that can be obtained. Yet, if allowed multiple opportunities for observation, home-cage assessments can effectively detect the presence/absence of play behavior but not the subtle differences in such behavior.

10.4.2. Scoring Juvenile Social Behavior

During normal scheduled clinical observations, juvenile social behaviors will be evaluated in group-housed animals over a 1-week period from PND 35 to PND 42. Pinning and pouncing are considered the main indices of social play behavior in rats because they strongly co-vary with other playful social behaviors, such as following and wrestling (Panksepp and Beatty 1980; Pellis et al. 2022; VanRyzin et al. 2020).

Play behavior will be assessed using a rating scale depicting the absence (1) or presence (2) of the specific behavior occurring within the home cage:

- Pinning behavior (without associated vocalizations)
- Pinning behavior (with associated vocalizations)
- Pouncing behavior
- Aggressive behavior (e.g., biting)

10.5. Locomotor Activity

An assessment of motor function by locomotor activity provides an indication not only of the activity level of the animal but, if decreased, an indication of concern for either general health or motor strength that could compromise subsequent behavioral evaluations. As an assessment of motor function, locomotor activity captured by automated photocell or video-capture detection systems allows for the evaluation of general motor activity; in addition, by using the stimulus of novelty, free exploration in the arena can be used to examine curiosity and exploration (Pisula and Modlinska 2020). Motor activity devices (photocell or video tracking) shall be capable of evaluating ambulatory motor activity in a two-dimensional manner (x, y planes) and rearing (z plane) in a time-interval manner and will be appropriate for the age and size of the animal. The

system shall be capable of measuring thigmotaxis and regional preference within the arena and of providing information on the ambulatory activity path length.

10.5.1. Configuration

Spontaneous locomotor activity shall be assessed within a defined arena using a commercially available system with documentation of usage and demonstration of biological response from positive control agents. The configuration of the system shall allow for data collection of activity within the entire arena, immediately along the perimeter at the chamber wall (margin zone), and within a defined smaller center area (center zone).

- Photocell open-field arena apparatus example: approximately 40 cm × 40 cm × 20 cm photocell device using a two-dimensional sensor array configuration (photocells at 1-inch intervals). Zones: margin (outermost infrared beam on each sensor, which would represent 1-inch margin at the wall of the arena) and center (5 × 5 inch) square. Video-capture systems would configure a similar arena and arena map for data capture. In addition, contrast distinction and lighting will be sufficient for uniform camera detection (e.g., white animal on a dark background).
- For photocell devices, the height placement of the photocell banks for horizontal measures shall be set to detect the midpoint of the body trunk as appropriate for the age and species of animal under study.
- For rearing behavior, the detection limit (photocell height, video recording height) will be at a height equivalent to at least three-quarters of the full rearing height of the animal to ensure accurate detection of full hindlimb rearing, exclusive of raising of the head, back, or slight rear, not requiring full weight to be placed on hindlimbs. (This height location shall be empirically determined for each species, strain, age, sex, and size of animal under study by confirmation of experimenter-observer counts compared with photocell detection).
- Parameters of the testing apparatus (height location of the photocells for ambulatory activity, empirically determined height location of photocell bank for rearing, arena zone definitions) shall be documented in the study file.
- At the beginning of each test session, each apparatus shall be calibrated for photocell alignment and function as instructed in the manufacturer's manual. In addition, each unit shall be tested to ensure accurate tracking by moving either the experimenter's hand or a controlled moving item (e.g., plastic ball) within the chamber and following the tracking pathway on the computer screen. The instrument shall provide a software diagnostic feature to be run prior to each session.
- Tests of motor activity shall be conducted under lighting conditions that maintain the normal home-cage room light/dark cycle. The lighting level within the front section of the home cage will be measured using a Lux meter. Standardization of lighting for each motor activity arena shall be similar to the level measured for the home cage. In configuration of the test room and apparatus placement, the uniformity of luminance shall be confirmed. The luminance shall be similar across each full arena to ensure no area of the arena is within shadows. Meeting these specifications may require modification of the actual luminance of the room lighting. The actual luminance of the room and in each of the arenas shall be tested using a Lux meter and recorded.

10.5.2. Initiation and Duration of Testing

- Animals shall be placed in the center of the activity arena. Each apparatus shall be programmed for the software to automatically start the test session with the detection of first movement within the chamber. If this is not possible, then the computer test session shall be started manually by the experimenter upon entry of animal into the chamber and clearance of the experimenter's hand.
- Standard Test: Measurements shall be collected in 5-minute epochs for a total of 30 minutes (preweanling/weanling <35 days of age) or 45 minutes (>45 days of age).

Endpoints for Collection

- For the entire arena, data shall be collected in 5-minute epochs for total activity, ambulatory activity (as defined by manufacture), fine movements, distance traveled, stationary time, rearing events, and time spent rearing.
- For the full arena, total session data shall be calculated for total activity, ambulatory activity, distance traveled, rearing events, and time spent rearing.
- In defined zones, data shall be collected in 5-minute epochs for ambulatory activity, distance traveled, time spent within defined zones (margin time [thigmotaxis]; center arena), and entries into zones.
- For the full arena, a pathway track or heat map for each animal shall be recorded for the entire session.
- Commercial supplier information defining how each endpoint is captured/determined and description of any calculations necessary for endpoint determination shall be provided in the study file.

10.5.3. Endpoints for Analysis

Total Session

- Full Arena: Total activity, ambulatory activity (as defined by manufacture), distance traveled, rearing events, and time spent rearing.
- Zones: Ambulatory activity, distance traveled, time in zone, and entries into zone.

Epochs

- Full Arena: Total activity, ambulatory activity, distance traveled, rearing events, and time spent rearing.
- Zones: Ambulatory activity.

10.5.4. Statistical Analysis

- Data will be examined for homogeneity of variance.
- Data transformations, such as logarithms, shall be considered for analysis over epochs, if necessary, to meet model assumptions.
- Data obtained across the full session, either in the full arena or in epochs, shall be analyzed with ANOVA, with dose as a factor.

- Data collected in 5-minute epochs shall be analyzed with RMANOVA, with dose and time as factors.

10.6. Forelimb and Hindlimb Grip Strength

To detect alterations within the peripheral nervous system or the spinal cord that would compromise limb strength or motor behavior, grip strength of the forelimbs and hindlimbs shall be assessed using a digital force gauge (Maurissen et al. 2003; Meyer et al. 1979; Takeshita et al. 2017).[2]

10.6.1. Configuration of Grip Strength Apparatus

- Animals shall be assessed for fore- and hindlimb grip strength using a strain-gauge system appropriate for the species and age of animal (screen, bar, overall length of platform, gauge strength size [kg]). Preference is given to equipment configuration that allows assessment of fore- and hindlimb grip strength in one pass and provides physical support for the animal.
- The apparatus shall be placed on a stable surface, away from drafts or vents that could disturb the measurement by the sensor.
- A bar shall be used for assessing fore- and hindlimb strength in adult rats. It is recommended that a screen grid not be used with adult rats due to the nature of the procedure and to prevent the error of the animal not being able to rapidly release the grid, thus altering the grip score.
- For juvenile rats and all mice, a screen grid or T-bar, sized appropriately, shall be used to assess fore- and hindlimb strength.
- Control animals should measure within the midrange of the meter, and the settings shall allow for detection of an increase or decrease in strength.

10.6.2. Grip Strength Protocol

- Confirm that the forelimb gauge is set to PULL mode and the hindlimb gauge is set to PUSH mode and that both gauges have been reset to zero.
- Place the animal on the center platform of the apparatus, facing the forelimb gauge. Set the forepaws on the screen (juvenile rats and mice) or bar (adult rats), attached to a strain gauge. Alternately, hold the animal by the "neck scruff" or base of the tail so only its front paws grip the grid platform/bar (handling method to be standardized across all animals in the test).
- Contact with the forelimb apparatus shall require all four digits of both limbs.
- Once a successful grip is observed, hold the animal by the base of the tail and gently pull horizontally and quickly with an even force in one continuous motion until its grip is released down the complete length of the grid/bar. The propensity is that the animal will cling onto the grid/bar until it can no longer resist the increasing force before it is released. Immediately following this and within the one smooth,

[2] https://www.mousephenotype.org/impress/ProcedureInfo?action=list&procID=1130; https://treat-nmd.org/wp-content/uploads/2016/08/MDX-DMD_M.2.2.001.pdf

continuous pulling motion for the forelimb assessment, the hindlimbs are allowed to grasp a bar (or grid) as the animal's body is quickly but smoothly pulled away. A slight pause may be necessary before beginning the pulling motion to assure the rat's digits are properly curled around the bar and the paws are not crossed.

- Do not allow the front feet to touch the hindlimb screen or bar.
- Do not allow the experimenter's hand to touch the screen or bar.
- Do not allow the toenails to catch in the mesh as this can result in a strong jerk motion and invalidate the reading.
- Three sequential trials shall be performed with an approximate minimum intertrial interval (ITI) of 10 seconds. Juvenile rats and mice may require a longer ITI to minimize fatigue, if more than three trials are required (to be determined by pilot study).
- The digital readouts on the gauge shall be recorded and the gauges reset.
- If necessary, two additional trials can be run to obtain three valid trials for averaging.
- If any animal fails more than two trials, that shall be noted.
- Unacceptable trials include: (a) the animal is aggressive and cannot be properly handled; (b) the animal fails to grip the mesh grid or T-bar with two paws; (c) the animal reacts in such a manner that the grid mesh or T-bar is released before the technician pulls the animal away from the gripping surface; (d) for some reason, the force gauge is accidentally activated by contact unrelated to the appropriate experimental procedure; and (e) the technician pulls the animal too strongly to mask animal response.

10.6.3. Endpoints for Collection

- Each individual gauge reading for forelimb grip strength
- Each individual gauge reading for hindlimb grip strength
- Average of three valid gauge readings for forelimb grip strength
- Average of three valid gauge readings for hindlimb grip strength
- If fewer than three valid readings, average of two valid trials can be calculated and the failed trials noted.

10.6.4. Endpoints for Analysis

- Mean forelimb grip strength for three valid trials
- Mean hindlimb grip strength for three valid trials

10.6.5. Statistical Analysis

- Data will be examined for homogeneity of variance.
- Data transformations shall be considered for analysis.
- Considerations of body weight difference as an influencing factor shall be made.

- Data obtained shall be analyzed with ANOVA, with dose as a factor.

10.7. Accelerating Rotarod (Motor Coordination)

The rotarod apparatus/protocol can be used to measure motor function, motor learning, coordination, and equilibrium in both rats and mice (Chapillon et al. 1998; Crawley 1999; Rustay et al. 2003a; 2003b). Assessment of motor coordination using the rotarod requires the animal to learn the novel task. To ensure all subjects have learned the task to the same degree, thus enabling experimenters to accurately measure differences in motor coordination and equilibrium, 2 weeks of training with three daily sessions of three trials per week are normally required in rats and mice.

10.7.1. Configuration of Rotarod Apparatus

- The automated apparatus shall be equipped with a rotating rod of a diameter appropriate for the size of animals tested. The rod will be grooved to allow for gripping by the animal.
- An apparatus that automatically records latency to fall and rotational speed (rpm) of the rod at time of fall under an accelerating or fixed speed is recommended.
- Before study initiation, data obtained within the previous year will be available to confirm the appropriate acceleration rate for the species, strain, sex, and age of the animal and the proficiency of the laboratory. The rate shall allow control animals to improve performance over trials without demonstrating a ceiling effect; the rate will not be so aggressive as to prevent control animals from adequately performing.
- Modifications to the apparatus can be employed to provide better traction/grip for the animal, with coarse rubber, Velcro, or fine grit (320 grit) sandpaper (this has been found to be helpful with mice or young rats) (Bohlen et al. 2009).
- The apparatus shall have partitions between animals and an enclosure to prevent animals from escaping after a safe landing.
- Calibration of rotational acceleration will be conducted within a year of study and data provided in the study report (Bohlen et al. 2009).

10.7.2. Rotarod Protocol

- Rats and mice may be placed on the rod by gripping the tail and/or by grasping the animal around the body. The limbs should be restrained as much as possible to minimize the animal grasping peripheral structures. An animal is placed on the rod, facing away from the direction of rotation, so it has to walk forward to stay upright. In mice, holding the animal by the tail at an angle of 40° below horizontal works well. On a multi-animal apparatus, animals are quickly placed on the rod, facing in the correct direction, and are in stable position at the start of timing.
- The first exposure to the rotarod serves as an initial training trial and is not included in the performance data analysis. With this first trial on the first day, the animal learns to balance on the stationary rod and then to maintain balance on the rotating rod. This first trial can be either (1) a rod constantly rotating at approximately 10 rpm for 1 minute or (2) on rotation parameters consistent with the testing trials. For the

training trial, the animals are placed on the low-speed rotating rod. If the animal falls off in less than a minute, it is immediately placed back on the rod until it stays on the rod for a full minute. The animal is allowed a 10-minute rest interval before the next trial.

- On day 1, the first exposure trial is followed by three trials. To ensure all subjects have learned the task to the same degree, thus enabling experimenters to accurately measure differences in motor coordination and equilibrium, 2 weeks of training with three daily sessions are normally required. Three trials shall be run per day, three times per week for 2 weeks, with a minimum ITI (rat: 10 minutes; mouse: 15 minutes) to minimize fatigue.
- ITI will be maintained across all animals. The rod will be rotating at 2 to 4 rpm (warm-up speeds). Any subject that falls or jumps from the rod during the first approximately 10 seconds of testing can be replaced, and the lane timer can be restarted.
- Overall test confounders include animals that cling to the rod but do not fall (passive rotations), animals that refuse the test and simply fall without any real evidence of altered muscle strength (exclude as outliers), and weight and size of animals—heavier and larger animals perform less well on the rotarod and fatigue with progressively longer latencies (confirm with fixed speed tests).

10.7.3. Endpoints for Collection

- Latency to fall
- Record the occurrence of jumping or passive rotations with speed or time of occurrence
- Record a latency of maximum cut-off for animals that did not fall off within the test interval

10.7.4. Endpoints for Analysis

- Latency to fall: individual trials and mean per day (excluding initial training trial)

10.7.5. Statistical Analysis

- The mean response of day 1 and day 2 shall be analyzed with ANOVA, with dose as a factor.
- Data across trials on each day shall be conducted with RMANOVA, with dose and trial as factors.
- The data across trials on each day shall be analyzed for pairwise comparison and trend across dose for each trial, using the F-test ANOVA.
- The mean response on day 1 and day 2 shall be analyzed by RMANOVA.
- If the data represent censored observations, the amount of censoring shall be reported and an appropriate analysis (e.g., time-to-event modeling) shall be considered.

10.8. Acoustic Startle Reactivity and Prepulse Startle Inhibition

The startle response is an unconditional reflex, characterized by the rapid contraction of skeletal muscles, in response to a sudden and intense startling stimulus (e.g., noise burst, air puff, light flash). In rodents, the acoustic startle response (ASR) can be used to study habituation, sensitization, classical conditioning, fear, and anxiety. Habituation to the startle response is a form of nonassociative learning and can also be viewed as a sensory filtering process as it decreases an organism's response to a nonthreatening stimulus. Habituation can be examined within a test session (short-term habituation) or across sessions (long-term habituation). Within a session, habituation normally occurs within the first 10 trials and over 4–5 days for across sessions (Pilz and Schnitzler 1996; Pilz et al. 2014; Valsamis and Schmid 2011). PPI describes the phenomenon in which a weak initial stimulus (prepulse) inhibits the startle response that is elicited by a strong stimulus. The level to which the prepulse stimuli inhibits the startle response increases with prepulse intensity. Animals shall undergo testing for startle response and PPI using a computer-assisted automated startle/PPI system. This system shall allow for the ability to view the continuous individual waveform responses and to examine the data post hoc based on shifting the millisecond time interval for recording a response. Recent articles on procedural methods and optimization considerations for ASR and PPI are available (Hormigo et al. 2019; Miller et al. 2021; Shoji and Miyakawa 2018; Valsamis and Schmid 2011).

10.8.1. Testing Units and Calibration

- All testing units shall be housed in individual sound-attenuated chambers within a testing room under normal animal facility environmental conditions.
- Calibration: Calibration of the sound (sound meter) and the movement (e.g., oscillation calibration device) sensors is critical for obtaining valid test results. A set background decibel level is essential when conducting experiments examining PPI as the prepulse levels are set relative to background. Thus, calibration shall be to a specific level and not to a range. This may require an extended time period to optimize calibration. It is recommended that the full calibration of each unit be scheduled to allow for adequate time prior to the start of any specific experiment. Once calibrated for offset (gain) and sound, the units should remain relatively consistent over time. If a unit is identified to drift over time, exclusion of that unit should be considered until recalibration from the commercial supplier. Confirmation of calibration shall be conducted within 24 hours prior to the start and 24 hours following cessation of a specific testing time for an experimental set of animals. Adjustments to sound or gain may be required based on species, strain, age, or animal tested.
    - Each sensing plate/unit shall be calibrated for mechanical and circuit offsets or gain using an oscillation calibration device following detailed instructions provided by the commercial supplier. The gain shall be set for each age/weight of the animal species and strain tested. Uniform readings (actual and not range) across units shall be confirmed. The actual value shall be recorded for each unit.
    - Using a sound meter, each unit shall be calibrated to the set background decibel level. The preferred level is 65 dB. Given the increase in fan noise

with aging equipment, if experimenters are unable to lower units to this level, then they will set the lowest level between 65 and 70 dB and record the background level. Set all units to the same background decibel. If, for any reason, a unit is unable to be set at the same background sound level, there can be a variability of ±1 dB for background across units (note how this will alter settings for prepulse decibels).

- The prepulse stimuli decibel levels will be set relative to the background decibel level for each unit. Using a sound meter, confirm the decibel level for each prepulse stimulus and for the 120-dB startle stimulus during the calibration stage. If calibration is required, balance the calibration for that unit across all decibel levels (background, prepulse, startle). Record any deviation.

10.8.2. Adaptation to Handling and Holding

- The startle apparatus requires that the animal be restricted for movement during the test session. The animal's species, age, and weight determine the appropriate size of the enclosure to restrict mobility as recommended by the manufacturer. The holding enclosure represents a novel environment for the animal and can alter an initial startle response. Unique handling is required to place the animal into the holder to minimize stress and allow for acclimation. For a tube type holder, the handling sequence involves gently squeezing the forepaws together so they cross on the underside of the animal, holding the hindquarters to prevent perambulation, introducing the animal into the holder or other suitable facsimile, and holding it there for 3 minutes (Geyer and Swerdlow 1998). In general, this procedure shall be conducted two times within the week prior to first startle test. For an open holding chamber, the animals can be placed into the holder and held for 3 minutes for one session within the week prior to the first startle.

10.8.3. Confirmation of Startle Decibel and Prepulse Intensities

- Before examination of animals on study, within 1 year of study initiation, the following will be conducted in animals (five/sex) for specific test ages, species, and strain to establish and confirm optimal intensities and absence of drift. Once established, these settings shall be confirmed on an annual basis prior to initiating testing on study animals.
    - An input/output function test shall be conducted. After a 5-minute acclimation period under constant background white noise of 65 dB, startle stimuli (20 milliseconds) shall be delivered on an ITI of 20 seconds. Startle stimuli events shall start at approximately 75 dB and increase by 5-dB increments until reaching 120–125 dB. Startle magnitudes shall be sampled each millisecond for 200 milliseconds beginning at the onset of the startle stimulus. These data shall be used to determine the maximum startle response (largest response within 200 milliseconds), provide information on the waveform, and average the response over the entire response window.
    - PPI stimulus intensities shall be identified to elicit intermediate levels of PPI to allow for treatment-induced increases or decreases in PPI to be observed. Maximum startle response to each of the individual PPI intensities shall be

determined. The required number and interval of PPI intensities shall be determined for the species, strain, and age of the animal.

10.8.4. Paradigm Configuration

- Background level: 65 dB
- PPI intensities set at 3, 6, 12, and 15 dB above background

10.8.5. Prepulse Auditory Startle Inhibition (PPI) Protocol

- Each session shall start with a 5-minute period of acclimation to the restrainer and chamber with continuous background noise (65 dB).
- Delivery of startle trials shall be under a fast rise time (<2 milliseconds) burst of noise presented for a 40-millisecond duration at an intensity of 120 dB.
- Trials will be delivered according to a variable ITI of 15 seconds, with a range of 7–23 seconds.
- The delivery of stimuli shall follow the sequence outlined in Table 10-1 that includes six initial 120-dB pulse-only trials followed by two "blocks" of trials that represent each of the paired prepulse intensities with a 120-dB trial five times each, five of the 120-dB pulse trials, and one to two no-stimulus trials presented in a pseudorandomized manner. These two blocks shall be followed by 10 of the 120-dB pulse trials.
- The full collection window shall be set at 500 milliseconds: (1) a sampling of 250 milliseconds preceding the 120-dB startle elicitation to confirm confounding with baseline activity and/or confirm absence of startle response elicited by the prepulse intensities followed by (2) a sampling of 100 milliseconds to collect the maximum startle response. Data for determining PPI will be obtained from the 100-millisecond sampling window measured from startle stimulus onset. The sampling during the 250 milliseconds preceding the 120-dB startle stimulus, but following the prepulse stimulus, shall be collected and examined to determine any group differences in response to each prepulse intensity.
- Prepulse stimuli (3, 6, 12, 15 dB above threshold unless empirically changed on the basis of the pilot study results) shall be presented for a 20-millisecond duration with an interstimulus interval of 65 milliseconds (mice) and 80 milliseconds (rats) before the onset of the 120-dB startle stimulus ().
- Peak response magnitude (i.e., V_{max}) on no-stimulus (NOSTIM) trials shall be recorded and reported as a sampling of excessive activity of the animal within the chamber.

Table 10-1. PPI Protocol for Stimuli Delivery

Trial No.	Block No.	Trial Name	Prepulse Level (dB)
1	0	P120	–
2	1	P120	–
3	1	P120	–
4	1	P120	–
5	1	P120	–
6	1	P120	–
7	**2**	**NOSTIM**	–
8	2	PP77P120	77
9	2	PP68P120	68
10	2	PP80P120	80
11	2	P120	–
12	2	PP71P120	71
13	2	PP71P120	71
14	2	PP80P120	80
15	2	PP77P120	77
17	2	PP68P120	68
18	2	P120	–
19	2	P120	–
20	2	PP77P120	77
21	2	PP71P120	71
22	2	PP68P120	68
23	**2**	**NOSTIM**	–
24	2	PP80P120	80
25	2	PP80P120	80
26	2	P120	–
27	2	PP77P120	77
28	2	PP71P120	71
29	2	PP68P120	68
30	2	PP77P120	77
31	2	PP68P120	68
32	2	PP80P120	80
33	2	P120	–
35	2	PP71P120	71
36	3	P120	–
37	3	PP77P120	77
38	3	PP80P120	80

Trial No.	Block No.	Trial Name	Prepulse Level (dB)
39	3	PP68P120	68
40	3	PP71P120	71
41	3	P120	–
42	3	PP80P120	80
43	3	PP71P120	71
44	**3**	**NOSTIM**	–
45	3	PP77P120	77
46	3	PP68P120	68
47	3	PP77P120	77
48	3	PP71P120	71
49	3	PP80P120	80
50	3	PP68P120	68
51	3	P120	–
52	**3**	**NOSTIM**	–
53	3	PP71P120	71
54	3	PP80P120	80
55	3	P120	–
56	3	PP68P120	68
57	3	PP77P120	77
58	3	PP77P120	77
59	3	P120	–
60	3	PP68P120	68
61	3	PP71P120	71
62	3	PP80P120	80
63	4	P120	–
64	4	P120	–
65	4	P120	–
66	4	P120	–
67	4	P120	–
68	5	P120	–
69	5	P120	–
70	**5**	**NOSTIM**	–
71	5	P120	–
72	5	P120	–
73	5	P120	–

NOSTIM = no stimulus.

10.8.6. Endpoints for Collection

- For startle and prepulse startle inhibition, the schedule of the stimulus presentations shall adhere to Table 10-1, and documentation will be provided.
- Peak response amplitude (i.e., $V_{max)}$ for each startle trial.
- Peak response amplitude for each prepulse stimulus intensity during the 250-millisecond sampling interval to determine whether there is an alteration in the prepulse threshold or a significant movement response that may affect the ASR.
- Time to maximum response (i.e., T_{max}) for each trial, excluding NOSTIM.
- If available: latency to onset of response, rise time of response as identified by the manufacturer's instructions.

10.8.7. Endpoints for Analysis

- First 120-dB trial: Peak response amplitude – representative of naive startle response.
- Peak response amplitude for individual animals and each 120-dB-only trial.
- The measured startle response is typically lognormally distributed across pulse types (Csomor et al. 2008) and often the median response over a block is more robust than the mean. Therefore, where applicable, both mean and median response will be calculated.
- Median peak response amplitude of 120 dB only occurring in the "blocks" of trials inclusive of prepulse stimulus intensities (Blocks 2 and 3) for each animal. This will be considered the "120-dB peak response" in calculating percent prepulse inhibition (% PPI) for that individual animal.
- Mean and median peak response amplitude for each prepulse intensity stimulus for each animal.
- PPI: For each individual animal and each PPI trial, the % PPI shall be calculated as [(120-dB peak response − prepulse peak response)/120-dB peak response] × 100. For each animal and each prepulse stimulus intensity, the average % PPI is calculated across all matched trials for the entire test session.
- Habituation: Calculated as the change in peak response amplitude over trials. Percentage change is calculated as a change of peak response of the last 120-dB trial compared with peak response of the first 120-dB trial for each individual animal.
- Data shall be represented as the actual metric recorded—e.g., Newtons (Amplitude [N]), static weights (Amplitude [g]), or volts (Amplitude [v])—per manufacturer's instructions.

10.8.8. Statistical Analysis

- Data shall be evaluated for assumptions of ANOVA (homogeneity of variance and normally distributed observations). Nonparametric methods should be considered if these assumptions are violated.
- Peak response amplitude of the first 120-dB ASR trial shall be analyzed using a one-way ANOVA.

- Peak response amplitude of 120 dB across trials shall be analyzed using RMANOVA (dose and trial as factors).
- Peak response amplitude for paired prepulse intensity per 120-dB trial for each prepulse intensity shall be analyzed by one-way ANOVA (dose as factor).
- Negative PPI values shall be set to 0 (the incidence of negative PPI values will be recorded).
- Calculated % PPI for each prepulse intensity shall be analyzed by one-way ANOVA (dose as factor).
- Calculated % habituation shall be analyzed with one-way ANOVA (dose as factor).

10.9. Morris Water Maze

Learning and memory will be assessed in the MWM using commercially available video tracking equipment and software that has demonstrated use in the published literature. Animals will undergo a sequence of training tests to evaluate performance in an MWM. On days 1 and 2, animals shall be familiarized to the tank, water, and swimming requirements of the test and assessed for nonspatial cued learning. This shall be followed by acquisition of a spatial hidden platform task (three training trials/day for 7 consecutive days). Twenty-four hours after completion of the hidden platform task, spatial reference memory shall be assessed in a probe trial. Forty-eight hours after completion of the probe trial, performance on a spatial reversal acquisition task shall be assessed (three trials/day for 3 days) (Gallagher et al. 1993; Gerlai 2001; Maei et al. 2009; Vorhees and Williams 2006).

10.9.1. Testing Environment

- Performance is dependent upon maze configuration. The use of multiple tanks requires uniformity of tank configuration and dimensional details of the room, a spatial-defined area, and spatial cues as they relate to the visual field of the animal. All specific details of physical properties of the tank configuration and cue placement shall be documented and provided in the study report. The tank shall be properly sized. The final report shall include all variables and detailed physical descriptions of the tank, platform, platform placement, and visual cues.

10.9.2. Tank and Platform

Tank

- The interior diameter of the standard circular tank shall be approximately 180 cm (6 ft.) (adult rats) or 130–150 cm (4–5 ft.) (immature rats and adult mice) with nonreflective interior surfaces. A large tank can be modified to the smaller size by the insertion of a circular ring to decrease diameter.
- When filled with water, the depth shall be sufficient to prevent the animal from touching the bottom (e.g., approximately 28–35 cm for a 70-day-old rat; 15–20 cm for mice) and with a surface-to-tank-lip distance of approximately 10–12 cm to prevent the animal from jumping out of the tank but allowing for line of sight to visual cues.

- The water temperature shall be equilibrated to the ambient room temperature (approximately 22°C). During training and testing, the water temperature shall be recorded at the start and end of each test day to confirm it is within this range.
- The tank shall have no prominent interior features that might provide proximal cues, such as markings inside the tank (e.g., welded seams shall be smoothed and painted to blend with the surrounding walls). The sides of the tank shall be smooth to minimize animals' attempts at climbing walls and should be a nonreflective matte finish. Location markers shall be placed on the exterior of the tank and will line up with markers on the floor to ensure identical location placement of the tank across trials/sessions.
- For albino (or light-coated) rodents, the tank shall be dark, and the platform shall be dark or clear to prevent visualization from the surface of the water. For pigmented rodents, a light-colored tank with a similarly colored or clear platform shall be used. If necessary, for animals with a dark coat, a white nontoxic water coloring (e.g., Crayola watercolor paint, tempera paint) can be used with a clear platform.
- The maze shall be designated into four equal quadrants (randomly identified as Northwest, Northeast, Southwest, and Southeast).

Platform

- The tank shall have the capability for using a hidden platform and a visible platform. The goal platform shall be approximately 10 cm in diameter for rats and 8 cm in diameter for mice. The platform shall be covered by a nonreflective textured material to allow for gripping by the animal to facilitate escape from the water.
- The platform shall be positioned in the respective quadrant (cued learning: NW; hidden platform: NE; reversal learning: SW), located approximately 36 cm (rat) or 30 cm (mice) from the interior wall of the tank (to minimize the chance of finding the platform simply by swimming along the tank wall), yet at a distance from the center. The platform shall remain within that quadrant for all animals during cued (visual) and hidden platform acquisition trials.
- Height of platform: For cued learning, the visible platform shall be at a height of approximately 1.5 cm above the surface of the water with a visible projection (10–12 cm^2 "flag") attached and rising approximately 13 cm above the visible platform. For spatial learning and reversal learning, the hidden platform shall be submerged below the surface of the water (approximately 1 cm for rats; approximately 0.5 cm for mice).
- The platform will be immobilized within the tank to prevent any shift over the day's testing.

Cleaning of Tank

- After each animal test, floating feces shall be removed and water dispersed to minimize urine scent near the platform or prior platform location.
- At a minimum, the tank shall be drained and rinsed clean at the end of each 5-day period, refilled, and allowed to equilibrate to ambient temperature. (Changing of the tank water can be conducted more often but must occur at least every 5 days.) Partial

changing of the tank water can be conducted within the 5-day period, if needed. The schedule of cleaning shall be constant across all test cohorts. If, for any reason, any one tank becomes soiled outside of the normal schedule and requires cleaning, this activity can be done without draining and cleaning the other tanks.

- Upon refilling of the tank, it shall be placed in its original position, and the camera shall be recalibrated to its original settings.

10.9.3. Experimental Environmental Cues

Visible Platform

- For nonspatial cued learning, minimizing visual spatial cues within the testing arena (enclosure) is critical to reduce the animal's access to visual cues that may be used to spatially navigate and to ensure the animal focuses on the platform as the cue.
- The tank shall be encircled by a curtained wall devoid of spatial cues.
- The experimenter shall remain at the start position during the trial to minimize movement or exit to the outside of the curtained wall.

Spatial Hidden Platform

- Visual cues within the testing room or within an enclosure are a major defining factor in the ability of an animal to learn the location of the platform via spatial processing.
- All tanks shall have an identical cue profile. If multiple testing areas are to be used, the environment shall be as identical as possible with regards to defined cues and architectural features.
- One specific cue set profile shall be used across all animals and shall be maintained over the entire course of the study.
- The rack of test animals within the test room is an odor and auditory cue. It can serve as a visual spatial cue, as well; thus, localization shall be standardized across test sessions and test rooms as dictated by room configuration. Location markers shall be employed as needed to ensure uniform rack placement across sessions.
- Room configuration. The room and walls shall be devoid of extraneous visual cues as much as possible, and any items remaining shall be considered part of the cue profile and thus, remain stationary across the study.
- Within a room, the background walls are light in color and, as an example, cues can consist of (1) two dark vertical lines from floor to ceiling (each line being 20 cm wide with a space of approximately 15 cm between them), (2) a large dark circle approximately 32 cm in diameter, and (3) a long dark horizontal line (20 cm in width) from the wall edge.
- Within-curtain enclosure. Curtains can be placed as walls around the tank to define a test enclosure. Curtains shall be placed a minimum of 2 ft. and a maximum of 3 ft. from the inner wall of the tank. The curtains shall be smooth and securely hung in a manner to minimize disturbance by testing room airflow. The curtains shall be at a sufficient distance from the tank to allow for experimenter mobility without movement of the curtain and disruption of the spatial cues. A permanent opening

shall be maintained for the experimenter to enter and exit and/or to remain (serving as a cue) during the trial to minimize any movement of the curtain and disruption of the spatial cues placed on curtain walls.

- The experimenter serves as a cue; thus, a mark on the floor shall be provided to indicate the exact location where the experimenter should remain after placing the animal into the water maze. The cues shall be placed within the animal's line of sight as it is swimming in the maze.
- A schematic of the room configuration or curtain dimensions and cues shall be included in the testing protocol (Figure 10-1).

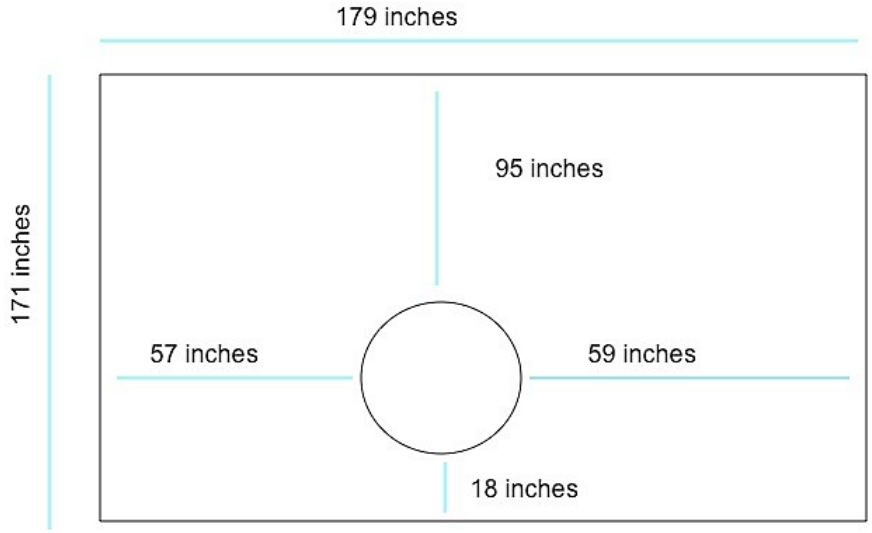

Figure 10-1. A Representative Example of Room Dimensions Relative to Water Tank

Lighting

The nature of lighting is critical to maximize accuracy of video tracking.

- Lighting shall be bright enough to allow for visualization of spatial cues.
- Lighting shall be bright enough to allow the video camera to track the animal.
- Lighting shall be arranged to prevent reflection on the water or in the video image (reflections can compromise video tracking as the software may confuse those with the animal).
- Lighting shall be arranged to prevent a shadow being cast into the tank interior from any surrounding structure, including the experimenter.
- Lighting shall be even, such as that obtained with a diffuse light source like a shaded fluorescent tube or globe-type incandescent bulb. Spotlights or uneven lighting shall not be used.
- Lighting shall be indirect and not in the direct line of sight of the camera. (One way this can be accomplished is by placing four to six globe bulbs around the pool, below the level of the water surface, outside of the line of sight of the camera lens.)

10.9.4. Configuration of Camera and Software

- The camera shall be positioned above the tank, perpendicular to the water surface. This position will be fixed and anchored above the tank. If this requires a stationary arm attached to the tank, that arm will be considered one of the visual spatial cues and thus, will be maintained in one position. Any wiring for the camera shall also be considered a spatial cue and be treated as such and included in the configuration schematic.
- The camera zoom setting shall be adjusted and the lens focused to display the entire experimental arena (in focus) on the computer screen.
- All camera automatic settings shall be disengaged.
- The camera aperture shall be adjusted for maximal contrast of the image.
- The entire setup shall be anchored during testing. The position of the arena, camera, and illumination shall be secured, relative to each other. If moved for tank cleaning, the system shall be recalibrated to its original position prior to testing.
- The camera zoom, focus control, and aperture setting shall be locked.
- Confirmation of the settings and quality of detection shall be conducted each day prior to the testing of the animals.
- The camera shall have a polarizing lens filter to minimize reflection.
- The visual field and lack of interference with capturing animals within that field (e.g., reflection) shall be confirmed each day prior to the start of the test session.
- Camera settings to capture the arena and animals shall be confirmed for alignment at the start of each test day.
- An automated video tracking system shall be used to capture MWM performance. The software algorithms used by the automated imaging program to define each of the endpoints shall be provided, per the commercial supplier's software manual. The software tracking system shall provide documentation of the testing protocol (arena parameters) and animal assignment and allow for post hoc evaluation of video images.

10.9.5. General Test Procedures

Placement and Removal of Animal from Tank

- The water shall be still before testing of any animal to minimize reflection that can interfere with video capture.
- The animal shall be removed from the transport cage and handled in a manner that provides support (e.g., placed in the crook of an arm, placed against body). With the animal supported by the palm of the hand or in a "carrier," it shall be placed into the tank by gently lowering the hand or carrier into the water. If an alternative to the experimenter's hand is used for animal placement into the tank or removal from the platform, this movement will be done in a manner that provides whole-body support to the animal and will be the method used for all animals in the study.
- The animal shall be placed in the tank with its nose facing the wall.

- If the animal cannot swim and sinks (not diving underwater), it shall be immediately removed from the tank and placed in the holding cage. For safety reasons, it is recommended that such retrieval be done with a container rather than by hand. This behavior shall be documented.
- Removal of animals from the platform shall be conducted with a whole-body method and not by use of the tail (unless necessary). The animal shall be first allowed to climb into the experimenter's hand or "carrier" prior to removal from the platform or from the water during the probe test. Upon removal, animals shall be placed on an absorbent towel. Effort shall be taken to place the animal in an area without air drafts to minimize discomfort. A "tested" animal shall not be placed into a cage with animals waiting for testing.

Start Location in the Tank

- A marking visible to the experimenter shall be placed on the outside of the tank to indicate starting location.
- Geographical nomenclature (N, NE, E, SE, S, SW, W, NW) is used to identify start locations (relative to cued/training goal/platform NE location) in the start location pattern provided. An alternative nomenclature can be used but must be consistent with the orientation and cross-referenced with geographical nomenclature.
- Cued learning (visible platform) start positions: SE to require a full transition across the tank to the platform in the NW quadrant.
- Different start quadrant locations will be randomized during spatial learning (hidden platform) and reversal learning. The sequences of start locations are designed to balance the right/left goal location (see Table 10-2 and Table 10-3 for example start positions).

Table 10-2. Start Locations for Acquisition with NE Quadrant as Hidden Platform Location

Day	Trial 1	Trial 2	Trial 3
1	S	W	NW
2	NW	S	SE
3	SE	NW	W
4	W	S	SE

Repeat sequence as needed.

Table 10-3. Start Locations for Reversal Learning with SW Quadrant as Hidden Platform Location

Day	Trial 1	Trial 2	Trial 3
1	N	E	SE
2	SE	N	NW
3	NW	SE	E
4	E	NW	N

Repeat sequence as needed.

Testing Schedule

- For each day of testing, a test schedule shall be provided, indicating the order that animals are to be tested and ensuring a counterbalance across dose groups. For any individual animal, this order shall be maintained across all test sessions.
- While animal performance will improve with training, the ITI between runs shall remain constant to maintain a uniform time for memory integration and learning.
- The testing schedule shall be appropriate for the species and age of the animal (e.g., adult rats perform well with consecutive training trials, whereas young rats and mice are more prone to hypothermia-induced performance effects; therefore, the latter's trials are separated by a significant ITI, approximately 30 minutes).

Visible Platform

- Animals will be trained on cued learning using a visible platform in the **NW** quadrant to ensure swimming ability, basic vision, and the ability to escape onto the platform.
- The task shall be performed under conditions that obscure the visibility of room spatial cues (e.g., decreased room lighting, curtain around tank with no cues attached).
- On day 1, the animal shall be placed on the platform for approximately 30 seconds to familiarize it with the platform before the initiation of the first trial.
- For each trial, the animal shall be placed into the periphery of the tank within a defined quadrant (Table 10-2), with its nose facing the wall of the tank.
- Animals will be allowed up to 90 seconds to find and escape onto the platform. If the animal does not find the platform within 90 seconds, the experimenter will gently guide the animal toward the platform by placing the hand behind the animal and allowing the animal to maintain a swimming action to reach the platform. The animal shall be allowed to escape the water by climbing onto the platform. This procedure will ensure that all animals learn to associate swimming and fully climbing onto the platform as the method of escape from the water. The animal will be allowed to remain on the platform for approximately 20 seconds. If the animal re-enters the water after climbing onto the platform, the same guidance procedure will be conducted, and the animal will be allowed to remain on the platform for approximately 10 seconds.
- Each animal will receive three sequential training trials per day for a total of 2 days with an ITI of at least 60 seconds for adult rats and a longer ITI for mice and young rats. The ITI length allows for integration of the learned event and minimizes the fatigue factor, thus decreasing variability in latency across trials. A relatively constant ITI will be maintained across the study for each animal in repeating the trial rotation for training.
- The start location sequence (SW, NE, SE) shall be followed in four sequential trials.
- If an animal "floats" or "circles" in early sessions, it may perform in later sessions and thus shall not be excluded. If an animal fails to swim (i.e., sinks but does not dive), this shall be noted. If this occurs on the second day, the animal shall be considered for removal from the study. Replacement of an animal at this point will

result in a lack of test history but shall be considered in discussions with the COR as an option to maintain sufficient n size.

- All animals shall achieve the performance criterion of reaching the visible platform before the 90-second cut-off before progressing to the hidden platform test. If an animal does not achieve the criterion within the number of sessions designated, a decision shall be made about additional training sessions.

10.9.6. Hidden Platform (Spatial Acquisition)

- Approximately 24 hours following completion of the visible platform test, the animal will be placed in the water maze and allowed 90 seconds to find the platform (NE quadrant). Animals shall remain on the platform for approximately 20 seconds, and they will be permitted to remain on the platform for approximately 20 seconds during all sessions of the first day and in the first trial of each subsequent day. In the second and third trial of subsequent testing days, the interval can be decreased to approximately 5 seconds.
- If the animal fails to find the platform within the maximum trial time, the experimenter will gently guide the animal toward the platform by placing the hand behind the animal and allowing the animal to maintain a swimming action to reach the platform. The animal shall be allowed to escape the water by climbing onto the platform. If the animal re-enters the water from the platform, the same guidance procedure will be conducted, and the animal allowed to remain on the platform for approximately 10 seconds. This procedure will ensure all animals learn to associate swimming and fully climbing onto the platform as the method of escape from the water as well as visual cues for spatial orientation to the platform. The animal will not be picked up from the water and placed on the platform. The animal will be allowed to remain on the platform for approximately 20 seconds.
- Over a 7-day interval, each animal will receive three sequential training trials per day with an ITI of at least 60 seconds (time constant across animals) for rats, with a longer ITI for mice or young rats (due to fatigue).
- It is expected that approximately 80% of control animals will reach the criterion of ≥50% decrease in either latency to platform or swimming distance to platform by the seventh day. If not, considerations shall be made to extend the hidden platform spatial learning phase of the assay.

10.9.7. Probe Trial (Reference Memory)

The MWM probe trial allows for the confirmation and assessment of spatial reference memory in performance of the task.

- At 24 ± 2 hours following each animal's last hidden platform test (acquisition), the animal will be assessed for reference memory using a single probe trial.
- Visual cues and lighting conditions shall remain as they were for the hidden platform task, but the platform will be removed from the tank.
- As described in the hidden platform testing, the animal shall be placed in the tank at the SW start location.

- The animal shall be allowed to freely swim for 90 seconds with data collected for each 30-second epoch. The animal shall be removed from the tank at the end of 90 seconds.

10.9.8. Reverse Platform (Reversal Learning)

Approximately 48 ± 4 hours after the probe trial (scheduling to maintain constant time interval across all groups), the animals will be evaluated for their ability to learn a new platform location.

- The tank setup and visual cues shall be the same as those for the hidden platform test, with the platform moved to the opposite quadrant location (**SW**).
- Animals will be individually placed in the water maze and allowed up to 90 seconds to find the platform. If an animal fails to find the platform, it will be guided to and placed upon the platform. Animals will be allowed to remain on the platform for approximately 20 seconds.
- Given that the animals have already learned the parameters of the task and are only shifting location, they shall be tested for three trials per day for 3 days with a minimal ITI of 60 seconds.
- If the performance fails to reach the level observed on the last day of the hidden platform acquisition (the last day prior to the probe trial), training shall continue for up to 2 additional days or until performance reaches the previous levels observed in the hidden platform test or at the decision of the COR.

10.9.9. Endpoints Collected

For the MWM, data collected for visible platform, hidden platform, and reversal learning shall be averaged over the daily trials for each animal for repeated measures analysis to demonstrate acquisition and, for the reversal learning, the ability to shift to a new location. For the probe test, data are collected to demonstrate preference for the quadrant previously containing the escape platform, relative to the other quadrants. It is also used to show that the animal can learn that the platform is not present and then shift their search strategy.

Visible Platform (Nonspatial Learning)

- Time to find platform (latency)
- Total distance traveled to platform (path length)
- Average swim speed
- Time spent floating (% trial duration)
- Percent thigmotaxis time (% trial duration when the subject was in the outer 10% of the pool diameter)
- Thigmotaxic tendency (proportional distance traveled within the outer 10% of the pool relative to total distance traveled)
- Daily averages calculated for trials within a session for individual animals
- Analysis: RMANOVA (dose and day as factors)

Hidden Platform (Spatial Acquisition)

- Time to reach platform (latency)
- Total distance to reach platform (path length)
- Average swim speed
- Time spent floating (% trial duration)
- Percent thigmotaxis time as % trial duration when the subject was in the outer 10% of the pool diameter
- Pathway tracking, as available, by commercial video tracking and analysis software, as instructed by manufacturer
- Daily averages calculated for trials within a session for individual animals
- Analysis: RMANOVA (dose and day as factors)

Probe Trial (Reference Memory)

Initial Response

- Initial latency to enter the quadrant containing previous platform location
- Initial latency to swim to a predefined annulus surrounding the previous platform target that is 1.5 times larger than the target itself
- Initial latency to swim to previous platform target site
- Distance traveled to the entry into the previous target platform quadrant
- Distance traveled to the target annulus
- Distance traveled to the previous platform target site
- Data analyzed by one-way ANOVA (dose as factor; or Kruskal Wallis/Dunn)

Total Session and 30-second Epochs

- Platform-site crossings: number of crossings over the previous escape platform location
- Platform-annulus site crossings: number of crossings over the annulus of the previous escape platform location
- Time in the target annulus site
- Total number of entries into each quadrant
- Quadrant time: the total time spent in each quadrant
- Quadrant distance traveled: the total swimming distance (path length) within each quadrant
- Calculated quadrant time percentage: the percentage of time spent in each quadrant
- Calculated quadrant distance traveled percentage: the percentage of distance traveled in each quadrant
- Total session swimming distance: the total swimming distance (path length) covered over the entire tank over the full session

- Search strategy: pathway tracing as provided by instrument manufacturer software analysis
- Data analyzed by one-way ANOVA (dose as factor, or Kruskal Wallis/Dunn): platform-site crossings, platform-annulus site crossings, time in target annulus site, in-goal quadrant-only total time spent; total distance traveled, number of entries; calculated % total time spent, % total distance traveled in-goal quadrant relative to other quadrants to demonstrate preference for the goal quadrant
- Analyses of time spent, distance traveled, or entries into quadrants other than goal quadrant are not of relevance to the assessment and shall not be conducted

Reversal Platform (Reversal Learning)

- Time to reach platform (latency)
- Total distance traveled to reach platform (path length)
- Average swim speed
- Time spent floating (% trial duration)
- Calculated percent thigmotaxis time as % trial duration when the subject was in the outer 10% of the pool diameter
- Pathway tracking by commercial video tracking and analysis software, as instructed by the manufacturer, shall be maintained for all MWM trials

10.9.10. Statistical Analysis

- Data shall be evaluated for homogeneity of variance and normally distributed observations. Transformation of data or nonparametric methods should be considered if these assumptions are violated.
- Independent group mean comparisons shall be conducted upon significant one-way ANOVA or Kruskal Wallis/Dunn results.
- Post hoc analysis shall be conducted in the presence of significant main effects and the absence of significant interactions.

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10.11. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapter 10 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was 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 10: Neurobehavioral Testing
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

Peer Reviewer

Kevin Crofton, Ph.D.
President
R3Fellows LCC
Durham, North Carolina, USA

11. Data Collection and Submission

J.M. Fostel[1], G.K. Roberts[1], K.A. Shipkowski[1] (Editors)

[1]Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Last Updated: March 2023

11.1. Computer System for Data Input (NTP Provantis)

In accordance with National Institute of Environmental Health Sciences (NIEHS) policy, all data collected shall be returned to NIEHS via electronic files. Data management shall adhere to relevant National Institutes of Health (NIH) and NIEHS Data Management and Sharing plans. A copy of the data is permitted to reside at the testing laboratory.

For in vivo data from animal studies, NIEHS supplies licenses to NTP Provantis, which is a commercial, hosted product configured for use by NIEHS and the testing laboratories to collect data for studies. NTP Provantis permits access by multiple laboratories but prevents laboratory personnel from viewing the content of studies performed in other laboratories. NTP Provantis is a computerized real-time data collection system that includes:

- Customizable protocol input for unique study design
- The ability to track maternal animals and individual pups from specific litters
- A hierarchical security structure to control access to individual data files (e.g., technician clearance, study director clearance)
- Real-time dose calculation for gavage studies based on body weights and dosing volume
- Integration of measuring apparatus that allows input of values directly into the system without manual reentry
- The ability to create summary reports for all major study endpoints during the course of the study, including standard parametric and nonparametric analyses for monitoring study progress

In compliance with the Food and Drug Administration's Good Laboratory Practice (GLP) guidelines, NTP Provantis is validated for use in a GLP environment, and all changes are subject to a revalidation process before release to the laboratories. The testing laboratory shall be responsible for documenting their acceptance of the use of NTP Provantis in their GLP environment and the qualification/calibration of balances and other equipment interfaces used for input to the data management system.

With the approval of the contracting officer's representative (COR), the testing laboratory may use a data collection system similar to NTP Provantis and arrange for all data collected to be entered retrospectively by the laboratory into NTP Provantis or returned in an electronically readable format with full metadata attached.

Some data are not suited for direct collection in NTP Provantis. In this case, the testing laboratory shall return the data in electronically readable files with all appropriate metadata. The COR shall obtain agreement from NIEHS for the format of these files before submitting the data. This data submission shall include the original (raw) instrument data files. As applicable and required by the individual protocol outline, analysis results shall be submitted electronically following approval of the study report by the COR.

Data collection shall adhere to the Division of Translational Toxicology (DTT) Data Dictionary (DDD)[1] when possible. The testing laboratory shall work with DTT to register new terms in the DDD prior to using the terms in data submissions to DTT.

If required to perform analysis on human study samples, sensitive data and all patient agreements covering the data shall be submitted to DTT in accordance with NIH policy,[2] which is available to contractors and staff with government identification badges and NIEHS network accounts.

11.1.1. Hardware

The testing laboratory will be expected to provide electronic balances with cables to interface with the computer. Specifications for compatible balances can be provided. Wireless connections are discouraged.

11.1.2. NTP Provantis Users

Access to NTP Provantis is governed by guidance documents. Users are required to employ the following:

- Guidance for NTP Provantis Users
- Guidance for NTP Provantis Training
- Guidance for NTP Provantis System Changes
- Guidance for NTP Provantis Information Technology

These guidance documents can be found in the Chemical Effects in Biological Systems[3] (CEBS) bin for NTP Provantis guidance documents. These documents are available to contractors and staff with government identification badges and NIEHS network accounts.

NIEHS also produces documentation that includes expectations of use for users of NTP Provantis, as well as hints, guide sheets, and tips on use of tables.

These documents can be found in the CEBS bin.[4]

11.1.3. Study Protocol

When a study is initiated, the study protocol is created in NTP Provantis under the NTP Provantis support contract at the request of the testing laboratory with COR approval. The testing laboratory then adds the protocol information, including information about the test article (e.g.,

[1] https://cebs.niehs.nih.gov/cebs/paper/15466
[2] https://junction.niehs.nih.gov/divisions/director/osim/policy/human-research/index.htm
[3] https://connect.niehs.nih.gov/cebs3/cebsbin/index.cfm?action=main.showBin&bin_id=430
[4] https://connect.niehs.nih.gov/cebs3/cebsbin/index.cfm?action=main.showBin&bin_id=715

name, CASRN, treatment groups); the animals studied (e.g., species, strain, sex, supplier, number per treatment group, cage allocation to racks); and the scheduled activities (e.g., collection of body weights, feeder weights, clinical signs). The testing laboratory enters the information into the protocol a week before study initiation.

11.1.4. In-life Data Collection

Animal body weights, food and water consumption data, clinical observations, and animal removal information shall be collected and stored in NTP Provantis. Data are checked for completeness and validity each day. If needed, corrections are made using NTP Provantis and a reason for the change is entered into the audit trail.

11.1.5. Pathology

Weights and observations made at necropsy and at tissue trimming are recorded in NTP Provantis. A standard terminology, the NTP Provantis lexicon, is used to describe gross and microscopic lesions. Data are entered into the computer by choosing the organs, sites, morphologies, and qualifiers from a menu on the computer screen. The study pathologist can review and change diagnoses at any time during the evaluation of a study.

11.1.6. Data from Instruments

The testing laboratory may use instruments not connected to NTP Provantis to collect data (e.g., instruments used in neurological testing or clinical pathology). These data are imported into NTP Provantis using the data import feature. The testing laboratory works with NTP Provantis support contract personnel to design a form for data import that the testing laboratory uses to import the data.

11.1.7. Non-Provantis Data

Some data are too large to be captured in raw form into NTP Provantis (e.g., profiles from clinical pathology instruments, next generation sequencing files). These files shall be loaded into a CEBS collection portal or CEBS bin. Before files are submitted, the COR shall work with NIEHS program leads and the CEBS administrator to ensure that all data and metadata needed for FAIR (findable accessibility, interoperability, and reusability) data standards are included. In general, these are raw data, any transformed or normalized data, outliers, methods used for transformation, links between data files and animal ID in NTP Provantis, and any reporting about the method and results.

11.1.8. Locking

The testing laboratory is responsible for locking each animal after pathology data entry is complete. After the study is complete, the testing laboratory requests that the NTP Provantis support contractor lock the study.

After the study is locked, the testing laboratory will no longer have access to the study other than reading access. The testing laboratory is responsible for saving a copy of the data before locking if such data are needed by the testing laboratory.

11.1.9. Reports

The testing laboratory uses data summaries from NTP Provantis to make the laboratory report. Table templates have been set up in NTP Provantis for this purpose, but can be modified by the testing laboratory at the direction of the COR.

NIEHS staff should set up separate table templates in NTP Provantis (and not use table templates that the testing laboratory has set up when running its reports). If modifications are made in table templates within NTP Provantis, the testing laboratory will support staff in replicating results.

As predetermined for a test program and agreed upon by the contractor (including Quality Assurance Unit), pathology data may be exported from NTP Provantis to the Histopathology Slide Review Module (SRM) for review and reporting. The testing laboratory shall document acceptance of SRM after review of verification documentation. When the data are in SRM, DTT tables shall be used to present the data for the laboratory report.

11.2. Submission of Reports and Data

11.2.1. Content and Format of Reports

Reports shall be prepared according to the instructions provided in this document. The content and format of reports that are not described in these Specifications are to be discussed with the program COR.

11.2.2. Submission of Reports

Laboratory reports (including final study reports, prestart chemistry reports, prestart inhalation reports, and toxicokinetic reports) shall be submitted to the COR. Final accepted reports shall be provided in PDF format. Digital signatures are acceptable. If any element of a report needs to be scanned, they shall be scanned at 300 dpi (black and white) to a single searchable PDF file. Password protection shall not be used, nor will any document property restrictions. If the report requires an amendment, the entire amended report shall be submitted to the COR.

11.2.3. Submission of Data to the NTP Archives

All original source documentation is the property of NIEHS and shall be sent to the NTP Archives. Only that correspondence related to the technical conduct of a study shall be included. Site visit and Annual Program Review reports and responses to action items shall not be submitted to the NTP Archives.

Inventory of the records sent shall be recorded on the NTP Archives Inventory/Index lists as shown on succeeding pages of this chapter. The Inventory/Index lists shall be prepared by the testing laboratory and shall accompany the records to the NTP Archives. Each section in the NTP Archives Inventory/Index and subpart thereof shall be separated by a stiff paper or cardboard divider or shall be contained in a file folder. A tab affixed to the divider or a label on the file folder shall identify the section and subpart. The testing laboratory shall provide the folders or dividers and boxes for the records they submit.

The records pertaining to a study shall be sent after the pathology materials are sent to the NTP Archives. If records from a study are shipped in parts at different times, Inventory/Index lists

shall accompany each shipment. The NTP Archives clerk will prepare and maintain a master copy of the Inventory/Index for this study to show all receipts of records from the testing laboratory, computer data forms, or other sources.

Pathology materials (slides, blocks, and tissues) shall be sent to the NTP Archives as specified in Chapter 8, Section 8.13 and in study specific SOWs under Milestones and Deliverables. Pathology materials shall not be placed in the same box with original study documents.

A copy of all final reports reviewed by the testing laboratory's Quality Assurance Unit for a study shall be submitted to the NTP Archives. A copy of each standard operating procedure (including revisions) in effect and used during the conduct of each study shall be submitted. Electronic copies, rather than hard copies, are acceptable if the laboratory prefers.

Archival materials shall be appropriately boxed and shipped prepaid to the NTP Archives. The following Inventory/Index organization scheme shall be used:

NTP Archive Inventory/Index – Archive Records for Each Study

- Section I. General
- Section II. Test Article Records
- Section III. Study Type (to be indicated on each set of forms; e.g., Repeated Dose, Subchronic, Chronic, Transgenic Study, Toxicokinetic Study, Separate Special Study)

The full archival inventory list is provided in Appendix A.

11.2.4. Electronic Submission of Data

All original study documentation and reports shall be organized and submitted to the NTP Archives using a secure file transfer service to transfer irreplaceable data from DTT-sponsored studies securely and reliably.

The data shall be organized according to the Archive Inventory/Index forms. Files shall be prepared as follows:

- Files shall be organized in folders according to the Archive Inventory/Index and presented in order. Each file name shall include sufficient information to identify the specific section of the NTP Archive Inventory/Index Form to which the data refer.
- The entire NTP Archive Inventory/Index shall be included as a separate file. The inventory file shall include experiment number, date, laboratory name, test article, study type and duration, species, CASRN, and Provantis study number or NIEHS study ID.
- It is not necessary to send data already deposited in CEBS or loaded into NTP Provantis.
- Data are to be sent in electronic formats such as JSON, Excel, or XML with sufficient metadata to permit reuse. Raw data, normalized data, and the results of statistical analysis shall be provided. Analysis methods shall be described in sufficient detail to permit replication, or the software shall be provided.

- Summary data tables may also be converted to PDF or scanned at 300 dpi (black and white) to searchable PDF files. The files are to contain document restrictions except for printing and content copying.
- All frames of data shall be positioned in either portrait or landscape orientation (whichever is appropriate for that page). Oversized pages shall be reduced or photographed to allow the data to fit on a single page. Chart recorded data shall be scrolled left to right or top to bottom, depending on how the script runs on the tracings.
- When data are missing, a file shall be submitted in the location of the missing data that explains why the data are missing. If study data records are of such quality that they cannot be scanned, a cross-reference shall be included in the file index that gives the location of the raw data or records.
- The electronic archival package shall be examined by the study laboratory for completeness, accuracy, and quality before submission to the NTP Archives.

11.3. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapters 1, 2, 3, 4, 11, and 12 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was charged to:

1. Peer review the following chapters 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 1: General Personnel Requirements
    - Chapter 2: Facilities
    - Chapter 3: Health and Safety
    - Chapter 4: Quality Program
    - Chapter 11: Data Collection and Submission
    - Chapter 12: Report Formats and Guidance
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

Peer Reviewer

Catherine Spong, M.D.
Professor and Chair, Department of Obstetrics and Gynecology
Chief of Maternal Fetal Medicine
UT-Southwestern Medical Center
Dallas, Texas, USA

Appendix A NTP Archive Inventory/Index

Test Article: _____ CASRN:_____

Laboratory: _____ Study No. (e.g., C #): _____

Address: _____ Provantis No. (e.g., C12345-01): _____

Contract No.: _____ Laboratory Study No.: _____

Table A-2. Section 1–General[a]

	Box # or Date Sent[b]	Other Location or Comment[c]	Archive Location[d]
A. Personnel (indicate time interval and job)			
1. List of key/critical personnel who participated in the study			
2. Identify consultants (by name and full address; specify work performed)			
B. List of subcontractors (by name and full address; specify work performed)			

[a]Records that pertain to multiple areas within one section or to multiple sections and cannot be separated conveniently to fit into the filing scheme are to be filed in one section and referenced in the other section(s) to which they pertain.
[b]Include the box number for the current shipment in which the listed data/materials are included. If the data/materials listed were submitted in a prior shipment, indicate the date of that prior shipment.
[c]If data/materials listed have been included in another location, the other location is to be identified here. This space is also included for additional explanatory comments.
[d]This column will be completed by NTP Archival staff when storing the listed data/materials.

Test Article: _____ **CASRN:**_____

Laboratory: _____ **Study No. (e.g., C #):** _____

Address: _____ **Provantis No. (e.g., C12345-01):** _____

Contract No.: _____ **Laboratory Study No.:** _____

Study Type(s): _____

Table A-3. Section 2–Test Substance Records

	Box # or Date Sent[a]	Other Location or Comment[b]	Archive Location[c]
A. Identity (Manufacturer, Lot[s], Date)			
B. Characterization			
C. Bulk Stability			
D. Shipment (from Analytical Contractor or Manufacturer)			
E. Receipt			
F. Storage			
G. Bulk Analyses (Identity and Purity Analyses)			
H. Vehicle Analyses			
I. Test Article/Vehicle Method Validation			
J. Test Article/Vehicle			
1. Dose preparation records			
2. Dose preparation and room dose analyses			
3. Homogeneity study			
4. Animal room dose analyses			
K. Inventory/Use Records for Bulk Test Article			
L. Shipment of Test Article Aliquot(s) to Analytical Contractor			
M. Related Correspondence			

[a]Include the box number for the current shipment in which the listed data/materials are included. If the data/materials listed were submitted in a prior shipment, indicate the date of that prior shipment.
[b]If data/materials listed have been included in another location, the other location is to be identified here. This space is also included for additional explanatory comments.
[c]This column will be completed by NTP Archival staff when storing the listed data/materials.

Test Article: _____ **CASRN:**_____

Laboratory: _____ **Study No. (e.g., C #):** _____

Address: _____ **Provantis No. (e.g., C12345-01):** _____

Contract No.: _____ **Laboratory Study No.:** _____

Study Type(s): _____ **Route:** _____

Date Dosing Initiated: _____ **Date Necropsy Completed:** _____

Table A-4. Section 3–Records by Study Type[a]

	Box # or Date Sent[b]	Other Location or Comment[c]	Archive Location[d]
A. Study assignment (including modifications pertaining to the technical aspects of each study); laboratory-approved protocol with amendments and deviations			
B. Vehicle Records (brand, source, dates purchased, lot no(s)., dates of use, and test article(s) for which used; for corn oil, peroxide analysis with indication of lot no(s). and dates of analysis)			
C. Dosing Records			
1. Dose preparation procedure(s)			
2. Dose preparation log			
3. Dose analysis			
4. Stability study			
D. Diet Analysis			
E. Water Analysis			
F. Water Treatment (commercial and in-house)			
G. Animal Records (prestudy)			
1. Species, strain, source, age			
2. Receipt			
3. Conditions of quarantine (caging, food, water)			
4. Health examination/clinical signs in quarantine			
5. Release for study			
6. Disposal of extra animals			
H. Animal Records (in-life) (for studies supported by Provantis)[e]			
1. Provantis in-life and pathology protocol validation reports			

	Box # or Date Sent[b]	Other Location or Comment[c]	Archive Location[d]
2. Room location			
3. Cage type and number per cage			
4. Randomization			
5. Identity code and confirmation records			
6. Bedding type, manufacturer, contaminants			
7. Cage filter type and source			
8. Feed type, source, lot no(s)., dates used, contaminants			
9. Type of water system and treatment			
10. Cage rotation			
11. Rack rotation			
12. Mortality checks			
13. Provantis in-life data			
a. Data collection forms			
b. Error corrections (when applicable) and exceptions reports			
c. Dosing records for gavage and dermal studies			
d. Perinatal exposure studies: dam, litter, and pup data			
I. Animal Records (in-life) (for studies not supported by Provantis)[f] Supply the records referred to in H.			
J. Records Unique to Inhalation Studies			
1. Chamber design and maintenance information			
2. Exposure system generation and monitoring description, operating procedures, and validation data			
3. Generator degradation results			
4. Chamber concentration versus time plots			
5. Chamber degradation results (without and with animals)			
a. Prestudy developmental data and results			
b. In-life study data and results			
6. Chamber atmosphere homogeneity results (without and with animals)			
a. Prestudy developmental data and results			

	Box # or Date Sent[b]	Other Location or Comment[c]	Archive Location[d]
b. In-life study data and results			
7. Particle/aerosol measurements (without and with animals)			
a. Prestudy developmental data and results			
b. In-life study data and results			
8. Generation and monitoring equipment maintenance and calibration records			
9. Chamber residual concentration/ overnight monitoring results			
10. Daily chamber exposure concentration data			
11. The prestudy developmental data and in-life study data are to be organized and indexed separately			
K. Animal Records (pathology)[e]			
1. IANRs			
2. Provantis slide, block inventory			
3. Necropsy log			
4. Organ weight records			
5. Histology processing records and associated records			
L. Animal Room Records			
1. Temperature raw data			
2. Humidity raw data			
3. Light cycle/intensity measurements			
4. Air changes/air flow			
5. Cleaning agents used			
M. Virology Screening Program Data			
N. Microbiological Testing Reports			
O. Genetic Monitoring Data			
P. Special Studies			
1. Clinical lab studies			
2. Other special studies			
Q. Final Report			
1. Introduction			
2. Materials and methods			
3. Results			
4. Discussion			
5. Appendices			

	Box # or Date Sent[b]	Other Location or Comment[c]	Archive Location[d]
R. SOPs in Effect for This Study Period Including Provantis SOPs			
S. Provantis			
1. Software version in use at time of study			
2. All administrative records relevant to study data processing			
3. Software receipt and maintenance records			
T. Internal Computer-generated Forms/Tables			
1. Toxicology			
2. Clinical chemistry			
3. Analytical chemistry			
4. Other			
U. Photographs Taken During the Study			
1. Gross observation documentation			
2. Microscopic observation documentation			
3. Other			
V. Incident (Experimental Impact) Reports			
W. Correspondence			

[a]Ancillary records may be kept by the study laboratory, but duplicate records should not be shipped to the NTP Archives.
[b]Include the box number for the current shipment in which the listed data/materials are included. If the data/materials listed were submitted in a prior shipment, indicate the date of that prior shipment.
[c]If data/materials listed have been included in another location, the other location is to be identified here. This space is also included for additional explanatory comments.
[d]This column will be completed by NTP Archival staff when storing the listed data/materials.
[e]The expectation is that in vivo studies shall be supported using NTP Provantis and that all in-life data and pathology records will be directly entered into NTP Provantis. The only records that shall be submitted with the archive file are those not directly captured or recorded in NTP Provantis. If paper records are utilized during the study (e.g., gavage dosing records, IANRs), planned or as a contingency, they shall be entered retrospectively into NTP Provantis and the original paper records submitted with the archive file.
[f]For in vivo studies that are not supported by NTP Provantis, the expectation is that all in-life data shall be submitted in a machine readable electronic format (non-PDF) to CEBS. If paper records are utilized during the study (e.g., gavage dosing records, IANRs), planned or as a contingency, they shall be entered retrospectively into an acceptable electronic format and the original paper records submitted with the archive file.

12. Report Formats and Guidance

G.K. Roberts[1], M.D. Stout[1] (Editors)

[1]Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Last Updated: March 2023

12.1. Lab Reports

This section describes the basic requirements for study reports submitted to NIEHS. The overall structure of the reports is flexible and can be determined by the laboratory. In addition to the requirements outlined below, if the study is conducted in compliance with Good Laboratory Practices, additional elements shall be included per 21 CFR Part 58.[1]

12.1.1. Front Matter

Lab reports shall include the following components.

Title Page

- Study title
- Lab study number
- NIEHS study number
- Chemical CASRN
- Date
- Contract number

Summary Table

Information that the study director considers to be toxicologically relevant, including data that are not different from controls but critical to interpret the study (e.g., survival, body weight), shall be included in the summary table. This information may or may not be statistically significant. This table shall not include information that is statistically significant unless it is also deemed to be biologically relevant.

Personnel

- Study director
- Contract principal investigator
- Applicable discipline leaders (e.g., pathology, clinical pathology, DART, quality assurance)
- Data coordinators
- Technical discipline and facility manager(s)

[1]https://www.ecfr.gov/current/title-21/chapter-I/subchapter-A/part-58

12.1.2. Introduction

A brief purpose statement for the study shall be provided. A literature review of the test material uses and potential exposure is not needed.

12.1.3. Materials and Methods

Information listed in this section shall be provided in the body of the report or in contributing scientist reports (CSRs) appended to the study report. In some cases, it will be acceptable to include information in SOPs and facility records, which must be discussed and agreed upon with the contracting officer's representative (COR). Throughout this section, applicable SOPs shall be cited. Where feasible, information shall be tabulated in a summary table; however, if necessary, text can be used to relay this information, but it shall not duplicate what is shown in a summary table.

Chemistry

The below information shall be reported. If information is duplicative of information provided in the prestart chemistry report, that report can be cited instead were appropriate.

Test Article

- Grade or other test article-specific information from the supplier
- Supplier/manufacturer name and address
- Storage conditions for bulk test article
- Lots used, including amount received, date received, dates used for each lot, and color picture of the bulk material compared with a color spectrum
- Test article identity and purity, as follows:
    - Information regarding initial bulk analysis, including name of laboratory performing the analysis, methods used, title of report from lab, and results, with lot number(s) clearly identified
    - Information regarding bulk test article reanalysis, with the applicable SOP used for reanalysis cited
    - A table of results of the initial and subsequent bulk reanalyses for each lot, including dates and analysis results (frozen reference, bulk sample, and calculation of relative purity)
    - A photograph compared with a color spectrum (see Section 12.3 and Figure 12-1 as an example)

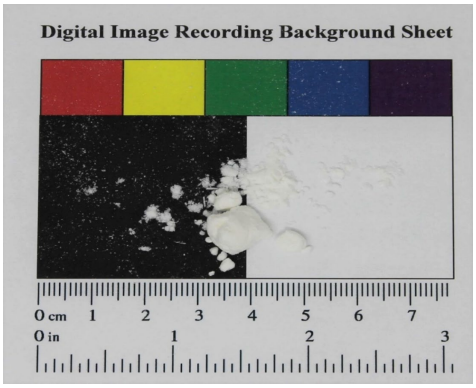

Figure 12-1. Example Color Spectrum Chart

Vehicle

- Vehicle or control article used (e.g., feed, water, corn oil, air)
- Grade/purity/test article-specific information from the supplier
- Name and address of supplier and manufacturer/producer
- Table showing dates and results of purity analyses, citing the applicable SOP used for purity analysis

Dose Formulation Procedure

- Mixing procedure, such as equipment and type of container used and duration of mixing, with the applicable SOP used for dose formulation cited
- Stability and homogeneity information on dose formulation (obtained from analytical lab or performed at testing lab)
- Storage conditions for dose formulations as appropriate for route of administration (e.g., temperature, humidity, protection from light, maximum length of time stored)

Dose Formulation Analysis

- Analysis procedure, with the applicable SOP used for analysis cited
- Formulation and analysis schedule for dose preparations and animal room samples
- Tabulated information showing dates that dose formulations were prepared, analyzed, and used; results of analyses (mean ± standard deviation) that include analysis before dosing (% high/low of theoretical), analysis after dosing for unused/stored formulations, and analysis of animal room samples (% high/low of theoretical and % of original formulation); if formulations were out of specification, whether these were remixed or not shall be indicated

Animals and Animal Husbandry

Animals

- Species and strain/stock
- Source of animals (supplier name and address)
- Examinations conducted to assure health of test animals

- Quarantine period
- Randomization procedure (for test group, rack, and cage)
- Animal identification procedure
- Age of animals upon receipt and age and weight of animals when tested
- Serologic analysis performed including sampling dates and results (see format for table of serology results in Section 12.1.

Animal Husbandry

- Description of cages, filters, racks, bedding, feeders, and water bottles or automatic waterers used, including the manufacturer's name and address for each item
- Description of cleaning method for each item listed above and, where applicable, the type of washing equipment and cleaning agent and the manufacturer's name and address for each item
- Schedules for changing cages, chambers, filters, racks, bedding, feeders, water bottles, etc.
- Description of barrier maintenance and disease control procedures
- Description of room sanitization and pest control procedures
- Temperature and humidity ranges, number of room air changes per hour, and type of air filtration used and name of filters (with manufacturer's name and address); light cycle and type of lights used; temperature/humidity excursions, including dates, ranges, and number of hours involved
- Description of study room(s)
- Number of animals per cage, relationship of control and treated group animals (including rack position), rotation of cages on racks, and rotation of racks within the room
- Feed used and storage conditions
- Source of water supply (city or well) and any water treatment used; discuss water analyses performed

Study Design

Design

- Route of administration and frequency of administration
- Number of animals per treatment group, number of groups, and dose levels/exposure concentrations
- Duration of exposure, including specific dates for first and last dose/exposure; details of perinatal dosing (gestation and lactation) for dams and pups for studies with perinatal exposure
- Specific details of special studies performed (e.g., clinical laboratory studies, immunotoxicity studies, neurobehavioral studies, internal dose assessment studies),

including details of tissues/samples taken, sample collection and handling, analysis methods or procedures for conducting special studies, and dates performed

- Identification by title/date all reports submitted under separate cover that are related to these studies (e.g., Prestart Chemistry Reports, Prestart Toxicokinetic Study Reports, Single Administration Toxicokinetic Study Reports, Proficiency Reports for Special Studies)

In-life Observations and Pathological Examinations

- Description and frequency of in-life observations performed
- Specific dates for interim sacrifices and terminal necropsies
- Handling of moribund animals
- Method of euthanasia
- List of tissues collected at necropsy and processed for histopathological evaluation
- Histological processes, preservation, embedding, sectioning, staining, and evaluation scheme (e.g., in the case a read-down is performed in which dose groups are only evaluated to a no-effect level)
- Statistical analyses, if conducted, with short paragraph of methods used

12.1.4. Results

Data for all endpoints described in the study protocol and collected during the study shall be presented in the lab report. In addition to Provantis (or subsequent sponsored data capture system) tables, other tables, figures, and images shall be included as required to provide context and increase the reader's ability to understand and interpret the study findings (see further details below). Findings that are considered related to the test article shall be presented in the main body of the report. Succinct in-text tables shall be created to present toxicity findings, if appropriate. Data for endpoints that were unchanged or not considered related to the test article shall be included in appendices.

For Provantis data, summary tables for all endpoints included in the lab protocol shall be provided in the appendices of the study report or as part of appended CSRs. For non-Provantis data, lab-generated summary tables shall be included in the appendices of the study report or as part of the appended CSRs. In addition, for non-Provantis data, individual animal data that have undergone quality control (QC) review shall be included in the lab report appendices and provided electronically in a machine-readable format. A data transfer and archival process and location shall be discussed with the COR before study initiation for non-Provantis data.

12.1.5. Discussion

A brief summary of salient findings, including biological significance of the data shall be provided. The summary should provide perspective that arises from having conducted the study and include a description of any problems encountered that would affect interpretation of data.

12.1.6. Appendices

In addition to methodology and data included in appendices, appendices shall also include the information below.

- Feed information, including sample feed tag, irradiation certificate, and table with lots used in the study, milling date, and date of initial use
- Temperature and humidity recordings for study room(s) (presented by study week) that include minimum and maximum readings for temperature and humidity, the number of readings within specification, and total readings for temperature and humidity
- Copy of work assignment and modifications
- Copy of laboratory protocol, deviations, and summary of amendments
- Lab reports generated by a subcontractor
- Individual animal data that cannot be collected in Provantis (also to be provided electronically)
- SOPs for nonstandard aspects of the studies, if requested by the COR

12.2. Prestart Chemistry Report

Before starting noninhalation toxicity studies, a prestart report is to be submitted that includes the following items (where applicable for individual test articles).

- Information on the source of the bulk chemical as well as manufacturer stability, storage requirements, and a color picture of the bulk material compared with a color spectrum
- Identity and purity data along with representative chromatograms and spectra
- Stability of the bulk chemical using methods developed by NIEHS or the testing laboratory as well as copies of the SOPs for the purity analysis methods
- Data on preparation, handling, stability (if performed by the testing laboratory), homogeneity, and dose formulation analysis as well as SOPs for formulation preparation and analysis methods
- Method performance evaluation procedures and validation data for dose formulation analysis, including representative chromatograms or spectra (see Chapter 5 [Chemistry], Section 5.3.2. Formulation Analysis)
- Health and safety procedures

Animals shall not be dosed until this report has been received and approved by the program COR.

12.3. Prestart Inhalation Report

A prestart inhalation report describing chemistry, exposure system, and health and safety methods and results shall be prepared and submitted before starting inhalation studies. Only one report shall be provided for a given exposure duration. For example, one report would be

provided for a 3-month study in rats and mice. This report shall include the following, as applicable for individual test articles.

- Information on the source and receipt of the bulk chemical as well as the manufacturer description of the bulk material, stability, storage requirements, identity, and purity data (including particle size and milling information if appropriate); study laboratory description and color photograph of the bulk material compared with a color spectrum (see Figure 12-1); the amount of test material remaining for conducting the study; and the intended disposition of the material at the end of the study.
- Methods, results, data, and conclusions, as appropriate, for the following:
    - Identity and purity, including representative chromatograms or spectra of the test article and reference material, if used; stability and physical properties (e.g., density, dimensions, surface area) of the bulk test material, if required
    - Description and schematics of overall exposure system (including exposure suite, chamber or carousel, generation, distribution, and monitoring systems) and exposure operations
    - Exposure concentration monitoring validation, including demonstration of performance of the method, sample collection efficiency/stability, calibration of online monitor, specificity of online monitor, accuracy/precision of online monitor, linearity of online monitor, and detection/quantitation limits of the online monitor
    - Test chemical purity and stability in the exposure system (reservoir, distribution line, chambers/carousels); evaluation of aerosol in exposure chambers (for vapors generated from liquids); exposure concentration and environmental stability over 3 days of exposure; exposure concentration uniformity; exposure concentration versus time; post-exposure monitoring; measurement of oxygen levels in exposure chambers/carousels (when inert gas used for distribution)
    - Concentration monitoring in the exposure room methodology, strategy, frequency, and results; effluent exhaust treatment monitoring methods/data and percent efficiency of the effluent exhaust treatment
    - Health and safety procedures (refer to appropriate section of specifications).
    - SOPs for test article identity and purity reanalysis; characterization, operation, and maintenance of the exposure, distribution, and monitoring systems; concentration monitoring in the exposure room and effluent exhaust monitoring; other SOPs specific to the test material

All corresponding data performed during the in vivo study shall be included in the respective study report.

12.4. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapters 1, 2, 3, 4, 11, and 12 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 expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was charged to:

1. Peer review the following chapters 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 1: General Personnel Requirements
    - Chapter 2: Facilities
    - Chapter 3: Health and Safety
    - Chapter 4: Quality Program
    - Chapter 11: Data Collection and Submission
    - Chapter 12: Report Formats and Guidance
2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

Peer Reviewer

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