Chapter 8. Anatomic Pathology

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

From: Roberts GK, Stout MD, editors. Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences. Research Triangle Park, NC: National Institute of Environmental Health Sciences; 2023.
https://doi.org/10.22427/NIEHS-00

8. Anatomic Pathology

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Last Updated: March 2023

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).

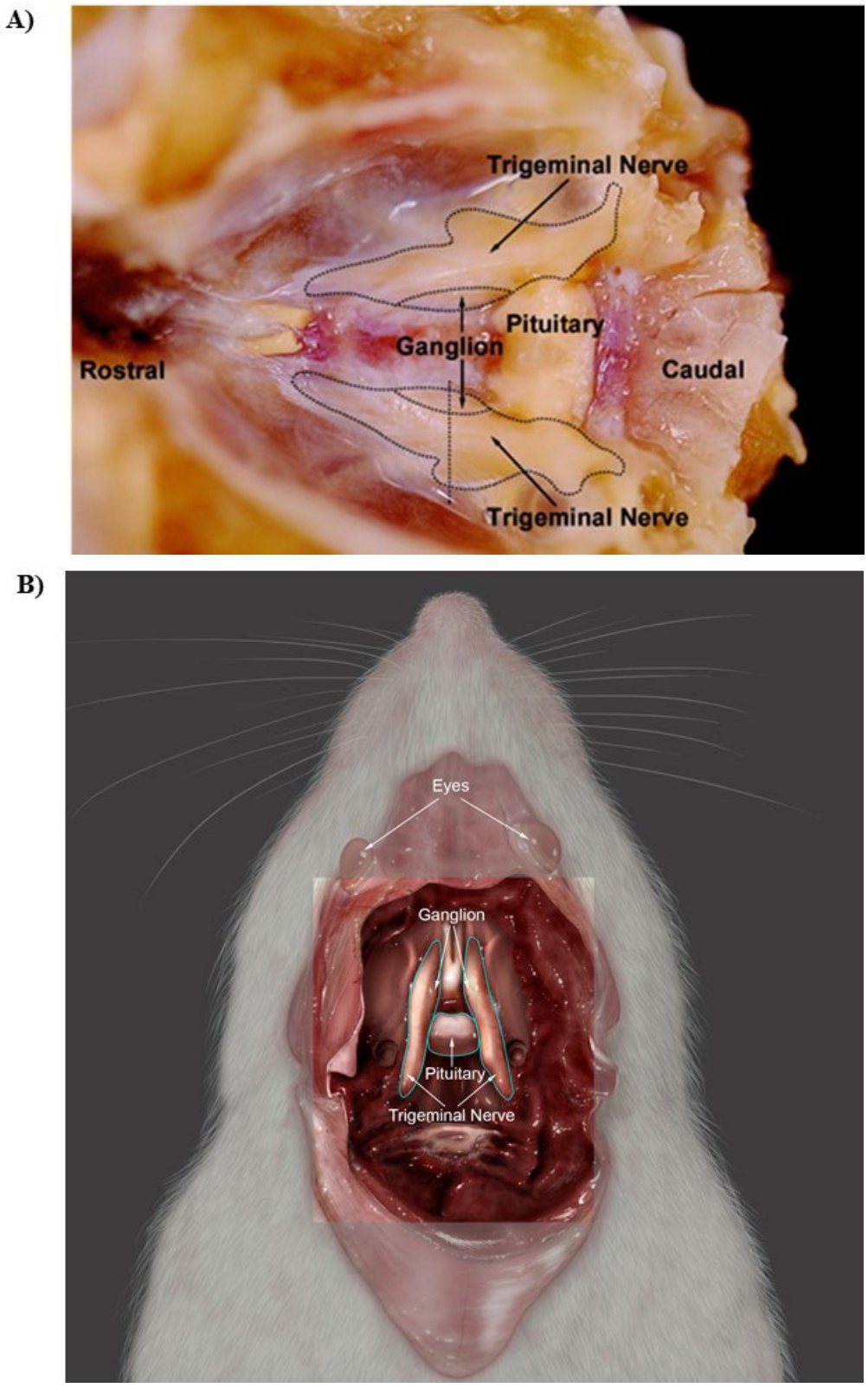


Figure 8-1. (A) Pituitary Gland and (B) Trigeminal Nerves with Ganglia

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.

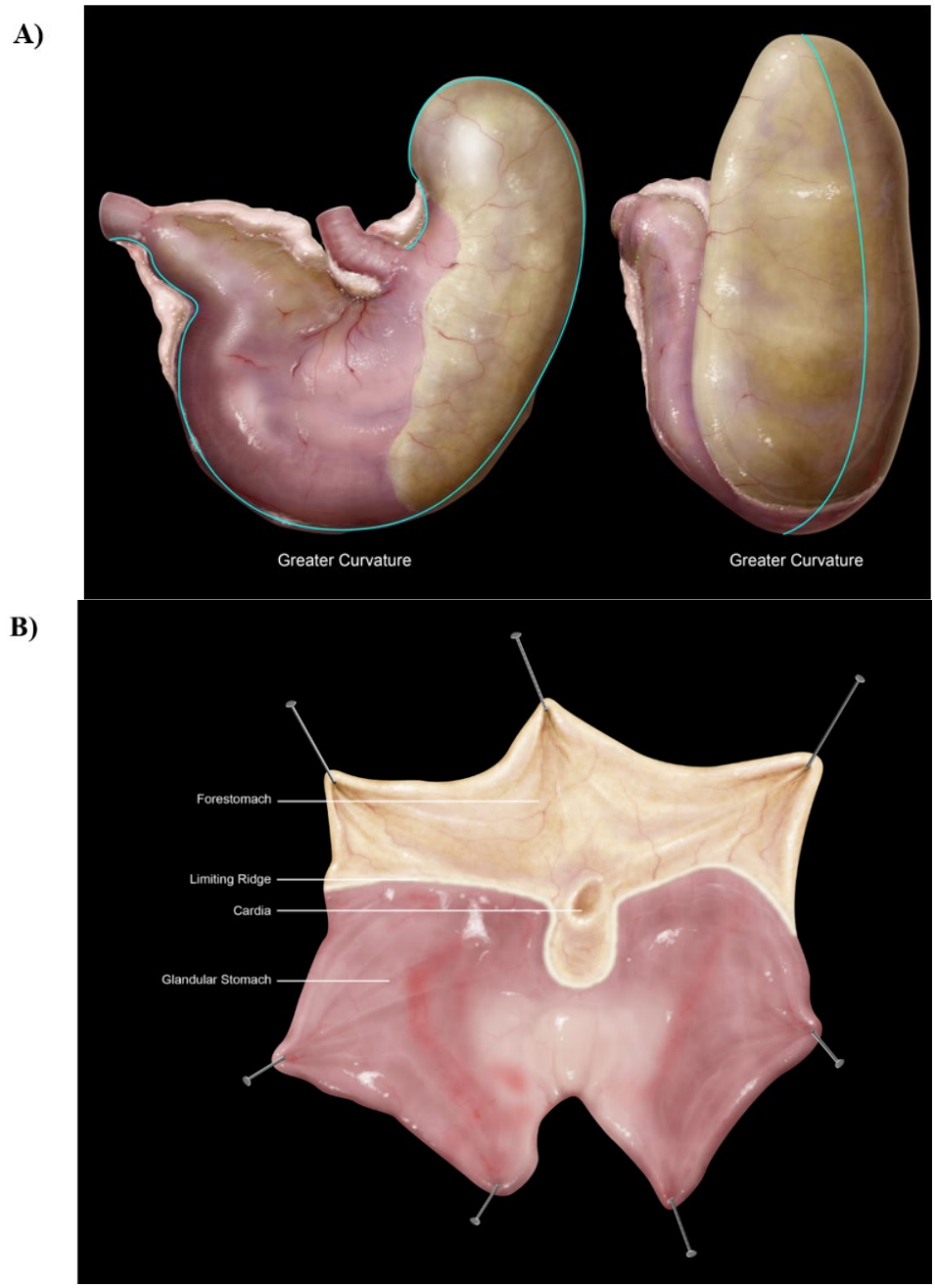


Figure 8-2. (A) Stomach Transection and (B) Pinning to Index Card for Fixation of the Glandular and Forestomach Regions

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.

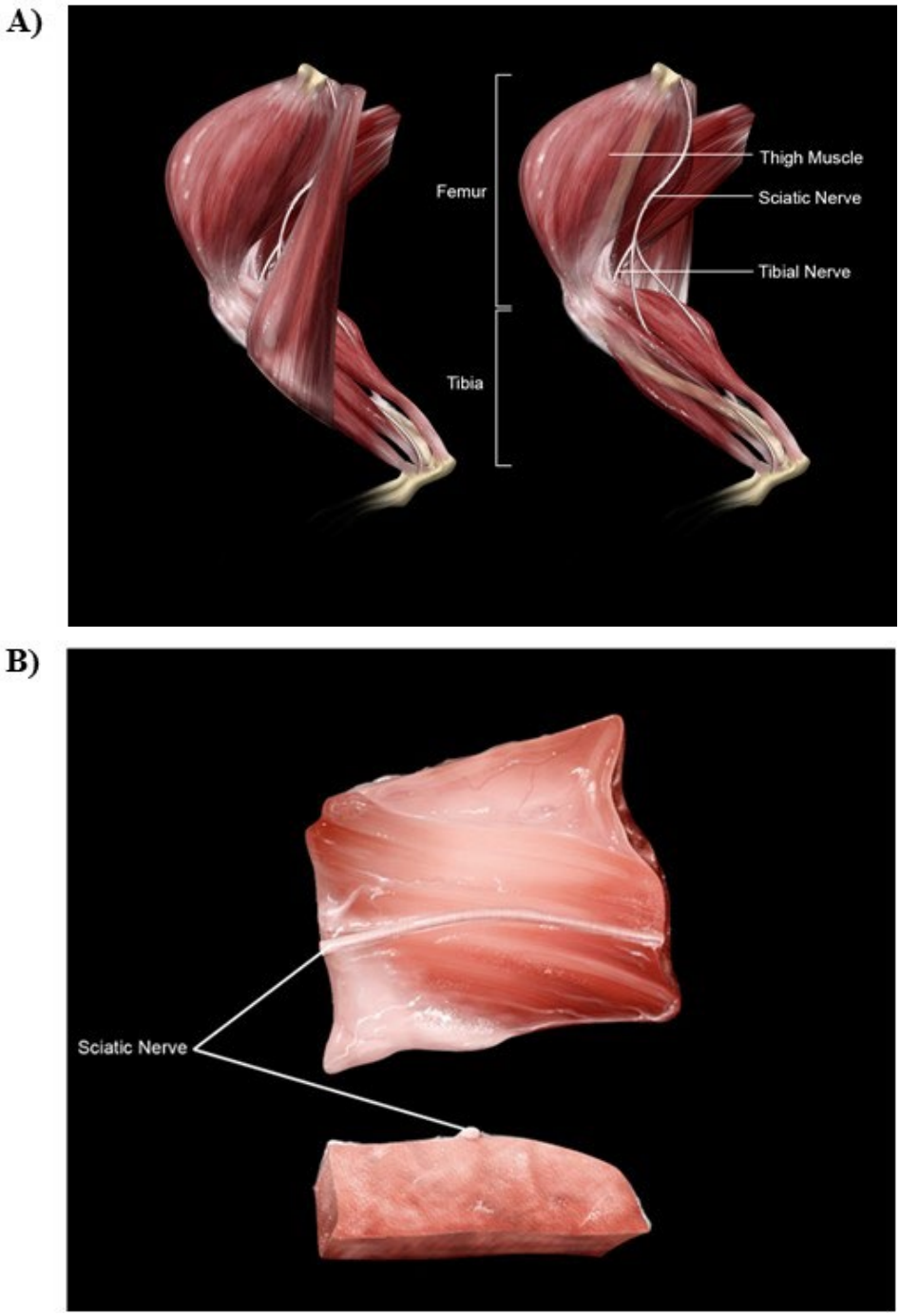


Figure 8-3. (A) Muscles of the Hind Limb Dissected Out from Lateral Surface to (B) Demonstrate the Sciatic and Tibial Nerves

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.

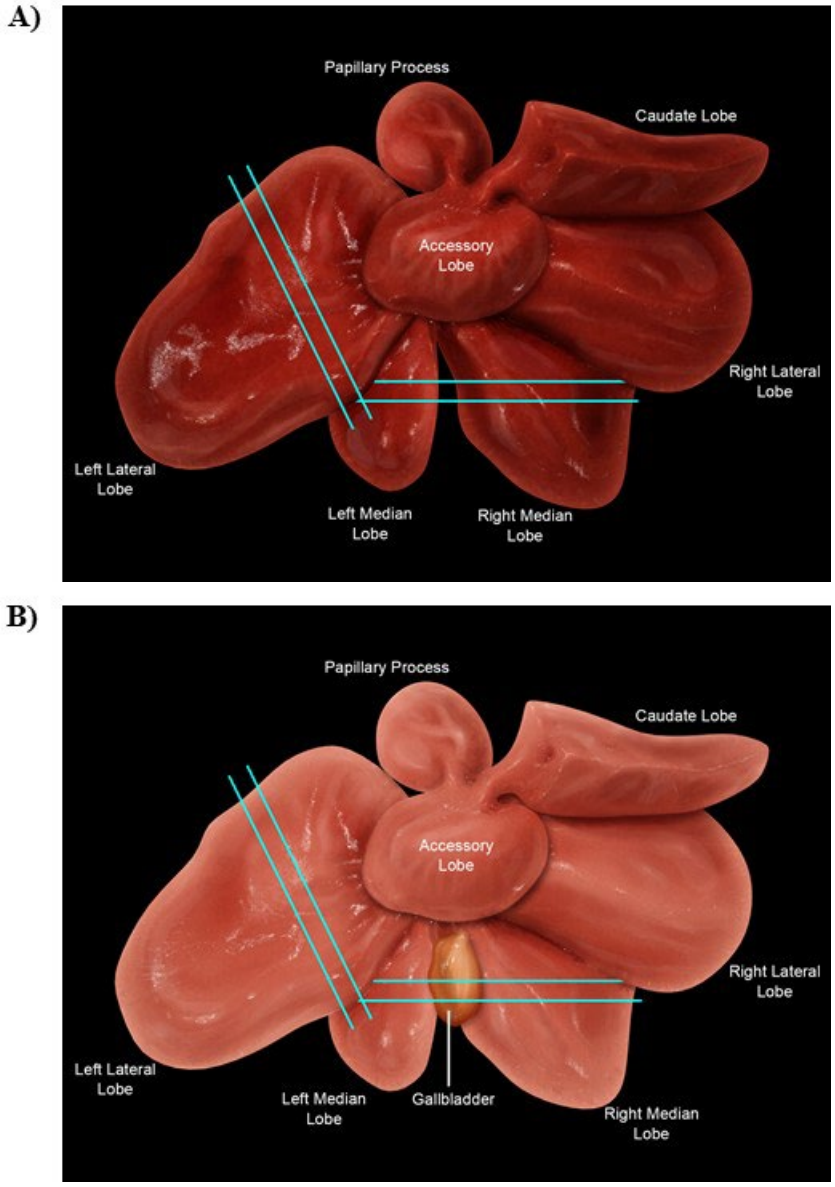


Figure 8-4. Ventral Surface of (A) Rat and (B) Mouse Liver Showing Left, Median, Caudate, Right Anterior, and Right Posterior Lobes

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.

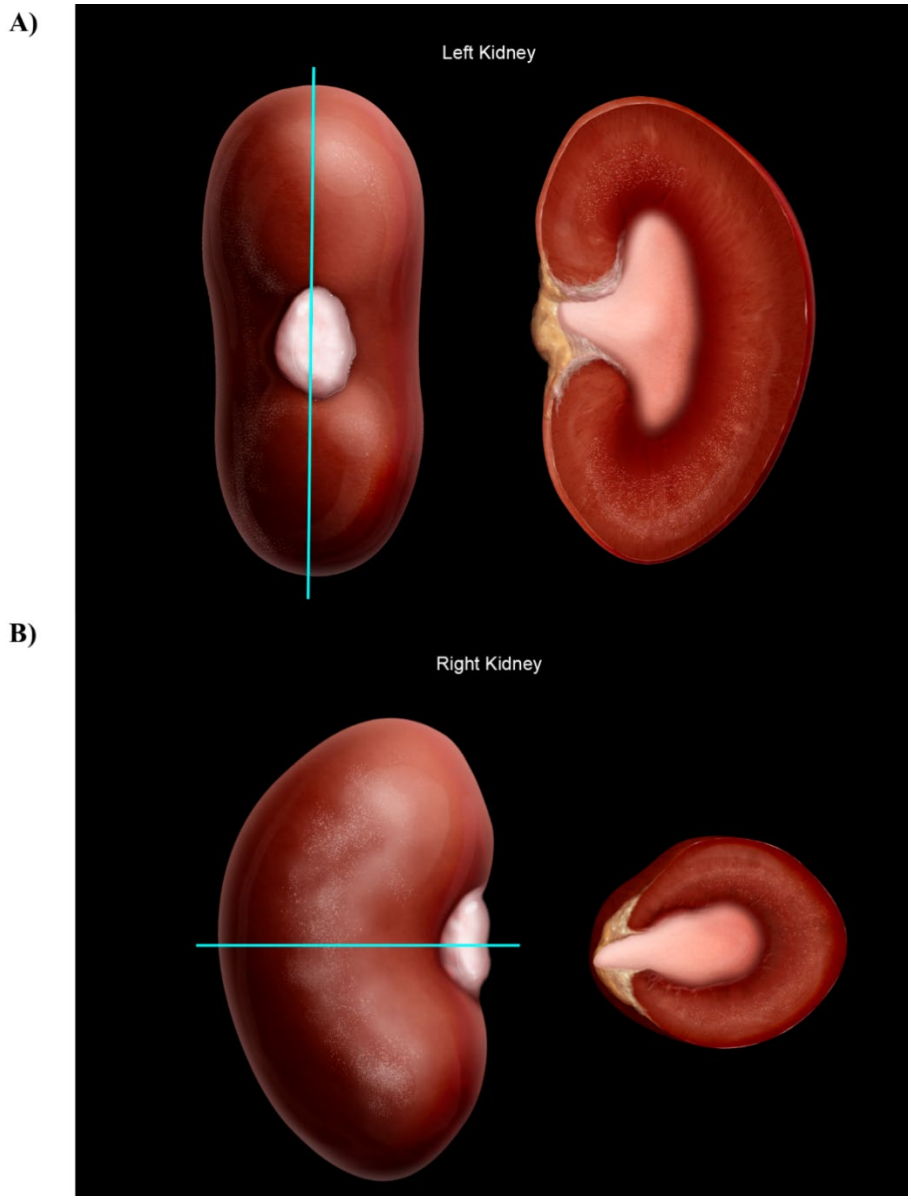


Figure 8-5. Bisection of the (A) Left and (B) Right Kidneys

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.

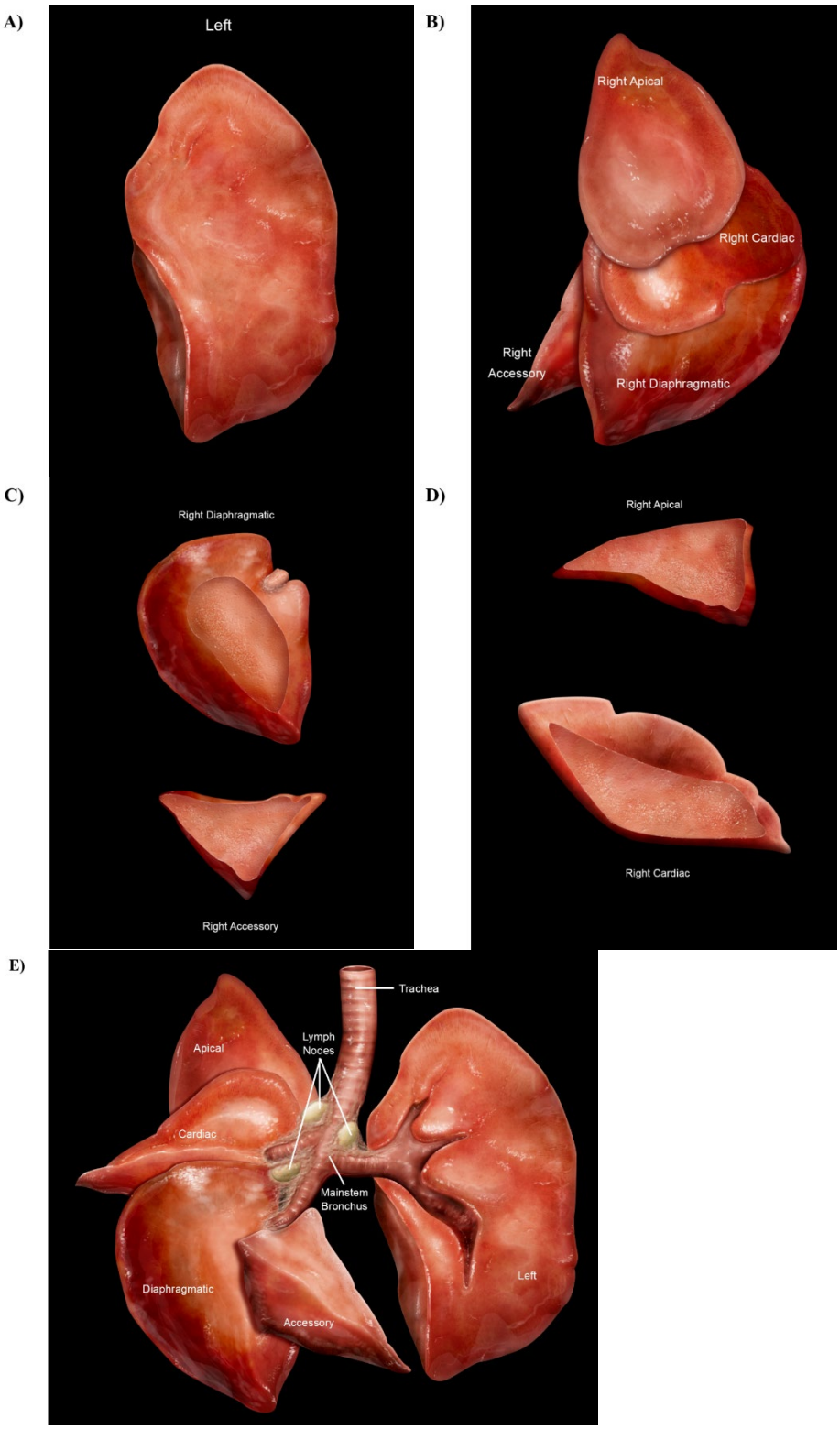


Figure 8-6. Lung Lobes of the (A–D) Rat and (E) Mouse

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

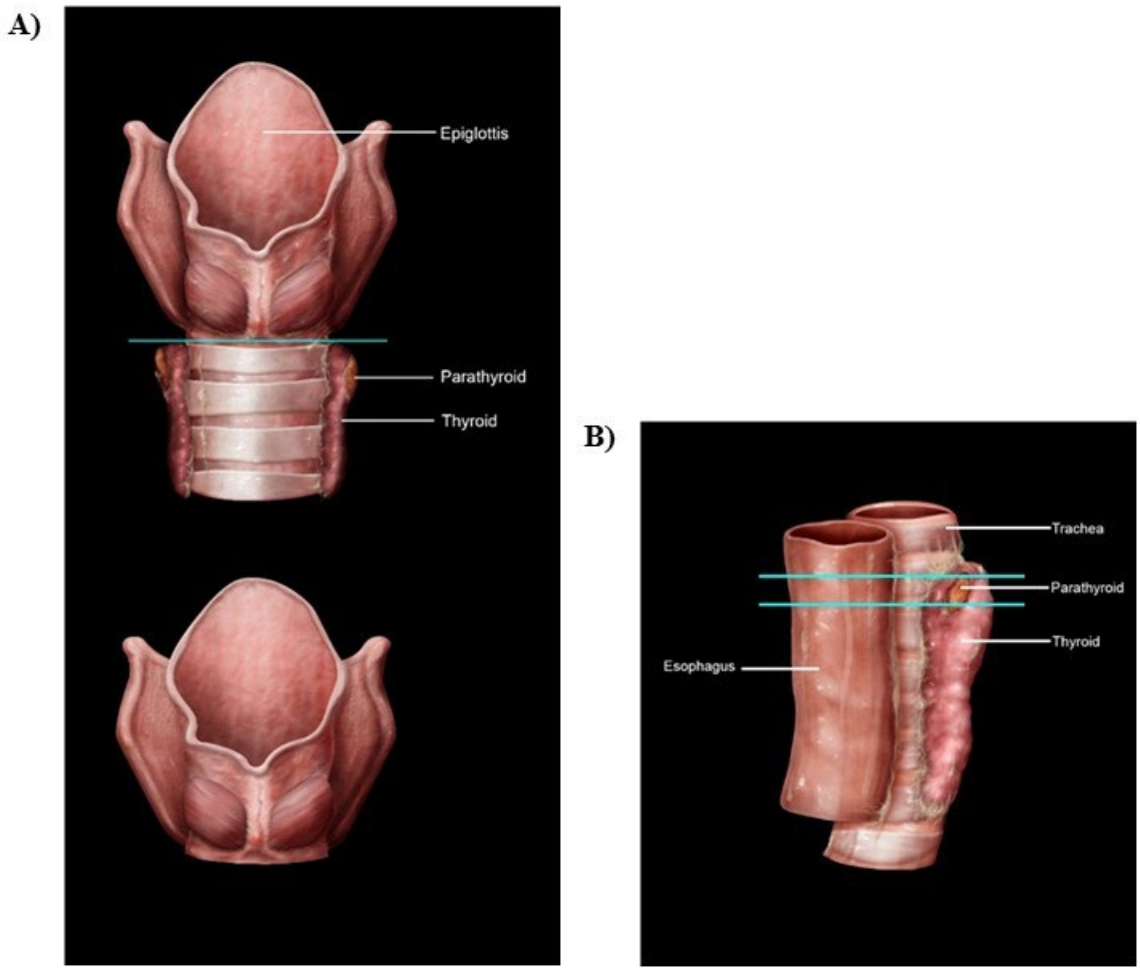


Figure 8-7. (A, B) Trachea, Thyroid Gland, and Larynx

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.

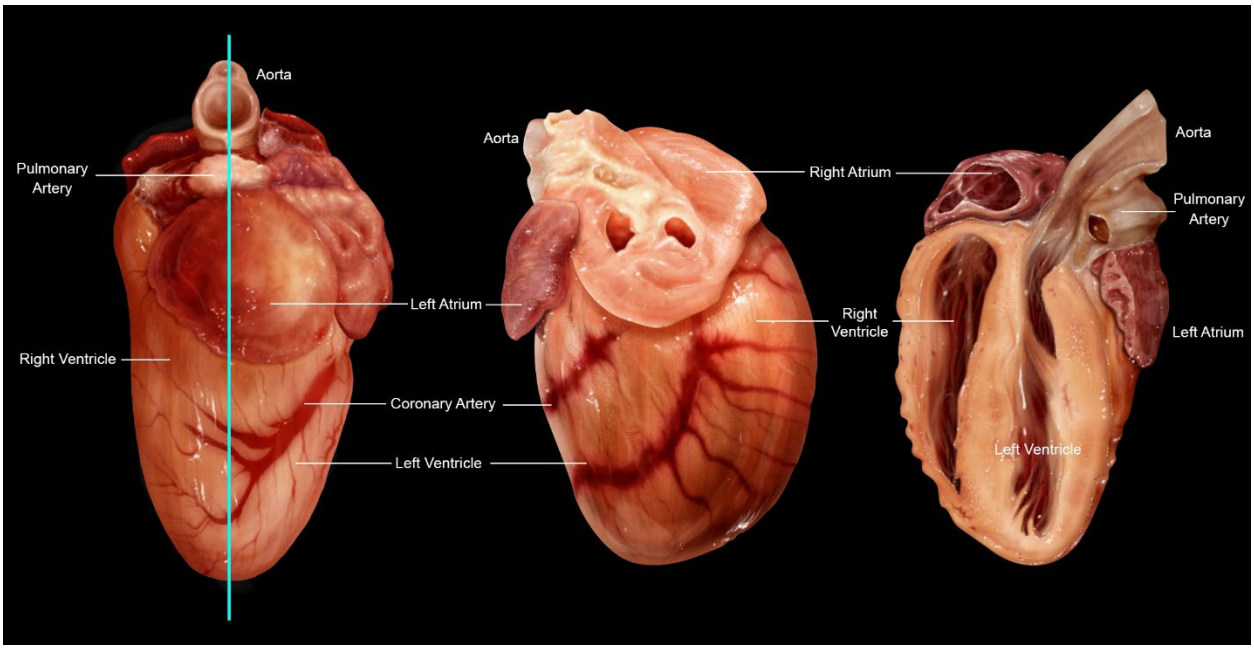


Figure 8-8. Anatomy and Positioning of the Heart and Great Blood Vessels to Demonstrate the Desired Plane of Section

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.

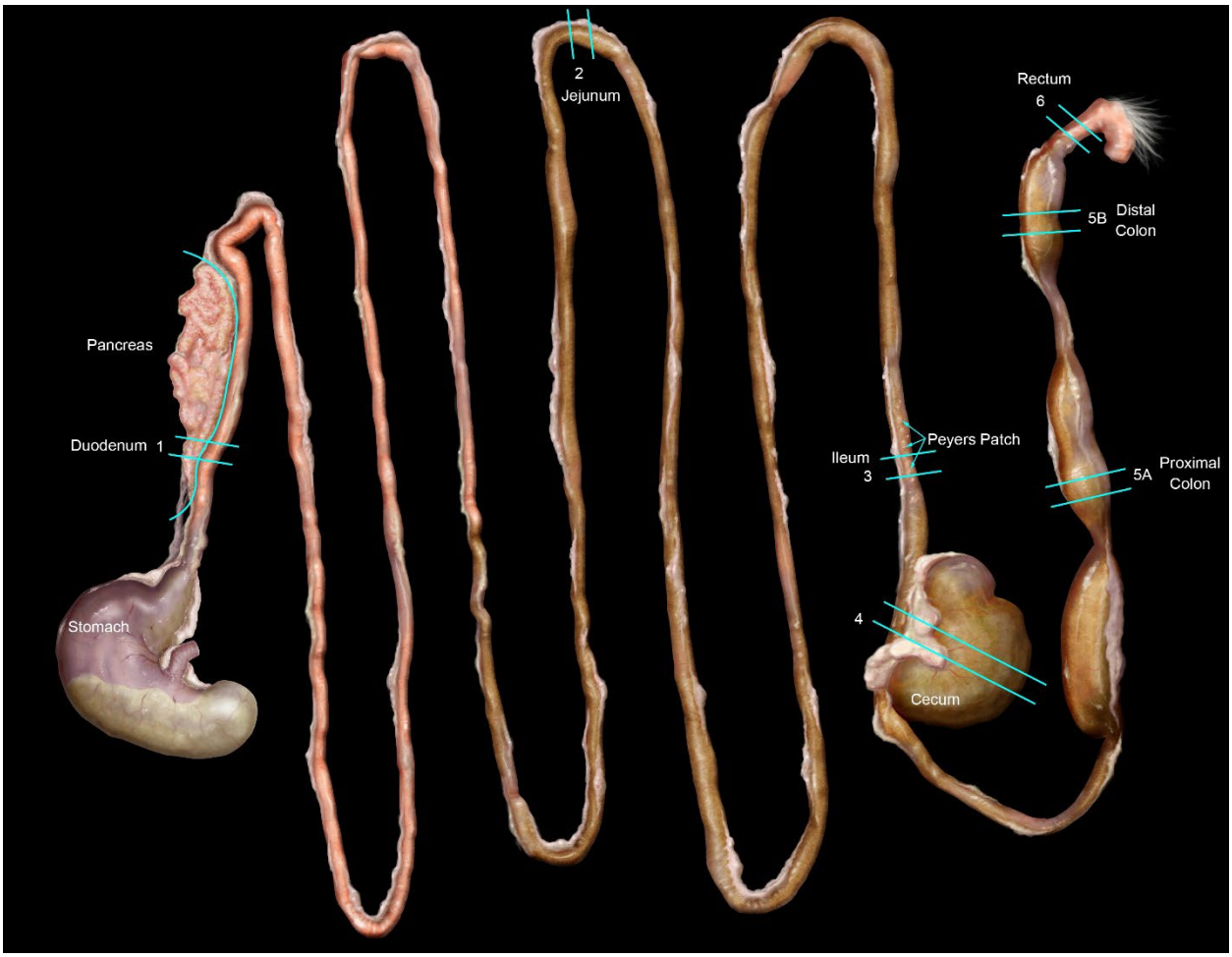


Figure 8-9. Small and Large Intestines

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.

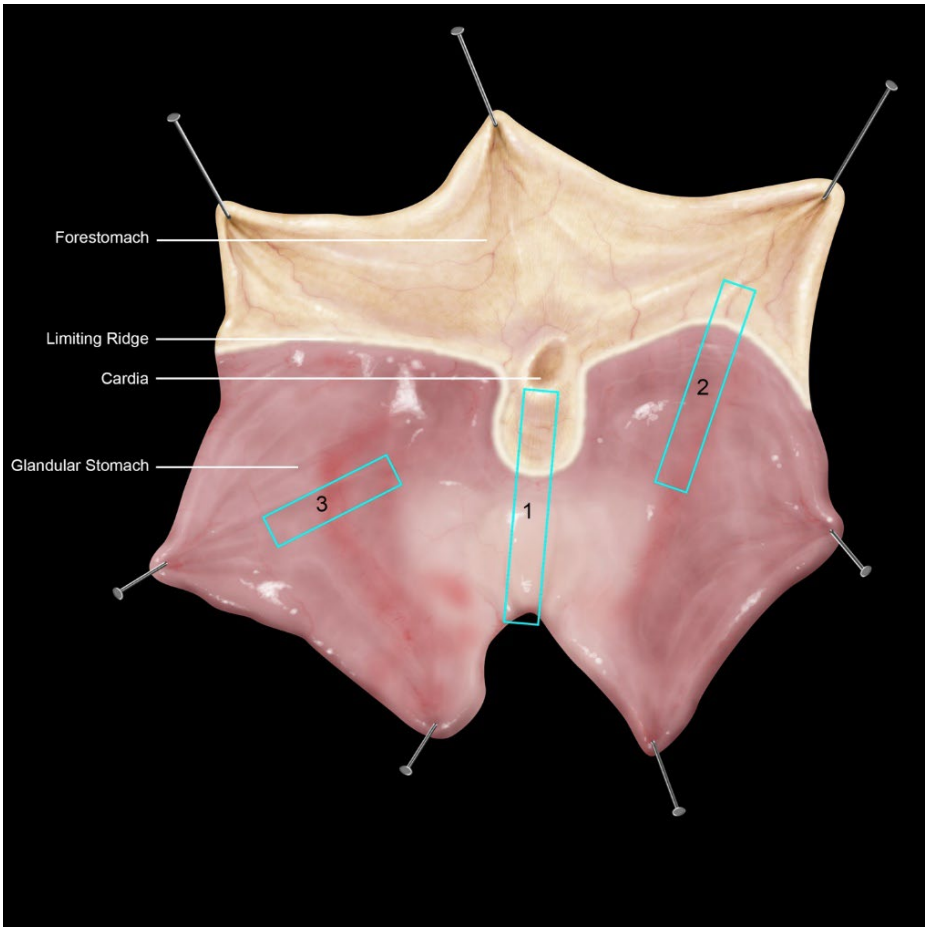


Figure 8-10. Trimming of the Stomach for Histology

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]).

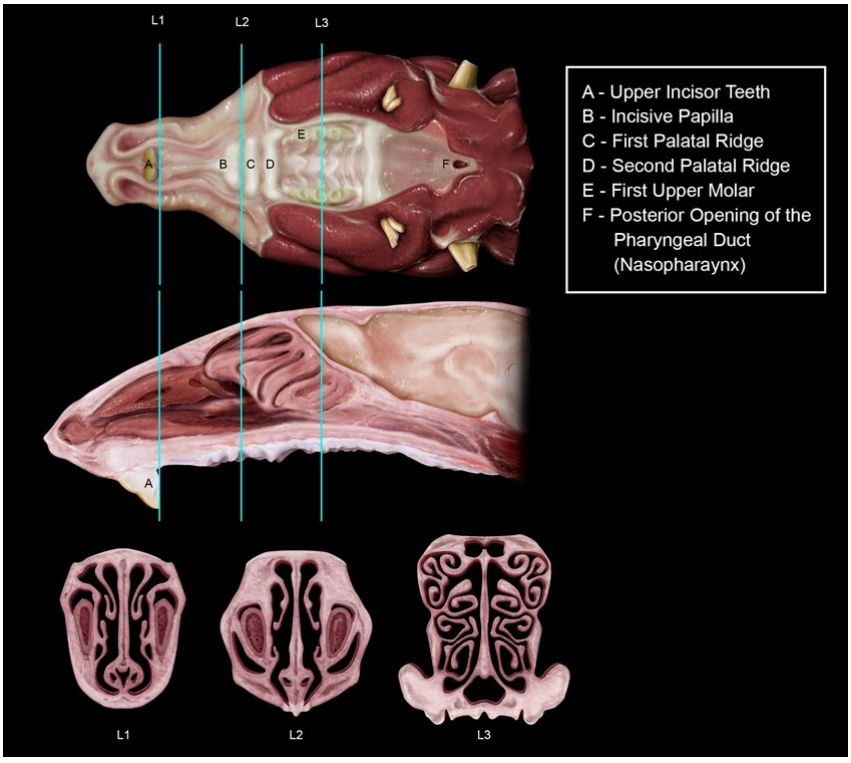


Figure 8-11. Nasal Cavity Sections

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)

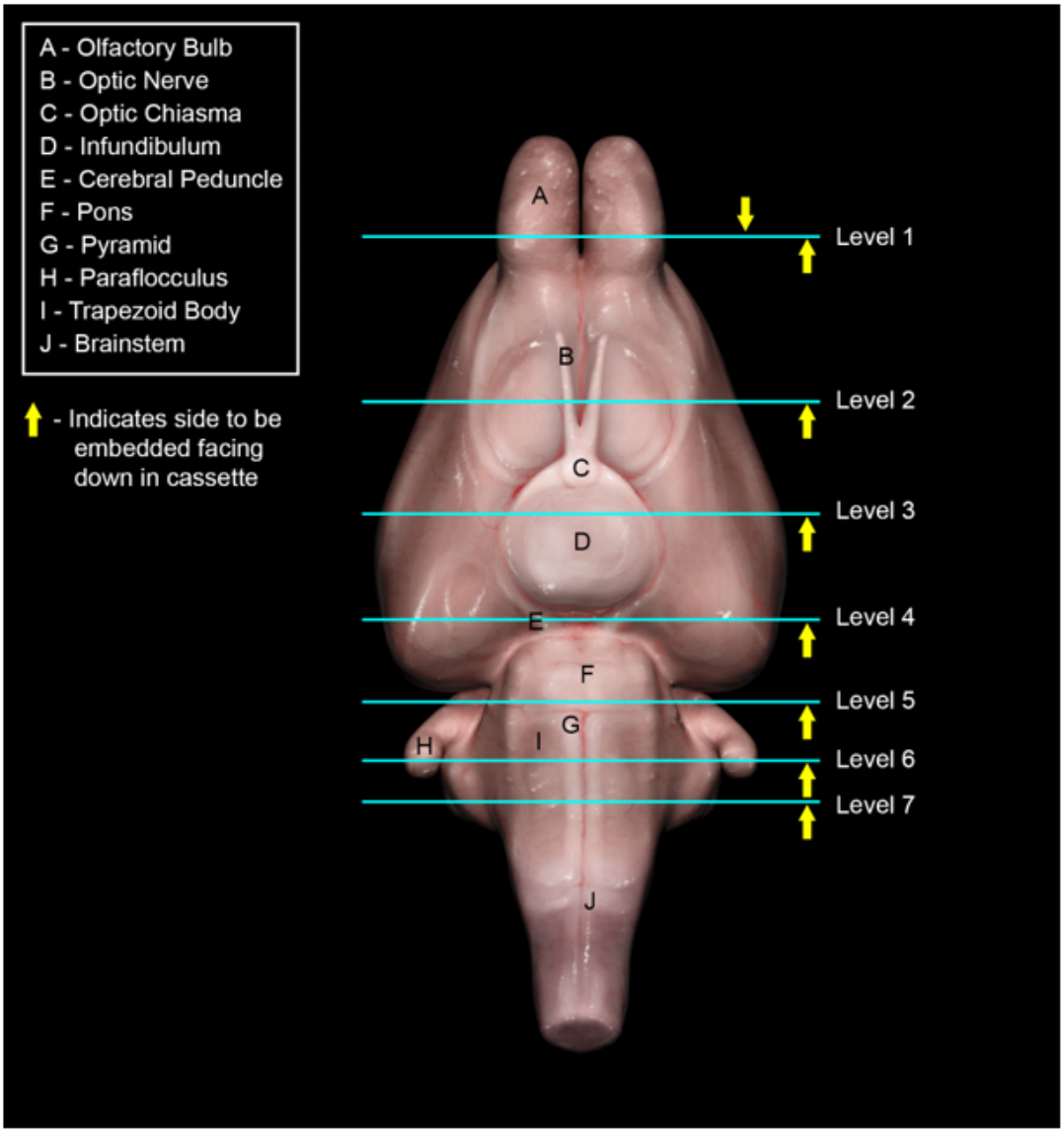
(5) Midpoint of posterior colliculus

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

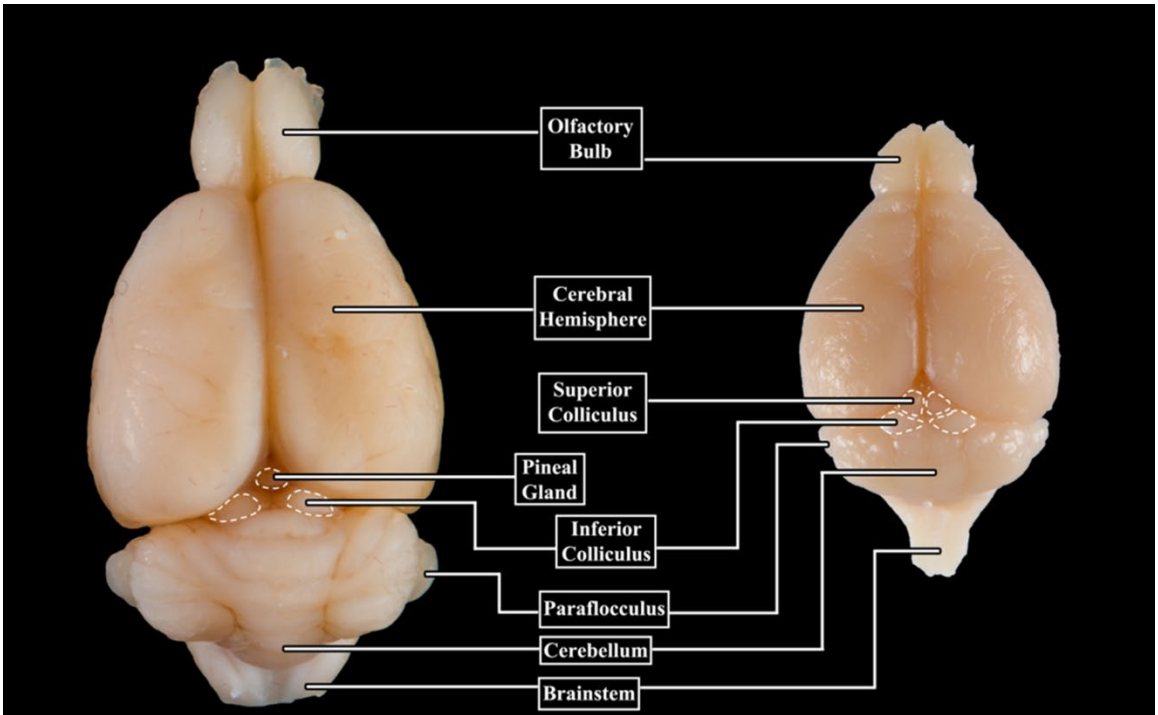
(7) 2–3 mm anterior to caudal termination of the cerebellum (posterior medulla through the area postrema)

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.

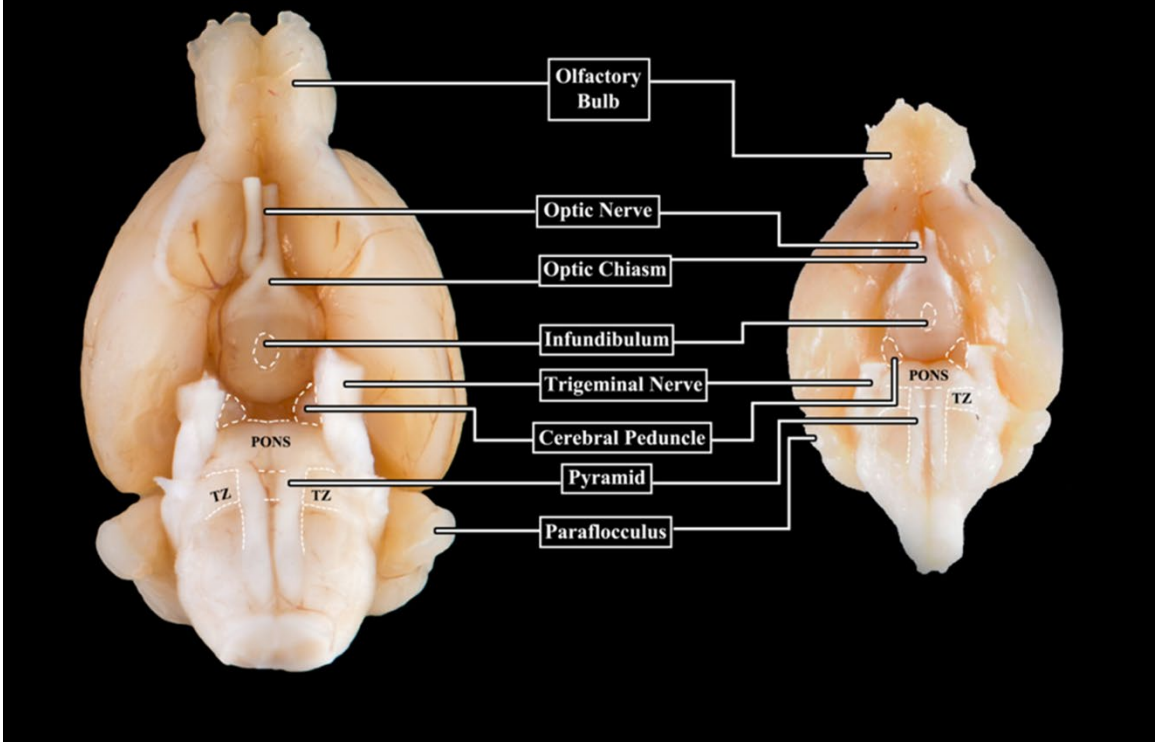
A)


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

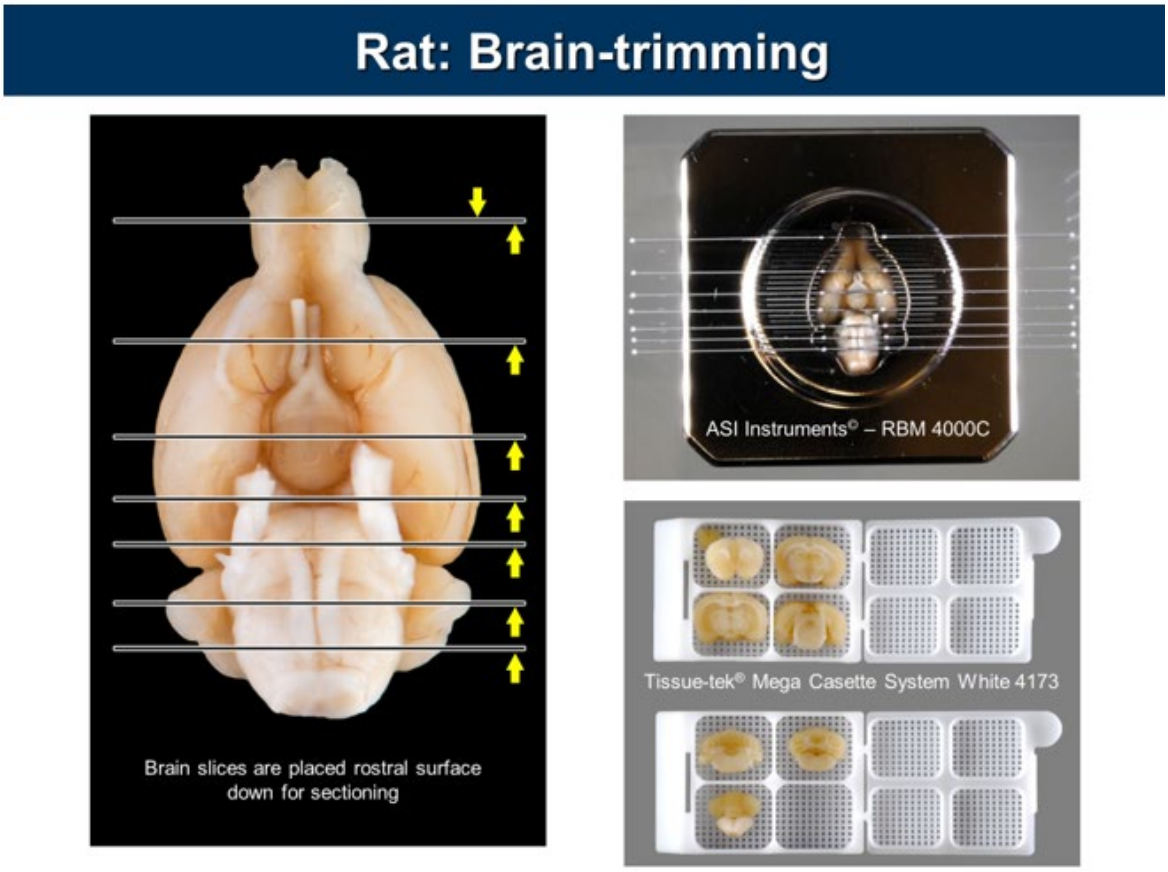
B)


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

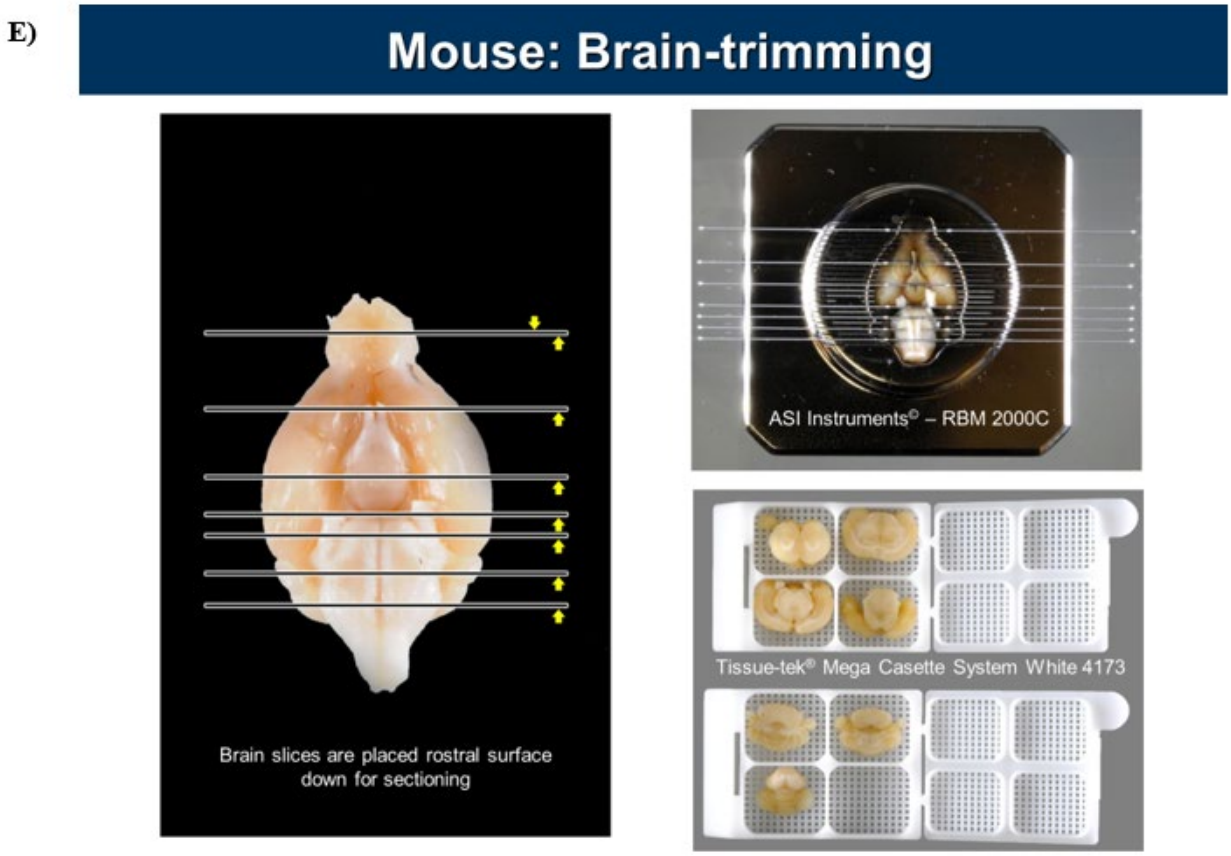


Figure 8-12. (A–F) Trimming and Sectioning Protocol for the Rat and Mouse Brain

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).

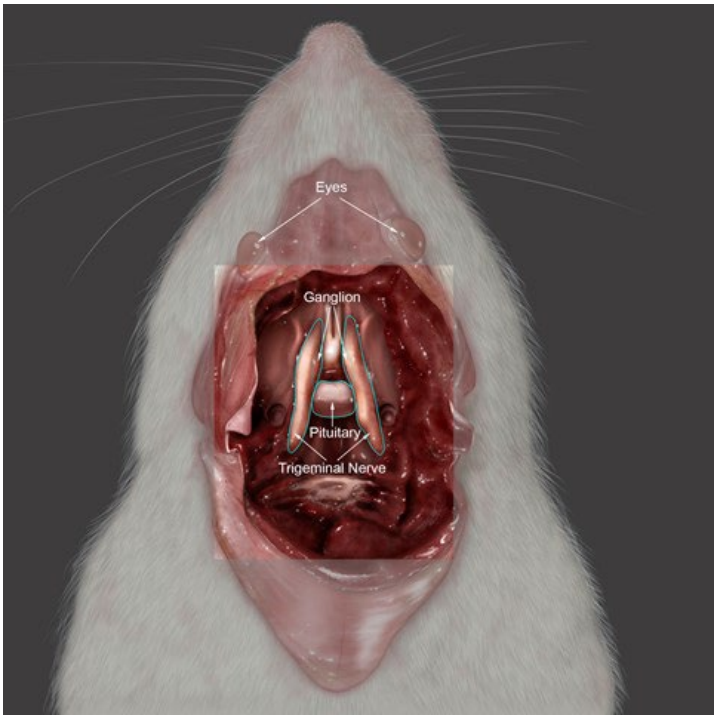


Figure 8-13. Trigeminal Nerve and Ganglion

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)

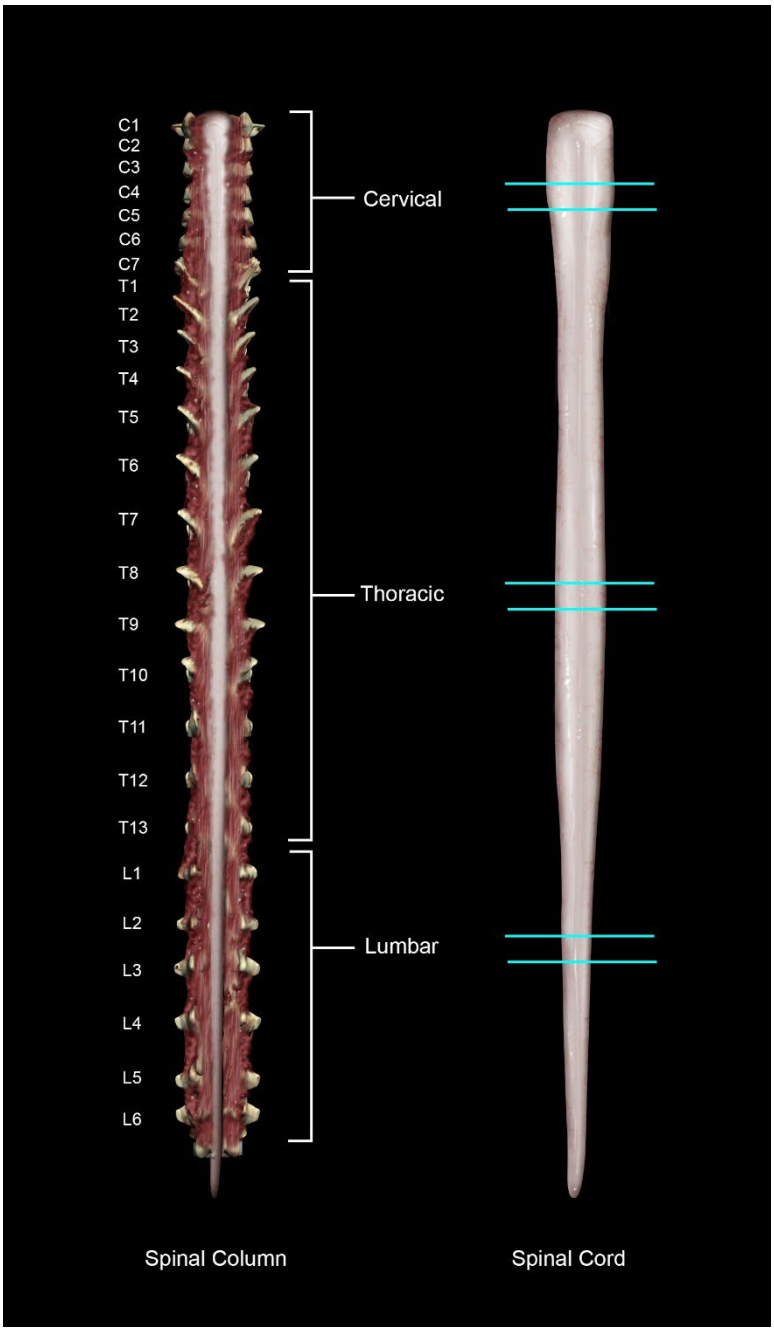


Figure 8-14. Spinal Cord

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).

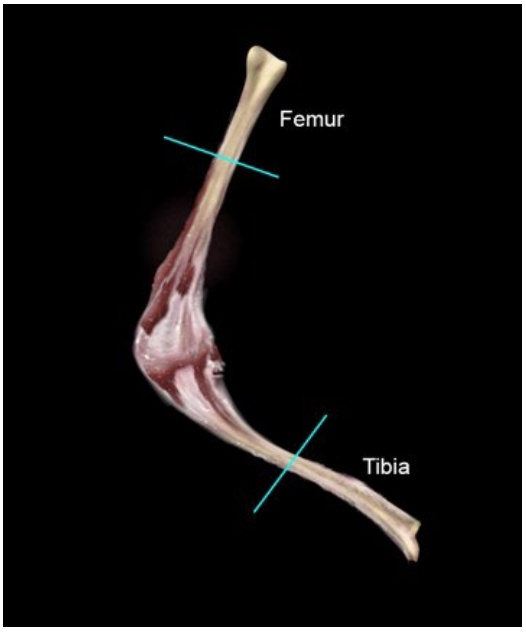


Figure 8-15. Femur and Tibia

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.

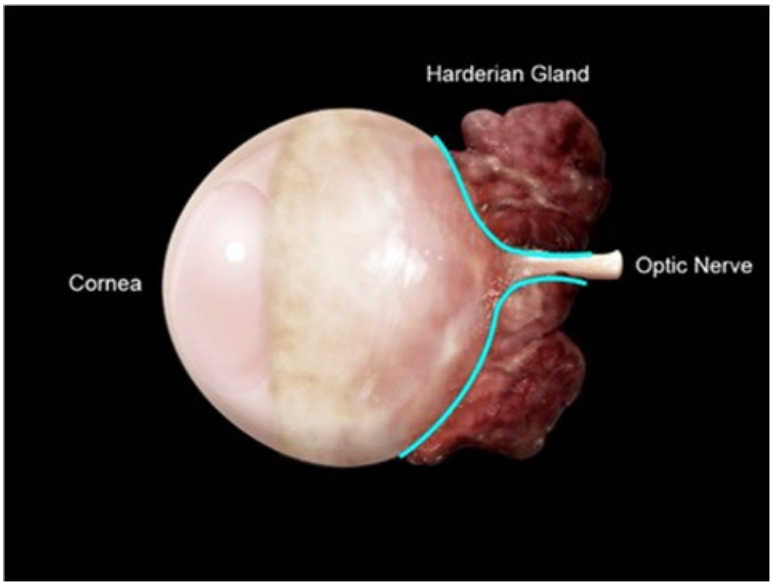


Figure 8-16. Eye and Harderian Gland

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).

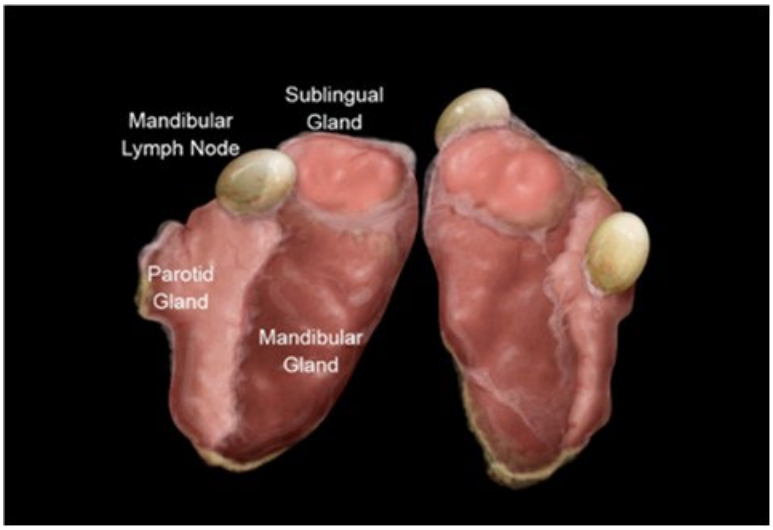


Figure 8-17. Profile of Required Sections of Salivary Glands and Mandibular Lymph Node

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.

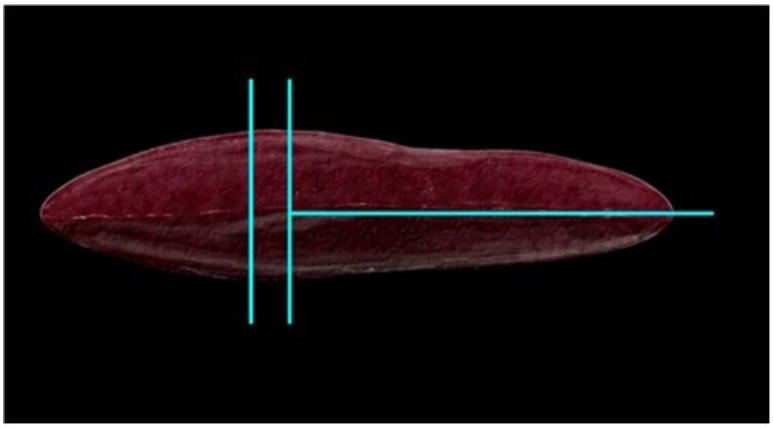


Figure 8-18. Spleen

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).

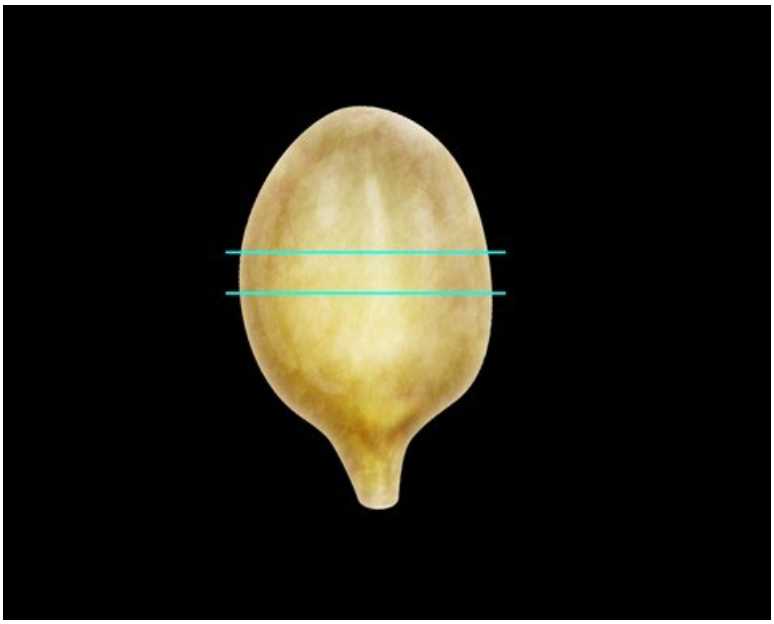


Figure 8-19. Urinary Bladder

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:

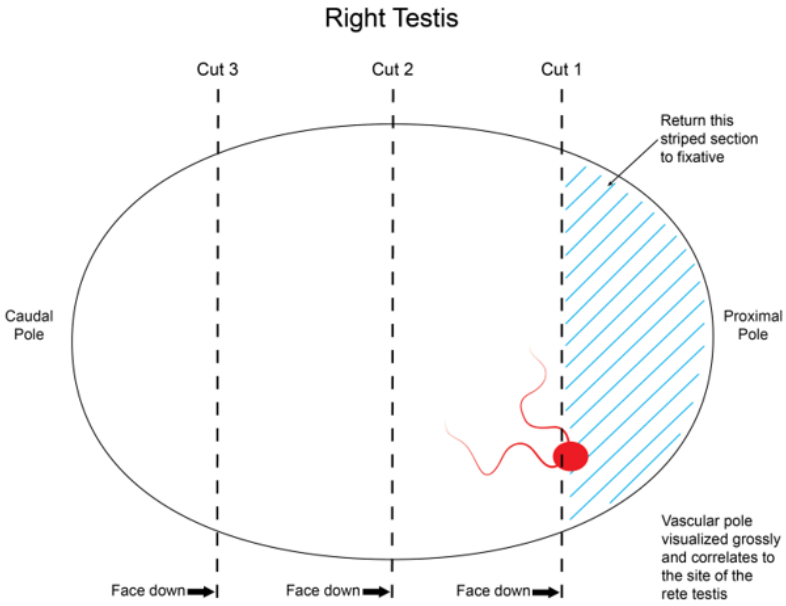
(1) At a point one-third distal from the proximal pole, to include the rete testis (same section as for adult-only exposure)

(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)

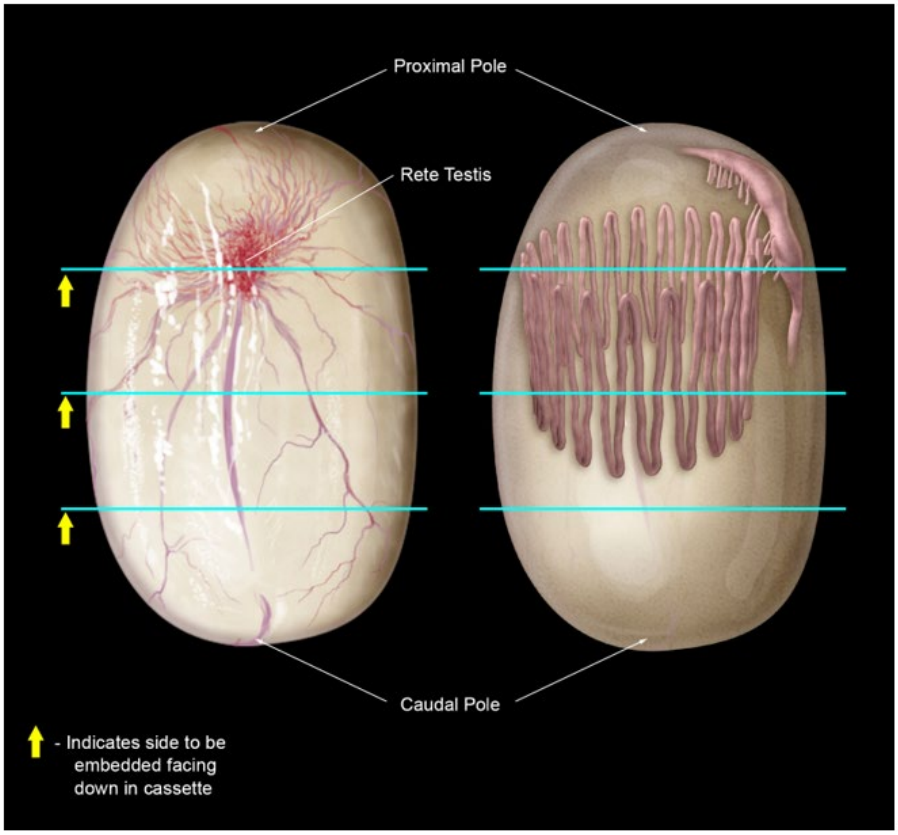


Figure 8-20. (A, B) Profile of Required Sections of Testis

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

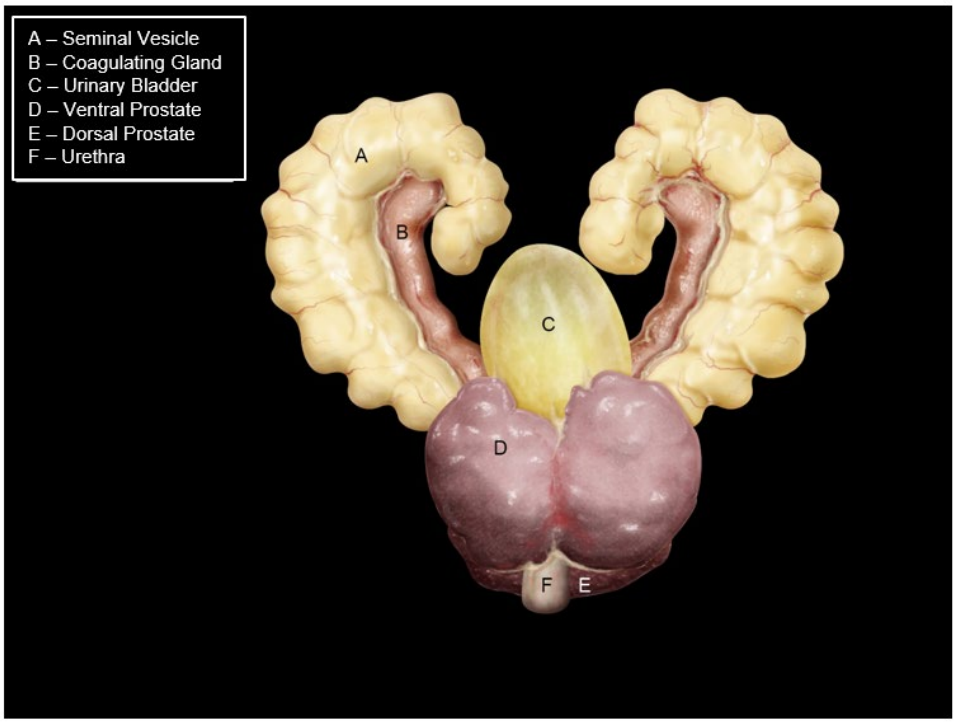
The seminal vesicles with the coagulating glands in situ shall be separated from the prostate glands, placed intact ventral surface down in a cassette for embedding, and sectioned along the longitudinal plane (Figure 8-21A, B) such that both lobes of the seminal vesicle with the coagulating glands attached 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

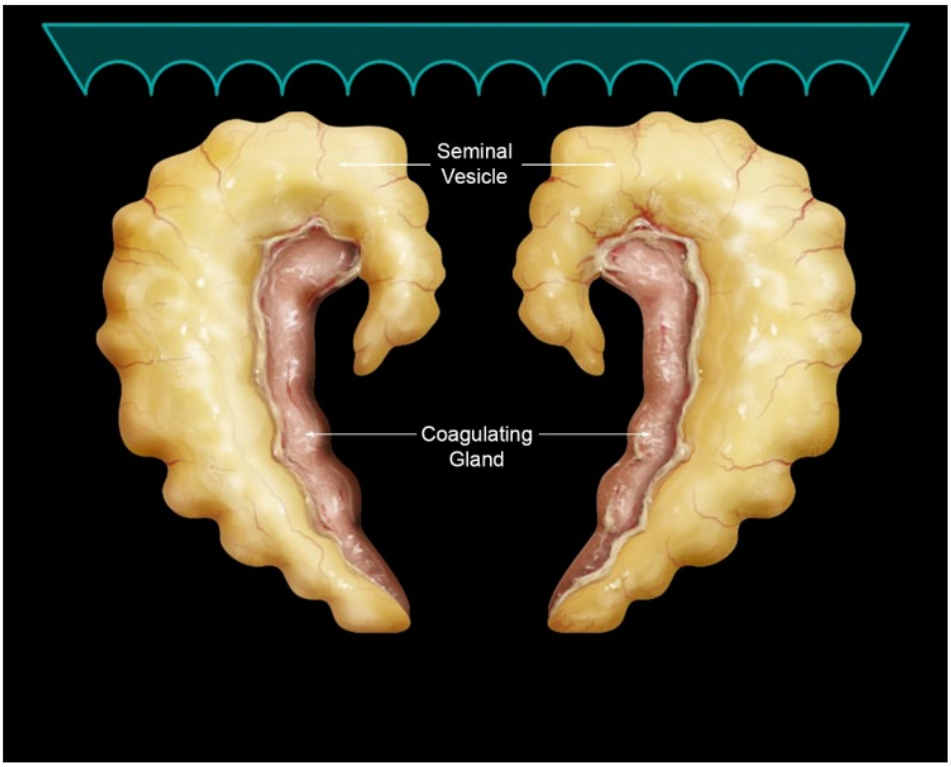
For standard studies, the lobes of the prostate gland shall be gently separated, spread horizontally (in butterfly fashion), placed flat with the ventral aspect down in a cassette for embedding, and sectioned along the longitudinal plane (Figure 8-21A, C) such that all lobes are visible in the histological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

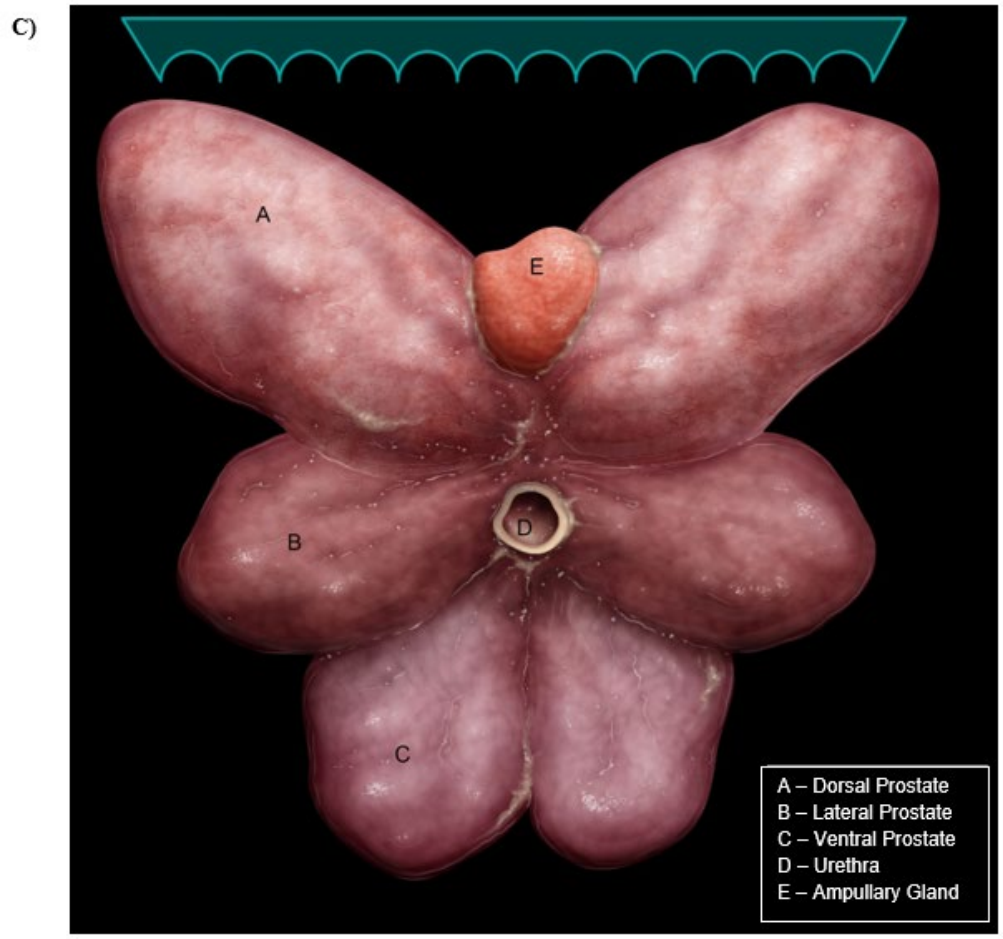


Figure 8-21 (A) Accessory Sex Glands, (B) Seminal Vesicles and Coagulating Glands, and (C) Prostate Gland

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).

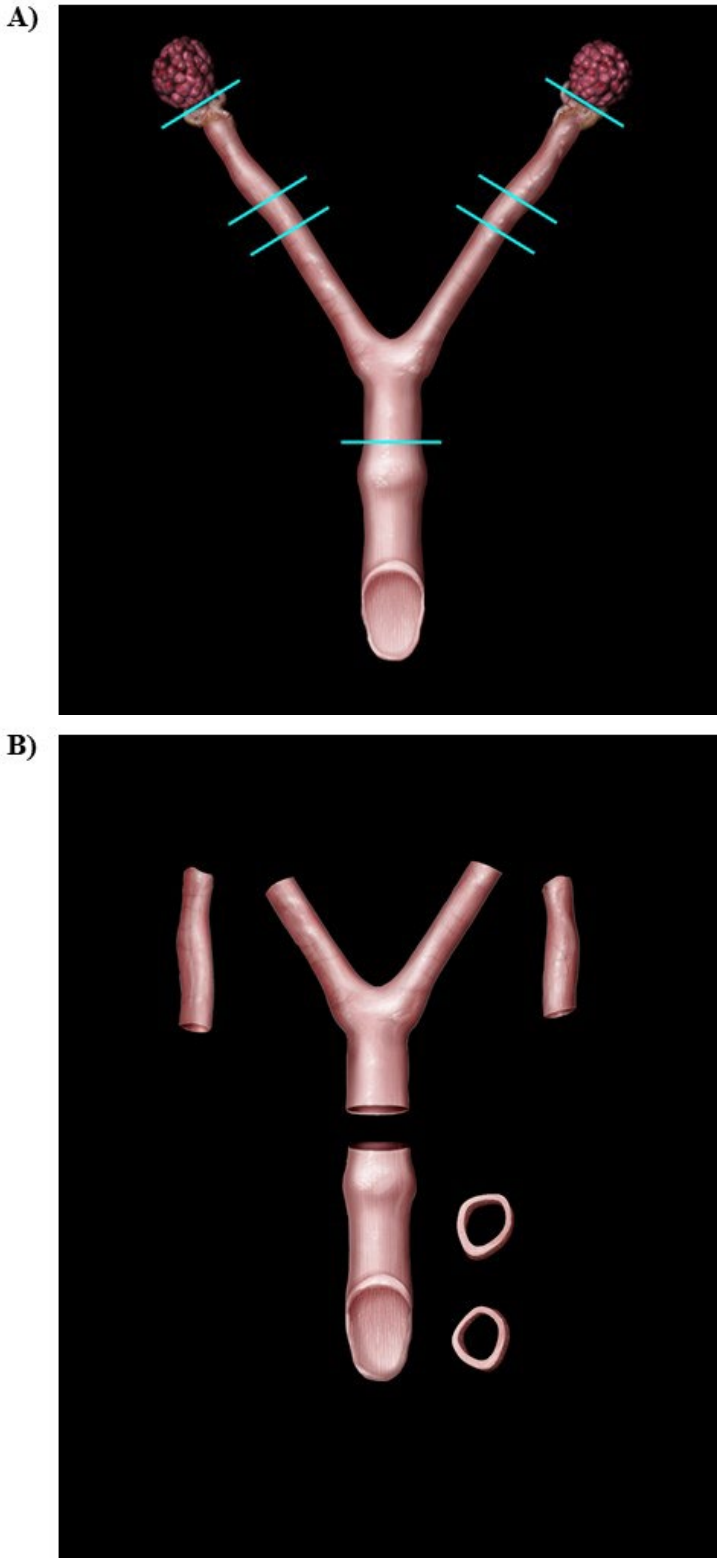


Figure 8-22 (A, B). Uterus, Cervix, and Vagina

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.

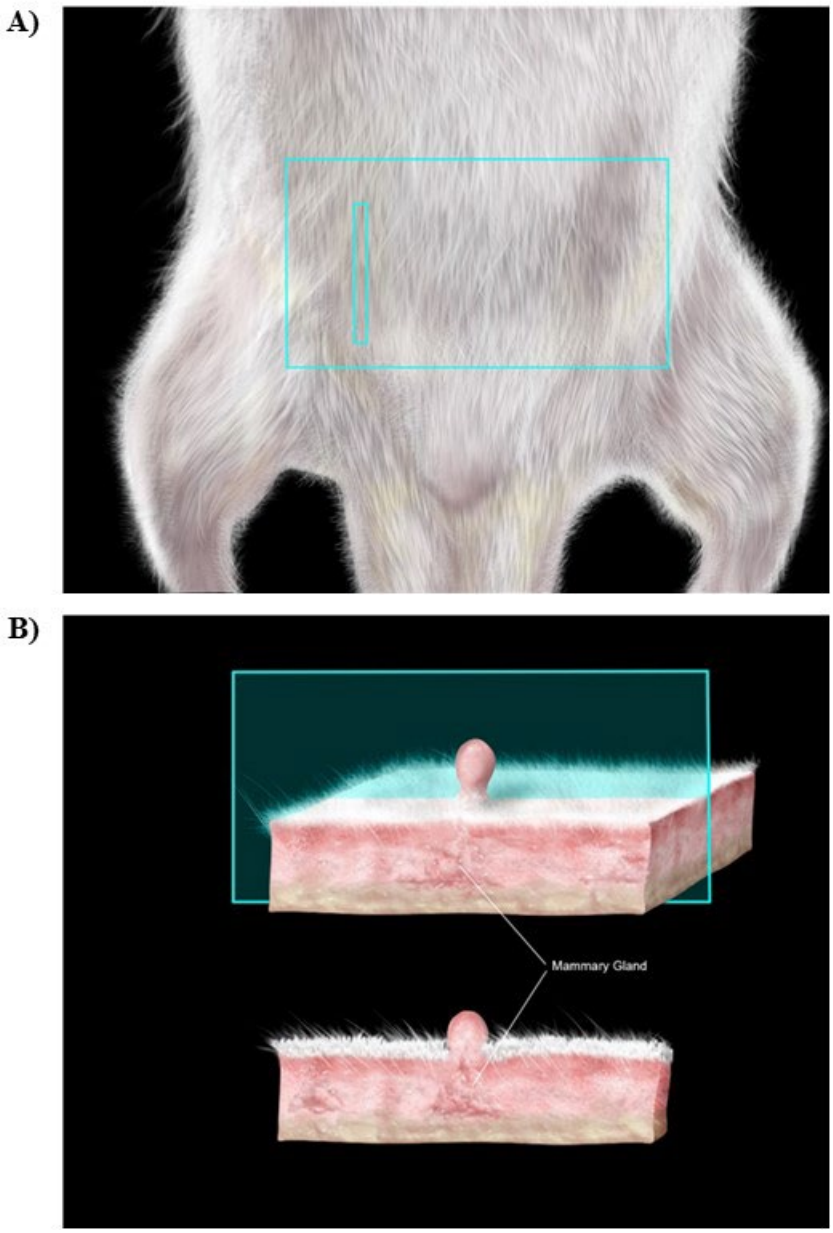


Figure 8-23 (A, B). Skin and Mammary Gland

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.

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