SPECIFICATIONS
FOR THE CONDUCT OF STUDIES
TO EVALUATE THE TOXIC AND CARCINOGENIC POTENTIAL
OF CHEMICAL, BIOLOGICAL AND PHYSICAL AGENTS
IN LABORATORY ANIMALS
FOR THE NATIONAL TOXICOLOGY PROGRAM (NTP)

October, 2006

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(For Double-side Printing)

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I. PERSONNEL

The Principal Investigator, Discipline Leaders for Toxicology, Chemistry, Inhalation Exposure, Laboratory Animal Management, Health and Safety, Quality Assurance, as well as Study Directors, shall be employees of the contract laboratory (e.g., not consultants or subcontractors). The daily interaction and constant coordination of efforts needed amongst these discipline areas throughout the in-life portion of the studies makes it critical that they be physically and organizationally together.

A. KEY PERSONNEL

Principal Investigator - The Principal Investigator, as key personnel, shall be considered essential to the contract and must be responsible for the daily management of the contract and in the communication between the NTP personnel and contract personnel. He/she shall be available to meet with NTP personnel during site visits. Site visits by the NTP Project Officer involving NTP discipline leaders, and quality assurance audits of studies in progress, are routinely conducted at laboratories with ongoing studies. The Principal Investigator must be knowledgeable and up to date in all aspects of the Program, including the status of toxicity studies, record keeping, reporting, and detailed analysis of cost for any or all functions which make up the total operation. General qualifications expected of a Principal Investigator include:

- A Doctorate, or the equivalent relevant experience in a key scientific discipline for toxicology/carcinogenesis testing (e.g., toxicology, pathology, veterinary medicine, biochemistry, chemistry)

- Significant experience in managing large-scale rodent toxicology programs requiring all disciplines

- Demonstrated capability for effective communication, written and oral

B. DISCIPLINE LEADERS

Individual(s) assigned to the following discipline roles are considered to be "critical staff" and must be capable of meeting the indicated requirements. The Discipline Leaders (DLs) are responsible for establishing scientific guidelines and procedures, training and supervision of professional and technical staff, and evaluation of results and performance within their discipline area relative to NTP requirements.

1. Discipline Leader for Toxicology - A Toxicologist shall be available to supervise and/or consult with each Study Director and professional and technical toxicology staff. Experience in studying the mechanisms of action of toxic agents is desirable since the Toxicologist, in addition to contributing to the implementation of basic prechronic and chronic experimental designs, must be familiar with any supplementary protocols to be included in studies for a particular agent. General qualifications of a DL for Toxicology include:

    - A Ph.D. or an equivalent in the Biological Sciences with relevant formal course work in disciplines such as toxicology, pharmacology, physiology, biochemistry, and related areas. Board certification (American Board of Toxicology) is desirable.

- Experience in the conduct of toxicity/carcinogenicity studies and the management of professional and technical toxicology staff.

- Experience with in vivo evaluation of toxicants.

- Experience with rodents (e.g., whole animal testing, treatment response) preferably in long-term toxicology studies

2. Discipline Leader for Chemistry - A Chemist shall be available for supervision of, and/or consultation with, personnel performing chemical analyses, dose preparations, and dose analyses. General qualifications of a DL for Chemistry include:

   - Ph.D. in chemistry with two years experience or M.S. in chemistry with five years experience.

   - Experience in dose mixing and dose analysis relative to those required in this Specifications plus experience in the use of analytical instrumentation including gas-liquid and high performance liquid chromatography, spectroscopy, instrumental and wet chemistry analyses.

   - For inhalation studies, experience in analyses of test agents in chamber atmospheres.

3. Discipline Leader for Inhalation Exposure - Professional staff member available to supervise and/or consult with staff involved in the design, development, and fabrication of generation and monitoring systems for inhalation studies for the contractor to be considered capable of this route of administration. General qualifications of inhalation exposure specialist include:

   - A minimum of a Bachelor's degree in engineering, chemistry, or related physical science with a minimum of five years experience.

   - Experience in designing and operating generators for maintaining stable inhalation chamber atmospheres for vapors, particulates, or liquid aerosols.

   - Experience in developing and operating a variety of monitoring systems for determining test article concentration in the chamber atmosphere.

4. Discipline Leader for Pathology - A veterinary or medical Pathologist with experience in laboratory animal rodent pathology, shall be responsible for oversight of professional and technical pathology staff and all pathology procedures, histopathologic evaluations, and reporting. General qualifications of a Pathologist include:

   - Formal training in a medical specialty, e.g., veterinary medicine or medicine.

   - Experience in rodent pathology, particularly tumor and toxicologic pathology.

   - Post-doctoral training and/or Board Certification in pathology.

5. Discipline Leader for Laboratory Animal Management - A veterinarian, with experience in laboratory animal medicine, must closely monitor the health of experimental animals and interact with the Principal Investigator and the NTP personnel concerning all phases of

the animal experiments. General qualifications of the DL for Laboratory Animal Management include:

- Graduate of Veterinary College recognized by the American Veterinary Medical Association.

- Diplomate of the American College of Laboratory Animal Medicine (preferred) or eligible by experience and education to take the examination.

- Previous experience in managing large colonies of laboratory animals, in particular, rodents in a toxicology setting.

- Previous experience that required multidisciplinary interactions such as toxicology studies.

6. Discipline Leader for Clinical Pathology - This person shall have professional responsibility for all clinical pathology procedures and be on-site during sample collection, processing, and evaluation of clinical laboratory indices. General qualifications of a clinical laboratory scientist include:

   - Doctorate-level, or equivalent experience in a directly relevant scientific discipline.

   - Previous experience in rodent hematology and clinical chemistry, and hormone assays.

7. Health and Safety Officer - A qualified Health and Safety Officer shall be designated to monitor worker health and safety conditions during all phases of the work. In his/her role as Health and Safety Officer, he/she shall be responsible to someone other than the Principal Investigator (PI) and the PI's subordinates, and shall have the authority to bring unsafe conditions to the attention of higher management. The Health and Safety Officer may have other responsibilities within the offeror's organization; however, the amount of time devoted explicitly to health and safety is to be commensurate with the scale of the offeror's operations. General qualifications of the Health and Safety Officer include:

   - Bachelor's degree (at a minimum) majoring in industrial hygiene, chemistry, biology, safety engineering or a closely related science or engineering field.

   - At least two years experience in occupational health and safety, along with completion of courses in general occupational health and hazard control indicating the acquisition of successively greater levels of knowledge regarding industrial hygiene. This experience shall have taken place within the last 4 years. (A Master's degree in industrial hygiene, safety engineering or a Bachelor's degree in industrial hygiene, safety engineering, with one year of experience, is an acceptable substitute for this experience.)

   - Training shall have been completed within the last eighteen months, and will be refreshed with additional training at an interval not exceeding eighteen months.

   - Recent experience in working with specific requirements of local, state, and federal statutes relating to occupational health and safety, environmental protection, and chemical monitoring.

- Demonstrated ability to deal effectively with the scientific and managerial staffs in responsibly implementing the health and safety program (including the identification of problem areas and the execution of corrective actions as required).

8. Quality Assurance Unit Officer - This individual shall be responsible for monitoring each study to assure management that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with the regulations in Part 58, "Good Laboratory Practices for Non-clinical Laboratory Studies (Federal Register, Friday December 22, 1978, Part II and any later interpretations published by the FDA). The Quality Assurance Unit Officer shall have management support and organizational independence from the personnel engaged in the direction and conduct of the study. General qualifications for a Quality Assurance Officer include:

   - A minimum of a Bachelor's degree, majoring in a biological science or chemistry.

   - Appropriate scientific experience to ensure understanding of the tasks or reports being inspected or audited in toxicology, chemistry, and histopathology.

   - The QAU Officer should have supervisory experience when the unit consists of more than one person.

C. OTHER CRITICAL STAFF

1. Study Directors - Individual(s) assigned as the GLP Study Director must have formal training in and experience with conducting studies according to regulations defined in Part 58, “Good Laboratory Practices for Non-clinical Laboratory Studies (Federal Register, Friday, December 22, 1978, Part 11 (and subsequent interpretations published by the FDA). The Study Director must be available to observe all toxicological animal room activities and must be capable of meeting the indicated requirements:

   - A Ph.D. or an equivalent in the Biological Sciences with relevant formal course work in disciplines such as toxicology, pharmacology, physiology, biochemistry, and related areas. Board certification (American Board of Toxicology) is desirable.

   - Experience with in vivo evaluation of toxicants.

   - Experience with rodents (e.g., whole animal testing, treatment response) preferably in long-term toxicology studies with the species and routes proposed, but at least prechronic experience with the species and routes proposed.

   - Demonstrated capability for effective communication, especially written.

2. Pathologists - Veterinary or medical Pathologists with experience in laboratory animal rodent pathology, responsible for the histopathologic evaluation and reporting of a study. General qualifications of a Pathologist include:

   - Formal training in a medical specialty, e.g., veterinary medicine or medicine.

   - Experience in rodent pathology, particularly tumor and toxicologic pathology.

   - Post-doctoral training and/or Board certification (American College of Veterinary Pathologists).

D. SUPPORT STAFF

1. Dose Formulation and Analysis Staff - A dose formulation supervisor with experience related to the testing program. Appropriately trained technical staff to perform dose formulation, and bulk test article and dose formulation analysis.

2. Inhalation Exposure Staff - Professional personnel to assist in the design, fabrication and development of generation and monitoring systems for inhalation studies and for the operation of the system during the study.

3. Animal Care/Toxicology Staff - Animal care/toxicology technicians with training and experience in the administration of test articles via the required routes of exposure, in providing appropriate animal care, in performing sperm morphology and vaginal cytology evaluations, and in evaluating clinical signs of toxicity.

4. Necropsy and Histology Staff - ASCP (HT) or ASCP (MT) registered technician to supervise the histology operations. Prosectors trained and experienced in the anatomy and dissection of laboratory animals, particularly rodents, able to recognize abnormal anatomic organs and to describe gross abnormalities. Appropriately trained histology technicians.

5. Clinical Pathology Staff - Registered technician(s) to supervise clinical laboratory studies. Appropriately trained technicians with experience conducting required clinical laboratory tests.

6. Quality Assurance Staff – Appropriately trained staff responsible for conducting audits and inspections of study activities.

7. Data Management Staff - Individual(s) responsible for data management activities involving TDMS and non-TDMS data, and archiving aspects.

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II. FACILITY

A. FLOOR PLAN

Floor plans shall show locations where all study activities will be conducted.

- Animal Facility:
  Floor plan indicating quarantine rooms, animal rooms, showers/change areas and rest rooms; storage areas for feed, bedding and general storage; cage and rack washers; emergency power source and those areas in which emergency power operates. This floor plan is to indicate traffic flow for personnel and equipment through the facility.
  A separate floor plan of the animal facility is to indicate room airflow directionality and indicate the location of all safety equipment, such as eyewash stations, safety showers, fire control equipment, etc.
  A floor plan indicating ventilation equipment and ductwork, including interior and exterior exhausts, shall be supplied. A floor plan for the roof(s) shall indicate the location of each of the building(s) general air intakes and exhausts and the location of the exhaust for each hood or vented enclosure.
- Chemistry:
  Indicate storage areas for bulk chemical and dose formulations, bulk chemical and dose formulation analysis, dose formulation, and supporting equipment and exhaust hoods.
- Clinical pathology:
  Indicate laboratory space and location where terminal bleeds are performed.
- Pathology:
  Indicate areas for necropsy, histology, pathology, and storage.
- Other areas:
  Indicate QA offices and filing space; data archives; waste storage; and special study facilities.

B. EMERGENCY FACILITY SUPPORT

Facilities shall have a tested back-up power source with automatic changeover equipment that is sufficient to preserve the integrity of the testing experiment. Emergency power must handle those areas critical to the study such as animal rooms, inhalation chambers, HVAC, storage freezers/refrigerators, waste storage, autotechnicons, etc. Essential mechanical equipment must be guarded or alarmed. Provisions for prompt maintenance response must be provided. Alternative air handling systems for inhalation studies are required.

C. ANIMAL FACILITIES

All facilities for the Testing Program must be approved by the NTP and will be evaluated with respect to criteria outlined in the "Guidelines for Carcinogen Bioassay in Small Rodents" (DHHS Publication No. (NIH) 76-801), "Long Term Holding of Laboratory Rodents" (ILAR News, XIX, #4, 1976), the "Guide for the Care and Use of Laboratory Animals for Research Involving Chemical Carcinogens" (DHHS Publication No. (NIH) 76-900), the "Guide for the Care and Use of Laboratory Animals NRC, 1996) and any other additions and exceptions thereof. Research is to be conducted in accordance with the Public Health Services Policy on Humane Care and Use of Laboratory Animals, Office of Laboratory Animal Welfare (OLAW). Accreditation of toxicology research and testing facilities by an external peer review organization such as AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) or CCAC (Canadian Council on Animal Care) is required. It is the responsibility of the testing laboratory to establish policy and procedures to address entry of

approved staff and visitors into the animal facility. Entry shall be prohibited to those individuals that have been in another animal facility within the last 48 hours regardless of disease status of that facility. The animal facilities shall be designed and managed to prevent contamination of animals with pathogenic organisms and to prevent contamination of personnel and the environment with test articles and also to prevent cross-contamination of animals with other test articles. A two-corridor system with intervening animal rooms is the preferred way to fulfill this requirement, given that:

1. All materials coming in contact with animals are sanitized to a clean state suitable for introduction to the supply (clean) corridor and the animal rooms.

2. Following use, these materials are removed from animal rooms by a return (dirty) corridor for disposal, destruction, or reprocessing.

3. The air pressure is adjusted so that the animal rooms are positive to the return corridor and negative to the "clean" one.

D. SUPPORTING FACILITIES

1. Animal Facility

   a. Facilities for sanitization of cages, racks, water bottles, and feeders must be available and properly located in relation to the study rooms.

   b. Clean, well-ventilated, vermin-free storage space must be provided for clean supplies and equipment, cage filters, and feed/bedding awaiting use.

   c. Ability to autoclave feed and bedding is desirable.

2. Pathology

   a. The necropsy facility should be in close proximity to the Pathologist(s) office(s). The necropsy facility must be equipped with adequate working surfaces, dissection boards, running water with drains, adequate lighting, ventilation, and exhaust hoods. Necropsy and microscopic photography capabilities are required.

   b. Refrigeration shall be available for holding dead animals until necropsy. Dead animals shall not be frozen prior to necropsy.

   c. The histology laboratory should be separated from the necropsy area and equipped with automatic tissue processor(s), microtomes, embedding and staining equipment, and with supplies and appropriate ventilation adequate for the expected volume.

   d. Acceptable storage space must be available for storage of residual archival and histologic materials that will be retained by the contractor prior to shipment to the NTP-designated recipient. These areas must have secured, limited access.

3. Chemistry and Hazardous Waste Storage

   a. The contractor must have the analytical instrumentation required for dose analysis and chemical purity checks. The specific types of equipment required will depend on the test article(s), but might include gas and/or liquid chromatographs, infrared

spectrophotometers, UV-visible spectrophotometers, and other similar instruments and apparatus.

b. Secured, controlled access bulk storage facilities must be available for retention of the test article at 5^{o} C $\pm$ 3^{o} or at ambient temperature as specified by the NTP. Also, cold storage at -20^{o} C $\pm$ 5^{o} must be available for analytical reference standards.

c. Suitable V-blenders with intensifier bars must be used for diet mixing.

d. Designated areas shall be provided and described for hazardous waste storage prior to disposal.

4. Data and Quality Assurance Archives

A secured, limited access area shall be provided for maintenance of all study records. In addition, Quality Assurance records must also be stored in a secured, limited access area.

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III. HEALTH AND SAFETY

A. ADMINISTRATIVE CONTROLS

1. Regulations and Guidelines

The NTP and/or its representatives may inspect, photograph, sample and monitor the laboratory and associated facilities used for its studies at any time to ensure that NTP minimum requirements and applicable regulations and guidelines (described below) are being followed. Any deviations to these requirements shall be approved by the NTP.

All work shall conform to applicable local, state, and federal statutes in effect at the time of award including the following federal regulations and updates:

OSHA:

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

DOJ:

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

EPA:

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

DOT:

- General Information, Regulations, and Definitions, 49 CFR 171
- Hazardous Material Table, Special Provisions, Hazardous Materials Communication Requirements and Emergency Response Information Requirements, 49 CFR 172
- Shippers, General Requirements for Shipments and Packaging, 49 CFR 173
- Carriage by Public Highway, 49 CFR 177

NRC:

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

DEA:

- Federal Requirements for Controlled Substance, 21 CFR 1300

For contract work involving infectious agents, the Centers for Disease Control Guidelines, Biosafety in Microbiological and Biomedical Laboratories (HHS Publication No. (NIH) 93-8395, 1999) and the NIH Guidelines for Research Involving Recombinant DNA Molecules (66 Federal Register 57970, 2001 and updates) shall be followed.

Where not superseded by this document, the American National Standard for Laboratory Ventilation, Z 9.5, published by the American National Standards Institute (ANSI) shall be followed.

Other consensus standards and publications may include: the current edition of the Threshold Limit Values for Chemical Substances and Physical Agents & Biological Exposure Indices published by the American Conference of Governmental Industrial Hygienists (ACGIH), Criteria Documents for various substances and the Recommended Exposure Limits published by the National Institute for Occupational Safety and Health (NIOSH), and the Workplace Environmental Exposure Levels (WEEL) published by the American Industrial Hygiene Association. Where there may be conflict in the acceptable exposure levels as compared to the OSHA Permissible Exposure Limit, the most stringent standard shall be used for worker's protection.

2. Health and Safety Plan (Chemical Hygiene Plan)

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

      No contract laboratory will participate in studies without a Health and Safety Plan that has been approved by the NTP. An updated Plan shall be submitted every 2 years to the NTP for review. In addition, the NTP shall be informed of any updates to the Plan during the course of the contract. If approval of the Plan is not granted at the time of award, the laboratory must submit a revised Plan for review within 30 days of the receipt of award notification. Revisions to the Plan shall be clearly indicated to facilitate reviewer approval.

      For all contract laboratories, a Chemical Hygiene Plan as required under the OSHA "Laboratory Standard" may be used in place of a Health and Safety Plan **provided it meets or exceeds ALL of the requirements outlined in this document.**

   b. Health and Safety Plan Content

      Written Policies. In addition to the SOPs outlined below, the Health and Safety Plan shall address (but not be limited to):

      - health and safety responsibilities, policies and organization
      - record keeping and archiving
      - initial and periodic employee training
      - engineering controls
      - personal and environmental monitoring
      - medical surveillance and biological monitoring

- respiratory protection program
- personal protective clothing and equipment
- general housekeeping
- eating and smoking policies and areas
- precautionary signs and labels
- chemical and biological storage
- fire protection and prevention
- emergency and evacuation contingencies
- locations (with schematic diagrams) of fire control equipment, and plumbed eyewash stations and emergency showers
- laboratory safety inspection
- waste management and disposal
- other pertinent personnel, operational, and administrative practices, and engineering controls necessary for the containment and safe handling of chemical, physical, biological, and radiological hazards.
- entry and exit to restricted areas
- visitors

3. Standard Operating Procedures

The laboratory shall be required to have written Standard Operating Procedures (SOPs) that have been reviewed and approved by NTP for at least the following activities:

- visitors access to test areas
- employee training
- medical surveillance and biological monitoring
- respiratory protection, mask-fit, cleaning/maintenance, and inspection
- eye and face protection
- personal protective clothing and equipment
- general housekeeping practices
- ventilation system maintenance
- storage, receipt, transport, and shipping of study materials
- hazardous material handling (e.g., in analytical chemistry labs)
- dose preparation (if applicable)
- entry and exit from the limited access areas (including traffic patterns of dose prep facility and animal handling and testing room) (if applicable)
- spill clean-up, accident, emergency response and evacuation (including natural disasters) and fires/explosions
- use of radio-labeled material, infectious agents, and/or controlled substances (if applicable)
- waste management and disposal

4. Exposure Evaluation and Control

a. Permissible Exposure Limits/OSHA-Regulated Substances

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

action level or the PEL, the exposure monitoring provisions of the relevant standard shall be complied with.

If a PEL has not yet been established for a study material, alternative acceptable exposure standards (e.g., TLV®, REL, WEEL) shall be used (Refer to Section III.A.1.). In situations where there is no known exposure standard for a proposed test article, a suitable interim exposure standard based on current toxicology and industrial hygiene literature shall be established when feasible.

b. Formaldehyde Monitoring in Histology, Pathology, and Anatomy Laboratories

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

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

c. Test article/Positive Control Monitoring

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

5. Occupational Medical Surveillance

Medical examinations for personnel who will be working with study materials or animals shall be performed at the time personnel are assigned to the program, before they are exposed to test article/positive controls.

Follow-up medical examinations shall be performed at least every 18 months and upon termination of an individual' s participation in the project.

The scope of the medical examination shall be specified in the laboratory's Health and Safety Plan. Persons who are required to wear respirators must obtain written medical clearance from an occupational health service provider (e.g., an occupational medicine

physician, a physician assistant or nurse practitioner who is supervised by the physician) for use of this equipment.

6. Injury and Incident Reports

A record shall be kept of all injuries or illnesses, including animal bites. In addition any record of an OSHA recordable incident shall include a full description of the incident, the test article/positive control involved, the medical attention required, any remedial actions taken, and planned follow-up to minimize the likelihood, or eliminate the potential for, reoccurrence (if pertinent). Copies of such incident reports shall be forwarded to NTP.

The Project Officer shall be notified IMMEDIATELY if a serious (as defined by OSHA) accident or incident occurs.

All occupational injuries and illnesses shall be recorded and reported according to the OSHA recording system.

B. TEST ARTICLE/POSITIVE CONTROL HANDLING AND SAFETY POLICIES

1. Receipt/Handling/Storage

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

Weighing of the test article and/or positive control shall be done using the smallest quantity needed. An analytical balance shall be used whenever possible to preclude the need for handling large amounts of chemical. This balance shall be placed at all times in an effective laboratory hood or a vented enclosure exhausted to the outside (see Section III.C.3.). Protocols shall be designed to use the minimum possible quantities of "neat" chemical in preparing solutions.

A non-breakable, secured secondary container shall be used for transfer of any test article and/or positive control.

Volatile test articles shall be handled properly (e.g., keeping lids on container when not in use, segregating from unintended contact with heat or high pressure, etc.) and stored in an area with adequate ventilation that is directly vented to the outside. All other test articles shall be stored in a secured, designated storage area(s). However, flammable liquids must be stored in a non-vented flammable liquid storage cabinet (see Section III.E.1.).

2. Hazard Communication

a. Training

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

b. Labeling

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

c. Health and Safety Documents

The contractor must have available for each study agent and positive control health and safety documentation that includes, but is not limited to, the supplier's MSDS*, which includes information on the material's hazards, properties and appropriate control measures. All employees handling the study material and/or positive control must be trained regarding the contents of the agent-specific health and safety data document.

MSDS shall be accessible at all times at designated locations known by the employees.

* If a chemical is produced for a user outside of the laboratory then the laboratory is required to develop an MSDS.

C. ENGINEERING CONTROLS

1. General Facility Requirements

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

2. Isolation and Access Restriction

a. General Requirements for Access Restriction

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

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

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

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

b. Requirements for Barrier Systems

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

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

Within the shower facility, the "clean" and "dirty" sides must be physically separated by the shower or by another physical barrier. The facility design and procedures shall be arranged so that it is not necessary to require entering the clean side prior to showering and to prevent returning to the dirty side after showering (e.g., to store or retrieve items such as shoes, towels, respirators, etc.).

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

c. Facility Design for Barrier Systems

Air exhausted from dose preparation areas involving the particulate form of the test materials shall be passed through HEPA filters. If volatile chemicals are handled, charcoal filters shall also be used. These filtration systems shall be periodically monitored and maintained and personnel performing maintenance shall wear the protective clothing described for neat test article handling. (See Section III.D.1.a.)

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

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

Emergency power generator systems shall be in place and emergency generator maintenance and testing shall be documented.

3. Hoods and Vented Enclosures

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

a. Hood/Enclosure Operations and Requirements

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

- All dose preparation operations (e.g., weighing, premix, micro encapsulation, mixing of dosing solutions), as well as diluting, or administering (gavage, dermal, intra-peritoneal injection, inhalation chamber administration) of study materials/positive controls
- Test article weighing in laboratories (e.g., analytical laboratories)
- Transfer/filling of dosed feed containers
- Unpacking, analysis, and other handling operations involving test article/positive control or other hazardous agents
- Necropsy, tissue trimming, tissue processing, and staining
- Handling tissues, fluids, and exhaled air collected from animals for evaluation
- Cage and feed container dumping
- Plastic-backed absorbent matting shall be secured inside of any hood wherever the test articles and/or positive controls (including dilutions) are being handled. After each working session in the hood, or sooner if there is known contamination, this matting shall be disposed of as hazardous waste.

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

1) Hoods for Weighing, Diluting, or Administering Test Articles/Positive Controls

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

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

2) Exhausted Enclosures/Hoods for Automatic Tissue Processing/Staining.

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

3) Exhausted Enclosures/Hoods for Necropsy, Tissue Trimming, Manual Tissue Processing, and Manual Staining.

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

b. Hood/Enclosure Venting

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

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

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

Re-circulation of air from local exhaust systems into occupied spaces shall not be permitted. The only exception to this will be for dosed feed container-filling hoods, cage dumping hoods, or vented enclosures for studies involving non-volatile, solid test articles. If re-circulation is desired in this case, the air discharged from hoods or vented enclosures must be equipped with HEPA filtration to clean air prior to its discharge to the study room. The HEPA filter shall be disposed of as hazardous waste. (See Section III.G.2.)

c. Hood/Enclosure Monitoring

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

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

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

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

D. PERSONAL PROTECTION EQUIPMENT SELECTION

1. Selection

a. Operations Involving Handling of the Neat Test Article/Positive Control and in Animal Rooms.

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

- Disposable full-body Tyvek® suit and disposable head covering, unless Tyvek® suit includes a hood

- Gloves

  If chemical-specific gloves cannot be identified, two pairs of dissimilar, disposable gloves (e.g., N-Dex®or equivalent, PVC, latex, natural rubber) will be worn when handling test article/positive control (as neat material or in formulated doses). Both pairs of the two dissimilar gloves shall be changed after any known chemical contact and/or after every two hours of handling test article/positive controls or dose formulations.

- Respirator

  Appropriate NIOSH-approved respirators shall be worn.

- Eye Protection

  Splash-proof safety glasses, goggles, or other eye protection specified by OSHA and ANSI.

- Footwear

  Disposable shoe covers, disposable boots, or facility-dedicated rubber boots.

b. Operations Not Involving Neat Chemical/Positive Control

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

- Single pair of disposable gloves

- Laboratory coat

- Splash-proof safety glasses, goggles, or other eye protection specified by OSHA and ANSI

c. Animal Barrier “Clean” Corridor

(1) All staff entering the clean corridor with the intention of entering animal rooms must follow PPE requirements as defined in section D.1.a.

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

2. Respiratory Protection

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

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

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

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

3. Usage and Storage Practices

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

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

Work clothing shall be removed upon exit from the laboratory on a daily basis.

Disposable clothing previously used in laboratories shall not be reused, and shall not be worn in common areas such as hallways and offices.

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

E. FIRE SAFETY

NOTE: Fire safety requirements for Inhalation studies are described in Section H. below.

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

1. Storage and Handling

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

All non-working quantities of flammable liquids shall be stored in storage cabinets approved by Underwriters Laboratories or Factory Mutual, or in a designated flammable liquids storage room with suitable fire protection, ventilation, spill containment trays, and with equipment meeting the requirements of OSHA. In either storage arrangement, the flammable liquids shall be segregated from other hazardous materials such as acids, bases, oxidizers, etc.

Flammable storage cabinets shall not be vented unless required by a chemical- specific OSHA regulation or by local authorities. Metal bung caps shall be used in place of flash arrestor screens. If it is necessary that venting be provided, the following shall be adhered to: (1) Remove both metal bungs and replace with flash arrestor screens. The top opening shall serve as the fresh air inlet. (2) Connect the bottom opening to an exhaust fan by a substantial metal tubing having an inside diameter no smaller than the vent. The tubing shall be rigid steel. (3) Ensure that the fan has a non-sparking fan blade and non-sparking shroud. It shall exhaust directly to the outside where possible. (4) The total run of exhaust duct shall not exceed 25 feet.

Class I flammable liquids shall not be stored in conventional refrigerators/freezers. If flammable liquids must be kept at low temperatures, they shall be stored in Underwriters Laboratory (UL) listed/Factory Mutual (FM) Global approved refrigerators/freezers designed for flammable storage. In a potentially flammable or explosive atmospheric environment, only those explosion-proof refrigerators/freezers listed for Class I. Division 1, Group C and D, and listed by UL as a "Special Purpose Refrigerator and/or Freezer" shall be used. All explosion-proof refrigerators shall be labeled as such.

Whenever flammable liquids are stored or handled, ignition sources shall be eliminated. This includes the prohibition of smoking.

Flammable liquid transfer shall be done in the designated storage room or over a tray within an effective laboratory hood. In the former location, all transfer drums shall be grounded and bonded and shall be equipped with pressure relief devices and dead man valves.

Safety cans shall be used when handling small (e.g., no more than 2 gallons) quantities of flammable liquids, unless chemical purity requirements require otherwise (e.g., distilled-in-glass grade, etc.).

2. Fire Safety Equipment

a. Fire Extinguishers

Fire extinguishers shall be conspicuously located where they will be readily accessible and immediately available in the event of fire as required by local, state, and federal regulations. Placement of portable fire extinguishers shall conform to OSHA 1910.157. The specific type and size of extinguisher shall be selected with consideration for the hazards to be protected and the strength of the personnel who might use the extinguishers. For the majority of laboratory applications, water and aqueous film forming foam (AFFF) extinguishers shall have a capacity of 2 1/2 gallons. Dry chemical, carbon dioxide, and foam extinguishes shall have 20-30 pound capacity.

b. Safety Showers

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

3. Training

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

F. EMERGENCY PROCEDURES

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

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

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

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

G. WASTE DISPOSAL/TEST ARTICLE SHIPMENT

1. Disposition/Shipment of Surplus/Residual Test Article

The following practices shall be adhered to concerning the disposition of surplus/residual test article:

Thirty days prior to shipment, the contractor shall notify the NTP of their intention to ship

surplus or residual test article, including the amount to be shipped, and complete details of the shipping procedures, including the contractor to be used.

Upon completion of testing and after receiving approval from the Project Officer, the contractor shall immediately ship excess quantities of test articles after the final bulk chemical analysis has been completed. In addition, a 100 gram aliquot of each batch of the test article is to be reserved and shipped separately to the designated NTP chemistry support contractor after the final bulk chemical analysis has been completed. For reactive chemicals, gases, etc. the Project Officer shall be contacted to determine if any test article is to be reserved and shipped.

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

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

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

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

2. Potentially Contaminated Material

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

temperatures and residence times), operating procedures, and information on licensing by local regulatory authorities shall be provided to NTP for evaluation. If a contract disposer is to be used, complete information on the firm's licensing and hazardous waste transporter shall be provided.

Where Toxicology Data Management System (TDMS) terminals are used, the terminal shall be decontaminated using a chemical-specific solution when removed from an animal room after each use. Terminals must be disconnected from any electrical power sources before decontamination, and care will be taken to ensure that any solvents used do not damage the plastic parts of the TDMS terminal.

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

H. INHALATION STUDIES

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

1. General Requirements for Inhalation Studies

The test atmosphere generation apparatus and flow meters through which test atmospheres pass shall be contained in enclosures exhausted to the outside (see section III.C.3.). All connections in the piping and/or ducting between the test atmosphere generator and the exhaust air filters shall be either compression-fitted, threaded, welded, or enclosed and vented and leak-tested before use. All equipment through which test article flows shall be electrically grounded and bonded according to the provisions of the National Electrical Code and the National Fire Protection Association Standard 77, "Recommended Practice on Static Electricity." Use of plastics, such as PVC, is not permitted. All piping, ducting and other materials must be compatible with the test article.

A full description of all safeguards, safety procedures, alarms, shutdowns, emergency plans, clean-up procedures and disposal methods must be reported to the Project Officer and be in-place before the start of all studies.

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

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

At least two NIOSH-approved, 30-minute positive pressure (pressure demand) self-contained breathing apparatus shall be available for use by trained in-house (or by an on-site, trained, emergency response contractor) if emergency entry into a study room

following a leak is required. These units shall be maintained and inspected as required under 29 CFR 1910.134 of the OSHA respiratory protection standard.

The personal protection requirements for inhalation studies as specified in section III.D. shall apply except as follows:

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

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

2. Combustible/Flammable Test articles

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

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

- Flow monitoring equipment shall be used to determine variations in the flows of the test article and carrier air. In the event that there is a 10% change in flow, system shutdown shall occur with an audible alarm signaling such action at a manned location.

- The instrumentation used to continuously monitor the inhalation chamber and chamber room for the test article shall be equipped with an audible alarm that signals a manned location when a concentration equal to 25% of the lower flammable limit or the minimum explosive concentration is detected.

- A flame arrestor shall be installed on the gas or vapor supply line. If the test article is a combustible dust, an optical flame detector must be located in the supply line and connected to trip a fast acting shut-off valve upstream.

- An alarm shall be in place to indicate when the air flow through the vented enclosure which surrounds the test article generation devices(s) falls below 85% of its nominal value.

- All equipment through which the test article flows shall be electrically grounded and bonded according to the provisions of the National Electrical Code and the National Fire Protection Association Standard 77, "Recommended Practice on Static Electricity."

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

- The inhalation chamber study room shall be isolated from the other operations by walls with a fire resistance of 1 hour such that it is solely dedicated to the testing of the flammable or explosive study material.

- Explosion venting shall be installed on each inhalation chamber. The recommended vent ratio is 1 ft^2/10 ft^3.

- All electrical equipment shall be suitable for a Class I, Division II (flammable gas or vapor) or Class II, Division II (combustible dust) location as defined by the National Electrical Code.

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

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

3. Reporting Requirements for Inhalation Studies

The following information shall be included in the Prestart Inhalation Report.

a. Effluent Exhaust Monitoring

Description of the method(s) to be used for effluent exhaust treatment during generation runs at the protocol-required concentrations, in all chambers under actual animal exposure conditions; data demonstrating the effectiveness of the effluent exhaust treatment unit immediately after the effluent treatment unit and/or at the point of exhaust from the building; percent efficiency of the exhaust treatment (the effluent exhaust treatment must be effective in removing the test article to an acceptable concentration (e.g., greater than 90% efficiency of removal by the treatment system and less than 50% of the TLV, if a TLV exists), or written documentation for a waiver from appropriate air regulatory agencies must be provided; determination of the lifetime expectancy of any proposed filtration/treatment units and the amount of treatment media that will be required; and confirmation that none of the exhausted test article is re-entrained.

b. Room Air Monitoring

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

c. Special Requirements for Particulate Studies

When a test article is a particulate (e.g., dust, mist or aerosol), NTP requires that the test system minimize the probability of, and the consequences associated with, fire

and/or explosion. The laboratory shall provide NTP with data on its test system that includes equipment and techniques for reducing the fire and/or explosion hazard. The laboratory shall also provide complete information on the test article that includes determination of the following: the minimum explosion concentration, minimum spark ignition energy, explosion severity, minimum ignition temperature of a layer, and the volume resistivity. As NTP recognizes that there may be alternative approaches for minimizing the risks due to fire and explosion, the Project Officer may grant approval to test system configurations that differ from the following provisions on a site-specific basis.

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

d. Standard Operating Procedures

All SOPs that are specific to the study are to be attached to the Prestart Inhalation report.

IV. CHEMISTRY

A. GENERAL REQUIREMENTS

1. NTP will typically supply the test article.

2. NTP will typically provide procedures for bulk chemical analysis, dose formulation and dose formulation analysis. Modest modifications may be made to suit existing instrumentation.

3. For inhalation studies the development of methods for generation and monitoring of the chamber atmosphere shall be the responsibility of the Contractor.

4. Toxicokinetic studies may be included in selected toxicology studies. In some cases, the contractor's responsibility may be limited only to the collection and shipment of tissue(s) for analysis by an NTP chemistry contractor. However, for some studies the Contractor shall be required to develop methods for determining the test article or metabolite in biological samples, analyze samples and provide an interpretation of the results.

5. Standard Operating Procedures (SOPs) shall be prepared for all chemistry, dose formulation, toxicokinetic studies, and inhalation technology operations.

6. The results of all analyses shall be reported to at least three significant figures unless the analytical methodology limits the data to fewer significant figures.

B. BULK TEST ARTICLE CHEMISTRY

1. Bulk Test Article Receipt and Storage

   The test article(s) and frozen reference(s), as well as storage conditions for the bulk test article, will be supplied by the NTP. Whenever feasible, a sufficient quantity of the test article will be procured so that only one lot of chemical will be needed to complete all of the contracted study phases. Contractors shall plan to have adequate storage at the specified condition(s) for bulk test article(s). A use log of the bulk test article shall be kept and submitted as part of the raw data at the end of the study.

   If appropriate storage conditions for the bulk test article are not provided by NTP, it may be possible to use the manufacturer's data to establish the stability and thus the storage conditions for the bulk test article without the need for additional studies by the Contractor. If stability studies are required they are to consist of triplicate analyses of the bulk test article after storage at - 20, 5, and 25^{o} C for two weeks in sealed vials that are protected from light.

2. Initial Identity and Purity

   Methods to confirm the identity and purity of the bulk test article are to be provided by NTP, and will generally involve two identity analyses and one purity determination.

   It shall be necessary to conduct re-analyses to confirm the purity of the test article while it is being used in the toxicology studies. The most efficient method for determining stability is to maintain a stable standard for comparison with the stored bulk test article.

Therefore, upon receipt of each batch of test article, the Contractor shall remove enough samples for all reanalysis tasks (typically 15 X 1 gram samples). Each sample shall be placed in a glass vial with a Teflon-lined top that is tightly closed. All reference samples shall be placed in a freezer and maintained at –20° C ± 5 for storage prior to analysis. Analyses of the bulk test article and the reference standard shall be run in tandem, so that the bulk chemical purity can be compared to that of the reference standard from the same batch. Each vial of reference test article shall only be used for one reanalysis. Any proposed repackaging of the test article by the contractor must be approved in advance by NTP. In specific cases, to be identified by NTP, additional frozen reference samples may be required to be taken from each individual bottle or drum of test article supplied, for use in bulk reanalysis of those specific containers while they are in use.

3. Bulk Test Article Chemical Reanalysis

   The Contractor shall check the purity of the bulk test article at 24 ± 2 week intervals while the article is at the Contractor's facilities. The bulk test article re-analyses will normally require the use of only one purity analysis method. These bulk chemical re-analyses shall include an analysis within thirty days prior to the start of any study. Additional test article re-analyses shall include an analysis within thirty days after sacrifice of the last animal for studies greater than 30 days duration.

   The Principal Investigator shall immediately via telephone or e-mail and in the next Monthly Progress Report notify the NTP Project Officer of any significant change in purity (e.g., a difference not explained by variability within the analytical procedure used) or appearance of the test article during the study.

C. DOSE FORMULATION

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

2. The stability of the dose formulations shall typically be established by the NTP. The dose formulations shall not be used beyond their stability period.

   If a stability study is to be the responsibility of the Contractor, the stability of the test article formulated with the dosing vehicle shall be conducted at the lowest concentrations specified for the toxicology study. If suspensions are to be formulated, the re-suspendability of the highest concentration formulation shall also be determined. Stability studies shall be run for a 42-day period, at 1 week intervals, with the dose formulations stored sealed and protected from light at -20, 5, and 25°C. Triplicate samples shall be analyzed. Stability determinations shall also be made under conditions that simulate the environmental conditions of dosing.

3. An inventory of each dose formulation shall be maintained. A record of the formulation date and use date for each formulation shall be kept. This record, which shall be signed by the dose formulation supervisor, shall contain information on the quantities of dose formulation prepared and identifying numbers for both the test article and dosing vehicle.

4. One archival sample of each batch shall be set aside at the time of preparation for possible dose analysis checks and stored in individually labeled, sealed containers. The containers shall be transferred to an archival storage area by dose formulation personnel. All aliquots shall be stored at –20° C ± 5° except for aqueous formulations that shall be stored at 5° C ± 3°. The quantity of the archival samples are: approximately 50 mL for

gavage and drinking water studies; 100 g for feed studies; and 25 mL for dermal studies. When a dose analysis of a formulation is planned, the analytical chemistry technician shall retrieve the archival samples from the storage area. Archival samples that are not selected for analysis shall be discarded as hazardous waste in accordance with federal, state and local regulations ninety days or more after preparation. (NOTE: Each time an aliquot of the bulk chemical is weighed and formulated with the vehicle, that formulation is defined as a batch. Therefore, for each dose prepared on each formulation day it is possible that two or more batches will be required.).

5. To ensure that homogeneous dosed feed or suspensions are prepared, homogeneity shall be checked prior to initiation of each study phase (e.g. 14-day study, 90-day study and 2-year study) at the highest and lowest doses, unless blend parameters (size and concentration) have not changed from the previous study. For feed studies, samples shall be taken for analysis from three different points in the blender (e.g. top left, top right, and bottom of the twin-shell blender; two samples per site). For suspensions, two samples shall be taken from the top, middle, and bottom of the container under simulated dosing conditions. All samples taken for homogeneity study shall be analyzed in duplicate. The formulation shall be considered homogeneous if the coefficient of variation of all samples taken for analysis is within 5%.

6. To prevent improper dosing of study animals:

   a. The containers shall be labeled with the test article name, number, other identifying data and intended species. The inclusive dates of use must appear on the containers.

   b. The labels on the containers shall be color coded for different dose groups, species, and sex (if different concentration used for species/sex).

   c. Control and dosed formulations shall be stored separately from the bulk test article.

7. For dosed water studies, water for control groups is to be taken from the exact same source and at the same time as the water used for the treated group formulations. The control water and dose formulations are to be stored in carboys in the refrigerator until it is time to dispense the formulations to water bottles and transport them to the animal rooms. For dosed feed studies, feed for the control groups is to be taken from the exact same source and at the same time as the feed used for treated group formulations.

D. FORMULATION ANALYSIS

1. Formulation analysis methodology shall typically be provided by NTP. However, in some cases this may be the responsibility of the Contractor.

2. The Contractor shall validate the method over the proposed range of doses. If doses required for subsequent study phases are outside the validated range from those used in the initial study, additional pre-start validation studies and reports shall be required.

3. As a quality control check, formulations shall be analyzed periodically (see IV.E.) by the Contractor. The results of analysis for feed formulations shall be reported as mg chemical/g vehicle. All other formulations shall be reported as mg chemical/mL vehicle. Analyses are to be carried out without the dose formulation personnel being told which formulations are scheduled for analysis.

4. Routine analyses of dose formulations shall be conducted using the following procedures:

   a. Prepare two standard stock solutions of the chemical from independently weighed samples. Use these solutions to prepare at least six vehicle standards at concentrations bracketing the concentration range expected for the samples such that alternate standards are prepared from each stock solution.

   b. Analyze the spiked vehicle standards and a vehicle blank. Duplicate analyses of spiked vehicle standards are not required.

   c. Calculate the regression equation and the correlation coefficient. Compare the regression equation with all previous equations. Any significant differences in slope or intercept shall be explained. The correlation coefficient (r) must be > 0.99.

   d. Analyze the submitted dose formulation samples in triplicate.

   e. Use the regression equation sample responses and any dilution factors to compute the concentration of the unknowns.

   f. For chromatographic analyses, only single injections are required.

   g. Relative Standard Deviation.

   h. A Q-test may be used to statistically identify outliers.

5. When formulation analyses are required analyses shall be completed prior to administering the dose formulations.

6. Precision and accuracy of the methods shall be such that values that deviate from the target concentration by more than 10.0% will be considered out of tolerance. There may be cases where a 10.0% tolerance limit cannot be attained and these will be addressed on an individual basis and must be approved by NTP. The cause of any deviation from the approved tolerance limit shall be discussed in the Monthly Progress Report. If the dose formulation is out of tolerance, the dose formulation shall not be given to the animals without NTP approval. Re-mixes shall be shown to be within the accepted tolerance limit before they are used for dosing. If a re-mix is necessary, aliquots will be analyzed according to the original procedure.

7. Reprocessing (diluting or adding test article to) out of tolerance dose formulations is not acceptable.

E. FORMULATION ANALYSIS REQUIREMENTS

1. Studies for less than 30 days

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

   b. Samples of the formulations shall be taken from the animal room according to the following scheme:

      1) The samples shall only be taken from formulations for which dose formulation samples have already been analyzed.

2) The sample shall be taken on the last dosing day prior to the expiration date of the batch.

3) The sample shall be taken at the end of the dosing day.

4) The sample submitted for analysis shall be the residual formulation in the original dosing vessel. For drinking water studies the sipper tube assemblies are to be removed and the bottles capped. For dosed feed studies the contents of the feeders are to be emptied into clear, interferent-free containers. In addition, for dosed feed and drinking water studies, samples of the unused formulation from which feeders or bottles are filled shall be collected and analyzed along with animal room samples in determining animal room sample stability.

5) Samples shall be taken from one sex of each species and each dose group (If dose groups for each sex are different then samples must be taken from each sex).

6) Each sample shall be analyzed in triplicate.

The results of these analyses shall be compared to the results of the original formulation room samples.

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

2. Studies greater than 30 days and equal to or less than 90 days

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

b. A homogeneity study is required for feed and suspension studies (See Section IV.C.5.).

c. In addition to the formulation room samples taken and analyzed at the beginning, midway, and end of the study, samples of these same preparations shall be taken from the animal room (upon completion of dosing) and analyzed as described in section IV.E.1.b. above.

3. Studies greater than 90 days

a. All batches prepared for the initial set of dose formulations shall be analyzed. Thereafter, these analyses shall be carried out every ten weeks, plus or minus two weeks.

b. A homogeneity study is required for feed and suspension studies (See Section IV.C.5.).

c. At the beginning of the study, samples of all initial dose formulations shall be taken from the animal room for analysis as described in section IV.E.1.b. above. Thereafter, similar animal room samples shall be taken during every third scheduled analysis period.

4. Recommended Sample Label Format

Chemical Name:_____

Vehicle: _____

Concentration: _____

Identification: (opt)* _____

Dates Prepared:_____Expiration:_____

Storage Condition:_____

* If a specific sample identification (e.g., testing laboratory's unique identifying code) is required in a report, it can be placed here.

DOSE FORMULATION ANALYSIS SAMPLE SUBMITTAL FORM

DATE _____

NAME OF ORGANIZATION _____

RETURN ADDRESS _____

NAME OF SUBMITTER _____

TEST ARTICLE _____

CAS # _____

TYPE OF STUDY _____

SPECIES/STRAIN _____

VEHICLE _____

TEST ARTICLE LOT NO _____

DATE MIXED _____

Sample Identification	Concentration	Approximate Amount Shipped*

* Minimum required:

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

F. ANALYSIS OF DOSING VEHICLES

1. The Contractor shall perform analyses of dosing vehicles to confirm the identity and purity. The protocols for the analyses are given in Appendix 2.

2. Frequency of the analyses shall be as follows:

   a. Any batch of corn oil shall be analyzed for peroxides before it is first used and at bimonthly intervals thereafter while it is in use. All corn oil must be stored at 5^{o} C or lower.

   b. Any batch of ethanol, acetone, methylcellulose or other vehicle shall be analyzed on receipt for identity and purity and then for purity every six months thereafter while it is in use. Sufficient material shall be purchased so that enough of a single lot is available for the entire study for which it is purchased. Ethanol (other than absolute) used for dose vehicle preparation must be demonstrated to be benzene free.

G. PRESTART CHEMISTRY REPORT

1. Prior to starting non-inhalation toxicity studies, a special report is to be submitted to the NTP to include the following items (where applicable for individual test articles):

   a. Information on the source of the bulk chemical as well as manufacturer stability, storage requirements, identity and purity data

   b. Confirmatory data on identity, purity, (including representative chromatograms and spectra of the test article and reference material, and if necessary, stability of the bulk chemical using methods developed by NTP or the Contractor as well as copies of the SOPs for the bulk chemical reanalysis methods

   c. Data on preparation, handling, stability (if performed by the testing laboratory), homogeneity, and analysis of dose formulations as well as SOPs

   d. Dose formulation analysis method performance evaluation procedures and validation data, including representative chromatograms or spectra (see Appendix 1)

   e. Health and safety procedures

2. Animals **shall not** be dosed until this report has been received and approved by NTP.

H. INHALATION STUDIES

1. Inhalation chambers may be of any design that can be demonstrated to provide uniform and reproducible exposure of all animals to the test article. However, the design shall be such that the chamber air-supply and the incoming test materials will be thoroughly mixed prior to entering the chamber.

2. The Contractor shall develop methodology for generation and monitoring chamber concentrations.

3. The specificity, precision, linearity, absolute recovery, measurement limits and relative error shall be established for the monitoring method.

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

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

6. The method of generation of the test atmosphere shall be reproducible so that the average chamber concentration does not vary by more than ± 10% from the target concentration from exposure period to exposure period. The daily average chamber concentration shall not vary by more than ± 10% relative standard deviation (RSD). Chamber concentrations and RSD shall be reported as time- weighted averages.

7. The uniformity of the concentration of the test article in the chamber shall be demonstrated so that the challenge to each animal is the same. This shall be done for each chamber during the development of the generation technique without animals and again at the beginning of each study with the animals in the chamber. The uniformity of the chamber concentration shall be rechecked once each ninety-days during long-term studies. The within port and between port variability shall not exceed ± 5% RSD.

8. Chamber concentration versus time plots shall be developed for each chamber during the developmental work (without animals) and for one day at the beginning of each study (with animals). This data will be used to evaluate:

    a. The time necessary to reach the target concentration

    b. The ability of the generation system to maintain a stable concentration over a full exposure period

    c. The length of time necessary to clear the chamber of test article

    d. The time necessary to reach 90% of the target concentration (T90) shall be estimated from chamber concentration versus time plots and compared to the theoretical T90. The exposure day shall be T90 plus six hours

    e. The time necessary to reach 10% of the target concentration (T10), after exposure termination, shall be estimated from chamber concentration versus time plots and compared to the theoretical T10

9. Stability studies to confirm the integrity of the generated chemical atmosphere shall be conducted by:

    a. Establishing that there is no degradation in the reservoir during the expected residence time in the reservoir. The reservoir sample shall be taken at the end of an exposure day. If the reservoir is not refilled daily, then the sample shall be taken immediately before a refill.

b. Establishing that the generator does not cause decomposition of the test article prior to introduction into the inhalation chamber. The generator sample shall be taken at a point after generation but prior to final dilution of the chemical stream before introduction into the exposure chamber.

c. Determining to what extent, if any, the test article is degraded after introduction into the chamber atmosphere. The chamber sample to be tested shall be taken during the last hour of the exposure.

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

11. The Contractor shall develop methods for analysis of known or suspected degradation products. The studies shall be carried out at the lowest and highest exposure concentrations during developmental work without animals in chambers and at the beginning of each study with animals in chambers. Methodology developed by the Contractor must be sensitive enough to detect the degradation products down to a level of 1% of the chamber concentration target.

12. When the test atmosphere of a liquid (at standard temperature and pressure) is to be generated as a molecular vapor of that test article rather than as an aerosol, the Contractor shall demonstrate by photometric or other appropriate means, that the test atmosphere does not contain aerosolized test article. This procedure shall be carried out during developmental work and at the beginning of each study at the highest exposure concentration with animals.

13. If the test atmosphere is an aerosol, the particle size distribution shall be controlled and monitored. The measurement method must provide the mass median aerodynamic diameter (MMAD) and the geometric standard deviation for the distribution. Particle size distribution of the aerosol shall be determined as part of the development of test atmosphere generation techniques during the first week of exposures and checked monthly during the in-life study and with each new batch. The initial particle size distribution determination shall be done by impactor and shall have a MMAD of less than 3 microns with a sigma g of less than 3.

14. The residual concentration of test article in the high chamber atmosphere shall be monitored overnight or until the concentration for two consecutive hours is < 1% of the target concentration. The overnight monitoring period begins immediately after exposures are completed. If possible, delay opening and servicing chambers until the concentration has decayed to <1%. This shall be done during the developmental phase and at the beginning of each study.

15. If an inert gas such as nitrogen is used to generate or transport the test article to the inhalation chamber, then the oxygen content in the highest concentration exposure chamber shall be measured during the developmental phase. A minimum oxygen concentration of 19% is required.

16. If the test article is to be generated as a particulate aerosol, then test article identity is to be confirmed in the generator, distribution line, and high and low chamber during the prestart effort, once during each prechronic study and annually during the chronic study.

I. PRESTART INHALATION REPORT

1. Prior to starting inhalation toxicity studies, a special report is to be submitted to the NTP to include the following items where applicable for individual test articles:

   a. Information on the source of the bulk chemical as well as manufacturer stability, storage requirements, identity and purity data (particle size and milling information if appropriate)

   b. Confirmatory data on identity, purity, including representative chromatograms or spectra of the test article and reference material and, if necessary, stability of the bulk chemical using methods developed by the Contractor as well as copies of the SOPs for the bulk chemical reanalysis methods

   c. Exposure system description including schematic of the entire generation and monitoring system and SOPs

   d. Generator, distribution system, and chamber atmosphere characterization data and SOPs

   e. Description of chamber atmosphere concentration monitoring procedure as well as method performance evaluation data and SOPs

   f. Health and safety procedures

2. Animals **shall not** be exposed until this report has been received and approved by NTP.

3. Addendum to Prestart Inhalation Report

   This addendum to the Report shall contain all data regarding uniformity of chamber concentration, T90, overnight monitoring, chemical degradation, and where appropriate, aerosol determination conducted with animals during the animal study.

J. TOXICOKINETICS

1. For some test articles, toxicokinetic studies may be conducted. These are usually designed to: 1) establish basic toxicokinetic parameters; 2) determine the actual internal doses; 3) determine the extent of bioaccumulation; 4) evaluate the effects of sex, age and long term exposure on toxicokinetic parameters. The data may be used to select doses, route of administration and vehicle for the toxicology studies and/or to evaluate the possible correlation of toxic effects with toxicokinetics. Ultimately, the data should prove useful in risk assessment.

2. Toxicokinetic studies will generally be conducted at three levels, any of which may be included in the study design for any given test article. The three levels are: 1) preliminary, 2) single administration, and 3) repeated exposure. The preliminary study is designed to aid in bioanalytical method development and in the selection of blood or tissue sampling times. It is conducted without validated methods according to a design provided by the NTP. The single administration study provides the definitive toxicokinetic parameters. It is designed from the results of the preliminary study. The repeated exposure study provides information of the effect of multiple exposures to test article and aging on the toxicokinetic parameters as well as an estimate of the internal doses during the toxicology study. The design for the repeated exposure study, e.g., exposure

concentrations and sampling time-points, will be specified by the NTP based on data from the previous two levels of toxicokinetic studies when conducted, or based on information from the literature when sufficient.

3. Toxicokinetic studies generally require the characterization of the blood/plasma concentration versus time profile after chemical administration. The quantitation of test article in other tissues at specified time-points may be included as well. NTP will specify whether the Contractor is to collect and ship samples to another laboratory for analysis, or is to collect and analyze the samples themselves.

4. If the Contractor is to analyze the biological samples and the anticipated concentration range of the analyte in the biological samples has previously been established, then the Contractor shall develop the quantitative analytical method and a method performance evaluation shall be conducted. The stability of the analyte in the biological matrix at a concentration approved by the NTP Project Officer shall then be established for the period of time over which the samples are to be stored prior to analysis.

5. If the anticipated concentrations in the biological samples obtained in the planned toxicokinetic studies are unknown, then the Contractor shall establish the expected concentration range by conducting a preliminary toxicokinetic study. NTP will provide the design for this study (typically in the SOW). The Contractor shall then develop, but at this time not optimize, a bioanalytical method for quantitation of the parent chemical or its metabolites in the biological matrix.

6. Dose formulations for preliminary toxicokinetic studies are not to be analyzed. The dose formulation method development work is to consist of only feasibility studies that will entail the non-quantitative evaluations of homogeneity and syringeability. Interim data from those analyses are to be submitted to NTP for review and together with the contractor, a decision will be made as to:

    a. the need for additional preliminary toxicokinetic studies

    b. the required ELOQ for the biological analysis method

    c. the range over which the method is to be validated

    d. the length of time over which the stability study in biological matrices will be conducted

    e. the concentrations for the future dose formulations to be used in the single administration studies

7. If the decision is made not to go forward with a Single Administration TK Study, the data from the preliminary study will be reported to NTP in a written document that includes:

    a. design of the preliminary toxicokinetic study and results

    b. bioanalytical method description

    c. detailed procedures for biological sample collection and processing

    d. description of dose formulation methods used

8. If the decision is made to go forward with a Single Administration TK Study, and the test article concentration in blood or in other biological tissues is within a range for which quantitation is technically feasible, the Contractor shall validate the bioanalytical method over the appropriate concentration range. (See Appendix 1.) In some cases, multiple standard curves may be needed to cover the required range. In other cases, samples with concentrations above the highest concentration standard may be diluted and only one standard curve may be needed. If dilution into the analytical range is proposed, this must be demonstrated with a matrix sample prepared at an expected concentration that can be analyzed using the method and, when the dilution factor is applied, give the expected analytical result within method variability. Any dose formulation methods not validated as part of the toxicology study, e.g., formulations for intravenous dosing, are now to be validated and any required stability or homogeneity studies conducted.

9. After validation of the bioanalytical method, the Contractor shall prepare a Prestart Toxicokinetic Report (prestart of the Single Administration Study), which shall be audited and include:

    a. design of the preliminary toxicokinetic study and results

    b. source of test article and storage conditions

    c. bioanalytical method description and validation data, including data demonstrating dilution into the analytical range, if proposed

    d. detailed procedures for biological sample collection and processing

    e. proposed concentrations for the quality control (QC) samples to be used during the biological sample analyses

    f. stability information of test article or its metabolites in the biological matrix

    g. description of dose formulation methods to be used in the single administration studies and data on the formulation characterization studies

    h. description of analysis methods for formulations to be used in the single administration studies and results of method validations

    i. stability of test article in dose formulations and, if necessary, a confirmation of homogeneity

    j. all appropriate SOPs

    k. protocols for the single administration studies

10. Once the prestart toxicokinetic report has been approved by NTP, the Contractor shall conduct the Single Administration TK Study. Animals will be dosed intravenously at one or two doses and/or by another route using three doses to be determined by NTP. (See SOW for specific requirements for each test article.) Rats and mice shall be 13 ± 2 weeks old and shall be purchased by the Contractor. Blood or other biological samples shall be collected at different time points to be determined by NTP and shall be processed and analyzed by the Contractor. (See IV.J.12.) Study results shall be sent to NTP in a Single Administration Study Report that shall include:

    a. a summary of the results of the preliminary toxicokinetic study

b. source of test article and storage conditions

c. design of the single administration toxicokinetic study

d. tabulated data and plots of log concentration versus time

e. estimates of half-life of elimination, Cmax, and area under the concentration versus time curve (AUC) as determined by trapezoidal rule, with and without end point correction

f. plot of AUC versus dose

g. dose formulation and analysis data

h. all appropriate SOPs

11. Based on the results of the Single Administration TK Study, special toxicokinetic animals may be added to the subchronic or chronic study, bled multiple intervals during the study, and at multiple time points at each interval (see SOW for details of study design for individual test articles). Samples shall either be analyzed by the testing laboratory or shipped to an NTP analytical contractor for analysis. Interim results shall be submitted to NTP. Study results shall be sent to NTP as part of the final study report (if analyses conducted by testing laboratory) to include:

    a. source of test article and storage conditions on receipt and at the study lab

    b. a summary of the results of the single administration studies including the aged animal study

    c. design of the repeated dose toxicokinetic study

    d. tabulated data and plots of log concentration versus time

    e. estimates of half-life of elimination, Cmax, and area under the concentration versus time curve (AUC) as determined by trapezoidal rule, with end point correction

    f. plot of AUC versus dose

12. Suggested variations in this standardized approach are to be proposed by the contractor in the prestart toxicokinetic report. The general scheme to be followed includes:

    a. The preparation of quality control (QC) samples on receipt of the toxicokinetic study samples. These QC samples are spiked blank biological samples prepared at two concentrations. The concentrations will generally be the next to the lowest standard and the next to the highest standard used in the spiked biological sample standard curve. Note that the proposed concentrations of the QC samples are to be listed in the prestart toxicokinetic report.

    b. Six spiked biological sample standards are to be prepared with each batch of study samples assuming only one standard curve will cover all expected sample concentrations. A blank biological sample is also to be prepared with each batch. If more than one standard curve is to be used to cover the expected sample concentrations then each additional segment is to consist of six spiked biological samples. However the high standard of the low segment can be used as the low

standard for the high segment. The blank and spiked biological samples are to be processed in conjunction with the study samples.

c. QC samples, as defined above, are to be processed with each batch of study samples. Generally the total number of QC samples analyzed is 10% of the number of study samples.

d. System suitability shall be established, including the evaluation of data from QC samples and spiked biological samples to determine if the analytical run should be allowed to go to completion. The run sequence is to be the spiked biological samples, QC samples, 7-15 study samples, QC samples, 7-15 study samples, QC samples, 7-15 study samples, QC samples, and so on with a QC sample and a set of spiked biological samples as the final injections. Criteria for acceptance of the analytical run will be predetermined and based on the precision and accuracy of standards and QC samples and specificity of blanks.

K. DISPOSITION OF SURPLUS/RESIDUAL TEST ARTICLE

1. Thirty days prior to the shipment of the test article, the contractor shall notify the NTP of their intention to ship surplus or residual chemical, including the amount to be shipped. Shipment is to be made within 30 days after the terminal sacrifice for the last study for that test agent. (See Section III.G. for complete details.)

2. A completed Surplus Test Article Aliquot Transmittal Form (provided below) shall accompany shipments of aliquots and surplus test article. In addition to the surplus chemical, a 100 g aliquot of each batch of chemical is to be reserved and shipped. The chemistry support contractor is to be notified (by e-mail or phone) at least 24 hours prior to arrival of the shipment. Information regarding the carrier and tracking number is to be provided when available. In addition, appropriate return address information is to be included on the package. If the shipment will arrive during non-working hours or requires special handling or storage conditions, the laboratory is to be contacted at least 48 hours prior to arrival so that arrangements can be made to receive and handle the shipment properly.

SURPLUS TEST ARTICLE TRANSMITTAL FORM

DATE _____

NAME OF ORGANIZATION _____

RETURN ADDRESS _____

NAME OF SUBMITTER _____

TEST ARTICLE* _____

CAS# _____

Provide information below for each lot used.

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

* Use full NTP test article name.

L. SUMMARY OF CHEMISTRY REQUIREMENTS FOR NON-INHALATION STUDIES

Category	Type	Anticipated Frequency	Reporting Requirement	Comments
Bulk Analyses	Identity and Purity Analyses	Once on receipt	Prestart Chemistry Report	Methods provided by NTP
	Stability Study		Prestart Chemistry Report	
	One Purity Analysis	24 ± 2-weeks	Study Report	
Corn Oil Analyses	Peroxide Level Determination	Bimonthly	Study Report	Method is provided by NTP
Vehicle Analyses	Identity and Purity Analyses	Once on receipt	Prestart Chemistry Report	See Appendix 2 for NTP supplied methods
	One Purity Analysis	Every 24 ± 2 weeks	Study Report	
Dose Analyses Method Performance Evaluation	Dose Analysis	Once	Prestart Chemistry Report	Using methods provided by NTP validate before initiation of first animal study. If concs change in subsequent studies, additional MPE's will be required
Dose Analyses for Studies less than 30 days	Formulation Room Dose Analyses	Initial only	Study Report	All batches at each dose
	Animal Room Dose Analyses	Initial only	Study Report	Each dose for each sex and species
	Homogeneity Study	Once	Prestart Chemistry Report	Prior to preparation of formulation designated for dosing (feed and suspension only); total of two samples from each of three sites
Dose Analyses for Studies greater than 30 days and equal to or less than 90 days	Formulation Room Dose Analyses	Initial, Middle, and Final	Study Report	Same as for studies less than 30 days
	Animal Room Dose Analyses	Initial, Middle, and Final	Study Report	Same as for studies less than 30 days
	Homogeneity Study	Once	Prestart Chemistry Report	Same as for studies less than 30 days
Dose Analyses for Studies greater than 90 days	Formulation Room Dose Analyses	Initial and every tenth ± 2 wks	Study Report	Same as for studies less than 30 days
	Animal Room Dose Analyses	Initial and every 3rd scheduled formulation room analysis	Study Report	Same as for studies less than 30 days
	Homogeneity Study	Once	Prestart Chemistry Report	Same as for studies less than 30 days

M. SUMMARY OF CHEMISTRY REQUIREMENTS FOR INHALATION STUDIES

Category	Anticipated Frequency	Report Requirement
Bulk Chemical Analyses		
Identity and Purity Analysis	Once on receipt	Prestart Inhalation Report
One Purity Analysis	24 ± 2 weeks	Study Report
Generator and chamber schematics	Once	Prestart Inhalation Report
Monitor development and validation	Once	Prestart Inhalation Report
Chamber distribution		
Without animals	Once	Prestart Inhalation Report
With animals	At the beginning of each study, and each 90 days in studies longer than 90 days	Addendum to Prestart Inhalation Report
Determine T90 (theoretical vs actual)		
Without animals	Once	Prestart Inhalation Report
With animals	At the beginning of study	Addendum to Prestart Inhalation Report
Generation and monitoring SOPs	Once	Prestart Inhalation Report
Chemical degradation study: generator, reservoir, chamber		
Without animals	Once	Prestart Inhalation Report
With animals	At the beginning of study	Addendum to Prestart Inhalation Report
For vapor generation from a liquid: check for particulates		
Without animals	Once	Prestart Inhalation Report
With animals	At the beginning of study	Addendum to Prestart Inhalation Report
For aerosols: determine particle size		
Without animals	Once	Prestart Inhalation Report
With animals	At the beginning of study, then monthly	Addendum to Prestart Inhalation Report
Overnight monitoring	At the beginning of any study	Addendum to Prestart Inhalation Report
Chamber concentration vs time plots		
Without animals	Once	Prestart Inhalation Report
With animals	At the beginning of study	Addendum to Prestart Inhalation Report
Description of effluent exhaust treatment, monitoring data, percent efficiency and SOPs	Once	Prestart Inhalation Report
Room air monitoring methods, strategy, data and SOPs	Once	Prestart Inhalation Report
Oxygen determination when Nitrogen is used	Once	Prestart Inhalation Report

V. LABORATORY ANIMAL MANAGEMENT AND TOXICOLOGY

A. ANIMAL FACILITY OPERATIONAL REQUIREMENTS

1. Humane Care of Rodents in NTP Studies

a. Animals shall be anesthetized to alleviate pain in procedures that may cause more than momentary or slight pain.

b. Animals with a) large masses or other conditions interfering with their eating and drinking, b) major injuries or ulcers related to husbandry and treatment, c) debilitating conditions or other conditions indicating pain or suffering as judged by the veterinarian or an experienced laboratory animal specialist shall be euthanatized immediately to avoid further pain and distress. (See Appendix 3.)

c. Moribund animals and animals scheduled for interim and final necropsies shall be euthanatized by personnel trained in methods and techniques established by the AVMA panel on euthanasia (J. Am. Vet. Med. Assoc. 218(5): 669-696. 2001), preferably euthanasia with compressed CO_2 gas in cylinders.

2. Facility Compliance

a. The testing facility shall comply with the Animal Welfare Act of 1966 (P.L. 89-544) as amended in 1970, 1976 and 1985 and other applicable Federal, state and local laws, regulations and policies.

b. The testing facility shall adhere to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" (NRC, 1996) and the NTP Specifications.

c. The testing facility must have a functional Animal Care and Use Committee.

d. The laboratory must have qualified Laboratory Animal Veterinarian(s) to supervise the care and health of the animals.

3. Emergency Notification Procedure

a. Each contractor shall keep an Emergency Notification Procedure that shows who to notify in the event of various types of potential emergency situations. This procedure shall be posted in a prominent location and on pertinent equipment (e.g. refrigerators, freezers, etc). Weekend duty personnel, in particular, shall be aware of its location. All personnel must read and initial, acknowledging they have read and understand the procedures.

b. In all cases, the Principal Investigator (or designated alternate, if the Principal Investigator is absent) shall be notified of emergency situations. The Laboratory Animal Veterinarian must be made aware of any emergency situation that impacts animal welfare.

4. Pest Control

Programs to control or eliminate insects, escaped or wild rodents, and other similar pests shall be in use before starting animal studies. Pesticides and traps may be used as

necessary in conjunction with a strict program of sanitary maintenance. However, to prevent toxic effects in research animals and possible interference with experimental procedures, pesticides, including insecticide impregnated plastic materials, shall not be used in the animal rooms, feed and bedding storage areas, and any other areas of the facility where animals, cages, racks, feed, bedding, and water may be exposed to the pesticides (when used) either in particulate or in vapor form.

5. Sanitization of Equipment and Animal Facility Rooms

   The sanitizing chemicals shall not contain essential oils, perfumes, fragrances, or any other chemicals expected to influence the metabolism of mammalian systems.

6. Movement of Animals Between Rooms

   a. Studies (study animals) shall not be moved to a different room during the course of the study except for the reasons stated below:

      - Specific statement in the protocol of the study requiring or permitting the move;
      - The physical condition (floor, walls, ceiling, fixtures, etc) of the room and/or its adjacent supporting area deteriorated to the extent that a safety hazard is judged to exist.
      - The physical plant or ventilation equipment and ventilation, or lighting equipment and fixtures deteriorated to cause highly variable environmental conditions in the room;
      - Due to change in physical factors in and around the study room, procedures to control the existing pest problem and/or procedures to control disease and microbial spread will not be effective.

   b. If a move is necessary, an attempt shall be made to accomplish this move at least sixty days before an important event such as detailed neurobehavior evaluation, clinical pathology evaluation, planned necropsy, etc.

   c. When a study is moved to a new or different animal room due to reasons listed above in 6.a., the equipment to control environmental conditions, health and safety conditions, and the conditions to control disease and microbial spread shall be substantially superior in the new room when compared to the previous animal room.

   d. Except in cases of emergency, approval for the move shall be obtained from the NTP Project Officer in advance. There shall be a detailed procedure for this type of move and the study report shall include reasons and approvals for the move, date of the move, and detailed procedure of the move.

7. SOPs for Laboratory Animal Management and Toxicology

   The test facility shall have specific SOPs for all laboratory animal management and toxicology procedures including, but not limited to, the activities listed below.

   a. Technician training

   b. Procedures for handling emergencies/disastrous situations in the animal facility

c. Testing and maintenance of emergency backup systems

d. Pest control procedures

e. Procedures for disease control and for prevention of microbial spread in the study facility

f. Sentinel Animal Program

g. Environmental conditions of study rooms including lighting

h. Sanitization of animal rooms before the receipt of animals

i. Sanitization of test facility and study rooms during the study

j. Sanitization of racks, cages, feeders, and watering system

k. Operation and maintenance of cage and rack washers

l. Watering System

m. Receipt and storage of feed including evaluation of nutrient and contaminant reports to satisfy the NTP standards

n. Receipt and storage of bedding including evaluation of physical quality from a randomly selected bag and contaminant report to satisfy standards

o. Feeding and change of feeders

p. Rack, cage, and bedding change

q. Rack and cage rotation

r. Receipt and examination of animals

s. Quarantine, health evaluation, and release of animals for study

t. Randomization of animals

u. Identification of animals

v. Weighing of animals

w. Observation of animals: Daily AM and PM check, detailed clinical observations

x. Evaluation of feed and/or water consumption

y. Handling of dead and moribund animals and criteria for moribund sacrifice

z. Custody transfer of animals from animal care/toxicology to necropsy/pathology for the interim and final sacrifice of study animals.

aa. Criteria for disposition of escaped animals

bb. Gavage treatment procedure

cc. Dermal treatment procedure

dd. Monitoring of sanitation practices

8. Emergency/Disaster Response and Management Plan

The facility shall have an emergency/disaster response plan specifically addressing the animal facility.

B. ANIMAL ROOM ENVIRONMENT

1. The ventilation system shall provide a minimum of ten complete changes of room air per hour without drafts. There shall be no recirculation of room air unless it has been treated to remove all particulates and toxic vapors by effective filters and where necessary, scrubbers to avoid spread of disease and to eliminate the recirculation of contaminants.

An automatic recording and alert system shall be used to monitor the ambient conditions in each animal room. If a completely automated system is used, the probes to determine room temperature and humidity shall be in the exhaust for each room. If a free-standing, portable temperature and humidity recording system is used, the equipment shall be located near the room exhaust at a level of three to four feet from the floor.

Each month the contractor shall record qualitative evidence of the correct direction of airflow in each animal room. Quantitative measurements of flow rate shall be made at least twice per year, once in the cooling and once in the heating season.

2. Temperature of the animal room shall be maintained at 72^{o} F ± 3^{o} F. The temperature shall not be below 67^{o} F or above 77^{o} F during the course of the study and there shall be an alarm system for warning of temperature fluctuations beyond the 67 to 77 degree range. If the temperature is below or above the 67 to 77 degree range, it shall be returned to the acceptable limits within two hours. The relative humidity of the animal room air shall be 35 to 65%. It shall not be below 30% or above 70% during the course of the study. If the relative humidity is below or above the 35 to 65% limits, it shall be returned to the acceptable limits within two hours. Thermometers shall be accurate within 2^{o} F or better. Accuracy of thermometers and hygrometers must be checked as often as necessary but not less than at quarterly intervals. Animal room temperature and humidity results shall be reported as either means ± relative standard deviation or as time-weighted averages.

3. The animal rooms shall be windowless (except for window in door) and uniformly lighted, preferably by diffuse lighting with fluorescent lamps. The light cycle in the animal rooms shall be twelve hours light and twelve hours dark, with the timing of the light/dark cycles varying no more than ± 15 minutes from day-to-day. Appropriate means must be taken to prevent light from entering the animal room during the dark cycle. The light cycle shall be controlled by automatic equipment with monitoring of proper functioning at two to three day intervals. The NTP requires a uniform light intensity of 30 ± 3 foot-candles at 3.3 feet (1.0 meter) from the floor for normal lighting of the animal rooms. During the observation periods, for convenience of the technicians, the light intensity may be increased to 45-55 foot-candles at 3.3 feet from the floor. To accomplish this lighting, the animal room may be equipped with two-stage lighting, both stages to be automatically turned off by an automatic timer. The first stage will be the normal lighting for the room

and it will be wired to turn on and off by an automatic timer. The second stage will be to facilitate observation of the animals. The second stage shall be wired to be turned off manually when not needed for observations. In the event the second stage lighting is not turned off, the automatic timer shall turn off not only the first stage but also the second stage lighting at the set time. The lights shall not be turned off during the light (day) phase or turned on during the dark (night) phase except in case of emergency. Emergency power must be connected to the light timers/controls and to some lights of the animal room.

C. DIET AND WATER

1. Diet

a. Irradiated, NTP-2000 open formula diet shall be used, unless a different diet is specified. Diet shall be stored at 80^{o} F or lower, 70% relative humidity or lower, and in a well ventilated area stored raised above the floor, and used for no more than one hundred twenty days post milling. Records shall be kept of the type of diet used for each study to include the batch number and when used, whether pellet or powder, and the source. Each batch of NTP-2000 diet shall be analyzed for contaminants, protein, fat, fiber, ash, moisture and heat-labile nutrients such as Vitamin A and Thiamine. Lists of contaminants with maximum acceptable levels are given in Appendix 3. A copy of the analysis records shall be included in the study records and sent to the NTP Archives. It is the responsibility of the contractor to verify that the diet meets the NTP standards prior to use. The testing laboratories may be required to ship a sample of the diet to the NTP or an NTP designated analytical laboratory for nutrient and contaminant analysis once a year.

b. Diet must be supplied to the animals in a feeder. Unless otherwise specified, pelleted feed shall be used for all routes of administration except dosed feed studies. Powdered food shall be used for dosed feed studies.

e. Clean feeders with fresh food must be supplied at least once weekly. However, fresh food shall be provided as often as necessary to ensure support of normal growth and maintenance. Food hoppers for powdered feed shall not be filled to the brim (filling to less than 80% capacity will help to decrease the spillage), but the animals must have food *ad libitum*, unless specified otherwise (e.g., see inhalation studies). Hoppers shall be dumped in the vented enclosure in the dirty cage wash area.

f. Dirty feeders are to be soaked when necessary, and then washed in at least one cycle of 180^{o} F water.

g. In order to avoid cross-contamination during washing, feeders used for all dosed feed studies shall be uniquely marked or labeled in order to identify the test article being dosed, each dose group, and the control group feeders.

2. Water

a. Municipal drinking water with 1-2 ppm chlorine shall be supplied *ad libitum*. Water shall not be hyperchlorinated or hyperacidified. The NTP may specify a suitable water treatment procedure for special cases.

b. Laboratories must demonstrate that water provided for animal use meets US/EPA National Primary Drinking Water Regulations. Appendix 3 contains a list of additional water components and contaminants to be determined and assessed. To satisfy this requirement, the laboratory must provide analyses of water from an animal room or a composite from several animal rooms at least once during the in-life phase of each subchronic study and at least once a year for chronic studies. For dosed water studies, water used for such analysis is to be taken from the specific source used to make the dose formulations. If any water treatment is performed by the laboratory, details of this treatment must be provided. For laboratories new to NTP, an additional report shall be provided to the NTP within thirty days of contract award. The testing laboratories may be required to ship a sample of water to NTP or an NTP designated analytical laboratory for contaminant analysis once a year. Water analyses must be performed by a laboratory qualified to conduct such studies on a local, state or interstate level.

c. When an automated watering system is used, the valve end shall be located outside the cage, which will require that a stainless steel grommet be affixed around the access port to the watering valve. Care must be taken to ensure that the animals can reach the valves, and that the valves are placed such that cages cannot be flooded in the event of a malfunction.

d. Water bottles may be used, although an automated watering system is preferred. Water bottles shall be of sufficient capacity so that no more than two per week per cage are routinely needed. Each cage shall be supplied at least twice weekly with a fresh sanitized water bottle, bottle cap, and sipper tube. Dirty (used) bottles shall be exchanged for clean bottles, **never refilled and reused**. Water bottle stoppers shall be made of an inert material and bottles shall be located in a position to prevent the stoppers from being chewed by the animals.

e. Dosed-water studies

For dosed water studies, water for control groups is to be taken from the exact same source and at the same time as the water used for the treated group formulations. The control water and dose formulations are to be stored at 5°C in carboys until it is time to dispense the formulations to water bottles and transport them to the animal rooms.

In order to avoid cross contamination during washing, water bottles used for all dosed water studies shall be marked indelibly or inscribed so as to identify the test article being dosed (symbol may be used), each dose group, and the control group water bottles.

f. Washing Water Bottles

Bottles, bottle caps, and sipper tubes must be soaked and washed promptly. Water bottles must be washed in water of at least 180^{o} F. Water bottles are to be:

- Washed with regular cage washing detergent using a brush apparatus, suitable for bottles being used; or,

- Washed in an automatic washing system wherein the water outlet for the bottle washing process is well within each bottle being washed.

- If the bottles are to be washed in a standard tunnel washer, each bottle at each dose (study water bottles only) shall be filled with tap water and rinsed twice prior

to washing. During washing, control bottles and those from each dose group shall be kept separate from each other and not washed with bottles from other dosed water studies.

D. CAGING, RACKS, BEDDING AND FILTERS

1. Caging

a. Cages for the NTP studies shall be program and test article specific. Cages shall be returned to the same test agent to avoid possible contamination. Each cage containing animals shall always be identified with a cage label that includes the study, cage number and animal number(s) within that cage. The cage identification card shall be attached to the cage and shall be transferred along with the animal(s) to a new cage throughout the in-life portion of the study.

b. Polycarbonate cages shall be used in a suspended cage rack system, unless otherwise specified by the NTP. The racks shall have provisions for placing filter fabric on the shelf above the cages.

c. During the quarantine period and prechronic studies, animals may be caged together according to the weight-space specifications recommended in "The Guide for the Care and Use of Laboratory Animals". For subchronic and chronic toxicity studies, animals shall be initially apportioned to cages as if they were in the upper weight range. Thus it will not be necessary to redistribute them later to larger cages in order to remain within the recommended weight-space specifications.

d. Rats and female mice shall be housed five per cage except in chronic studies where male rats shall be housed three per cage. In inhalation and dermal studies rats and mice shall be housed individually. For group-housed rats, the cages shall measure approximately 22" L, 12.5" W and 8" H. For individually housed rats in dermal studies, the cages shall measure approximately 9" L, 8" W and 8" H. For group housed female mice, the cages shall measure approximately 12.5" L, 9.25" W and 6" H. For individually housed male or female mice, the polycarbonate, solid bottom cages shall measure approximately 9.25" L, 6" W, and 6.125" H. If an automated watering system is used S-shaped stainless steel watering manifolds shall be used to facilitate sanitization at the time of rack sanitization. The rack watering manifolds shall be flushed or drained at least once a day to prevent matter and bacterial accumulation.

e. Group housed animals shall be changed to a sanitized cage twice weekly and individually housed animals shall be changed to a sanitized cage once weekly, or as often as necessary to keep the animals clean and dry. Remaining cage groups shall not be combined. If cage changing becomes more frequent than the above schedule on a continual basis, the NTP is to be notified as this might indicate a treatment-related effect.

f. Dirty cages shall not remain in the animal rooms. After changing, they must be washed promptly in a machine that provides one rinse cycle of at least 180^{o} F water. The wash cycle shall include a detergent.

g. Animal allocation for chronic starts:

1) Animal allocation shall be accomplished as follows: Randomly assign animals from weight classes to cages. Randomly assign cages to treatment/dose groups. Rearrange cages in a rack within like treatments/doses in a vertical column. Randomly assign location of treatment/dose columns in racks.

2) Cage rotation shall be accomplished as follows: Once every two weeks, or each time racks are cleaned, rotate each rack of cages vertically within a treatment/dose column. Also, rearrange racks within the original room configuration.

3) Sentinel animals for the animal disease screening program shall be included in procedures "1" and "2" above as though they were another treatment group.

2. Racks

a. Stainless steel, suspended type racks shall be used, unless otherwise specified.

b. Racks shall be capable of being moved to the wash area for periodic machine sanitizing, or if they are fixed racks, sanitization must be provided for each of these racks.

c. Racks must be kept clean while in use and, in particular, the wheel surfaces must be cleaned 360 º when the floor is being cleaned.

d. It is preferred that racks are run through a rack washer which includes one cycle of 180^{o} F water, or they shall be moved to a wash area, hosed and washed using a suitable detergent, and hosed down under high pressure.

e. Racks shall be sanitized at least every other week.

At the time of sanitization of racks, the automatic water manifolds:
- shall also be sanitized by flushing with hot water at 180^{o} F or higher for at least one minute preferably after flushing with warm detergent solution to remove organic matter, OR
- shall be sanitized by flushing/exposure to a sanitizing solution (chlorine) for 30-60 minutes followed by flushing with water for at least 2 minutes.

f. Water manifolds on each rack shall be flushed daily (at least 60 seconds) and each watering valve shall be checked for proper water flow.

3. Bedding

a. Irradiated, heat treated hardwood bedding that meets the NIH standards for physical quality and the NTP standards for chemical and microbiological contaminants is available from commercial manufacturers. (See Appendix 3 for maximum acceptable level of contaminants.) The contractor shall obtain the analysis data from the supplier for each shipment of bedding. It is the responsibility of the contractor to make sure that the bedding meets the NIH and NTP standards. The contractor is not expected to perform additional analyses on the bedding. The testing laboratories may be required to ship a sample of the bedding to the NTP or an NTP designated analytical laboratory for contaminant analysis once a year.

b. The NTP may specify the brand of bedding to be used in special cases.

c. Fresh bedding shall be supplied in clean sanitized cages as specified above.

4. Filters

a. Non-woven, synthetic fiber filters shall be used on the cages.

b. A fresh filter sheet shall be supplied at least every other week.

E. ANIMALS

1. Strains and Source

a. The B6C3F1 (C57BL/6N X C3H/HeN MTV ¯) hybrid mouse and the Fischer 344 (F344/N) rat will be the study animals used in these studies, unless otherwise specified by NTP, and will be supplied by the NTP.

b. When animals are shipped by air, they shall be met at the airport by study laboratory personnel and transported to the quarantine area without delay or arrangements will be made by the study laboratory with a local Air Freight agency to transport the animals without delay to the testing laboratory. All shipments, regardless of route, from the NTP suppliers containing dead, moribund, or otherwise unsatisfactory animals must be reported immediately to the NTP Project Officer and Laboratory Animal Management. If a shipment of animals is not received, the NTP Laboratory Animal Management shall be notified as early as possible to trace the shipment.

2. Animal Receipt and Quarantine

a. Shipping cartons/crates and the filter fabric shall be examined for damage that occurred during transit. Do not use animals in damaged cartons. Thoroughly wipe the entire outside of the shipping cartons with a disinfectant such as dilute sodium hypochlorite. Disinfected shipping cartons are to be separated from shipping cartons that have not been disinfected. Do not spray on and around the shipping cartons. Disinfected, unopened shipping cartons shall be taken directly to the door of the specific animal room, but shall not be taken into the animal room. During unpacking and transfer of animals to cages, the animals and the person in the animal room removing the animals from the carton shall not come in contact with the outside surfaces of the shipping cartons. Rats and mice from the same shipment and supplier or shipments received within 3 days from the same supplier may be maintained together. The Laboratory Animal Veterinarian shall examine the animals within 3 days of arrival to assess their health status.

b. Animals shall be quarantined/acclimated for a minimum of ten days to a maximum of fourteen days under conditions simulating those in the study situation. At the end of the quarantine/acclimation period the animals shall be examined and released from quarantine (if healthy) for study by the Laboratory Animal Veterinarian.

1) Animals shall be housed one to five per cage to simulate the housing to be used during the testing phase of the study.

2) If automated watering is used in the testing facility, it shall be used during the quarantine/acclimation period. Group housing is permitted for up to 7 days at the beginning of the quarantine period for acclimation to automated watering.

3) The animals shall receive the same textured feed (meal or pellets) from the same source during quarantine as they will receive during the study period.

c. The health of the animals shall be assessed during the last few days of quarantine (3-4 days prior to putting on study). Unsuitable animals, as determined by a veterinarian, shall be discarded. Before discarding, at least five of these unsuitable animals (all if less than five) in the worst condition shall be sacrificed and necropsied. If there are no unsuitable animals, randomly selected healthy animals (five per sex per specie) from each shipment shall be sacrificed and necropsied one to three days before release from quarantine. All the necropsied animals shall be examined grossly for disease and parasites by the Laboratory Animal Veterinarian and/or Veterinary Pathologist. Diagnosis of lesions seen at necropsy shall be confirmed by histopathological examination and/or microbiological culture. The results of these observations shall be included in the next Monthly Progress Report. **UNUSUAL DISEASE CONDITIONS RECOGNIZED IN QUARANTINED ANIMALS SHALL BE REPORTED TO THE NTP PROJECT OFFICER AS SOON AS DIAGNOSED**. Slides and blocks developed for histopathologic examination shall be sent to the Archives at the end of the study. Study animals shall be formally released from quarantine by the Laboratory Animal Veterinarian

3. Animal Assignment to Study

a. During the last 1-3 days of quarantine/acclimation, animals shall be assigned to test/control groups following formal randomization routines.

b. If sufficient healthy animals are available, these animals shall be randomly assigned to weight distribution groups. The weight distribution range of the animals selected for the study should be as narrow as possible and no more than $\pm$ 20% from the mean body weight (by sex) of all the animals available for the study at randomization. If it is necessary to use a few animals outside the $\pm$ 20% range, approval by the Project Officer is to be obtained.

c. It is necessary that in all studies the body weight means of the groups within a sex and species be close to each other. To achieve this objective, it is required that, before randomization to treatment, the animals shall be divided into weight classes and all outliers removed. Animals are to be distributed into stratified weight classes using 5 gram intervals for rats and 1 gram intervals for mice and then randomized into treatment groups. Extra animals shall be removed from the study room and accounted for in the raw data. There shall be no animal substitutions after a study starts. Extra animals may be used as sentinel animals if necessary or for training of technical personnel.

4. Animal Identification

Animals shall be uniquely and consecutively numbered. Once a number is used for an animal in a study, it shall not be repeated in the same study. Tattoo at the base of the tail with black pigment is the preferred method for all studies with albino and pigmented rats and mice. Double strength black pigment is recommended for tattooing pigmented (B6C3F1) mice. Some tattoos of pigmented mice may fade and they may have to be retouched or re-tattooed once or twice during the course of a two-year study. All the rats and mice in a study shall be serially numbered. The method of identification shall be approved by the NTP. The scheme used for identification shall become a part of the raw data. The tail, or other body part bearing the identification marks, shall be fixed with the tissues at necropsy.

F. ANIMAL DISEASE SCREENING PROGRAM

An Animal Disease Screening program is carried out by the NTP. Contractor participation is mandatory.

1. Study Requirements for Subchronic Studies

   The contractor shall collect ten serum samples from rats (five per sex) and from mice (five per sex) from the untreated or vehicle controls at the scheduled sacrifice from each subchronic study. If serum samples are collected from animals used for clinical laboratory studies and the Animal Disease Screening Program, then the anesthetic, site of bleeding and blood collection technique specified for clinical laboratory studies shall be used. Serum samples are shipped to an NTP designated Rodent Disease Diagnostic Laboratory for disease screening.

2. Study Requirements for Chronic studies

   a. Each chronic study requires the addition of fifteen animals/sex/species as untreated sentinel controls. Sentinel animals must be clearly marked as sentinel and used only as sentinel animals and not as part of the animals used for the study. The rats and female mice shall be group housed and the male mice housed singly. These cages shall be randomized in the racks along with the other control and treated animals. Blood shall be collected (and serum separated) from five rats and five mice of each sex (each animal taken from a different cage) at the sixth, twelfth, and eighteenth months. Animals bled at 6 and 12 months shall be returned to their cages after bleeding if there is no indication of infectious disease. If there is indication/suspicion of disease, animals shall be necropsied and examined for gross lesions following the collection of blood samples. At 18 months, all surviving sentinels shall be necropsied and examined for gross lesions. Blood is to be collected from orbital sinus, or via cardiac puncture or abdominal vessels (at necropsy) under CO_2 /O_2 anesthesia.

      At terminal sacrifice for chronic study animals, serum samples shall be collected from five rats and five mice of each sex in the high or middle dose for disease screening. Lesions shall be processed for histopathologic examination and the results reported to the NTP Project Officer as soon as they are available.

      At the direction of the Project Officer the testing laboratories may be required to collect additional serum samples (up to 10) and ship these serum samples to an NTP designated Rodent Disease Diagnostic Laboratory for further evaluation of an infection or disease.

      For each chronic mouse study, the contractor shall collect fecal samples from 5 male and 5 female sentinel mice at 18 months and ship the samples to an NTP contractor for evaluation of *Helicobacte*r infection. (See Appendix 3 for procedures for the collection and shipment of fecal samples.)

   b. Weighing

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

c. Moribundity/Mortality Checks

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

d. Pathology

Any sentinel animals lost to the study shall not be replaced. Sentinels that die or are sacrificed during the course of a study shall receive complete necropsies and shall be examined to determine if animal disease is present. Selected histopathology (to include all lesions and grossly abnormal organs) shall be done on dead and moribund sentinel animals (as well as those necropsied at 18 months) and the results reported to the NTP as soon as they are available, but no later than ten work days after the dead or moribund animal was found.

Two copies of the Individual Animal Necropsy Records (IANRs) for the dead and moribund sentinel animals shall be submitted to the Contract Coordinator of the NTP. Data from these forms shall not be entered into TDMS. The slides, blocks, and tissues shall be labeled with a non-TDMS label containing the experiment, the animal identified as sentinel, date, tissue, etc. The slides, blocks, and wet tissues of the sentinel animals shall be sent to the NTP Archives along with the rest of the tissues from the study.

3. Collection, Processing and Shipping of Sera

a. A volume of at least 1.0 ml, preferably 1.5 ml, of diluted serum at a 1:5 dilution (1 part serum and 4 parts normal or phosphate buffered saline) is necessary for a 10-virus serologic assay. Blood shall be collected from anesthetized animals from the retro-orbital sinus or from the abdominal vessels (terminal sample).

b. The contractor shall submit serum samples for screening for the presence of antibodies to murine pathogens. The screening tests shall include but not be limited to the following:

Mice: Pneumonia virus of mice (PVM), reovirus type 3 (Reo 3), Theiler's encephalomyelitis virus (GD VII), ectromelia, Sendai, mouse hepatitis virus (MHV), mouse parvo virus (mouse minute virus and mouse parvo virus), and lymphocytic choriomeningitis virus (LCM).

Rats: PVM, Sendai, rat coronavirus-sialodacryoadenitis virus (RCV-SDA) and rat parvo virus. All samples positive for rat parvo shall be retested for Kilham rat virus (KRV) and Toolan's H-1 virus (H-1).

c. All serum specimens must be submitted to the NTP designated Rodent Disease Diagnostic Laboratory pre-diluted 1:5 in saline in labeled, one dram, screw cap vials. The sera shall be heat inactivated at 56^{o} C for thirty minutes and not treated in any other way. Sera shall be stored at 4^{o} C, or colder, until shipment.

d. For shipment, vial labels shall be paper or opaque tape marked legibly with waterproof pencil, ink or type and held in place with cellophane tape encircling the vial. Each label must correlate to a corresponding line on the Serum-Sampling Form. Serum samples collected at the end of 24 month studies shall include the following note on the serum sampling form: "Chronic Study - Terminal Samples - Test for Mycoplasma Also". Vial lids shall be secured with rubberized tape or Parafilm (if necessary) applied after the labels are taped in place. Cellophane tape used to secure the lids is not acceptable. Vials of sera must be packaged tightly to prevent breakage. Individual wrapping or clustering in groups of three to five with rubber bands and/or envelopes is satisfactory. Serum shall be transported at ambient temperature in an insulated container via an overnight delivery service.

e. The Project Officer will provide the address of the diagnostic laboratory and the data forms to use, or to be received. The diagnostic laboratory will mail a copy of the results to the submitting laboratory and these data shall be included in the raw data submitted to the NTP Archives at the end of the study.

G. GENETIC MONITORING OF B6C3F1 AND TRANSGENIC MICE

Each testing laboratory shall collect tissue biopsies (tail or ear) from ten B6C3F1 or transgenic mice (equal numbers from each sex if possible) received for each chronic or transgenic study (up to 4 studies per year per strain) for the purpose of genetic monitoring to be conducted by the NTP. These tissue biopsies shall be shipped prepaid to the NTP genetic monitoring contractor within one week of their collection. The mice used to obtain the biopsies may be used as sentinels for the study. At the time of shipment, the testing laboratory shall mail a copy of the packing note and Custody Transfer Record that accompanies the shipment to the NTP Project Officer and Laboratory Animal Management of the NTP. Detailed collection and shipping procedures are presented in Appendix 3.

H. SPECIAL REQUIREMENTS FOR SPECIFIC ROUTES OF ADMINISTRATION

1. Inhalation

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

a. Exposure Room

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

2) Animals for these studies shall be quarantined in the inhalation chamber or in a separate room. If quarantined in a separate room, cages, feeding and watering systems shall be compatible with the systems in the inhalation chambers, and the room temperature shall be maintained at 75 ± 3^{o} F with 40 to 70% relative humidity. The animals shall be identified by tattoo.

3) If the animals are housed outside the chambers during the non-exposure periods, (due to special requirements of the test article) they shall be in the same room or in a room across from or adjacent to the chamber room. One room shall be allocated for each chemical. If the animals are housed outside the chambers during the non-exposure periods, the procedures used for transport, caging, feeding, and watering shall be described in detail and approved by the NTP.

b. Chambers

1) Inhalation chambers shall be of a design that can be demonstrated to provide uniform and reproducible exposure of all animals to the test article. Intake air to the chambers shall be filtered through absolute (HEPA), Purafil® and charcoal filters. Intake air is to be analyzed at the conclusion of exposure system development and installation and at least once during each 90-day and 2-year study to ensure the quality of the air entering the exposure chambers meets or exceeds human breathing air standard E set forth by the Compressed Gas Association Commodity Specification G7.1. Air flow rate, temperature, and relative humidity must be checked and recorded at least every three hours or, preferably, continuously. Chamber pressure (negative relative to room pressure) must be checked frequently and recorded at least daily. Flow meters shall be calibrated with regard to pressure drop on a routine basis at least once every two months or as often as necessary to maintain the required flow.

2) All animals must be individually caged in stainless steel wire mesh cages with secure latches during exposure. The cages must be of adequate size such that all animals in a particular dose group can be exposed in a single chamber. The animals themselves must account for no more than 5% of the total volume of a chamber.

3) For chronic inhalation studies in **rats:** House female rats in cages with approximately 40 sq. in. floor space and male rats in cages with approximately 60 sq. in. floor space from the start of the study. For a chronic study, for example, if Hazleton 2000 chambers are used, there will be 2 x 24 cage batteries for females and 3 x 16 cage batteries for males. The manufacturer has designed a cage battery for 10 males and 10 females. Thus each chamber can house 58M + 58F. Two males and two females per chamber will be set aside as sentinels. There will be 12 to 16 (4/chamber) sentinel animals/study. Obtain blood samples from sentinel rats by orbital bleeding at 6 and 12 months for viral serology profiles and return all sentinel animals to their respective cages. At 18 months, collect blood from all sentinels (under anesthesia) and necropsy all sentinels according to the Specifications.

Chronic inhalation studies in **mice**: House all mice in cages 24 to 30 sq. in. floor space from the start of the study. Sentinel mice will be distributed evenly throughout each exposure and control chamber.

4) The cage racks shall be rotated clockwise daily on exposure days of the repeated dose study and weekly during the subchronic and chronic studies. The cages and the chambers shall be washed and sanitized at least once a week and more often, if necessary. If cages are mounted on more than one tier in the chamber, pans for collection of excreta will be required between tiers during the exposure as well as non-exposure periods. To decrease ammonia production in the chambers, plain cage board or pan paper shall be used during the non-exposure periods. Cage board (pan paper) may be used in the excreta collection

pans of the chambers during exposure, provided the test article is not going to react with the cage board or is not absorbed by the cage board during exposure and released during non-exposure periods. The cage board or pan paper shall be changed once a day.

5) Animals remaining in the chambers during non-exposure periods are generally removed from the chambers briefly for maintenance and moribundity/mortality checks twice daily and periodically for clinical observations. Only one chamber shall be opened at a time for routine maintenance, clinical observations, etc. Care must be taken to avoid exposure of animals to pathogens in the chamber room.

c. Water and Diet

Water shall be available by automatic watering system during the non-exposure as well as the exposure periods. The automatic watering system shall be checked daily so that in case of malfunction or air locks, the animals will not be without water for more than a day. The feed shall be provided *ad libitum* in feeders or hoppers during the non-exposure periods. Fresh feed shall be provided at least at weekly intervals. The animals shall be housed in the chambers with doors closed and locked during the non-exposure periods unless special requirements of the test article make this impractical.

d. Environmental Conditions and Animal Acclimation

The study animals shall be acclimated in the chambers for at least three days simulating exposure conditions before initiation of chemical exposure. The chamber ventilation system shall provide 15 ± 2 air changes per hour and the design of the chamber must afford opportunity for equal exposure to each animal. The temperature of the chamber shall be maintained at 75 ± 3^{o} F. If the temperature is above or below the 75 ± 3^{o} F limit, it shall be returned to the acceptable limits within two hours. The temperature shall not be below 70^{o} F or above 80^{o} F during the course of the study. There shall be an alarm system for warning of temperature fluctuations below 70^{o} F and above 80^{o} F. The relative humidity of the chamber atmosphere shall be 40 to 70% and it shall not be below 35% or above 75% at any time during the course of the study. There shall be a back-up ventilation system in the event of the failure of the primary system supplying conditioned air to the chambers. Chamber temperature and relative humidity results are to be reported as means ± relative standard deviations or as time-weighted averages.

2. Dermal Studies

a. Animal Care

All animals shall be individually caged.

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

b. Skin Application

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

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

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

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

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

c. Skin Histopathology

All skin sections shall be cut from animals so that the orientation is well defined (anterior to posterior). In this respect, it is very useful to cut the anterior (cranial) border with an arrow shape. Excised skin shall be laid on a piece of index card (labeled with indelible ink) and placed in fixative with other tissues, flat (dermis side down). Tumors for histopathology (up to five) shall include those selected at the gross observation; also, the contractor shall select samples of skin from a non-tumor section of the skin-paint area and of skin from a consistent location away from the site of application. Care shall be taken when removing selected samples from the excised skin for histology to retain the remainder of the excised skin intact. Always trim tissue from anterior to posterior (cranial to caudal) and approximately 1 cm in length for slides. In trimming tumors, attempt to include non-tumorous adjacent skin (edge of tumor) in the section. The non-tumor treated section is important for assessing non-neoplastic changes, e.g. dermatitis, hyperplasia, etc. compared with control animal skin. It is important that non-neoplastic diagnoses be made on this non-tumor area. Skin away from the site of application will be important to assess any systemic effects on skin.

3. Gavage Studies

   a. In gavage studies, for each dose group, the concentration of the dose formulation will remain constant throughout the study, with the required dose provided by varying the volume administered based on animal body weight. The total volume of material given per animal per treatment shall not exceed 5 ml/Kg for rats, or 10 ml/Kg for mice without consultation with and written approval of the Project Officer. The volume selected must remain constant throughout all studies for a test article.

   b. All animals of a gavage study shall be treated during a "consistent, specified time" of the morning on each treatment day. The "specified time" shall be approved by the Project Officer. An entire dose group is to be dosed before moving to the next group. The treatment sequence of control and dose groups for each treatment day shall be randomized to avoid a control first and high dose last bias.

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

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VI. CLINICAL PATHOLOGY

A. CLINICAL PATHOLOGY ASSESSMENTS

At a minimum, the laboratory must be capable of performing the following required clinical assessments/measurements in a satisfactory manner:

1. Hematology

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

   a. Erythrocyte count

   b. Hemoglobin concentration

   c. Hematocrit (Packed cell volume)

   d. Mean corpuscular volume

   e. Mean corpuscular hemoglobin

   f. Mean corpuscular hemoglobin concentration

   g. Leukocyte count

   h. Leukocyte differential count

   i. Reticulocyte count

   j. Platelet count

   k. A spun (manual method) hematocrit (Packed cell volume) must be performed.

   l. A morphological assessment (microscopic evaluation) of erythrocytes, leukocytes and platelets must be performed and documented. Nucleated erythrocyte (nRBC) counts (nRBC/100 leukocytes) must be reported.

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

   n. Instead of an automated reticulocyte count, a reticulocyte count determined by microscopic examination (manual method) of a supravitally-stained blood smear (for example, new methylene blue) is acceptable. The manual reticulocyte count must be reported as an absolute number based on the proportion of reticulocytes in 1,000 erythrocytes or by use of a Miller disc.

Platelet, reticulocyte and leukocytic cell counts will be expressed as absolute counts. The raw data will be determined by electronic or laser optic methods. The reporting of data based upon percentages, estimates and manual counts is not acceptable.

At those times during NTP studies when blood samples are collected for hematologic analyses, the volume of packed red blood cells (VPRC or HCT) will be determined by manual (spun) micromethods. This procedure is in addition to the measurement (or calculation) of the hematocrit (HCT) performed by the hematology analyzer. Results of the manual determinations will be included with those of automated analyses in the data submissions. The plasma column in each microhematocrit tube will be inspected for the presence of hemolysis and a positive finding will be recorded and reported with these data.

2. Clinical Chemistry

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

a. Total protein concentration

b. Albumin concentration

c. Urea nitrogen concentration

d. Creatinine concentration

e. Alanine aminotransferase activity

f. Sorbitol dehydrogenase activity

g. Alkaline phosphatase activity

h. Total bile acid concentration

i. Glucose concentration

j. Creatine kinase activity

3. Urinalysis

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

a. Urine appearance

b. Urine volume

c. Urine specific gravity or osmolarity

d. Microscopic assessment of urine sediment

e. Urine protein concentration

f. Urine glucose concentration

g. Urine creatinine concentration

h. Activities of urine enzymes as specified for individual studies (e.g., N acetyl- β-glucosaminidase, lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase and gamma glutamyl transferase)

B. CLINICAL PATHOLOGY LABORATORY REQUIREMENTS

1. The clinical laboratory scientist is responsible for all phases of sample collection, handling (including transportation, recording, evaluation and preparation), analysis and storage. The training or approval of personnel to assume these tasks is also the responsibility of the clinical laboratory scientist. The clinical lab scientist must review and sign off on data at the end of each day, prior to discarding unused samples.

2. The laboratory shall have in place all equipment necessary to perform the above listed tests at a minimum.

3. The laboratory shall have **routine** capability to collect and analyze blood, serum and urine samples from rats and mice at a level of sixty samples for each study day. Non-routine situations may require collecting and analyzing sixty to one hundred twenty samples per day.

4. SOPs for performing the above referenced clinical tests shall be made available to the NTP for review and approval. The SOPs shall be accompanied by documented performance capability and ability to interpret the results.

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

6. The laboratory shall have written procedures for documenting incidents and deviations from protocol and SOPs and must follow the Westgard rules for rejecting sample runs. (Westgard, J.O., Barry,P.L., Hunt, M.R., A multi-rule Shewhart chart for quality control in clinical chemistry. Clin. Chem., 27:493-501, 1981.) The degree to which such deviations and incidents may influence the clinical laboratory measurements shall be identified. Examples of such deviations and incidents include: technician error, variation in reagent lots, equipment drift or failure, and the procedure that will be followed to determine the acceptance or rejection of data based upon the concurrent analysis of control samples. Based on Westgard rules, documentation of rejection and repeat of sample runs must be made along with corrective action.

7. Whenever a test article specific protocol calls for hematology, clinical chemistry, etc., the blood samples shall be obtained in random order (not by dose group) for a given sex and species. The samples shall be run in that same random order in the clinical laboratory. The laboratory shall have written procedures for collecting and processing

specimens in a randomized order. This requires an appropriate scheme for identifying and tracking specimens throughout all procedures.

8. Blood collection procedures shall be clearly identified. Blood collection sites, procedures, and anesthetics used shall be defined for interim as well as terminal sampling. All blood samples from rats and mice shall be collected from the retro-orbital sinus using a carbon dioxide/oxygen mixture as the anesthetic. Sample volumes taken at interim bleeds shall not exceed 2.0% body weight for rats and 2.5% body weight for mice. Animals shall not be fasted prior to sample collection. For dermal, gavage, and inhalation studies, at each collection time point, animals shall be treated for a minimum of two consecutive days (within 24 hours) prior to sample collection. Animals are not to be treated on the morning of collection for these routes, unless the protocol requires it. Blood samples for analysis in the clinical pathology laboratory shall be collected from the appropriate animals the morning of sample collection during a 3 hour period. This includes samples for analysis of routine hematology, clinical chemistry, methemoglobin, urinalysis, and hormone variables. Methods for harvesting serum and plasma shall be described. All males and females of a given species shall be treated the same number of days before collection of samples and all animals of a sex shall be bled on the same day. Overnight urine collection procedures must be clearly explained. As part of the demonstration of capability to collect blood and harvest serum, each laboratory must submit a listing of the total volumes per animal of whole blood, serum, and plasma that can be routinely obtained from rats and mice of six weeks, seventeen weeks, and six months of age, utilizing the retro-orbital bleeding technique. Interim, as well as terminal sacrifice volumes, shall be listed.

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

10. Automated hematology measurements, blood smear preparations and methemoglobin determinations shall be made within 6, 2 and 0.5 hours of sample collection, respectively. Constituents in serum (or plasma, if specified) shall be assayed the same day of sample collection. During the period when the samples are not being assayed, they shall be kept tightly sealed at 4^{o} C. With specific permission from NTP, samples may be frozen at -20^{o} C or colder for subsequent analysis. The freezing of samples for storage prior to the performance of routine assays is not acceptable. Samples collected for routine hematology assays (EDTA) shall not be stored on ice before analysis.

11. The laboratory shall have sufficient facilities for frozen storage of biological samples at -60^{o} C or below. Such samples may be retained for up to six months following NTP receipt of the relevant study report(s). Stained and cover-slipped peripheral blood, reticulocyte, and bone marrow smears shall be appropriately identified (same as for histology slide identification) and packaged for delivery to the NTP Archives at the conclusion of each phase of the study as specified in Section VII.I.1.

C. REPORTING REQUIREMENTS

1. Submission of Preliminary Pathology Results

Individual animal data and the results of the concurrent analysis of control samples for all interim sample collections of routine chemistry assays, automated hematology analyses,

methemoglobin, and urine chemistry determinations shall be submitted to the NTP Project Officer and Pathology Coordinator **within 7 calendar days** of sample collection. Prompt submission of data generated after bleeding is needed for evaluation before the next bleeding point so that any adjustments required can be made in a timely manner. For terminal sacrifice collections, data are to be submitted within 21 calendar days of sample collection. White blood cell differentials, reticulocyte counts and morphologic evaluations of blood smears are to be included in the final report.

Data to be reported include original and repeat sample assays for individual animals as well as quality control results. Copies of raw data may suffice for part of this requirement. It is **not** necessary for these data to be subjected to internal quality assurance prior to submission to the NTP. The data are to be tabulated and organized by species, sex, and treatment group.

For quality control data, show mean and measure of variability (and source of values) used in testing control materials to determine acceptability of animal values. If the mean and standard deviation of the controls were established in the contractor's laboratory, both in-house and manufacturer's values shall be provided.

For prompt processing and review of preliminary clinical pathology results by the NTP, a Clinical Pathology Study Information Sheet (shown below) is to be attached to the front of each data set.

2. Final Study Report

Individual animal data are to be included as an appendix to the final study report. Data shall be organized by species, sex and treatment group. Notations of any observation and/or action taken to confirm or explain atypical data points are to be included (example: diluted and reanalyzed to confirm or establish values that exceed linearity of assay, or sample reanalyzed to confirm low values). Relevant comments concerning sample quantity (QNS) and quality (example: lipemia, hemolysis, icterus, etc.) are to be included. Measures of central tendency in tabular form shall be reported for the measurements made at all time-points. The methodology used to obtain samples and measure analytes must be described in Materials and Methods Section of the study report. Interpretation of the biological significance of the results shall be presented in the Discussion Section of the study report and shall include correlations between clinical laboratory findings and anatomic pathologic changes and/or clinical signs exhibited by the study animals.

NTP CLINICAL PATHOLOGY STUDY INFORMATION SHEET

Laboratory Name:		
Study:		
Type: (example: prechronic, chronic)		
Time Point: (example: 5 day, 21 day, 90-day)		
Route of Administration: (example: gavage, dosed water)		
Species:		
Sex: (circle) Male Female		
Date Collected:		
Date Analyzed:		
Anesthetic:		
Site of Sample Collection:		
	Equipment Used for Analysis	Technician(s) Performing Analysis
Hematology:		
Clinical Chemistry:		
Other: (example, urinalysis, methemoglobin)		
Comments:		

VII. HISTOPATHOLOGY

A. CAPABILITY

1. The Pathologist assigned to the study shall examine the study protocol required tissues from **all** the animals of a given species in that study including early deaths. The assigned Pathologist for the chronic study shall perform the histopathologic interpretation on all interim sacrifice animals, if an interim sacrifice is included.

2. After review of the pathology slides and report by the NTP, the NTP may send slides back to the laboratory Pathologist for additional review and updating of TDMS necropsy or microscopic pathology data. Alternatively, the laboratory Pathologist may be asked to attend the Pathology Working Group review of the pathology at NTP and make appropriate corrections to the data.

B. NECROPSY

1. Necropsy Requirements and Sequence

   Scheduled necropsies shall be performed in the presence of and under the supervision of the assigned Pathologist. Unless an animal is necropsied shortly after the last dose, damage to some organs could be repaired and the significance of body weight determinations could be clouded. Scheduled necropsies and necropsies of moribund sacrifice animals shall be initiated within five minutes after an animal is sacrificed.

   Unscheduled early death animals shall be necropsied as soon after death as possible. Ideally, dead animals shall be refrigerated for no longer than eight hours prior to necropsy. Animals shall not be frozen. 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 error, accidental death, infectious disease, treatment-related toxicity/carcinogenicity.

   It is better to dose animals extra days in order to schedule necropsies in a timely manner than to have exactly the prescribed number of study days and delayed necropsies. Alternatively, if a laboratory has a limited necropsy staff, it would be better to stagger the start of an experiment so that actual scheduling is left to the laboratory because of differences in capabilities. However, the following requirements are spelled out to assure that necropsy occurs promptly after the sacrifices.

   a. Repeated Dose Study

      1) For all inhalation, gavage, or dermal studies, animals shall be dosed for two consecutive days before the day of necropsy. The date and time of the last dosing period are to be recorded. Feed and water, including dosed feed or dosed water, if appropriate, shall be provided until the actual time of sacrifice.

      2) A minimum of one species shall be necropsied per day. Necropsies shall be performed on controls first, then high dose animals working down to the low dose last. Necropsy shall be initiated within five minutes after an animal is sacrificed.

b. Subchronic Study

1) For all inhalation, gavage, and dermal studies animals shall be dosed for two consecutive days prior to the day of necropsy. The date and time of the last dosing period and date of necropsy shall be recorded. Feed and water, including dosed feed or dosed water, if appropriate, shall be provided until the actual time of sacrifice.

2) For all standard subchronic studies with 10 animals/dose group and five dose groups plus controls (e.g. 60 animals per sex/species), the necropsy of one complete sex/species shall take place on one day, and both sexes of one species within two consecutive days. The order in which animals are necropsied (within a species/sex) shall be randomized. Necropsy shall be initiated within five minutes after an animal is sacrificed.

c. Chronic Study

1) All chronic and interim sacrifice study animals shall be sacrificed without a recovery period. Interim sacrifice animals shall be dosed for two consecutive days prior to the day of necropsy. Feed and water including dosed feed and dosed water, if appropriate, shall be provided until the actual time of the sacrifice. Organ weights shall be performed only on animals sacrificed for interim evaluation.

2) One sex of a given species may be dosed for an extra week to allow necropsy of males and females in consecutive weeks if it is not possible to necropsy both in one week.

3) All necropsies shall be performed within seven consecutive workdays. The order in which animals are necropsied (within a species/sex) shall be randomized. Necropsies shall be initiated within five minutes after an animal is sacrificed.

2. Necropsy Standards

a. A complete necropsy shall be performed on all animals from all treatment groups in all studies that die or are sacrificed during and at the end of the experiment. Sentinels that die or are sacrificed during the course of a study shall receive complete necropsies (see section V.F.2.d. for requirements for sentinels). Animals removed with removal reasons of **"other"** are **not** to be necropsied. Animals on special studies shall be sacrificed according to that specific protocol. All potential lesions observed are to be recorded in the raw data.

b. Complete necropsy is defined as external examination of the animal including body orifices and examination and fixation of **all** of the following organs/tissues from animals from all treatment groups in all studies for histopathologic examination:

Adrenal glands
Brain
Clitoral glands
Esophagus
Eyes
Femur
Gallbladder *(mouse)*
Gross lesions
Harderian glands
Heart and aorta
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Kidneys
Liver
Lungs and mainstem bronchi
Lymph nodes
- mandibular and mesenteric
- bronchial and mediastinal *(inhalation studies)*
Mammary gland with adjacent skin
Muscle, thigh
Nerve, sciatic
Nose (nasal cavity and nasal turbinates)
Oral cavity, larynx and pharynx
Ovaries
Pancreas
Parathyroid glands
Pituitary gland
Preputial glands
Prostate
Salivary glands
Seminal vesicles
Skin *(dermal studies only)*
Spinal cord
Spleen
Stomach (forestomach and glandular)
Testes, epididymides and vaginal tunics of testes
Thymus
Thyroid gland
Tissue masses
Tongue
Trachea
Urinary bladder
Uterus
Vagina
Zymbal glands

3. 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. The exceptions to this are that the eyes (Davidson's solution) and testes, vaginal tunics of testes and epididymides (modified Davidson's solution) are fixed for 24-72 hours in their respective solution, and then transferred to 10% NBF. All organs (with the possible exception of skin, mammary glands, bone and muscle) shall be saved and fixed in their entirety, e.g. **no** part of the organ shall be discarded. Eosin shall be added to the stock formaldehyde solution in sufficient quantity to impart a pink tinge to the 10% neutral buffered formalin. Tissues saved for histopathology shall be fixed at a thickness not to exceed 0.5 cm except as stated below. 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 formalin with the animal tissues. If ear tags or other identifying methods are used for any reason, these too shall be saved in formalin with the animal tissues.

a. The number of grossly visible nodules/masses in the lung shall be recorded up to five per lung; after the five largest nodules/masses have been counted and sampled, the IANR shall show under "Notes" the number as "greater than five" for that organ.

b. The trachea and lungs shall be infused by introducing 10% buffered formalin (approximately 1-2 ml for mice and 4-8 ml for rats) into the trachea until the lungs are completely filled to normal inspiratory volume then the trachea shall be tied and the organs placed in formalin.

c. The calvarium shall be removed for examination of the brain and pituitary. The brain shall be removed for fixation and the pituitary left *in situ* for fixation. The nasal bones

shall not be removed. The head is to be retained for decalcification and sections of the nasal cavity taken as specified in Section VII.C.4.j.).

The turbinates and tissues of the nasal cavity shall be fixed by gently inserting a blunt needle attached to a syringe into the nasopharyngeal duct and instilling formalin in the nose until drops appear at the external nares.

d. Collect and fix prostate, seminal vesicle, coagulating gland and urinary bladder together in a manner that will minimize twisting and curling. Remove adipose tissue surrounding these tissues when collecting. Distended urinary bladders shall be fixed as is. Contracted, empty bladders shall be partially distended with formalin. Care shall be taken to insert the needle into the lumen of the bladder. Insertion of the needle into the bladder wall may result in severe artifact. Urinary bladders shall be opened and examined after fixation at trimming.

e. The number of grossly visible nodules/masses in the liver shall be recorded up to five per liver; after the five largest nodules/masses have been counted and sampled, the IANR shall show under “Notes” the number as "greater than five" for that organ. The liver shall be sliced to ensure fixation.

f. The kidneys shall be bisected, and the cut surfaces examined before fixation. The left kidney shall be bisected longitudinally and the right kidney crosswise.

g. The oropharynx, esophagus, stomach, small and large intestines, and rectum shall be opened only as described below:

1) Oral cavity, pharynx and larynx shall be carefully examined grossly. If any abnormalities, including tumors and/or lesions, are noted, the tissues shall be examined microscopically. The larynx shall be examined microscopically for all inhalation studies.

2) Remove mandible to allow visualization of the tongue and posterior pharynx.

3) Take a routine cross section of esophagus with thyroid, parathyroid trachea, and esophagus in one section. Open remaining esophagus and examine.

4) Split the pelvis and remove the entire gastrointestinal tract including the anus.

5) Transect stomach at the junction of the pylorus and duodenum and inject stomach with formalin (mouse = 2 ml; rat = 5 ml). Stomach shall be opened and examined at trimming.

6) For all studies, take cross sections consistently from the same area for each tissue: duodenum (to be taken exactly 1 cm from the pylorus), jejunum, ileum, cecum, colon, and rectum. Include Peyer's patches with these sections where possible. For chronic studies, the rest of the intestinal tract shall then be opened and examined. Areas with lesions shall be individually identified and pinned flat on paper or cardboard or placed in a cassette for fixation, labeled as to location, and recorded on the IANR. For prechronic studies the entire intestine does not need to be opened, however sections of suspect lesions shall be taken.

h. The spinal cord is to be saved in its entirety and fixed in 10% formalin at necropsy (may be left in vertebral column or removed from it at this point). It is not necessary to grossly examine the spinal cord unless the animal has exhibited neurological signs

or the protocol specifically requires it. Fixation and gross examination (if necessary) of the cord may be accomplished by one of several alternative methods, but must provide quality sections free of excess artifact. Gross examination of the entire spinal cord (when required) may be done at necropsy or at trimming.

i. A section of the heart shall be sliced from the base through the apex so that all four chambers are visualized.

j. Eyes (in Davidson's), harderian glands, preputial or clitoral glands, and thymus (plus lymph nodes if necessary) shall be dissected free and placed in labeled cassettes to avoid being lost.

k. Multiple representative portions of large or heterogeneous tissue masses including surrounding unaffected tissues must be fixed. Masses less than 0.5 cm diameter may be fixed in their entirety.

4. All gross lesions shall be described by the supervising Pathologist and recorded using the terminology in the TDMS Pathology Code Table (PCT) inclusive of morphologic lesion; anatomic site; quantity; size or volume in millimeters or milliliters; number; shape; color; and consistency. Each gross observation shall be correlated with a microscopic evaluation.

5. Those organs typically weighed are: liver, thymus, right kidney, right testicle, heart, and lungs. These organs shall be weighed to the nearest 10 mg except for testicle and thymus which shall be weighed to the nearest 1.0 mg. Organ/body weight ratios shall be calculated. Specific trimming instructions for these organs are as follows:

   a. Liver - shall be free of adjacent tissues and the gall bladder shall be opened in mice.

   b. Thymus - shall be carefully dissected from adjacent tissues.

   c. Right kidney - shall be dissected from perirenal fat and adrenal gland. If only one kidney is available it shall be weighed. If one kidney is grossly affected, both shall be weighed separately.

   d. Right Testicle - epididymis shall be removed prior to weighing. The left testicle shall be fixed with the epididymis intact (in modified Davidson's). If SMVCE is to be conducted, the left testis is to be used for SMVCE and the right testis for histopathologic evaluation.

   e. Heart - shall be removed at base and be free of pericardial sac. The heart shall be gently squeezed to remove blood from chambers. If blood has clotted, chambers shall be opened and clots removed before weighing.

   f. Lungs - one-half of the trachea shall remain attached to the lung. After weighing this is used to perfuse the lungs with fixative.

6. Following necropsy, the carcasses of rats shall be placed in a properly labeled container and fixed in 10% buffered formalin. Carcasses of **rats only** shall be discarded **after** the histopathology evaluation **and** the audit of the residual wet tissues have been completed, and it is determined that no lesions were missed at necropsy. Disposal of the rat carcasses shall require the approval of the Principal Investigator, Study Director, and Quality Assurance Officer. Carcasses of mice shall be placed in the container with the tissues and fixed in 10% buffered formalin. Carcasses of mice shall not be discarded, but

shall be submitted to the NTP with the residual formalin fixed tissues at the end of the study.

7. Color images of selected representative gross lesions in target tissues shall be prepared. All unusual lesions shall be photographed. All color images are the property of the NTP. Each color slide shall be identified with contract number, chemical number, TDMS number, sex, treatment group, animal number, and histopathologic diagnosis.

C. TISSUE TRIMMING

1. Tissue trimming shall be supervised by the assigned Pathologist and chief of the histology laboratory, although their continued presence is not required. A copy of the IANR for each animal shall be available for the technician at the time of tissue trimming. Any additional gross observations shall be recorded during the trimming procedure.

2. Tissues of the following animals are to be trimmed for possible microscopic examination.

   a. Repeated Dose Studies

      Trim all organs/tissues of animals on repeated dose study showing treatment-related gross lesions in all dose groups and corresponding tissues in controls of both sexes and all species. All trimmed tissues shall be embedded and H & E stained slides prepared.

   b. Subchronic Studies

      Trim all organs/tissues required for complete histopathologic examination including gross lesions from all control and all treated animals of both sexes and all species. All trimmed tissues shall be embedded and H & E stained slides prepared.

   c. Chronic Studies

      All early death animals and all scheduled sacrifice animals shall be handled in the same manner at the time of trimming. All gross lesions, and all organs/tissues required for complete histopathology shall be trimmed, embedded, and H & E stained slides prepared. This applies to all doses in the chronic study, both sexes and all species.

3. Trimming in tissues as soon as possible after death is preferred for optimal preservation and possible utilization of tissues for immunohistochemical or other analyses. Tissues shall be trimmed within a period of not less than forty-eight hours nor greater than 6 months from the day of necropsy for early death and moribund sacrifice animals and within 3 months of the terminal sacrifice date for all animals. Tissues are to be placed in blocks in a consistent manner so that the same tissues are in the same numbered blocks for all animals.

4. Specific methods for tissue trimming are as follows:

   a. Multiple portions of tumors or masses shall be trimmed and submitted if these are large or variable in appearance. Surrounding, normal tissue shall be included if possible with the tumors. Careful documentation of the number of sections taken per mass shall be maintained on the IANR so multiplicity can be determined.

b. Parenchymal organs, e.g., liver shall be free of adjacent tissues and trimmed to allow the largest cross- section surface area possible for microscopic examination. For liver and lung, one section of each nodule (tumor) shall be prepared up to five for each organ. The five largest tumors shall be sectioned if there are more than five lesions. Adjacent normal tissue shall be included along with the tumor. Two sections of normal liver including sections through the left and median lobes shall be prepared. The sections shall be transverse sections taken midway along the greatest dimension. That of the median lobe shall be taken about 0.5 cm right and lateral to the fissure. If the sections are greater than 2.5 cm in length, one end may be trimmed slightly. The location, approximate size (for an adult F344 rat), and appearance of the expected sections are shown in Figure 1. In the mouse, the section of gall bladder must be taken separately and apart from the two liver sections.

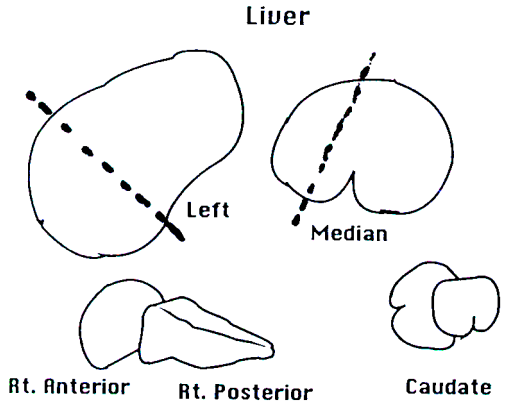

Figure 1. Liver of rat showing left, median, caudate, right anterior and right posterior lobes. Dashes represent location of sections to be taken.

c. Longitudinal sections of each preputial or clitoral gland shall be prepared.

d. Mid-longitudinal section (left kidney) and cross section (right kidney) through the entire cortex, pelvis and medulla of each kidney shall be submitted.

e. Three cross-sections of the brain shall include; (a) frontal cortex and basal ganglia; (b) parietal cortex and thalamus; and (c) cerebellum and pons. If any lesions are observed after sectioning, they shall be noted on the IANR. If neurologic signs were observed clinically, then extended evaluation of CNS shall be discussed with the NTP Project Officer.

f. Entire coronal (perpendicular to a sagittal plane and parallel to the long axis of the body) section of both right and left lungs including mainstem bronchi shall be submitted.

g. Hollow organs shall be trimmed and blocked to allow a cross-section slide from mucosa to serosa. Since the stomach is infused with formalin at necropsy, it will be opened and the mucosa examined for gross lesions at trimming. The stomach must be cut first through the midsagittal plane thereby dividing it into two equal halves (Figure 2). The section taken for histology shall include the entire greater curvature of the stomach to include forestomach, glandular stomach and pyloric region. The section can be further divided into two or three pieces to allow for

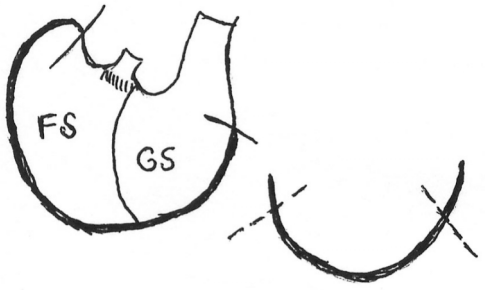

Figure 2. Profile of stomach through a midsagittal plane on the left. Forestomach (FS) and glandular stomach (GS). Shaded area represents location of section. Profile of required section is shown on the right.

convenient placement on the slide. Any gross lesions must also be embedded and sectioned.

h. Following fixation, the trachea shall be opened to the level of the hilus and grossly examined. Any tumors or abnormalities in the trachea shall be trimmed and examined microscopically.

i. Following fixation, the pituitary shall be carefully removed and trimmed to allow a coronal section.

j. After decalcification of the head, three separate sections of the nasal cavity shall be taken at (1) the level of the incisor teeth, (2) midway between incisors and first molar, and (3) middle of second molar (olfactory region). The remainder of the nasal cavity and turbinates shall be carefully examined for gross lesions at this time. The gross lesions shall be recorded on the IANR. (See Maronpot, Ed. (1999). *Nose, Larynx and Trachea in Pathology of the Mouse*, p. 261. Cache River Press.

k. The pancreas shall be trimmed to allow the largest surface area possible for examination. A portion of pancreas (about 1 cm^2 for rats and 0.5 cm^2 for mice) shall be embedded flat to provide a section through the frontal plane of the organ rather than a transverse plane.

l. For inhalation studies, a transverse section of the larynx shall be taken at the base of the epiglottis just anterior to the laryngeal saccule.

m. For dermal studies, skin at and away from the site of application shall be cut from animals so that the orientation of anterior and posterior edges is well defined. For example, the cut edge (dermis) of the anterior edge of the sample can be marked with indelible ink (India) or trimmed in the shape of an arrow. Before placing in formalin, the skin shall be placed with the dermal surface down on a piece of index card to keep the sample flat during fixation. The section of mammary gland with skin attached (taken from the inguinal area) shall be kept in a separate labeled cassette throughout the study to avoid confusing it with the application site skin when the blocks and slides are prepared.

n. The (sub)mandibular and major sublingual salivary glands are closely associated and together comprise an oval dorsoventrally compressed structure in the ventral cervical region. The mandibular lymph node(s) and parotid 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 can be embedded as a single unit and must be sectioned through the frontal plane to include the three organs (Figure 3).

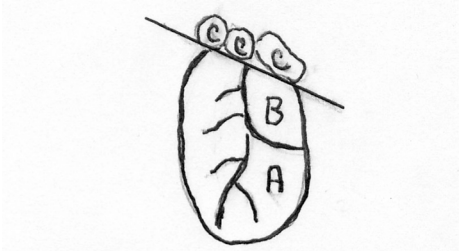

Figure 3. Profile of required sections of salivary glands and mandibular lymph node. Mandibular salivary gland (A), major sublingual salivary gland (B), and mandibular lymph nodes (C).

o. Sections of adrenal gland shall include the cortex and medulla.

p. A single transverse section of the spleen must be taken at the point of greatest width. If the spleen is diffusely enlarged due to leukemia or lymphoma, the transverse section can be trimmed on one side to allow placement on the slide.

q. A single transverse section at the midpoint of the testes is required.

r. The epididymis is to be removed from the right testis, bisected along the midsagittal plane and include the head, body, and tail (Figure 4). The sample can be cut at the midpoint of the body to embed in two pieces to allow convenient placement on the slide. For the left testis, a cross section shall be taken, with the epididymis still attached.

Figure 4. Profile of required section of epididymis (as bisected through the sagittal plane) including head (A) and tail (B).

s. Make a mid-transverse section (approximately 3 mm in thickness) to include dorsal, lateral and ventral lobes of the prostate and ampullary gland. The cutting-line shall include a border between ventral prostate and urinary bladder at the mid-sagittal area, in order to obtain the ampullary gland, and this section is to be embedded with the anterior surface down (Figure 5). Make a mid-transverse section (approximately 3 mm in thickness) of seminal vesicle and coagulating gland bilaterally. The cutting-line should be about midway between the anterior and posterior ends.

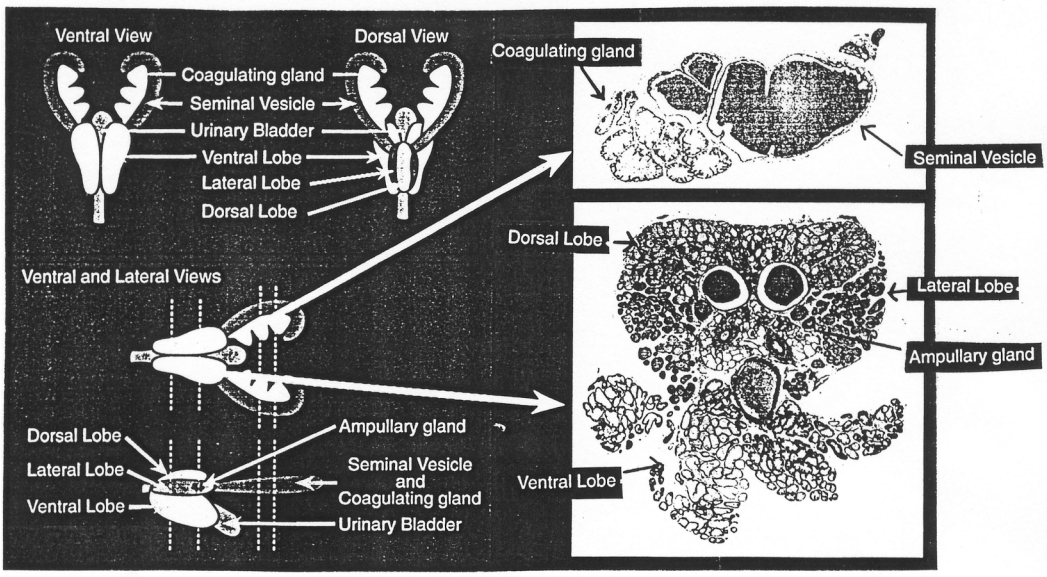

Figure 5. Trimming for histology of prostate and seminal vesicle.

t. A transverse section through each uterine horn approximately 0.5 cm from the cervix/body of the uterus is required.

u. The distal 1.5 cm (minimum length for rat) or 1 cm (minimum length for mouse) of the femur must be sectioned through the frontal plane to include the articular cartilage and articular surface, the femoral condyles with epiphyseal plate, and diaphysis with bone marrow. Sections of bone must include the joint surface and marrow to be considered complete.

v. The eyes are to be embedded intact, in a block by themselves, and shall be step-sectioned until a section includes both the lens and optic nerve. The harderian glands are to be embedded in a separate block, either alone or with other tissues of similar texture, e.g., adrenal glands.

5. Tissues must be trimmed to a maximum thickness of 0.5 cm for processing. Small (less than 0.4 cm) endocrine organs, lymph nodes, and tissue masses may be submitted intact. One cross section shall be prepared from each thyroid (2 lobes per animal), adrenal (2), pituitary (1), ovary (2), and testis (2). The thyroid lobes can be trimmed *in-situ* along with the trachea. Left testis shall include the epididymis; right testis shall be trimmed to remove the epididymis.

6. All residual tissues from all animals shall be double bagged in 10% buffered formalin following trimming. The animal identification label shall be included with the tissues for subchronic and chronic study animals.

D. HISTOLOGY

1. A unique histology number shall be assigned to each animal that is to receive histologic work-up. At the time of assignment, this number shall be entered in a permanent log and cross-referenced to the TDMS animal identification number.

   a. This histology number shall appear on the label placed on the tissue block, on the slide, and on the label between the two bags containing the wet tissues. This label shall also show the group and animal number.

   b. The format that shall be used for labeling slides (tissue slides, blood smears, cytology smears, etc) is shown below. Each block and slide shall be sub-numbered from 1,2,3.....n to show which number that block/slide is for that animal.

   c. The label on the paraffin tissue block and the label between the tissue bags shall not have the contractor's name shown. Instead, the NTP designated letter code (acronym) for the laboratory will precede the histology number.

2. All trimmed tissues shall be processed (dehydrated and infiltrated with paraffin) using appropriate chemicals, by an automatic tissue processor, sectioned and stained as indicated in the following sections.

FORMAT FOR SLIDE LABELS

Line l: Laboratory acronym/Pathology subcontractor acronym (if appropriate)/NTP [Acronyms will be supplied by the Project Officer]
Line 2: 7 Digits of TDMS Number = Experiment (5) - Test (2)
Line 3: Treatment/treatment group designation and individual animal number
Line 4: Histology number - Slide number

Treatment/Treatment Group Designations

The treatment/dose group designation will consist of two letters. The first letter will represent the treatment/dose group and the second letter will be M for male or F for female. The letter designations for treatment/dose group are as follows:

Prechronic Studies / Transgenic Studies

X = Untreated, Chamber or Vehicle 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 = High Dose/Exposure Concentration (study with 6 treated groups)
G = High Dose/Exposure Concentration (study with 7 treated groups)
P = Positive Control

Chronic Studies

U = Untreated or Chamber Control
V = Vehicle Control
L = Low Dose/Exposure Concentration
I = Intermediate Dose/Exposure Concentration (studies with 4 treated groups)
J = Low Intermediate Dose/Exposure Concentration (study with 6 treated groups)
K = Low Intermediate Dose/Exposure Concentration (study with 8 treated groups)
M = Medium Dose/Exposure Concentration
N = High Intermediate Dose/Exposure Concentration (study with 5 treated groups)
O = High Intermediate Dose/Exposure Concentration (study with 7 treated groups)
H = High Dose/Exposure Concentration

Examples:

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)

Sample Slide Label:

NE/NTP or NE/PI/NTP
05921-01
UF048
881750-9

3. After tissues are processed, they shall be embedded in paraffin blocks.

4. Tissues shall be cut 4-6 microns in thinness. Blocks shall be resealed using a warm spatula to melt the surface wax. Blocks shall be clearly identified with the designated letter code, plus the laboratory's histology number and sub-numbered from 1 through n to indicate the block number for that animal.

5. Tissue slides shall be stained with hematoxylin and eosin, after which they shall be cover-slipped, with glass cover-slips. Each slide shall be permanently paper labeled, or labeled using an approved alternate method.

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

7. Slides shall be compared to 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.

8. A Histology Processing Record shall be completed for each animal for which histology slides are prepared, and shall be submitted to the NTP with the Individual Animal Necropsy Record. The Histology Processing Record shall include, but is not limited to, the following information:

    a. Header information to include test article, histology accession number, species and sex.

    b. List of tissues trimmed, number of cassettes prepared and verification by trimming technician with initials and date.

    c. List of tissues embedded, number of paraffin blocks prepared and verification by embedding technician with initials and date.

    d. Number of blocks sectioned, number of slides prepared and verification by microtomy technician with initials and date.

    e. Number of slides stained and cover-slipped with verification by technician with initials and date.

    f. Number of slides checked out during quality control procedure and verification by technician with initials and date.

    g. Number of block re-cuts and/or wet tissue re-cuts and verification by technician with initials and date.

    h. Notes documenting deviations from protocol, missing tissues, missing gross lesions, problems, and/or comments.

    i. Signature of histology laboratory supervisor indicating review and approval of Histology Processing Record.

E. HISTOPATHOLOGIC EVALUATION

1. One Pathologist shall examine microscopically and diagnose all tissues including controls of one animal species for that test article. It is preferable that the same Pathologist conduct the histopathology on all phases of the studies and for both species when possible.

2. Microscopic evaluation by the Pathologist shall be performed on the following:

   a. Repeated Dose Studies

      Histopathologic evaluation is generally conducted on **all organs showing evidence of treatment-related gross lesions plus corresponding organs from control animals**.

   b. Subchronic Studies

      A complete histopathologic evaluation inclusive of treatment-related gross lesions shall be done on all early death animals regardless of dose group, all control animals, and all animals in the highest dose group with at least 60% survivors at the time of sacrifice plus all animals in higher dose groups. **Treatment-related lesions (target organs) shall be identified and these organs plus gross lesions shall be examined to a no-effect level.**

   c. Chronic Studies

      Complete histology (making of stained slides) and histopathologic evaluation may be required on early deaths or moribund sacrifices within 30 days after notification, when there is unexpected mortality and such action is deemed necessary. This decision shall be made by the Project Officer in consultation with the Principal Investigator. Generally, histopathologic evaluation can be postponed to the end of the study, unless the NTP requests otherwise. It must be noted, however, that tissues must be trimmed no later than 6 months post necropsy. **A complete histopathologic evaluation inclusive of gross lesions shall be done on all animals in the control group and all treatment groups in chronic studies.**

3. Complete histopathologic evaluation is defined as histologic evaluation of the tissues listed below:

TISSUES FOR COMPLETE HISTOPATHOLOGIC EVALUATION

Adrenal glands
Brain (3 sections including frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons)
Clitoral glands
Esophagus
Eyes
Femur, including diaphysis with marrow cavity and epiphysis (femoral condyle with epiphyseal cartilage plate, articular cartilage and articular surface)
Gallbladder *(mouse)*
Gross lesions
Harderian glands
Heart and aorta
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Kidneys
Larynx *(inhalation studies)*
Liver (2 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 with adjacent skin
Muscle *(if neuromuscular signs were present)*
Nasal cavity and nasal turbinates (3 sections)
Ovaries
Pancreas
Parathyroid glands
Pituitary gland
Preputial glands
Prostate
Salivary glands
Seminal vesicles
Skin *(dermal studies)*
Spinal cord and sciatic nerve *(if neurologic signs were present)*
Spleen
Stomach (forestomach and glandular)
Testes with epididymides
Thymus
Thyroid gland
Tissue masses
Trachea
Urinary bladder
Uterus

F. RECORDING OF HISTOPATHOLOGY RESULTS

All pathological findings for each animal shall be entered into the Toxicology Data Management System (TDMS) which utilizes a combination of manual forms and computer terminals to capture raw data generated during prechronic and chronic studies. See discussion of TDMS (Section III.A.) for more details.

1. The IANR shall be used to record necropsy observations. Descriptive narratives at necropsy shall be provided for all animals on the IANR. The number as well as description of tissue masses shall be included (see Appendix 4).

   a. The IANR shall be used to record the findings of the person performing the necropsy. At the completion of the necropsy, page 1 (and page 2 if used) of the IANRs shall be signed and dated by the necropsy prosector and the attending Pathologist.

b. IANRs containing necropsy description shall be available to the technician during the trimming process. Any additional gross observations shall be recorded during the trimming procedure, with a separate signature and date for comments added by the trimming technician.

c. For dermal studies, skin diagnoses for samples of different topography shall be clearly separated, e.g., treated skin or control skin.

2. The Pathologist or data clerk shall use the computer terminal to record all microscopic findings in prechronic and chronic studies.

a. Histopathologic diagnosis of all lesions shall be entered under Organ and Diagnosis. Indicate primary versus metastatic tumors, e.g., 1) liver hepatocellular carcinoma; and 2) lung, hepatocellular carcinoma, metastatic. Use the NTP terminology in the Pathology Code Tables (PCT) supplied by the NTP.

b. Designated non-neoplastic lesions shall be graded using a four-grade system of minimal, mild, moderate, and marked. The TDMS PCT shall be used for NTP approved nomenclature.

G. QUALITY CONTROL OF PATHOLOGY ACTIVITIES AND DATA

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

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, shrinkage, etc. Histology records for all animals shall be audited to ensure that all protocol-required tissues and gross lesions have been sectioned or otherwise accounted for, and that sections and slides have been prepared according to NTP guidelines.

2. Residual Wet Tissues

After all slides have been prepared (e.g. all study protocol required tissues 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 ten percent (10%) random sample of animals of each treatment group shall be examined according to the following guidelines:

a. 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 nodules/masses that are potential tumors. 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, the determination that small nodules may be tumors 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. That is why a Pathologist must confirm the presence of untrimmed potential tumors

before animals are sampled or additional histologic sections are performed. All potential tumors found as a result of this quality control review shall be trimmed and sections prepared for microscopic examination by the designated study Pathologist.

b. 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 to 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 NTP Project Officer and an attempt shall be made to resolve the discrepancies, but bags shall not be relabeled.

3. The completed IANR forms for all animals shall be reviewed for thoroughness of completion, documentation consistency, conformance to NTP Specifications, and for correlation of gross observations with microscopic diagnoses and agreement with TDMS. 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 laboratory QA Unit and subsequent submission to the NTP.

4. If computer entry of histopathology is performed from an audio-recording, all entries must be confirmed by a second person (the second person can be the study Pathologist). A record of the primary entry shall be retained and confirmation of its accurate transcription shall be made on the IANR forms. Similarly, if computer entry is performed from a written worksheet, the worksheet shall be retained within the study records and confirmation of its accurate transcription shall also be documented on the IANR forms.

5. The TDMS histopathology reports must be reviewed within the laboratory to confirm the correct header information, the correct selection of protocol required tissues, and the pathology data entry.

6. 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 tumors or non-tumor 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, then diagnoses must be changed to consolidate the data in an appropriate manner.

H. SUBMISSION OF PATHOLOGY DATA

1. Repeated Dose Studies

For repeated dose studies, slides, blocks, slide inventory, IANRs, Histology Processing Records, and if applicable, blood smears, reticulocyte preparations and bone marrow preparations, shall be submitted to the Archives at the time the subchronic report and materials are submitted. If no subchronic study is to follow, then these materials are to be submitted at the time the repeated dose study report is submitted. Note: Disposition of wet tissues from repeated dose studies is described in Section VII.I.2.a.

2. Subchronic Studies

   For subchronic studies, the pathology submission to the NTP Archives shall consist of: the histology slides, blocks, wet tissues, hematology slides (if blood smears or bone marrow smears are prepared), slide inventory, Histology Processing Records, IANRs, and notification that the pathology is complete and request for a lock-date (data transmittted to mainframe computer at NIEHS).

3. Chronic Studies

   Two copies of the IANRs for animals that die or are sacrificed during the chronic study (early death animals) must be submitted to the NTP Contract Coordinator on the first and fifteenth day of each month. The original shall be retained until the end of the study and submitted with the records for the remainder of the animals in the chronic study. For chronic studies, the pathology submission to the NTP Archives shall consist of: the original IANRs, slides, blocks, wet tissues, slide inventory, the Histology Processing Records, and notification that the pathology evaluation is complete and request for a lock-date (data transmitted to mainframe computer at NIEHS).

I. SUBMISSION OF HISTOPATHOLOGY MATERIALS (SLIDES, BLOCKS AND WET TISSUES)

1. All slides, blocks, wet tissues, stained cover-slipped blood smear slides, reticulocyte preparations and bone marrow preparations (if required by the protocol) of 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.

   a. 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 shown on this form.

   b. 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.

   c. 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.

   d. A letter of intent to ship showing how many boxes of what kind of histopathology material(s) and on what date(s) shall be directed to the NTP Archives with a copy to the Project Officer and Pathology Coordinator at least 7 calendar days in advance of shipment. This is required to aid in tracing lost or misdirected shipments.

2. Wet Tissues

   a. For 90-day and 2-year studies, all residual 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. For 14-day studies, wet tissues are to be retained at the laboratory until submission of the 90-day report (if a 90-day study is to be performed), at which time the Project Officer is to be contacted for disposition of these tissues. (Note: It is anticipated that in most cases the wet

tissues shall be disposed of at the laboratory once the 90-day study report has been received and reviewed.) If there is no follow-on 90-day study to be performed, the decision regarding disposition of the wet tissues shall be made following submission and review of the 14-day study report.

b. Wet tissues (residual from harvested tissues) shall be stored in two separately sealed plastic bags, each 4.5 mils thick, one inside the other so that there is no leakage, and organized by sex, species, group, and animal number. A permanent ink label (not ballpoint pen) shall be placed between the two bags showing the study number, laboratory, group number, and animal number. 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 Archives. Once the bags are organized, they shall be packed in two layers, separated by a piece of cardboard, within double-wall cardboard boxes (350 lb.-test/51ECT) approximately 15" x 18" x 7.5 " with a plastic liner in each box. The boxes shall be marked on one end to show:

Name of contractor
Contract Number
Experiment number (C #)
Animal group number(s)
Histology numbers in that box

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. An 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 contractor, 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 prechronic or chronic report or when otherwise specified. Special handling procedures may be required in extreme weather conditions.

3. Blocks shall be resealed with a warm spatula and organized by histology number. Blocks shall be labeled or permanently marked with a laboratory's letter code and the histology number. When histopathology is complete and the residual material, including blocks and slides prepared during prechronic and chronic evaluations, are to be prepared for shipment to the Archives, blocks shall be placed in animal order by treatment group into single-wall cardboard boxes the size of approximately eighty blocks. Rows of blocks shall be separated by dividers, in case of partial boxes, spacers will be used to maintain the order of the blocks, and then these smaller boxes (7.5" x 9" x 1.75") shall be taped, and placed into double-wall cardboard containers (350 lb.-test/51ECT) approximately 15" x 18" x 7.5". 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.

4. All slides (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 and uncover-slipped blood smears shall not be shipped to the Archives.

a. 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" x 18" x 7.5", separated by abundant packing material, for shipment to the NTP Archives. An Inventory Listing shall accompany the shipment. (The listing produced by TDMS shall be used for those studies on TDMS.). 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.

b. Each plastic shipping box shall be marked to show the phase of the study, the treatment, and treatment group/animal numbers and the name of the study laboratory.

c. Each cardboard box shall contain a packing list identifying the name of the contractor, 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.

5. Supplies for the shipment of residual material to the Archives shall be procured by each testing laboratory.

J. RELEASE OF SLIDES

1. Histologic slides prepared routinely to support these studies shall not leave the contractor's facility without the specific permission of the NTP. 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 contractor's slide file.

2. If it is desired to use sample tissues from these studies for workshops or other purposes where the slides would have to leave the facility, the study laboratory shall first obtain permission from the NTP Project Officer. After permission is obtained the laboratory shall prepare a separate set of slides and label them in the prescribed manner adding the words "Study Set". Since diagnosis from slides such as these are not recorded on TDMS, the slides shall not be shipped to the Archives upon completion of the study.

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VIII. QUALITY ASSURANCE

A. GOOD LABORATORY PRACTICE (GLP) REQUIREMENTS

1. The NTP requires that studies be conducted in compliance with FDA GLP regulations as specified in Part 58 "Good Laboratory Practices for Non-clinical Laboratory Studies" (Federal Register, Friday, December 22, 1978, Part II and any later interpretations published by FDA). There are areas where NTP has specific requirements that extend or are slightly different from the FDA requirements. In these areas, the laboratory is expected to provide NTP with those requirements as described in these Specifications. For example, the NTP requires separate protocols and separate reports for each species per NTP study.

2. There are NTP studies for which the EPA GLP Standards are more appropriate than the FDA GLP Regulations. To meet the need for compliance, it may be necessary to conduct NTP studies in compliance with the requirements of EPA as well as FDA and NTP. (The EPA, "Toxic Substances Control; Good Laboratory Practice Standards; Final Rule", Federal Register, Tuesday, November 29, 1983, Part III.)

3. The review and revision of Standard Operating Procedures (SOPs) are a continuing process. Along with the protocol, SOPs are considered essential to the successful conduct, documentation, inspection, and auditing of a study. For this reason, all SOPs must be reviewed at least bi-annually. New or revised SOPs need to be prepared, reviewed and approved before they are implemented.

4. A laboratory can expect to have Quality Assurance (QA) monitoring site visits periodically. During the visit, NTP QA staff will evaluate the organization and function of the laboratory's Quality Assurance unit (QAU). Additional follow-up visits will be made, if needed. A team of two to four QA professionals (support contract and NTP staff) may also visit a laboratory to audit and inspect representative ongoing studies.

B. THE QAU

1. If a laboratory or facility has multiple QAUs, based on the division of operation [e.g. Biology, Chemistry, subcontract (as for pathology), or other disciplines], that QAU serving the basic animal studies shall be responsible for assessing the quality of all aspects of the studies. Whether or not SOPs are prepared specific to the NTP program is to be determined by laboratory management. In either case, SOPs used in connection with each NTP study shall be maintained as NTP records for archiving purposes. These SOPs are subject to review by NTP personnel as to interpretation of what is to be done.

2. The QAU for the laboratory shall develop and maintain specific QA SOPs. These SOPs are to cover QA activities that are repetitive in nature and deal with the independent conduct of inspections, audits, and related activities associated with NTP studies performed by the contract laboratory. An historical file of these SOPs and all revisions thereof, including the dates of such revisions, shall be maintained.

3. The laboratory shall maintain a file of QAU reports (inspections, audits, periodic status) and responses to them in connection with NTP studies. As the sponsor for the studies, NTP management, QA personnel, and the Project Officer shall have access to the file for review purposes. The confidential, proprietary, and pre-decisional information contained in this file shall not be divulged by NTP reviewers. The file shall not be revealed to any

outside parties, including QA support contractors for the NTP or GLP compliance inspectors for the FDA or EPA. When a study is completed, the QAU reports file shall **not** be part of the study record and shall **not** be submitted to the NTP Archives.

C. AUDITS AND INSPECTIONS

Phases are to be inspected and resulting data audited according to frequencies based on the nature of the data collected, the extent of quality control (QC) review, and the potential impact of errors. These factors and the monitoring frequency are to be considered and established in concert with the critical staff and the laboratory's management. The schedule of inspections and audits reflects a dynamic process and will be influenced by a variety of factors such as previous findings, follow-up activities, workload and quality issues expressed by management, inspectional findings from outside reviewers, etc. The following sections outline the approach that shall be used for organizing inspections and audits of NTP studies by the laboratory QAU.

1. Prestart activities conducted during phase 1 are to be inspected and data/reports audited. These include, but are not limited to: receipt of test article; storage and handling of test article; purity and identity of test article; method performance evaluation; SOPs; prestart reports for chemistry, inhalation, toxicokinetics; special proficiency studies, etc.

2. The study protocol and amendments must be reviewed by the laboratory's QAU for compliance with contractual requirements, the NTP Specifications, and GLP regulations. Furthermore, the laboratory's QA officer is required to sign and date the study protocol prior to the initiation of the study to document that the study protocol has been received and reviewed by the laboratory's QAU.

3. Those phases of a study that occur only once and include procedures or conditions that can be directly observed are to be inspected at the time they occur and resulting data audited. Examples of such phases include animal quarantine, randomization, and individual identification; release to study, study start; clinical pathology; special study events and; scheduled necropsy; tissue trimming, and slide preparation. If a once-only phase undergoes a formal and documented QC review by technical staff, the QAU may limit its responsibility to inspecting the procedures of the quality control review for that phase.

4. Repetitive or routine procedures that impact the generation, collection, and handling of study data need to be subjected to inspection and audit on a periodic basis for each study. A few examples of such procedures include the control of environmental conditions; dose formulation, analysis, and administration; individual animal weights, feed or water consumption, clinical signs, and survival; animal husbandry practices; necropsy and processing of unscheduled-death animals; and documentation practices and error corrections.

5. All data and statements of fact included in study reports submitted to the NTP are to be audited by the laboratory's QAU, with the exception of the Monthly Progress Reports and preliminary data submissions that are defined in each contract.

I X. GENERAL STUDY PROTOCOLS

The design of these studies may be varied as needed and may encompass details not specified in this specifications. Routes of exposure will typically include dosed feed, dosed water, gavage, dermal application or inhalation. Parenteral routes, such as intra-peritoneal, subcutaneous, intravenous, etc. may be required for some studies. Specific requirements will be outlined in the individual test article SOWs.

A. REPEATED DOSE STUDY

1. The doses for the repeated dose study will be provided in the individual SOWs. Five fractional doses plus an untreated or vehicle control group will be initially tested. Vehicle controls instead of untreated controls are used for gavage and dermal studies.

2. The animals to be used shall be five to six weeks of age at the time of release from quarantine and start of this study. Five animals per dose group of both sexes and both species will usually be required for the repeated dose studies. All animals shall be uniquely, consecutively numbered.

Animals		Sex		Species		Dose Groups		Total
5	x	2	x	2	x	6 *	=	120

* Includes 5 treatment groups plus an untreated or vehicle control group

Animals shall be caged separately by sex, by dose group, and by species. Rats and female mice shall be housed five animals per cage except for inhalation and dermal studies where they shall be individually housed. Male mice shall be individually housed for all studies.

3. For dosed feed and dosed water treatments, animals shall be treated daily with the test article for fourteen continuous calendar days after which they will be sacrificed. For inhalation, gavage, and dermal studies, the schedule shall be twelve treatment days, not including weekends or holidays, with five consecutive treatment days at the beginning of the study and at least two consecutive treatment days immediately prior to terminal sacrifice day. Treatment days missed due to holidays shall be made up before sacrifice. Inhalation exposure shall be six hours $\pm$ T90 per dose day. For inhalation studies food shall be removed during exposures but water shall be supplied *ad libitum* at all times.

4. Individual animal body weights shall be determined on all animals on day one on study prior to initial treatment, weekly and at necropsy.

5. Food and water consumption shall be measured weekly (for a 7 day period) for control and treated animals for test articles in feed or drinking water.

6. Animals shall be observed two times daily (once in the early morning and once in the late afternoon at least six hours apart, and no later than 10:00 AM in the morning and no earlier than 2:00 PM in the afternoon, including holidays and weekends) for moribundity and death. Formal clinical signs shall be recorded daily by animal number. Animals whose condition makes it unlikely that they will survive until the next observation, based upon criteria established by the Laboratory Animal Veterinarian in concert with the Study

Director, shall be sacrificed immediately, necropsied, and tissues retained in formalin for possible histopathologic evaluation.

7. At terminal sacrifice, complete necropsies shall be performed on all animals. Histopathologic evaluation shall be performed in accordance with the histopathology requirements in Section VII.E. Generally, histopathologic evaluation is conducted on all organs showing evidence of gross lesions, plus corresponding organs from control animals.

8. Organ weights are generally taken from all animals surviving to the end of the study. (See Section VII.B.5.).

9. Data from the in-life and pathology phase of the repete dose study shall be entered on TDMS.

B. SUBCHRONIC TOXICITY STUDY

1. The purpose of the subchronic toxicity study is to determine the toxic effects of the test article and to estimate the high dose for each sex of each strain and species to be tested in the chronic toxicity study. Administration of the test article shall be by the same route to be used in the chronic carcinogenicity study.

2. Five dose groups (plus a control group) will be selected from results of the repeated dose study or based on other available data.

3. The animals to be used shall be five to six weeks of age at the time of release from quarantine and at start of this study. Ten animals of both sexes and both species per dose group shall be required. All animals shall be uniquely, consecutively numbered. Vehicle controls shall be used for gavage or dermal routes of administration; untreated controls shall be used for dosed feed and dosed water subchronic studies. For inhalation studies, controls shall be exposed to filtered air unless otherwise specified in the protocol. Thus, the total subchronic animals required are:

Animals		Sex		Species		Dose Groups		Total
10	x	2	x	2	x	6 *	=	240

* Includes 5 treatment groups plus an untreated or vehicle control group

4. Rats and female mice shall be caged separately by sex, by dose group, and by species, five animals per cage except for inhalation and dermal studies where animals shall be housed individually. Male mice shall be individually housed in all studies by all routes.

5. Animals shall be treated with the test article over a period of at least ninety, but for not more than ninety-seven days (or as specified in the individual SOW for the test article), according to the treatment regimen for that specific test article, after which they shall be sacrificed. Dosed-feed or dosed-water shall be provided 7 days/week *ad libitum* until the animals are sacrificed; inhalation, dermal and gavage administration shall be five times per week (weekdays only and exclusive of holidays) unless otherwise specified. All animals in inhalation, dermal, or gavage studies shall have at least two consecutive treatment days immediately prior to terminal sacrifice day. No treatment is required for these routes on the day of necropsy, unless otherwise specified. The daily inhalation exposure time shall be six hours plus T90. Feed and water shall be provided *ad libitum*

unless otherwise specified. During inhalation studies, food shall be removed during the exposure period.

6. Individual animal weights shall be recorded on day one on study prior to initial treatment, weekly and at necropsy.

7. Food and water consumption shall be measured weekly (for a 7-day period) for control and treated animals for test articles tested in dosed feed or drinking water.

8. Animals shall be observed two times daily (once in the early morning and once in the late afternoon at least six hours apart, and no later than 10:00 AM in the morning and no earlier than 2:00 PM in the afternoon, including holidays and weekends) for moribundity/mortality. Animals whose condition makes it unlikely that they will survive until the next observation, based upon criteria established by the Laboratory Animal Veterinarian and Study Director, shall be sacrificed immediately, necropsied, and evaluated histopathologically. Animals shall be examined for detailed clinical signs weekly, unless otherwise specified. The Study Director, in consultation with the DL for Toxicology, Laboratory Animal Veterinarian, or Pathologist shall periodically visit animal rooms and examine animals to confirm, correct, or expand the clinical observations made by technicians. This shall be done as often as necessary but at least once every two weeks, preferably once a week.

9. An animal survival report shall be submitted on the first and fifteenth day of each month for all test articles administered by gavage.

10. Animals shall be dosed for two consecutive days before necropsy. In the event that all of the animals cannot be sacrificed within the specified time limits, the NTP Project Officer must be notified at least fourteen days prior to the end of the subchronic study. Histopathology shall be performed in accordance with the histopathology requirements indicated in Section VII.E. Generally, complete histopathologic evaluation of all required tissues is conducted on all early death animals regardless of treatment group, on all control animals and all animals in the highest dose group with at least 60% survival at the time of sacrifice, plus all animals in higher dose groups. Treatment-related lesions (target organs) are identified and these organs plus gross lesions are examined in lower doses to a no-effect level .

11. Organ weights shall be determined at necropsy for all animals surviving until the end of the study. (See Section VII.B.5.)

12. Ten serum samples from rats and ten from mice shall be collected for the Animal Disease Screening Program from the untreated controls or from the vehicle controls at scheduled sacrifice of each subchronic study.

13. Special studies

    Specific toxicologic parameters such as hematology, clinical chemistry, urinalysis, SMCVC, blood collection for micronuclei evaluation, neurobehavioral studies shall be evaluated as specified in the individual test article SOW. Such evaluations may require separate groups of special study animals treated at the same time and in the same manner as the core study animals and shall be so stipulated in the SOW.

    For SMCVC procedures see Appendix 5.

14. Data from the in-life and pathology phases of the subchronic study shall be entered on TDMS.

C. CHRONIC TOXICITY AND CARCINOGENICITY STUDY

The design of these studies may be varied as needed and may encompass details not described in the general specifications. Details for each test article shall be provided in the individual SOWs.

1. This study is used to determine the chronic toxicity and carcinogenic potential of a test article in animals.

   The high dose will be estimated by the NTP from the subchronic toxicity study and the lower doses will be a fraction of it. In those instances in which the test article is administered in the diet, the high dose shall not exceed 5% (50,000 ppm) of the diet.

2. Animals to be administered test article in feed or water shall be provided dosed feed or dosed water *ad libitum*. For inhalation studies, food shall be removed during the exposure period; water shall be supplied *ad libitum* at all times. Animals to receive test article (including vehicle controls) by gavage, dermal or inhalation shall be treated five days per week on weekdays only, unless specified otherwise in the NTP SOW. Treatment days missed due to holidays do not have to be made up in chronic studies. Inhalation exposure time shall be six hours per day plus T90. All remaining animals shall be on the specified treatment regimen for 104 weeks, with no recovery period, unless specifically directed to do otherwise by the NTP. For dermal, gavage, inhalation and parenteral routes, no treatment is required on the day of necropsy.

   Control animals of both sexes are required for all studies. The control animals may be untreated (as for most dosed feed and dosed water studies) or vehicle controls (as for most gavage or dermal studies). The NTP may specify both untreated and vehicle controls in some studies. The type of control animals will be specified in the individual SOWs for each test article. Control animals for inhalation studies shall be in accordance with the specific SOWs.

3. The animals to be used shall be five to six weeks of age at the time of release from quarantine and start of this study. Fifty animals per dose group of both sexes and both species shall be started in each study group routinely. Three dose groups of test article plus controls shall be used routinely, although the number of dose groups and/or animals may vary and shall be specified in the individual SOWs for each test article. In some cases, there may be one or more interim sacrifices, or stop exposure groups. These shall be specified in the individual SOWs.

   The animals of each sex and species must be randomized to the study groups by a formal randomization procedure and assigned individual consecutive numbers.

   The animals required for a typical three-dose study are as follows:

Animals		Sex		Species		Dose Groups		Total
50	x	2	x	2	x	4*	=	800
15	x	2	x	2		Sentinels	=	60
								860

* Includes 3 treatment groups plus an untreated or vehicle control group

Rats and mice shall be caged separately by sex and by dose group. Male rats will be housed three per cage and female rats and female mice will be housed five per cage, except for inhalation and dermal studies where all animals shall be housed individually. Male mice shall be housed individually in all studies.

4. During the study, individual animal weights for treated and control groups shall be recorded on day one on study prior to initial treatment; weekly for the first 13 weeks; and at 4 week intervals thereafter, until the appearance of life-threatening tumors or a significant number of deaths occur in the groups, at which point weights shall be taken and recorded every two weeks. (This change shall be done only with the approval of the NTP Project Officer.) It is estimated that animals will be weighed every two weeks for the final 3 months of the chronic study. The animals shall also be weighed at necropsy.

5. For dosed feed studies, food consumption shall be measured per cage for a one-week period (7 days) every 4 weeks. For dosed water studies, water consumption shall be measured per cage for a one-week period (7 days) every 4 weeks. More frequent measurements may be required for some test articles during the first few weeks of study and will be specified in the individual protocol outlines provided. The report shall specify the inclusive dates during which measurements were made. Obvious spillage or wasting of feed shall be noted. Routine collection of food consumption data is generally not done for routes other than dosed feed.

6. Each animal shall be formally examined for clinical signs of toxicity at four-week intervals and these observations will be recorded on TDMS. Signs of toxicity detected at times other than the formal four-week observation shall be noted and recorded. If terminals are not available the data shall be collected as hard copy and subsequently entered into TDMS. The Study Director and DL for Toxicology shall review clinical observations frequently to assure that the information is properly recorded as well as used during the study and to see that they are being made consistently from one observation time to another. The Study Director, in consultation with the DL for Toxicology, Laboratory Animal Veterinarian, or Pathologist shall visit the animal rooms periodically and examine the animals to confirm, correct, or expand the clinical observations made by the technicians. This shall be done as often as necessary but at least once every two weeks, preferably once a week. Clinical observations made on an animal shall not be open-ended or have gaps during the course of study.

7. Animals shall be observed two times daily (once in the early morning and once in the late afternoon at least six hours apart, before 10:00 AM and after 2:00 PM, including holidays and weekends) for moribundity/mortality. Animals whose condition makes it unlikely that they will survive until the next observation, based upon criteria established by the Laboratory Animal Veterinarian and Study Director, shall be sacrificed immediately, and necropsied. The NTP Project Officer shall be notified immediately of perceived potential threats to the health of the colony. One copy of the IANR form, with necropsy information, for all early death animals, including sacrifices, shall be forwarded to the NTP Contract Coordinator on the first and fifteenth day of each month. The IANR must be signed by the prosector and the reviewing Pathologist and must include the probable cause of death based on gross observations.

8. An Animal Survival Report shall be submitted on the first and fifteenth day of each month during the course of the study for all test articles administered by gavage.

9. Histopathology shall be performed in accordance with Section VII.E. Generally, complete histopathologic evaluation of all required tissues is conducted on all moribund sacrificed

and early death animals, all controls and all animals in all treatment groups at terminal necropsy.

10. Data from the in-life and pathology phases of the chronic study shall be entered on TDMS.

11. For studies that include interim sacrifices:

    There shall be no recovery period prior to sacrifice of animals for a designated interim sacrifice. Animals sacrificed for interim evaluation shall not be pre-designated unless they are treated differently during the in-life phase. The NTP Project Officer must be contacted prior to an interim sacrifice for any specific instructions related to selection of animals, etc.

    Specific requirements for interim sacrifice animals shall be described in the individual SOWs and may include complete histopathologic evaluation and organ weight determination. Hematology and/or clinical pathology may also be required.

D. GENETICALLY MODIFIED MODEL STUDY

The purpose of the genetically modified model (GMM) study is to identify chemicals for toxic and carcinogenic potential using a GMM. These studies generally involve 26 or 39 weeks of exposure.

1. One or more GMM strains, i.e. Tg.AC, tp53 def, p16 INK4a, may be used and the route(s) of administration will be dependent upon the strain(s) used. Details shall be provided in the individual test article SOW. For feed or water studies, dosing shall be 7 days per week. For dermal, gavage or parenteral routes of administration, dosing shall typically be 5 days per week using a constant concentration of test article varying the volume to adjust the dosage for changes in body weight. For dermal or gavage studies the dosing volume shall be as specified in Sections V.H.2.b. or V.H.3.a. or as specified in the individual study SOW. For parenteral routes dosing volume shall not exceed 10 ml/Kg.

2. Typically fifteen to twenty-five animals of both sexes per dose and control group shall be required. Typically 3 to 5 dose groups of test article and one control dose group shall be used. Additional groups may be added, such as positive controls, when appropriate. Animals shall be housed separately by sex, by dose, and by strain. Females shall be group housed except for dermal studies where they shall be housed individually. Male mice shall be individually housed in all studies by all routes.

3. Individual animal weights shall be recorded on day one on study, after seven days and weekly thereafter, and at necropsy.

4. Food and water consumption shall be measured weekly (over a 7-day period) for control and treated animals for feed or drinking water studies.

5. Animals shall be observed two times daily (once in the early morning and once in the late afternoon at least six hours apart, and no later than 10:00 AM in the morning and no earlier than 2:00 PM in the afternoon, including holidays and weekends) for moribundity/mortality. Animals in poor health or with life-threatening lesions shall be isolated. Animals whose condition makes it unlikely they will survive until the next

observation, based upon criteria established by the Laboratory Animal Veterinarian in concert with the Study Director, shall be sacrificed immediately, necropsied, and diagnosed histopathologically when appropriate. Animals shall be examined for detailed clinical observations weekly, unless otherwise specified. The Study Director, in consultation with the DL for Toxicology, Laboratory Animal Veterinarian, or Pathologist shall visit the animal rooms periodically and examine the animals to confirm, correct, or expand the clinical observations made by the technicians. This shall be done as often as necessary but at least once every two weeks, preferably once a week. For animals treated topically, the recording and tracking of observations of the skin shall be performed weekly.

6. An animal survival report shall be submitted on the first and fifteenth day of each month for all gavage studies.

7. Animals shall be treated with the test article over a period of either 26 or 39 weeks (specific details provided in individual SOWs) according to the treatment regimen for that specific test article, after which they shall be sacrificed. All animals shall remain on the treatment regimen until the day of necropsy, except for dermal, gavage inhalation and parenteral routes, where X.no treatment is required on the day of sacrifice.

8. A complete necropsy shall be performed on all treated and control animals that either die or are sacrificed, and all tissues listed in Section VII.E.2.b.) shall be saved in formalin. Additionally, a tissue sample of the tail or ear is taken (typically at necropsy), frozen and shipped to an NTP designated laboratory for possible genotyping. Histology and histopathology requirements may differ depending upon the strain used or specific study objectives, and therefore these requirements will be described in the individual study SOWs.

9. Ten serum samples shall be collected for the Animal Disease Screening Program from the untreated or vehicle controls at scheduled sacrifice of each study. (see Section V.F.)

10. Data from the in-life and pathology phases of the study shall be entered on TDMS.

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X. DATA COLLECTION AND SUBMISSION

A. COMPUTER SYSTEM FOR DATA INPUT (TDMS)

Certain NTP study data are stored and maintained on computer files. Since the source of study information is the testing laboratory, each laboratory is required to provide data to the NTP computer system. The Toxicology Data Management System (TDMS) is the NTP computer system. The testing laboratory in-life subsystem of TDMS is called the Laboratory Data Acquisition System (LDAS) and includes animal room, histopathology and administrative components. Computer equipment, instructions, and liaison support for TDMS and LDAS shall be supplied by the NTP. Each laboratory will need to write standard operating procedures to accommodate incorporation of TDMS/LDAS into its operations.

1. Purpose

LDAS is used to collect, store, and report selected information produced from the in-life and histopathology portions of prechronic and chronic studies.

2. Organization

TDMS is maintained and operated under contract to the NIEHS. NIEHS/NTP determines the system software and hardware to be provided to the contract laboratory and is responsible for the functional capabilities of TDMS as well as for the LDAS-related procedures to be used by the laboratories. Technical instruction and support for operating LDAS are arranged for by NTP and provided to the testing laboratory by the TDMS contractor. It is the responsibility of each laboratory to organize operations so that TDMS/LDAS functions in compliance with FDA Good Laboratory Practice (GLP) guidelines. The system is validated prior to release and all changes are subject to a revalidation process before release to the laboratories.

3. Hardware

Computer workstations with the LDAS software installed are provided to the laboratory by NTP. They may be used only for TDMS unless NTP instructs otherwise. The animal room computer is a hardened version enclosed in a sealed chamber. It is on casters and has fold down shelves. The chamber can be completely wiped down after use. The testing laboratory will be expected to provide electronic balances with cables to interface to the computer. Specifications for compatible balances can be provided. At the present time each animal room workstation must serve more than one in-life study and shall be decontaminated each time it is removed from an animal room.

Pathologists who use direct entry are provided with a separate computer while they are evaluating slides for histopathologic changes. An attached desktop printer is available for Pathologists who require it. An administrative workstation is used for data tracking and transmission, and report generation. It is equipped with a high-speed printer. Data are transmitted from the laboratory to a TDMS mainframe computer located at NIEHS. TDMS reports can be requested directly from the NIEHS/NTP web-site.

4. System Components

TDMS is currently organized around four separate but interrelated components. Each of these is described in a detailed manual that is provided along with on-site technical

instruction. A brief overview is provided below.

a. In-life and Pathology Protocol

The In-life and Pathology Protocol is used to collect information about the test article (e.g. name, CAS number, treatment groups), the animals on study (e.g. species, strain, sex, supplier, number per treatment group, cage allocation to racks), and the scheduled procedures (e.g. collect body weights, feeder weights, clinical signs). The testing laboratory enters the information into TDMS one month prior to study start. After the laboratory verifies the data entered, utilizing the In-life and Pathology Protocol Verification Reports, they request a download to the administrative workstation.

b. In-life Data Collection

A disk containing the study database is inserted in an animal room workstation before the workstation is moved into the animal room. Animal body weights, food and water consumption data, clinical observations, and animal removal information are collected and stored on the disk. When the session is complete new information is transferred to the disk and taken to the administrative workstation for storage and transmission. After the data entered during the session are transferred to the administrative workstation, transaction files for transmission to the TDMS are built and the data administrator prints out the data on a transaction log and other local reports. The data are checked for completeness and validity after each session. Corrections may be made and transmitted to the TDMS at any time after data collection. Corrections are made following GLP guidelines. Data are to be transmitted to the TDMS within one workday after collection.

c. Histopathology

Observations made at necropsy and tissue trimming are recorded on the Individual Animal Necropsy Record form and are not presently entered into the computer system. Histopathologic observations are usually entered directly on a pathology workstation by a Pathologist and copied to the administrative workstation for transmission to the TDMS weekly or more frequently. Alternatively data may be recorded on forms for later entry by a clerical aid with 100% verification by the Pathologist. A standard terminology, the Pathology Code Table, is used to describe gross and microscopic lesions. Data are entered into the computer by choosing the organs, sites, morphologies, and qualifiers from a menu on the computer screen. For each study the Pathologist can store a personal dictionary of observations built from the menus and assign a two key combination to each. Then he/she just enters the key combination to record the observation for an animal. The study Pathologist can review and change diagnoses at any time during the evaluation of a study. A search and change feature is available to facilitate correction. Following NTP acceptance of the final TDMS pathology tables, the lock date is set and changes can be made only by authorized personnel.

d. Reports

Following transmission of data to the TDMS computer, a number of both in-life and pathology summary and individual data reports can be generated and printed at the testing laboratory. The laboratory is expected to periodically compare a sample of the data listed on the TDMS reports to the transaction logs to verify transmission and

processing. The TDMS tables and listings will also help the laboratory scientists to monitor the study and to prepare study reports.

5. System Security

In accordance with GLPs an operator identification code as well as the date and time of collection are associated with each data point collected. Changes to the data are made only by authorized personnel and the date, operator and reason associated with the change are recorded. As GLPs require, the original data are retained in the data base.

Access to TDMS is limited to authorized personnel. Operator IDs and passwords are assigned to individual laboratory personnel by the lab security administrator. The security administrator also determines which TDMS functions an operator may use. Access to the TDMS also requires an individual identification code and a password.

B. SUBMISSION OF REPORTS AND DATA

1. Study Reports

a. Content and Format of Reports

Reports shall be prepared according to the instructions provided in this document:

Prestart Chemistry Report	IV. G. 1.
Prestart Inhalation Report	IV. I. 1.
Prestart Toxicokinetic Study Report	IV. J. 9.
Single Administration TK Study Report	IV. J. 10.
Prechronic & Chronic Study Reports	XI. B. & C.

The content and format of reports other than those listed above are to be discussed with the Project Officer.

b. Submission of Reports

Laboratory reports (including final study reports, prestart chemistry reports, prestart inhalation reports, and toxicokinetic reports) shall be submitted electronically on a CD-R CD along with a single paper copy of each report. For some studies, the laboratory may be instructed to submit more than one hard copy of the report; this will be noted in the individual study SOWs. CDs shall be prepared as follows:

Reports are to be scanned at 300 dpi (black and white) to a single searchable PDF file. Do not use password protection or set any document property restrictions. The file is then burned to a CD.

The original CD shall be examined by the study laboratory for completeness, accuracy, and quality prior to submission to the NTP. The PDF file shall contain either a memo with signature or a digital signature from the study director indicating that the PDF is an exact copy of the report. This shall be the first page or on the first page of the PDF file.

CDs shall be submitted boxed and labeled to prevent mix-up and damage. Each CD must be labeled with appropriate title, CAS#, C#, TDMS# (if applicable), and laboratory name.

Should the report require amendment then the report and an updated CD with all revisions are to be submitted to the NTP.

2. Study Data

a. Submission of Data to the NTP Archives

1) **All original** source documentation is the property of the NTP and shall be sent to the NTP Archives. Only that correspondence related to the technical conduct of a study shall be included. Site visit and Annual Program Review reports and responses to action items shall not be submitted to the NTP Archives.

2) Inventory of the records sent shall be recorded on the NTP Archives Inventory/Index lists as shown on succeeding pages. The Inventory/Index lists shall be prepared by the study laboratory and shall accompany the records to the NTP Archives. Each Section in the NTP Archives Inventory/Index and subpart thereof shall be separated by a stiff paper or cardboard divider or shall be contained in a file folder. A tab affixed to the divider or a label on the file folder shall identify the section and subpart. The laboratory shall provide the folders or dividers and boxes for the records they submit.

3)`The records pertaining to a study shall be sent after the pathology materials are sent to the archives. If records from a study are shipped in parts at different times, Inventory/Index lists shall accompany each shipment. The Archives clerk will prepare and maintain a master copy of the Inventory/Index for this study to show all receipts of records from the contractor laboratory, computer data forms, or other sources.

4) Pathology materials (slides, blocks, tissues) shall be sent to the NTP Archives as specified in Section VII.I. and in study specific SOWs under Milestones and Deliverables. Pathology materials shall not be placed in the same box with original study documents.

5) A copy of **all final reports** reviewed by the laboratory's QAU and submitted to the NTP for a study shall be submitted to the NTP Archives. Amendments to reports must also be submitted to the Archives.

6) A copy of each SOP (including revisions) in effect and used during the conduct of each study shall be submitted. (Electronic copies rather than hard copies are acceptable if the laboratory prefers.)

7) Archival materials shall be appropriately boxed and shipped prepaid to the Archives. The following Inventory/Index organization scheme will be used:

NTP Archive Inventory/Index - Archive Records for Each Study

Section I. General

Section II. Test Article Records

Section III. Study Type (to be indicated on each set of forms:

Example - Repeated Dose, Subchronic, Chronic, Transgenic Study,

Toxicokinetic Study, or Separate Special Study

NTP Archive Inventory/Index

TEST ARTICLE_____ CAS NO._____

LABORATORY_____ EXP NO. (C #) _____

ADDRESS _____ TDMS NO. _____

CONTRACT NO._____

SECTION I. - **GENERAL**

BOX # or Date Sent | Other Location or Comment | Archive Location

COLUMN HEADERS AND THEIR DESCRIPTIONS:

Box # or Date Sent
Include the box number for the current shipment in which the listed data/materials are included. If the data/materials listed were submitted in a prior shipment, indicate the date of that prior shipment.

Other Location or Comment
If data/materials listed have been included in another location, the other location is to be identified here. This space is also included for additional explanatory comments.

Archive Location
This column will be completed by NTP Archival staff when storing the listed data/materials.

	BOX # or Date Sent	Other Location or Comment	Archive Location
A. Personnel (indicate time interval & job) 1. List of Key/Critical Personnel who participated in the study 2. Identify Consultants (by name and full address; specify work performed)			
B. List of Subcontractors (Idenitfy each by name and full address; specify work performed)			
NOTE: Records which pertain to multiple areas within one section or to multiple sections and cannot be separated conveniently to fit into the filing scheme are to be filed in one section and referenced in the other section(s) to which they pertain.			

NTP Archive Inventory/Index

TEST ARTICLE_____ CAS NO._____

LABORATORY_____ EXP NO. (C #)_____

ADDRESS _____ TDMS NO._____

CONTRACT NO._____

SECTION II. - **TEST SUBSTANCE RECORDS**

STUDY TYPE(S) _____	BOX # or Date Sent	Other Location or Comment	Archive Location
A. Identity (Manufacturer, Lot(s), Date)			
B. Characterization			
C. Bulk Stability			
D. Shipment - (From NTP Analytical Contractor or Manufacturer)			
E. Receipt			
F. Storage			
G. Bulk Analyses - Identity and Purity Analyses			
H. Vehicle Analyses			
I. Test Article/Vehicle Method Validation			
J. Test Article/Vehicle 1. Dose Preparation Records 2. Dose Preparation & Room Dose Analyses 3. Homogeneity Study 4. Animal Room Dose Analyses			
K. Inventory/Use Records for Bulk Test Article			
L. Shipment of Test Article Aliquot(s) to NTP Analytical Contractor			
M. Related Correspondence			

NTP Archive Inventory/Index

TEST ARTICLE_____ CAS NO._____

LABORATORY_____ EXP NO. (C #)_____

ADDRESS_____ TDMS NO._____

CONTRACT NO._____

SECTION III - **RECORDS BY STUDY TYPE**

STUDY TYPE _____ ROUTE _____
DATE DOSING INITIATED_____
DATE NECROPSY COMPLETED_____

	BOX # or Date Sent	Other Location or Comment	Archive Location
A. NTP SOW (including modifications pertaining to the technical aspects of each study); Laboratory Approved Protocol with Amendments and Deviations			
B. Vehicle Records Brand, source, dates purchased, lot no(s). dates of use, and test article(s) for which used; for corn oil, peroxide analysis with indication of lot no(s) and dates of analysis			
C. Dosing Records 1. Dose preparation procedure(s) 2. Dose preparation log 3. Dose Analysis 4. Stability Study			
D. Diet Analysis			
E. Water Analysis			
F. Water Treatment - Commercial and In-house			
G. Animal Records – Prestudy 1. Species, Strain, Source, Age 2. Receipt 3. Conditions of Quarantine (Caging, food, water) 4. Health Examination/Clinical Signs in Quarantine 5. Release for Study 6. Disposal of Extra Animals			

NTP Archive Inventory/Index

CONTRACT NO_____ LABORATORY_____ TEST ARTICLE_____

SECTION III - **RECORDS BY STUDY TYPE**

	BOX # or Date Sent	Other Location or Comment	Archive Location
H. Animal Records - In-Life, for studies supported by TDMS 1. TDMS: In-life and Pathology Protocol Verification Reports 2. Room Location 3. Cage Type and Number per Cage 4. Randomization 5. Identity Code and Confirmation Records 6. Bedding Type, Manufacturer, Contaminants 7. Cage Filter Type and Source 8. Feed Type, Source, Lot No(s), Dates used, Contaminants 9. Type of Water System & Treatment 10. Cage Rotation 11. Rack Rotation 12. Mortality checks 13. TDMS: In-life Data a. Data Collection Forms b. Error Corrections (when applicable) and Exceptions Reports c. Dosing records for gavage and dermal studies			
I. Animal Records - In-life, for studies not supported by TDMS - Supply the records referred to in H., but maintained in study-specific laboratory notebooks and/or forms.			
J. Records Unique to Inhalation Studies 1. Chamber Design & Maintenance Information 2. Exposure System Generation and Monitoring Description, Operating Procedures, and Validation Data 3. Generator Degradation Results 4. Chamber Concentration versus Time Plots 5. Chamber Degradation Results (without and with animals) a. Pre-study, Developmental Data and Results b. In-life Study Data and Results			

NTP Archive Inventory/Index

CONTRACT NO_____ LABORATORY_____ TEST ARTICLE_____

SECTION III - **RECORDS BY STUDY TYPE**

	BOX # or Date Sent	Other Location or Comment	Archive Location
6. Chamber Atmosphere Homogeneity Results (without and with animals) a. Pre-study, Developmental Data and Results b. In-life Study Data and Results 7. Particle/Aerosol Measurements (without and with animals) a. Pre-study, Developmental Data and Results b. In-life Study Data and Results 8. Generation and Monitoring Equipment Maintenance and Calibration Records 9. Chamber Residual Concentration/ Over-night Monitoring Results 10. Daily Chamber Exposure Concentration Data 11. The Pre-study Developmental Data and In-life Study Data are to be organized and indexed separately			
K. Animal Records - Pathology 1. IANRs 2. TDMS slide, block inventory 3. Necropsy Log 3. Organ Weight Records 4. Histology Processing Records and associated records			
L. Animal Room Records 1. Temperature Raw Data 2. Humidity Raw Data 3. Light Cycle/Intensity Measurements 4. Air Changes/Air Flow 5. Cleaning Agents Used			
M. Virology Screening Program Data N. Microbiological Testing Reports O. Genetic Monitoring Data			

NTP Archive Inventory/Index

CONTRACT NO. _____ LABORATORY_____ TEST ARTICLE_____...

SECTION III - **RECORDS BY STUDY TYPE**

	BOX # or Date Sent	Other Location or Comment	Archive Location
P. Special Studies			
1. Clinical Lab studies			
2. Other special studies			
Q. Final Report			
1. Introduction			
2. Materials and Methods			
3. Results			
4. Discussion			
5. Appendices			
R. SOPs in Effect for This Study Period including TDMS SOPs			
S. For All Phases Of TDMS			
1. Type of equipment and software version in use at time of study			
2. Processing Logs and All Administrative Records Relevant to Study Data Processing			
3. Hardware and Software Receipt and Maintenance Records			
T. Any Internal Computer-Generated Forms/Tables			
1. Toxicology			
2. Clinical Chemistry			
3. Analytical Chemistry			
4. Other			
U. Photographs Taken During the Study			
1. Gross Observation Documentation			
2. Microscopic Observation Documentation			
3. Other			
V. Incident (Experimental Impact) Reports			
W. Correspondence - Incidents, etc.			

b. Electronic Submission of Data

All original study documentation and reports shall be organized and submitted electronically on CD-R CDs. The usefulness of CDs depends on the quality and legibility of the files, as well as the organization of study data and documents. These qualities are the responsibility of the testing facility. The data shall be organized according to the Archive Inventory Index Forms. CDs shall be prepared as follows:

1) The CD files shall also be organized according to the Archive Inventory Index and presented in order on each CD under "View as list". Each file name shall include sufficient information to identify the specific section of the NTP Archive Inventory Index Form to which the data refer. A separate printed copy of the file names shall be included with the CD. If more than one CD is required, the list of file names by section shall clearly indicate on which CD each of the files is located. The entire Archive Inventory Index shall be included at the beginning of the first CD.

2) Portions of data that contain a large data-set may be divided into more than one file to prevent having large files. Each file shall be clearly identified as to its contents.

3) Data are to be scanned at 300 dpi (black and white) to searchable PDF files. The files are to contain document restrictions of printing and content copying or extraction only. The files are then burned to CDs.

4) All frames of data shall be positioned in the same orientation held for normal reading, i.e. portrait or landscape, whichever is appropriate for that page. Oversized pages shall be reduced or photographed to allow the data to fit a single page. Chart recorded data shall be scrolled left to right or top to bottom depending on how the script runs on the tracings.

5) When data are missing, a blank page with an explanation of the missing data shall be filed at the location of the missing data. If study data records are of such quality that they cannot be scanned, a cross reference shall be included in the file index giving the location of the raw data or records.

6) The original CD shall be examined by the study laboratory for completeness, accuracy, and quality prior to submission to the Archives. Each CD shall contain either a memo with signature or a digital signature (study director) indicating that the PDF is an exact copy of the data. This shall be the first page or on the first page of the first PDF file on each CD.

7) CDs shall be submitted boxed and labeled to prevent mix-up and damage. Each CD must be clearly labeled as to files contained on it. In addition chemical, CAS#, C# and laboratory name are to be included on each CD.

8) Once CDs are submitted to the Archives, follow-up correspondence or data changes shall be submitted to the Archives with an amended index on additional CDs.

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XI. REPORT FORMATS

A. FORMAT AND GUIDELINES FOR MONTHLY PROGRESS REPORT

Title Page

Monthly Progress Report

Reporting Period

List of Active Task Order Contract Numbers

Name and Address of Contract Laboratory

Table of Contents*

Section	Page
1. Administration	xx
2. Chemistry	xx
3. Laboratory Animal Management	xx
4. Toxicology	xx
5. Pathology	xx
6. Data Management	xx

* A table of contents shall be provided by the testing laboratory for each Monthly Progress Report. Only those topics that have information to be discussed are to be included in each Monthly Report.

Part 1. **ADMINISTRATION**

a. Status of Cost Proposals

List all change orders (a) received, but not yet equitably adjusted by signed modification, and (b) those negotiated during the past month. (Note: the Contractor shall not remove an item from this list until negotiations have been completed and the contract amount has been adjusted and reported the previous month.)

Contract Number	Change Order (Mod) Number	Purpose	Date Rec'd By Lab	Date Proposal Submitted	Proposed Amount	Mod No. for Adjustment	Date of Mod	Negotiated Amount

b. Overall Status of Each Contract

The Contractor shall provide a table as follows:

Contract Number	On Cost Target	On Schedule Target
	(yes or no)	(yes or no)

Any "no" responses concerning the cost target shall be explained. The Contractor shall indicate the projected overrun amount and the reason for it.

Any "no" responses concerning the schedule shall be explained and the need for possible contract extension shall be detailed.

c. Other Problems

The Contractor shall briefly discuss any other issues of contract administration not discussed above.

Part 2. **CHEMISTRY**

a. A brief summary of activities performed that month for each test article, i.e. bulk analysis, dose formulation, dose formulation analysis, etc. with summary of the results and discussion of results when they are out of spec.

b. A brief discussion with sufficient supporting documentation to include general problems, reason(s) for problems and approach for resolving and/or preventing them from recurring.; deviations from the contract, SOW, protocol, SOPs, and/or milestones. A brief discussion of special or unique scientific findings.

c. Test article Inventory: A listing of test articles shall be provided in each Monthly Progress Report. (**See Attachment 1**)

Part 3. **LABORATORY ANIMAL MANAGEMENT**

a. Animal Shipments

1) Provide narrative report of incidents on receipt or during quarantine. (Include name of supplier)

2) Animal Disposition - Submit a completed animal utilization report (**See Attachment 2**). If animals were used for purposes other than the one originally designated, so indicate at the bottom of the appropriate column.

b. Animal Health

1) Provide narrative report of clinical and/or pathological observations having potential animal health significance. If unique, specify chemical, test phase, species, and sex. List special examinations performed, if any, and provide results if available.

2) Provide a cumulative summary of incidence of health related clinical and/or pathological observations by chemical. The observations could include utero-ovarian infection, preputial gland infections, overgrown incisors, abscesses by region of organ, et cetera each month.

3) Provide a table of serology samples submitted since the last report for murine viral serology including 888 code number, chemical, date bled, species, number of each sex and if quarantine, terminal, sentinel, or moribund animals.

Serology Samples Submitted

Code	Study	Study phase/Status	Date	Species	Number (M/F)
888yy/yy/yy	ABC	Chronic/18 month	9/5/98	Mice	5/5
888zz/zz/zz	XYZ	90-Day/TSAC	9/15/98	Mice	5/5
888xx/xx/xx	ZZZ	Chronic/TS	9/25/98	Rats	5/5

4) Provide a cumulative summary of viral serology profiles by sampling date and age for each chemical each month, including results:

Serology Results

Rats Study/ Phase	Start Date	Test Phase (months)	PVM	Send	RCV/SDA	Parvo	KRV	H-1
ZZZ/Chronic	9/25/96	0	-	-	-	-	NT	NT
		6	-	-	-	-	NT	NT
		12	-	-	-	-	NT	NT
		TS	S	S	S	S	S	S

Mice Study/ Phase	Start Date	Test Phase (months)	PVM	Reo3	GDVII	Ectro	Send	MHV	Parvo	LCM
ABC/ Chronic	3/5/97	0	-	-	-	-	-	-	-	-
		6	-	-	-	-	-	-	-	-
		12	-	-	-	-	-	-	-	-
		18	S	S	S	S	S	S	S	S

- = negative += positive NT = Not tested S = Submitted/results pending

Any positive results, or borderline results that might require retest, are to be discussed in the text.

c. Animal Support

1) Provide narrative report of significant incidents or observations related to animal care.

2) Environmental Control

(a) Provide narrative description of observations that are at variance with NTP specifications and a statement of corrective actions taken.

(b) Provide summary table for all rooms in which test animals are located. (**See Attachment 3)**. Also provide a floor plan designating direction of airflow at each animal room entrance/exit and each air lock, with animal room numbers clearly identified.

d. NTP-2000 or other diet information

1) Provide narrative report of incidents in receipt, quality or use of diets.
2) Provide table of current feeding status as follows:

Chemical/Test Phase	Diet/Form	Lot No. and Date of Milling

e. Accreditation/Licensure

1) Describe current status of AAALAC accreditation, NIH assurance, animal care committee, USDA registration, and state and local licenses.

2) Provide date of next accreditation or licensure site visit when known and a narrative statement of the site visit report that pertains to the NTP when the information is available.

Part 4. **TOXICOLOGY**

A brief discussion with sufficient supporting documentation to include general problems and deviations from the contract, SOW, protocol, SOPs, and/or milestones. A brief discussion of relevant and significant scientific findings to include body weight effects, clinical observations, effects on food or water consumption, etc. Provide a table of all animal removals for each test article. If no animals are removed from a study, or for a treatment group within a study, then dose/removal numbers are not needed. (**See attachment 4.)**

Part 5. **PATHOLOGY**

A brief discussion with sufficient supporting documentation to include general problems and deviations from the contract, SOW, protocol, and/or SOP requirements that affects pathology data, documentation, and/or milestones. A brief discussion of relevant and significant scientific findings to include observance of or change in pathological findings, lesions, survival, chronic study interim sacrifice results, etc.

Part 6. **DATA MANAGEMENT**

A brief discussion with sufficient supporting documentation to include general problem areas relative to the NTP-supplied or in-house systems, system support contractor, hardware maintenance, report production, and deviations from the contract, SOW, protocol, and/or SOP requirements. A brief discussion of unique or special findings.

Attachment 1 Test Article Inventory Status: January 2004

Test Article	Lot/Batch#	Current Inventory	Weekly Usage	Weeks Remaining on Test	Additional Quantity of Test Article Needed for Completion	Approximate Depletion Date of Current Inventory
RAS	8266-F4	3.7 Kg	72 g	23	None	--
DCV	SDC-092179/01	1.9 Kg			Studies Completed	
FN	OF20-M/02	15 Kg [a]	125 g	104	None	
FL	Q112979/01	4.3 Kg	28 g	82	None	--
GBL	600-BLO/01	33.8 Kg	690 g	19	None	--
BEA	005-0120/03	12.2 Kg	610 g	67	30 Kg	5/04
BDCM	250-1/01	0.035 Kg	1.8 g	6	None	
PR 23	UB2158/02	222.1 Kg	11,750 g	3	None	

[a] Not repackaged (approximate figure)

Prepared by: _____ Date: 01/28/04

Attachment 2 MONTHLY ANIMAL UTILIZATION REPORT

Laboratory Identification	1. Test Article		2. Laboratory						4. Today's Date	
			3. Project Officer				3. Principal Investigator			
Animal Identification	5. Strain or Stock									
	6. Special Requirements									
	7. Supplier									
	8. Sex									
Animals Received this Month	9. Date Shipped									
	10. Date Received									
	11. Number Shipped									
	12. Number Received									
	13. Boxes Shipped									
	14. Boxes Received									
	Number Discarded on Arrival	15. Dead								
		16. Sick								
	17. Balance (No. Rec'd minus No. Discarded)									
Animals Discarded From Holding Cages During Quarantine This Month	18. Dead									
	19. Sick									
	20. Underweight									
	21. Overweight									
	22. Overage									
	23. Other									
	24 . Total Discarded									
Animals Used This Month	25 . Testing									
	26. Surplus(18+19…+24)									
	27. Total Used (25+26)									

Attachment 3 SUMMARY OF ENVIRONMENTAL CONDITIONS - - May 1, 2004 – May 31, 2004

Room #	Chemical / Phase / Species / Weeks on test	T °F Mean ± SD or TWA ± SD	T Min	T Max	Readings in Spec/ Total Readings	RH % Mean ± SD or TWA ± SD	RH Min	RH Max	Readings in Spec/ Total Readings
1	Chemical “ABC”/ C / R / 42	72 ± 1	71	76	718/720	46 ± 3	38	73	717/720
2	Chemical “XYZ”/ SC / R&M / 6	72 ± 0	71	76	719/720	47 ± 4	38	64	720/720

Attachment 4 ANIMAL REMOVAL SUMMARY - - May 1, 2004 – May 31, 2004

Room Number	Chemical / Phase Dose units	Study week Rats / Mice	Dose	Removals Current month / cumulative			
				Rats		Mice	
				Male	Female	Male	Female
5	TFE / C ppm	76/74	0 1 2 4 8	7/19 6/18 9/21 18/30 NA [a]	3/12 4/13 7/18 15/29 NA	3/8 NA 4/8 9/12 21/32	2/7 NA 3/9 7/11 17/26
10	APR / SC mg/Kg	2/2	-- [b]	--	--	--	--
11	CEB / SC ppm	10/10	0 100 200 400 800 1600	-- [c] -- -- -- 1/1 3/4	-- -- -- -- -- 1/1	-- -- -- -- -- --	-- -- -- -- -- --

Examples above:

[a] NA - No animals treated for this dose group

[b] No animals removed in this study

[c] No animals removed in this dose group

B. PRECHRONIC STUDY REPORT FORMAT

I. SUMMARY TABLE OF SIGNIFICANT TREATMENT-RELATED EFFECTS (**See Table 1.**)
(Include only effects considered treatment-related, not merely statistically significant results.)

II. INTRODUCTION

Brief statement not to exceed 2 pages on test article use, production, exposure route, known toxicity and significance of human exposure.

III. MATERIALS AND METHODS

A. CHEMISTRY

1. Test Article

a. Grade or other test article specific information
b. Supplier/manufacturer, name and address
c. Storage conditions for bulk test article
d. Lots used, giving amount received, date received and dates used for each lot
e. Test article identify and purity
(1) Provide information regarding initial bulk analysis, including name of laboratory performing the analyses, methods used, title of report from lab, and results. Clearly identify lot number(s).
(2) Provide information regarding bulk test article reanalysis, including the number of the SOP used for reanalysis.
(3) Provide a table of results of the initial and subsequent bulk reanalyses for each lot including dates and analysis results (frozen reference, bulk sample and calculation of relative purity).

2. Vehicle

a. Vehicle used (feed, water, corn oil, etc.)
b. Grade/purity/test article specific information
c. Supplier, Manufacturer/Producer, name and address
d. Table showing dates analyzed and results of purity analyses; include the number of the SOP used for purity analysis

3. Dose Formulation Procedure

a. Mixing procedure, including type of container used, duration of mixing, etc. Include the number of the SOP for dose formulation
b. Stability and homogeneity information on dose formulation (from analytical lab or performed at testing lab)
c. Storage conditions for dose formulations as appropriate for route of administration, i.e. temperature, humidity, protection from light, maximum length of time stored, etc.

4. Dose Formulation Analysis

a. Describe the analysis procedure. Include the number of the SOP used for analysis.
b. Describe the formulation and analysis schedule for both dose preparations and animal room samples.
c. Provide a table showing dates dose formulations were prepared, dates analyzed, and dates used; and analysis results (mean $\pm$ S.D.) to include analysis prior to dosing (%high/low of theoretical), and analysis after dosing for unused/stored formulations and animal room

samples (%high/low of theoretical & % of original formulation analysis). If formulations were out of spec, indicate if these were remixed or not.

B. ANIMALS AND ANIMAL HUSBANDRY

1. Animals

a. Species and strain
b. Source of animals – supplier name and address
c. Examinations to assure health of test animals
d. Quarantine period
e. Randomization procedure (to test group, rack, cage)
f. Animal identification procedure
g. Age of animals upon receipt and age and weight of animals when placed on test
h. Serologic analysis performed including dates samples were taken and results (See format for table of serology results in Monthly Report Format.)

2. Animal Husbandry

a. Describe cages, filters, racks, bedding, feeders, water bottles or automatic waterers used, including the manufacturer's name and address of each item.
b. Describe method of cleaning each item listed above, and where applicable, include the type of washer, cleaning agent, and manufacturer's name and address.
c. Provide schedule for changing cages, filters, racks, bedding, feeders, water bottles, etc.
d. Describe barrier maintenance and disease control procedures. (See Monthly Report for format).
e. Describe room sanitization and pest control procedures.
f. State temperature and humidity ranges, number of room air changes per hour, type of air filtration used, name of filters, manufacturer name and address; light cycle and type of lights used; indicate temperature/humidity excursions, including dates, ranges, number of hours involved.
g. Identify study room(s).
h. Describe number of animals per cage; relationship of control and treated group animals (including rack position); and describe rotation of cages on racks and racks within the room.
i. Identify feed used and storage conditions.
j. Provide source of water supply (city or well), any water treatment used and discuss water analyses performed.

C. STUDY DESIGN

1. Design

a. Specify route of administration and frequency of administration.
b. Indicate number of animals per treatment group, number of groups and exposure concentrations.
c. Specify duration of exposure – provide specific dates for first and last treatment.
d. Provide specific details of special studies performed, such as clinical laboratory studies, organ weights, immunotoxicity, neurobehavioral, tissue analysis. Include details of tissue/sample taken, sample collection and handling, analysis method or procedure for conducting special studies, and dates performed.
e. Identify by title all reports submitted under separate cover which are related to these studies, i.e. Prestart Chemistry Reports, Prestart Toxicokinetic Study Reports, Single Administration Toxicokinetic Study Reports, Proficiency Reports for Special Studies, etc.

2. In-life Observations and Pathological Examinations

    a. Describe in-life observations performed and frequency
    b. Specific dates for interim sacrifices and terminal necropsies
    c. Handling of moribund animals
    d. Method of euthanasia
    e. List of tissues collected at necropsy and processed for histopathological evaluation
    f. Histological processes, preservation, embedding, sectioning, staining
    g. Statistical analyses, if conducted, to include a short paragraph of methods used

D. SUMMARY TABLE

Provide a summary table to include details of experimental design and animal maintenance (**See Table 2**).

IV. RESULTS

A. Tables/Curves

1. TDMSE Animal Removal Summary by Treatment Group (E01)
2. TDMSE Survival Curves (P40). Explain all accidental deaths in text.
3. TDMSE Body Weight Curves (E03)
4. TDMSE Table of Body Weights (E04)
5. TDMSE Table for Feed or Water Consumption with Estimate of Test Article Consumed (E08) (if applicable)
6. Table of Organ Weight and Organ/body Weight Ratios (**See Table 3**)
7. Tables of Clinical Pathology Data (**See Table 4** for sample format for clinical chemistries; submit similar table for hematology)
8. Table of Treatment-related Histopathology Results (**See Table 5**)
9. Table of Other Special Toxicological Data, if appropriate

B. Text

1. Provide a summary of treatment-related clinical signs, body weight, mortality, etc.
2. Present results of special studies, including clinical pathology, tissue analysis, etc. as appropriate.
3. Describe pathology findings, including potential target tissues and any life-threatening lesions; compare pathologic findings with other toxicological effects

V. DISCUSSION AND SUMMARY

A. Provide a discussion of findings, including biological significance of the data

B. Describe any problems encountered that would impact interpretation of data

C. Provide a brief summary of salient findings

VI. QUALITY ASSURANCE UNIT STATEMENT

(**See Table 6**)

VII. APPENDICES

A. Feed

- One Sample Feed Tag and Irradiation Certificate
- Provide a table that includes lot #, date of milling, and inclusive use dates for feed.

B. Water Analysis Reports

C. Weekly Temperature and Humidity Recordings for Study Room (See Monthly Report Format for example of format)

D. Individual Animal Data for all non-TDMS collected data, i.e.:

- Clinical lab studies (**See Tables 7 & 8**)
- Organ weights, organ/body weight ratios
- Other special studies, i.e. tissue analysis, enzymatic activity, cell proliferation, etc.

E. TDMSE Table of Non-neoplastic Lesions (P18)

F. Copy of Contract SOW and Modifications

G. Copy of Laboratory Protocol and Amendments

H. Study Deviation Reports

Table 1

Summary of Significant Toxicological Effects in Male Rats
90-Day Gavage Study of Chemical "XYZ"

SAMPLE

Treatment Group (mg/Kg)	Mortality	Relative Body Weight Change	Clinical Observations	Organ Weight % Difference from Control	Clinical Pathology/ Special Studies	Histopathology
Vehicle Control	0/10	NA [a]	NE [b]	NA	NE	NE
37.5	0/10	+ 6 %	NE	NE	NE	Not performed
75	0/10	+ 2 %	NE	NE	NE	Not performed
150	0/10	-5 %	Ruffled fur 1/10	Liver + 2.5 %	NE	NE
300	1/10	- 8%	Ruffled fur 4/10 Lethargy 4/10	Liver + 12.5 % Thymus - 4.7 %	NE	Liver necrosis 3/10 Thymic atrophy 2/10
600	8/10	-22 %	Ruffled fur 10/10 Lethargy 10/10	Liver + 28.5 % Thymus - 16.5 %	+ 22% ALT + 19% SDH	Liver necrosis 7/10 Thymic atrophy 6/10

[a] NA = Not applicable
[b] NE = No effect observed

Table 2. Materials and Methods for Subchronic Studies of Fischer 344/N Rats Fed Diets Containing Chemical "XYZ"

Experimental Design	
Size of Test Group	Core study - 10 males and 10 females Special study (clinical pathology) –10 males & 10 females
Doses	0, 3125, 6250, 12500, 25000, or 50000 ppm in feed
Duration of Dosing	Core males 3/17/02 - 6/17/02 Core females 3/18/02 - 6/18/02 Special study males 3/17/02 - 4/8/02 Special study females 3/18/02 - 4/9/02
Type and Frequency of Observation	Observed twice daily for moribundity and mortality; body weights, clinical observations and food consumption, recorded every week
Special Studies Conducted	Clinical pathology on special study rats on day 5 and 23, and on core study rats at terminal sacrifice; hepatic CYP1A1 activity on special study rats at day 30; SMVCE.
Necropsy and Histological Examination	Necropsies and histology performed on controls and high dose, and read down to a no-effect level
Animals and Animal Maintenance	
Species	F344/N rats
Date Animals received	3/6/02
Animal Source	XXX Laboratories, Anywhere, USA
Time Held Before Start of Test	males 11 days; females 12 days
Age When Placed on Study	males 38 - 44 days; females 37 - 43 days
Age at Terminal Necropsy	males and females 18 - 19 weeks
Method of Sacrifice	CO_2 asphyxiation
Method of Animal Distribution	Animals of each sex randomized into cage groups, and then cages randomized to dosed and control groups by a table of random numbers
Animal Identification	Tail Tattoo
Feed	Irradiated NTP-2000 meal; ABC Inc, Nowhere, USA
Feeders	Stainless steel troughs made by XXX Roofing & Sheet Metal Co, Somewhere, USA; changed twice weekly

Table 2. Materials and Methods for Subchronic Studies of Fischer 344/N Rats Fed Diets Containing Chemical XYZ (continued)

Maximum Storage Time for Feed	120 days post milling
Storage Conditions for Feed	55-73^{o} F; 24-56% RH
Water	Automatic Watering System; XYZ, Inc, Anywhere, USA; sanitized biweekly
Animals per cage	Five
Cages	Polycarbonate cages; XYZ, Inc., Anywhere, USA; changed twice weekly
Cage filters	Spun-bonded polyester filter; XXX #24; AAA Inc., Smalltown, USA; changed biweekly
Bedding	Irradiated Hardwood Chips, XYZ, Inc. Anywhere, USA; changed twice weekly
Racks	Stainless steel; XYZ, Inc., Anywhere, USA; changed biweekly
Cage Washer	Tunnel type (4 cycles); ABC Co., Somewhere, USA
Rack Washer	Cabinet type (2 cycles); ABC Co., Somewhere, USA
Cage & Rack Washing Compound	AAA Compound; AAA Inc., Smalltown, USA.
Animal Room Environment	Actual range 69-76 ^{o} F; 2,502/2,520 readings within Spec) Actual range 24-83 % RH; 2,495/2,520 readings within Spec) 12 hours of fluorescent light per day; 10 - 12 room air changes per hour
Room Air Filter	Fiberglass roughing filter; XYZ, Co; Anywhere, USA changed biweekly
<u>Test Article Vehicle Mixture</u>	
Mixture Preparation	Weighed portion of Chemical "XYZ" and mixed with a weighed amount of NTP-2000 diet to make up selected doses. Mixture blended for 15 minutes in a YYY twin-shell V blender.
Maximum Storage Time	35 days
Storage Conditions	Stored in airtight, opaque plastic pails at room temperature (75 $\pm$ 2^{o} F), 60 % relative humidity

Table 3

Mean Organ Weights and Percent Organ-to-body Weight for Core Males
During the 90-day Gavage Toxicity Study of Chemical "XYZ" in Fischer 344 Rats.

SAMPLE

		Liver		Heart		Lung		Kidney, Right		Thymus		Testis, Right	
Treatment Groups (mg/Kg)	Terminal Body Wt (g) (n)	Absolute Wt. (g)	Relative Wt. [a]	Absolute Wt. (g)	Relative Wt.	Absolute Wt. (g)	Relative Wt.	Absolute Wt. (g)	Relative Wt.	Absolute Wt. (g)	Relative Wt.	Absolute Wt. (g)	Relative Wt.
0	354.2 ± 18.1 (10)	13.201 ± 0.906	3.725 ± 0.113	1.006 ± 0.072	0.284 ± 0.014	1.670 ± 0.227	0.471 ± 0.053	1.131 ± 0.061	0.319 ± 0.011	0.306 ± 0.057	0.086 ± 0.014	1.506 ± 0.045	0.426 ± 0.015
1000	364.6 ± 26.4 (10)	13.201 ± 0.906	3.801 ± 0.182	1.036 ± 0.101	0.284 ± 0.011	1.710 ± 0.293	0.468 ± 0.062	1.147 ± 0.108	0.315 ± 0.018	0.291 ± 0.032	0.080 ± 0.011	1.534 ± 0.078	0.421 ± 0.019
2000	351.3 ± 14.1 (10)	13.201 ± 0.906	3.712 ± 0.126	1.033 ± 0.042	0.294 ± 0.009	1.773 ± 0.182	0.505 ± 0.047	1.172 ± 0.073	0.334 ± 0.011	0.311 ± 0.036	0.088 ± 0.009	1.498 ± 0.112	0.426 ± 0.033
3000	345.4 ± 14.5 (10)	13.201 ± 0.906	3.789 ± 0.135	0.982 ± 0.079	0.284 ± 0.014	1.648 ± 0.189	0.477 ± 0.050	1.126 ± 0.061	0.326 ± 0.013	0.302 ± 0.042	0.087 ± 0.012	1.500 ± 0.055	0.435 ± 0.017
4000	353.9 ± 19.8 (10)	13.201 ± 0.906	3.850 ± 0.211	0.989 ± 0.906	0.280 ± 0.012	1.673 ± 0.205	0.472 ± 0.053	1.200 ± 0.064	**0.339 * ± 0.013**	0.310 ± 0.051	0.084 ± 0.015	1.522 ± 0.073	0.431 ± 0.023
5000	339.2 ± 24.0 (10)	13.201 ± 0.906	**3.986 ** ± 0.129**	0/988 ± 0.088	0.291 ± 0.014	1.609 ± 0.178	0.475 ± 0.036	1.185 ± 0.089	**0.350 ** ± 0.022**	0.292 ± 0.038	0.086 ± 0.009	1.395 ± 0.305	0.402 ± 0.111

[a] Relative weight = mg organ weight/g B.W. (mean ± SD)

* $p \leq 0.05$
** $p \leq 0.01$

Table 4

Ninety-Day Study of Chemical XYZ in Female Rats

Clinical Chemistry
(Mean ± S.D., N = 10)

SAMPLE

Treatment Groups (mg/Kg)	Albumin g/dl	Protein g/dl	CK u/L	Creatinine mg/dl	ALP u/L	ALT u/L	SDH u/L	Bile Acid umol/L	BUN mg/dl	GGT u/L	Glucose mg/dL
					Day 22						
0	4.4 ± 0.2	6.3 ± 0.2	268.1 ± 15.6	0.72 ± 0.04	431 ± 22	45.1 ± 5.2	20.2 ± 2.2	31.5 ± 4.3	12.3 ± 0.9	1.2 ± 0.4	139 ± 5
250	4.4 ± 0.1	6.3 ± 0.2	270.8 ± 15.1	0.73 ± 0.04	435 ± 20	48.3 ± 7.1	22.1 ± 3.1	34.2 ± 3.0	12.4 ± 0.8	1.4 ± 0.6	142 ± 7
500	4.3 ± 0.2	6.5 ± 0.2	289.4 ± 12.3	0.75 ± 0.03	442 ± 32	43.6 ± 8.9	25.0 ± 5.1	34.6 ± 2.9	13.0 ± 0.8	1.3 ± 0.5	141 ± 6
1000	4.3 ± 0.1	6.4 ± 0.1	285.2 ± 13.6	0.7 3± 0.02	425 ± 15	46.1 ± 7.1	23.2 ± 3.4	30.2 ± 4.6	12.0 ± 0.9	1.5 ± 0.5	136 ± 10
2000	4.4 ± 0.2	6.3 ± 0.1	292.6 ± 28.5	0.74 ± 0.02	446 ± 43	45.7 ± 10.3	24.4 ± 4.6	33.8 ± 4.0	11.8 ± 0.7	1.7 ± 0.7	140 ± 6
4000	4.2 ± 0.2	6.4 ± 0.2	278.8 ± 19.5	0.75 ± 0.05	442 ± 48	**72.2 ± 12.1 ****	26.1 ± 7.1	**44.1 ± 3.3 ****	12.7 ± 1.1	**3.2 ± 1.1 ***	143 ± 7

* $p \leq 0.05$
** $p \leq 0.01$

NOTE: Table is to include results for each time point samples were analyzed. Similar table is to be included for hematology parameters

Table 5 Treatment-related Lesions in Male Rats in the 90-day Study of Chemical ”XYZ”

SAMPLE

Organ/Diagnosis	Incidence of Lesions (Mean Severity Treatment Groups (ppm)					
	0	8	16	32	62	125
NOSE	**10** [a]	**NE**	**NE**	**10**	**10**	**10**
Inflammation, suppurative	0 [b]	--	--	0	3 (1.3) [c]	10 (1.6)
Turbinate, necrosis	0	--	--	0	0	2 (2.0)
Olfactory epithelium, atrophy	0	--	--	0	7 (1.1)	10 (1.9)
Respiratory epithelium, hyperplasia	0	--	--	0	9 (1.2)	10 (2.3)
Respiratory epithelium, metaplasia , squamous	0	--	--	0	1 (2.0)	10 (2.1)

[a] Number of tissues or animals examined
[b] Number of diagnoses made
[c] Severity: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked

NE = Not examined

Table 6

QUALITY ASSURANCE STATEMENT
Ninety-Day Toxicity Report
Gavage Study of Chemical XYZ In F344 Rats

Study #81-20 (Ninety-Day Toxicity Study/Rats)

Listed below are the phases and/or procedures included in the study described in this report which were reviewed by the Quality Assurance Unit, the dates the reviews were performed and findings reported to management. (All findings were reported to the study director or his/her designee at the time of the review.)

Phase/Procedure Reviewed	Review Date	Date of Report To Study Director/ Management
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
Bulk Chemical Reanalysis:		
In-process Inspection	01/22/02	01/28/02
Data Audit	01/22/02	01/28/02
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -

SAMPLE

- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
Clinical Lab Studies (Day 4)		
In-process Inspection	4/22/02	4/27/02
Data Audit	4/22/02	4/27/02
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
- -	- - - - - - -	- - - - - - -
Final Report	11/01-11/29/02	11/29/02

Quality Assurance Officer_____ Date _____

Table 7 Clinical Laboratory Data Form

Laboratory _____ Date Samples Collected _____ Date Assayed _____
Contract Number _____ Instrument Used _____
Test Agent _____ Laboratory Technician _____
(initials and date)
Clinical Laboratory Scientist _____
(signature and date)

Study Information:
Length of Study _____ Current Time-point _____ Route (vehicle) _____
Sample Site _____ Anesthetic _____

Animal Information:
Species _____ Sex _____ Intensity _____

HEMATOLOGY

Animal No.	RBC 10^6/uL	Hct %g/d	Hb fL	MCV pg	MCH g/dL	MCHC 10^3/uL	Retic /100WBC	NRBC 10^3/uL	WBC 10^3/uL	Seg 10^3/uL	Band 10^3/uL	Lymph 10^3/uL	Mono 10^3/uL	Eos 10^3/uL	Baso 10^3/uL	Plat	Comments
1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	
9																	
10																	

Comments:
1 - Animal died
2 - Insufficient sample
3 - Clotted
4 - Anisocytosis (1-4)
5 - Polychromasia
6 - Howell-Jolly bodies
7 - Heinz bodies
8 - Atypical cells
9 - Smudge/degenerative cells
10 - Toxic change
11 - Poikilocytosis
12 – Other

Table 8 Clinical Laboratory Data Form

Laboratory _____ Date Samples Collected _____ Date Assayed _____
Contract Number _____ Instrument Used _____
Test Agent _____ Laboratory Technician _____
(initials and date)
Clinical Laboratory Scientist _____
(signature and date)

Study Information:
Length of Study _____ Current Time-point _____ Route (vehicle) _____
Sample Site _____ Anesthetic _____

Animal Information:
Species _____ Sex _____ Intensity _____

CLINICAL CHEMISTRY

Animal No.	ALT U/L	ALP U/L	SDH U/L	Bile Acids umol/L	CK U/L	TP g/dL	Alb g/dL	BUN mg/dL	Creatinine mg/dL	Glucose mg/dL	Comments
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											

Comments:
1 - Animal died
2 - Insufficient sample
3 - Hemolysis (1-4)
4 - Lipemia (1-4)
5 - Icterus (1-4)
6 - Dilution _____
7 - Other _____

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C. CHRONIC STUDY REPORT FORMAT

I. INTRODUCTION

Brief statement not to exceed 2 pages on test article use, production, exposure route, known toxicity, and significance of human exposure.

II. MATERIALS AND METHODS

A. CHEMISTRY

1. Test Article

   a. Grade or other test article specific information
   b. Supplier/manufacturer, name and address
   c. Storage conditions for bulk test article
   d. Lots used, giving amount received, date received and dates used for each lot
   e. Test chemical identify and purity
      1) Provide information regarding initial bulk analysis, including name of laboratory performing the analyses, methods used, title of report from lab, and results. Clearly identify lot number(s).
      2) Provide information regarding test article reanalysis, including the number of the SOP used for reanalysis
      3) Provide a table of results of the initial and subsequent bulk reanalyses for each lot, including dates and analysis results (frozen reference, bulk sample and calculation of relative purity).

2. Vehicle

   a. Vehicle used (feed, water, corn oil, etc.)
   b. Grade/purity/chemical specific information
   c. Supplier, manufacturer/producer, name and address
   d. Table showing dates analyzed and results of purity analyses; include the number of the SOP used for purity analysis

3. Dose Formulation Procedure

   a. Mixing procedure, including type of container used, duration of mixing, etc. Include the number of the SOP for dose formulation.
   b. Stability and homogeneity information on dose formulation (from analytical lab or performed at testing lab)
   c. Storage conditions for dose formulations, as appropriate for route of administration, i.e. temperature, humidity, protection from light, maximum length of time stored, etc.

4. Dose Formulation Analysis

   a. Describe the analysis procedure. Include the number of the SOP used for analysis.
   b. Describe the formulation and analysis schedule for both dose preparations and animal room samples
   c. Provide a table showing dates dose formulations were prepared, dates analyzed, and dates used; and analysis results (mean $\pm$ S.D.) to include analysis prior to dosing (%high/low of theoretical), and analysis after dosing for unused/stored formulations and animal room samples (%high/low of theoretical & % of original formulation analysis). If formulations were out of spec, indicate if these were remixed or not.

B. ANIMALS AND ANIMAL HUSBANDRY

1. Animals

a. Species and strain
b. Source of animals – supplier name and address
c. Examinations to assure health of test animals
d. Quarantine period
e. Randomization procedure (to test group, rack, cage)
f. Animal identification procedure
g. Age of animals upon receipt and age and weight of animals when placed on test
h. Serologic analyses performed, including dates samples were taken and results; Helicobacter results. (See format for table of serology results in Monthly Report Format.)
i. Genetic monitoring of mice

2. Animal Husbandry

a. Describe cages, filters, racks, bedding, feeders, water bottles or automatic waterers used, including the manufacturer's name and address of each item.
b. Describe method of cleaning each item listed above, and where applicable, include the type of washer, cleaning agent, and manufacturer's name and address.
c. Provide schedule for changing cages, filters, racks, bedding, feeders, water bottles, etc.
d. Describe barrier maintenance and disease control procedures. (See Monthly Report for format.)
e. Describe room sanitization and pest control procedures.
f. State temperature and humidity ranges, number of room air changes per hour, type of air filtration used, name of filters, manufacturer name and address; light cycle and type of lights used; indicate temperature/humidity excursions, including dates, ranges, number of hours involved.
g. Identify study room(s).
h. Describe number of animals per cage; relationship of control and treated group animals (including rack position); and describe rotation of cages on racks and racks within the room.
i. Identify feed used and storage conditions.
j. Provide source of water supply (city or well), any water treatment used and discuss water analyses performed.

C. STUDY DESIGN

1. Study Design

a. Specify route of administration and frequency of administration
b. Indicate number of animals per treatment group, number of groups and exposure concentrations
c. Specify duration of exposure – provide specific dates for first and last treatment
d. Provide specific details of special studies performed, such as clinical laboratory studies, organ weights, immunotoxicity, neurobehavioral, tissue analysis. Include details of tissue/sample taken, sample collection and handling, analysis method or procedure for conducting special studies, and dates performed.
e. Identify by title all reports submitted under separate cover which are related to these studies, i.e. Prestart Chemistry Reports. Prestart Toxicokinetic Study Reports, Single Administration Toxicokinetic Study Reports, Proficiency Reports for Special Studies, etc.

2. In-life Observations and Pathological Examinations

   a. Describe in-life observations performed and frequency
   b. Specific dates for interim sacrifices and terminal necropsies
   c. Handling of moribund animals
   d. Method of euthanasia
   e. List of tissues collected at necropsy and processed for histopathological evaluation
   f. Histological processes, preservation, embedding, sectioning, staining

D. SUMMARY TABLE

Provide a summary table to include details of experimental design and animal maintenance (**See Table 1**)

III. RESULTS

A. Tables/Curves

1. TDMSE Animal Removal Summary by Treatment Group (E01)
2. TDMSE Survival Curves (P40). Explain all accidental deaths in text.
3. TDMSE Body Weight Curves (E03)
4. TDMSE Table of Body Weights (E04)
5. TDMSE Table for Feed or Water consumption with Estimate of Chemical Consumed (E08) (if applicable)
6. Table of Treatment-related Non-neoplsatic and Neoplastic Lesions (incidence and severity). (**See Table 2)**

B. Text

1. Describe interim sacrifice findings (if applicable).
2. Provide a summary of treatment-related clinical signs, body weight, mortality.
3. Present results of special studies, including clinical pathology, tissue analysis, etc. as appropriate. If clinical laboratory studies are performed during the 2-year study, present results in tabular format as described under Prechronic Study Report Format.
4. Describe pathology findings, including relation of non-neoplastic and neoplastic lesions to other toxicologic data.

IV. DISCUSSION AND SUMMARY

A. Provide a discussion of findings, including where appropriate, comparison with results observed in prechronic study

B. Describe any problems encountered that would impact interpretation of data

C. Provide a brief summary of salient findings

V. QUALITY ASSURANCE UNIT STATEMENT

See Prechronic Study Report Format for details.

VI. APPENDICES

A. Feed

- One Sample Feed Tag and Irradiation Certificate
- Provide a table that includes lot #, date of milling, and inclusive use dates for feed.

B. Water Analysis Reports

C. Monthly Temperature and Humidity Recordings for Study Room (See Monthly Progress Report for format)

D. Individual Animal Data for all non-TDMS collected data when conducted:

- Clinical lab studies (See Prechronic Study Report Format)

- Other special studies, i.e. tissue analysis, enzymatic activity, cell proliferation, etc.

E. TDMSE table of non-neoplastic lesions (P18)

F. TDMSE table of neoplastic lesions (P2)

G. Copy of Contract SOW and Modifications

H. Copy of Laboratory Protocol and Amendments

I. Study Deviation Reports

Table 1. Materials and Methods for Chronic Studies of Fischer 344/N Rats Fed Diets Containing Chemical "XYZ"

Experimental Design	
Size of Test Group	Core study - 50 males and 50 females Special study (tissue analysis) –10 males & 10 females
Doses	0, 12,500, 25,000, or 50,000 ppm in feed
Duration of Dosing	104 weeks - males (3/20/04 - 3/18/06) 105 weeks - females (3/21/04 - 3/21/06)
Type and Frequency of Observation	Observed twice daily for moribundity and mortality; body weights, clinical observations and food consumption, recorded every 4 weeks
Special Studies Conducted	Collected blood at 2 weeks, 3, 6 and 12 months for analysis of test agent
Necropsy and Histological Examination	Necropsies and histopathological examinations performed on all core study animals
Animals and Animal Maintenance	
Species	F344/N rats
Date Animals Received	3/10/04
Animal Source XXX Laboratories,	Anywhere, USA
Time Held Before Start of Test	11 days for males; 12 days for females
Age When Placed on Study	Males - 38 - 44 days Females - 37 - 43 days
Age at Terminal Necropsy	108-109 weeks
Method of Sacrifice	CO_2 asphyxiation
Method of Animal Distribution	Animals of each sex randomized into cage groups, and then cages randomized to dosed and control groups by a table of random numbers
Animal Identification	Tail Tattoo
Feed	Irradiated NTP-2000; ABC Inc, Nowhere, USA
Feeders	Stainless steel troughs made by XXX Roofing & Sheet Metal Co, Somewhere, USA; changed twice weekly

Table 1. Materials and Methods for Chronic Studies of Fischer 344/N Rats Fed Diets Containing Chemical "XYZ" (continued)

Maximum Storage Time for Feed	120 days post milling
Storage Conditions for Feed	53-76 ^{o} F; 26-61% RH
Water	Automatic Watering System; XYZ, Inc, Anywhere, USA; sanitized biweekly
Animals per cage	Five for females, three for males
Cages	Polycarbonate cages; XYZ, Inc.Anywhere, USA; changed twice weekly
Cage filters	Spun-bonded polyester filter; XXX #24; AAA Inc., Smalltown, USA; changed biweekly
Bedding	XYZ, Inc. Anywhere, USA; changed twice weekly
Racks	Stainless steel; XYZ, Inc., Anywhere, USA; changed biweekly
Cage Washer	Tunnel type (4 cycles); ABC Co., Somewhere, USA
Rack Washer	Cabinet type (2 cycles); ABC Co., Somewhere, USA
Cage & Rack Washing Compound	AAA Compound; AAA Inc., Smalltown, USA.
Animal Room Environment	Actual range 69-78 ^{o} F; 18,176/18,225 readings within Spec) Actual range 24-83 RH; 18,173/18,201 readings within Spec) 12 hours of fluorescent light per day; 15 room air changes per hour
Room Air Filter	Fiberglass roughing filter; XYZ, Co; Anywhere, USA changed biweekly
<u>Test Article Vehicle Mixture</u>	
Mixture Preparation	Weighed portion of Chemical XYZ and mixed with a weighed amount of NTP-2000 diet to make up selected doses. Mixture blended for 15 minutes in a YYY twin-shell V blender.
Maximum Storage Time	35 days
Storage Conditions	Stored in airtight, opaque plastic pails at 75± 2 ^{o} F; 60% relative humidity

Table 2 Treatment-related Lesions in Male Rats in the Two-year Study of Chemical "XYZ"

SAMPLE

Organ/Diagnosis	Incidence of Lesions (Mean Severity Treatment Groups (mg/Kg)			
	0	62.5	125	250
Kidney	**50** [a]	**50**	**50**	**50**
Renal tubule carcinoma	0 [b]	1	3	3
Renal tubule adenoma	1	3	3	4
Cortical tubules, atypical hyperplasia	0	3 (2.3) [c]	7 (2.5)	9 (2.5)
Cortex, infarct	0	0	1 (4.0)	2 (4.0)
Chronic nephropathy	44 (2.3)	48 (2.6)	46 (3.5)	49 (3.7)
Urinary bladder				
Transitional cell carcinoma	0	0	1	1
Transitional cell papilloma	0	0	0	3
Transitional epithelium, hyperplasia	2 (2.0)	3 (1.7)	5 (2.0)	7 (2.3)

[a] Number of tissues or animals examined
[b] Number of diagnoses made
[c] Severity: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked

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APPENDIX 1

DOSE ANALYSIS AND METHOD PERFORMANCE EVALUATION

Contents

Dose Analysis and Method Performance Evaluation

1. Rationale
2. Key Elements
3. Spiked Vehicle Standard Preparation
4. Solvent Standard Preparation
5. Calculations

Appendix 1. Prestart Chemistry Report.

Dose Analysis and Method Performance Evaluation:

Methods developed by the contractor for dose analysis shall be described. A prestart method performance evaluation shall be conducted according to the following general protocol and included in the report.

1. Rationale:

The purpose of the protocol is to provide a uniform procedure for investigators to confirm satisfactory performance of analytical chemistry methods specified by the NTP prior to initiating their studies. These tests shall be conducted over the dose concentration range used for the toxicity tests.

2. Key Elements:

The key elements for the performance evaluation include the following:

a. An indication of the precision of the method at specified concentrations.

b. Confirmation by statistical and visual inspection that the response versus concentration is linear over the specified concentration range.

c. An indication of blank vehicle contribution to responses seen in spiked vehicle determinations.

d. Estimation of recovery (percent) of the chemical from spiked vehicle at specified concentrations.

e. Determination of the percent relative error.

f. Estimates of the measurement limits.

To aid in the execution of the statistical requirements, all the computational formulae will be given in the text.

3. Spiked Vehicle Standard Preparation

Prepare spiked vehicle standards at six different concentrations using two independently prepared stock standards of different concentrations (Stock A and Stock B). Make spiked vehicle standards in triplicate at each concentration. Prepare a vehicle blank in triplicate. The concentrations of the spiked standards should be arranged so that each standard comes from alternate stock solutions (Table 1). Measure the response from a single analysis of each vehicle standard and blank.

Table 1:

Concentration of Vehicle Blank	Concentrations of Vehicle Standards					
X_{O_1}	X_{B_4}	X_{A_7}	$X_{B_{10}}$	$X_{A_{13}}$	$X_{B_{16}}$	$X_{A_{19}}$
X_{O_2}	X_{B_5}	X_{A_8}	$X_{B_{11}}$	$X_{A_{14}}$	$X_{B_{17}}$	$X_{A_{20}}$
X_{O_3}	X_{B_6}	X_{A_9}	$X_{B_{12}}$	$X_{A_{15}}$	$X_{B_{18}}$	$X_{A_{21}}$

Where the subscripts A and B denote the stock standards, A and B, which were used to prepare the vehicle standards and O signifies the blank.

Compute the mean response, Y_{f_i}, for each concentration, X_{f_i}, of the standards and the blanks (Table 2). Compute the standard deviation, S_i, for each concentration (equation 1).

Table 2:

Response of Vehicle Blank	Responses of Vehicle Standards					
Y_{O_1}	Y_{B_4}	Y_{A_7}	$Y_{B_{10}}$	$Y_{A_{13}}$	$Y_{B_{16}}$	$Y_{A_{19}}$
Y_{O_2}	Y_{B_5}	Y_{A_8}	$Y_{B_{11}}$	$Y_{A_{14}}$	$Y_{B_{17}}$	$Y_{A_{20}}$
Y_{O_3}	Y_{B_6}	Y_{A_9}	$Y_{B_{12}}$	$Y_{A_{15}}$	$Y_{B_{18}}$	$Y_{A_{21}}$
$\bar{Y}_{O_j} \pm S_j$	$\bar{Y}_{B_j} \pm S_j$	$YA_j \pm S_j$	$\bar{Y}_{B_j} \pm S_j$	$\bar{Y}_{A_j} \pm S_j$	$\bar{Y}_{B_j} \pm S_j$	$\bar{Y}_{A_j} \pm S_j$

Where j represents a set of three standards at one concentration (j=0–6).

So, the standard deviation, S_j for each triplicate standard set is given by:

$$S_j = \left\{ \frac{1}{(n-1)} \left[\sum_{k=a}^{b} Y_{f_k}^2 - \frac{1}{n} \left(\sum_{k=a}^{b} Y_{f_k} \right)^2 \right] \right\}^{1/2} \qquad [1]$$

where a and b are 1 and 3 (for the blanks), or 4 and 6 (for the first set of triplicate standards), or 7 and 9, 10 and 12, 13 and 15, 16 and 18, or 19 and 21 for each of the other sets of triplicate standards.

4. Solvent Standard Preparation

Prepare a series of solvent standard solutions with the same final concentrations (i.e. after any extraction, dilution, or concentration step) as were used for measurements on the vehicle standards (Table 3). Use the same standard stock solutions (A and B), prepared in E. I. B.1., for making these solutions. Prepare a reagent blank in the same solvent as the standards. Measure the responses on the same analytical system used for the vehicle standards (Table 4).

Table 3

Reagent Blank Concentration	Solvent Standard Concentrations					
X_{S_O}	X_{S_1}	X_{S_2}	X_{S_3}	X_{S_4}	X_{S_5}	X_{S_6}

Table 4

Reagent Blank Responses	Solvent Standard Responses					
Y_{S_O}	Y_{S_1}	Y_{S_2}	Y_{S_3}	Y_{S_4}	Y_{S_5}	Y_{S_6}

5. Calculations

a. Calculate the linear regression equations for the vehicle and solvent standard curves. Do not correct the response for experimental blank values. For spectrophotometric determinations, zero the instrument using the solvent only, then compare the blank value with the Y intercept (b_0) obtained from the linear regression equation. Linear regression parameters, the slope (b_1), and the intercept (b_0), are calculated as follows (equations 2 and 3):

$$b_1 = \frac{\sum_{k=1}^{21} X_{f_k} Y_{f_k} - \frac{\left(\sum_{k=1}^{21} X_{f_k}\right)\left(\sum_{k=1}^{21} Y_{f_k}\right)}{n}}{\sum_{k=1}^{21} X_{f_k}^2 - \frac{\left(\sum_{k=1}^{21} X_{f_k}\right)^2}{n}} \qquad [2]$$

$$b_0 = \frac{1}{n}\left(\sum_{k=1}^{21} Y_{f_k} - b_1\left(\sum_{k=1}^{21} X_{f_k}\right)\right) \qquad [3]$$

Where:

X_{f_k} = Prepared concentrations of the standards

Y_{f_k} = Values of the response corresponding to X_{f_k}

b. Calculate the correlation coefficient, r, for the solvent and vehicle standard curve data as follows (equation 4):

$$r = \frac{\sum_{k=1}^{21} X_{f_k} Y_{f_k} - \frac{\sum_{k=1}^{21} X_{f_k} \sum_{k=1}^{21} Y_{f_k}}{n}}{\left[\left(\sum_{k=1}^{21} X_{f_k}^2 - \frac{\left(\sum_{k=1}^{21} X_{f_k}\right)^2}{n}\right)\left(\sum_{k=1}^{21} Y_{f_k}^2 - \frac{\left(\sum_{k=1}^{21} Y_{f_k}\right)^2}{n}\right)\right]^{\frac{1}{2}}} \qquad [4]$$

For clarification of the variables used in the above equations, see Figure 1 below:

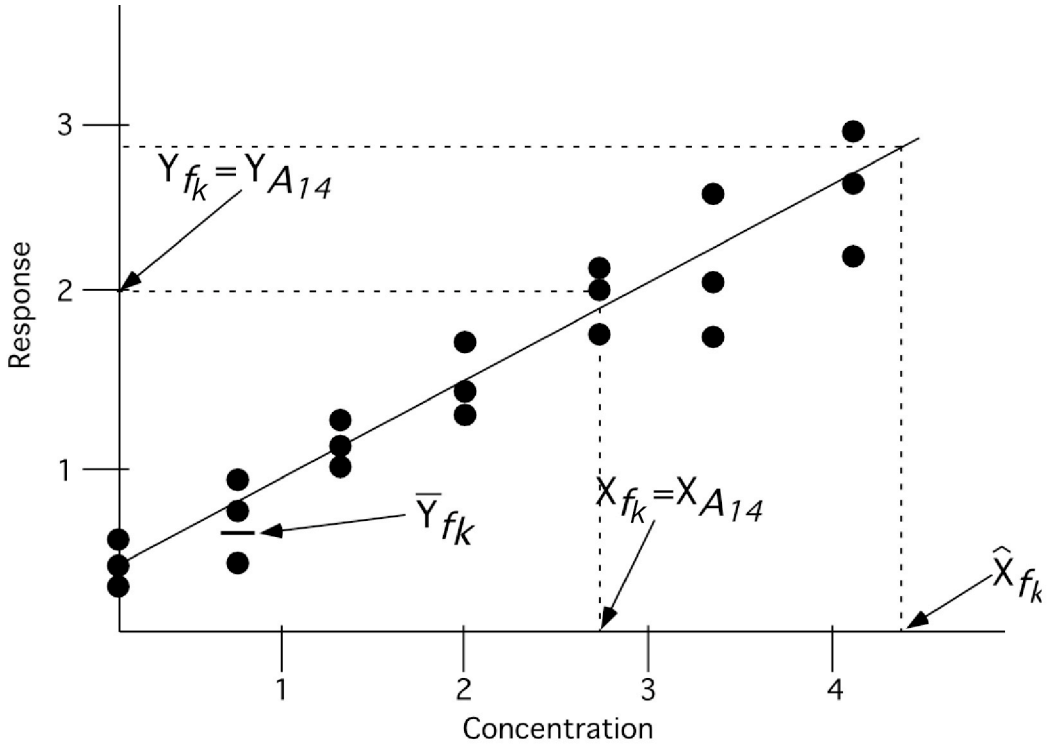

Figure 1

c. To estimate precision, calculate the percent relative standard deviation (% RSD) for each vehicle standard analyzed in triplicate (equation 5).

$$\%RSD = \left[\frac{\left[\sum_{j=1}^{3} Y_{f_j} - n\overline{Y_{f_j}} \right]^{\frac{1}{2}}}{\frac{n-1}{\overline{Y_{f_j}}}} \right] \times 100 \qquad [5]$$

where $\overline{Y}_{f_i}$ is the average response for each i[th] set of vehicle standards at a particular concentration or blank; I represents each replicate standard.

d. Determine the percent recovery at each concentration as follows (equation 6):

$$\% \text{ Recovery} = \frac{Y_{f_k}}{Y_{S_i}} \qquad [6]$$

where Y_{f_k} are the responses for the 18 vehicle standards (do not include the blank) and Y_{S_i} are the responses for the 6 corresponding solvent standards.

For the vehicle standards analyzed in triplicate determine the % recovery for the mean response in addition to the recoveries for the individual responses.

e. Determine the relative error of each found (calculated) concentration compared to each prepared concentration as follows:

i.) Calculate the concentration from the measured responses for the vehicle standards using the regression line (equation 7). Use the mean responses for the vehicle standards and the blanks to calculate the mean found concentration.

$$\hat{X}_{f_k} = \frac{Y_{f_k} - b_0}{b_1} \qquad [7]$$

where $\hat{X}_{f_k}$ s the found concentration of the standards, and Y_{f_k} is the instrument response for each standard (or the mean response for triplicates).

ii) Calculate the relative error as follows (equation 8):

$$\text{Relative Error} = \frac{\hat{X}_{f_k} - X_{f_i}}{X_{f_i}} \times 100 \qquad [8]$$

where X_{f_i} is the prepared concentration of each vehicle standard.

f. Determine the measurement limits as defined below:

The limit of detection (LOD) is defined as three times the standard deviation of the blank (if there is no blank value the standard deviation of the lowest standard concentration is to be used).

The limit of quantitation (LOQ) is defined as ten times the standard deviation of the blank (if there is no blank value the standard deviation of the lowest standard concentration is to be used).

The experimental limit of quantitation (ELOQ) is defined as the lowest vehicle standard concentration that has been analyzed that has a relative error of within 10% of target and a relative standard deviation of $\leq$ 10%.

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APPENDIX 2

PROTOCOLS FOR THE ANALYSIS OF DOSING VEHICLES USED IN NTP TOXICITY STUDIES

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I. Analysis of Corn Oil for Peroxide

A. Rationale:

Any batch of corn oil used in the testing program shall be analyzed for peroxides before it is first used and at bimonthly intervals thereafter while it is in use. Corn oil must be purchased in batches no smaller than two gallons (7 Kg) per chemical for which it is the dose vehicle, unless substantially less than this quantity is being used monthly. Purchase of lesser quantities needs to be approved by the Project Officer. All corn oil must be stored at $5^{\circ}C \pm 3^{o}$.

The following is a standard analytical procedure that should be used for these analyses. The method employed is the Official Method of the A.O.C.S. (1972). (Alternate methods need to be approved by the NTP Project Officer.) It determines all substances, in terms of milli-equivalents (meq) of peroxide per thousand grams of sample, which oxidize aqueous iodide under the conditions of the test. These are generally assumed to be peroxides or other similar products of fat oxidation. The method is highly empirical, and any variation in procedures may affect the results.

B. Method:

A 5.0 gram sample of the corn oil to be analyzed is quantitatively transferred into a 250 ml titration flask and 30 ml of glacial acetic acid:chloroform (60:40 v/v) is added. The mixture is stirred until the corn oil has completely dissolved. One-half ml of a saturated aqueous potassium iodide solution is added. The test solution is stirred thoroughly and allowed to stand for exactly one minute, after which 30 ml of distilled water is added. The iodine liberated by the peroxides in this solution is titrated potentiometrically with standard 0.005 N sodium thiosulfate solution, stirring vigorously to ensure thorough mixing. An automatic titrator with platinum disk working electrode and silver - silver chloride reference electrode is convenient. Titrations shall be run in triplicate. A blank titration of reagents will be run on the day the oil sample is analyzed; the blank will not titrate more than 1.0 ml of the 0.005 N sodium thiosulfate standard solution. Peroxide number, expressed in milli-equivalents of peroxide per kilogram of oil (meg/Kg), is calculated as follows:

$$\text{Peroxide Number} = \frac{(V-B) \times N \times 1000}{W}$$

V = volume (in ml) of thiosulfate solution required for titration of oil sample

B = volume (in ml) of thiosulfate solution required for titration of reagent blank

N = normality of sodium thiosulfate solution

W = weight of oil sample in grams

C. Additional Requirements:

The Contractor shall develop an analytical procedure (or SOP) based on this methodology. When reporting results, the SOP can be cited by reference. When reporting follow-up analyses, the dates and results of all previous analyses of the batch of corn oil shall be included for comparison. These data shall be summarized in the first Monthly Progress Report after the analysis. The report shall indicate which test chemical or chemicals the peroxide analysis is for and a copy of the analysis shall be included in the raw data submitted to the Archives for each chemical (microfiche copies are acceptable.)

D. Limits:

Corn oil with a peroxide number equal to or greater than 3 meq/Kg shall be considered rancid for purposes of this program and must be replaced immediately.

E. Characteristics and approximate composition of corn oil (Table 1):

Table 1

Calories per gram		8.9
Iodine value		127
Peroxide value (meq/L)		1.6
Anisidine value		2.3
Saponification value		191
Color		2.3 R/14 Y
Components		
Glycerides*		>98.7 %
Unsaponifiable matter*		1.25 %
Free fatty acids		0.04 %
Phosphorus		0.5 ppm
Sodium		0.1 ppm
Calcium		0.1 ppm
Magnesium		<0.1 ppm
Organo-chloride pesticide residues		<10 ppb†
Aflatoxin		<0.5 ppb†
Heavy Metals (Pb, Cu, Ni, Fe)		<0.1 ppm†
Estrogenic activity	Not detected	<5 ppb†
Fatty Acid, grams/100 grams of corn oil		
Total fatty acids		94.3
C12:0		trace
C14:0		trace
C16:0		9.5
C16:1		0.2
C18:0		2.3
C18:1		25.4
C18:2		55.1
C18:3		1.0
All others		0.8
Essential fatty acid (lipoxydase)		56.8
Unsaponifiables *, % of oil		1.25
Phytosterols		>1.0
Stigmasterol		0.07
Beta-Sitosterol		0.8
Gamma-Sitosterol or Campesterol*		0.2
Tocopherols - total		0.098
Alpha-Tocopherol		0.014
Gamma-Tocopherol		0.084
Delta-Tocopherol		<0.001
Ubiquinone (coenzyme Q-9)*		0.02
Squalene*		trace
Carotenoids*		trace

*From past historical experience; not analyzed
†Limits of detection

II. Protocols for the Analysis of Ethanol used in Toxicity Studies

A. Sources of Ethanol and Chromatographic Columns

The dose vehicle analysis of ethanol has been performed successfully using columns from two different manufactures. DB-Wax columns produced by J&W Scientific are available from several distributors such as Alltech[1] or Fisher Scientific[2]. A column with comparable performance[3] is the Supelcowax 10, available from Supelco[4].

Based on information received and a screening analysis for benzene, the recommended supplier for both 05% and anhydrous ethanol is Transchemical Inc.[5], a distributor for ethanol produced by Equistart Inc. They provide 95% and anhydrous synthetic ethanol, dried over molecular sieves.

1 Alltech Associates, Inc. 2051 Waukegan Road, Deerfield, IL 60015, (800) 254-8324, www.altechweb.com Cat. No. 93414.

2 Fisher Scientific, 585 Alpha Drive, Pittsburgh, PA 15238, (800) 766-7000. www.fishersci.com &W No.125-7032, Fisher Cat. No. 05-600-601.

3 Dose Vehicle Analysis of Ethanol, NIEHS Contract No. N01-ES-55385, ETP Task No. CHEM2363, MRI Project No. 4300, MRI Task No. 792, January 29, 1997.

4 Supelco Chromatographic Products, Supelco park, Bellefonte, PA 16823, (800) 247-6628, www.supelco.sial.com Cat. No. 25301-U.

5 Transchemical Inc., 419 East Desoto Ave., St. Louis, MO 63105, (314) 231-6917; Specify “Equistar Only”.

B. Identity by Infrared Spectrophotometry

1. Prepare a thin film of the sample by placing 1 to 2 drops between silver chloride plates. Be sure no air bubbles are trapped in the cell.

2. Obtain an IR spectrum of the sample from 600 to 4000 cm^{-1} using a suitable infrared spectrophotometer. Adjust the instrument settings or cell thickness to obtain a baseline of approximately 80% transmission, keeping the largest absorbances greater than or equal to 10% transmission.

C. Purity Assessment by Chromatography

1. Sample Preparation

Prepare and analyze two test article (ethanol) solutions. Prepare solution A by volumetrically pipetting 0.5 mL of test article and 0.5 mL of cyclohexanone (internal standard) into a 100-mL volumetric flask. Dilute the contents of the flask to volume with water and mix by inversion. Prepare solution B by delivering a 0.5-mL portion of cyclohexanone into a 100-mL volumetric flask, diluting the contents of the flask to volume with test article and mixing by inversion. Prepare an internal standard blank solution by pipetting 0.5 mL of cyclohexanone into a 100-mL volumetric flask. Bring the contents of the flask to volume with water and mix by inversion.

2. Analysis

Analyze the two solutions (A and B), the internal standard blank, a portion of the neat test article, and water blank. Use the instrument system and parameters described below.

3. System Suitability

Evaluate the analytical system described above for precision, theoretical plates, resolution, and tailing factor, according to USP guidelines. Calculate precision using the average response ratio for the ethanol peak obtained from six injections of solution A. Evaluate the tailing factor at 5% base height for the ethanol peak of a single injection of solution A. Evaluate resolution for the ethanol and cyclohexanone peaks of a single injection of solution A. Calculate theoretical plates for the ethanol peak of a single injection of solution A.

4. Instrument System and Parameters

Instrument:	Varian 3700 with Varian 8000 autosampler (or equivalent)
Column:	DB-Wax, 30 m x 0.53 mm ID, 1-mm film thickness, fused silica
Temperature Program:	40° C (5-min hold) to 220°C (5-min hold) at 10°C/min
Injection Volume:	1 μL
Mode:	Direct
Detector:	Flame ionization
Attenuation:	32×10^{-11}
Temperatures:	
Inlet:	150° C
Detector:	220° C
Carrier Gas:	Helium
Flow Rate:	10 mL/min
Makeup Gas:	Nitrogen
Flow rate:	20 mL/min
Air Flow Rate:	300 mL/min
Hydrogen Flow Rate:	30 mL/min
Retention Times:	
Ethanol: 2.3 minutes	
Internal standard: 9.9 minutes	

Set the attenuation so that a 60% to 80% pen deflection is obtained for the internal standard peak (approximately 32×10^{-11} AFS). Use the chromatograms from injection of the neat ethanol sample and solution A to correlate observed peaks with their respective retention times. Also use the chromatograms from the neat ethanol sample and the water solvent to determine that there are no interferences on the internal standard peak.

5. Calculations

Calculate the relative response factor (RRF_A) for the ethanol peak observed from solution A using the following formula:

$$RRF_A = \frac{\text{Peak area of ethanol} \times 200}{\text{Peak area of internal standard}}$$

Calculate the relative response factors for each impurity observed from injection of solution B using the following formula:

$$RRF_i = \frac{\text{Peak area of impurity}}{\text{Peak area of internal standard}}$$

Calculate the relative concentration of each impurity in the sample of ethanol using the following formula:

$$\text{Relative concentration (\%)} = \frac{RRF_i \times 100}{RRF_A}$$

Report the retention times and relative concentrations for any impurities with relative concentrations greater than or equal to 0.1%.

6. Limits

Ethanol IR spectrum must match with a library reference spectrum. Any detectable benzene is not acceptable for NTP studies. Material less than 99.9% is not acceptable.

7. Benzene Content Screening Analysis

If ethanol is obtained from a source other than the one recommended, the vehicle shall be screened for benzene content using the following method. A sample of the neat test article should be analyzed along with a series of benzene standards, prepared at concentrations from approximately 1 to 10 ppm. This would only be necessary on the initial receipt of each lot of vehicle. The DB-5 column, produced by J.& W. Scientific, is available from the same distributors as the DB-Wax column.

Instrument:	Varian 3700 with Varian 8000 autosampler (or equivalent)
Column:	DB-5, 30 m x 0.53 mm ID, 1.5-mm film thickness, fused silica
Temperature Program:	40° C (3-min hold) to 200°C (3-min hold) at 10°C/min
Injection Volume:	1 mL
Mode:	Direct
Detector:	Flame ionization
Attenuation:	32×10^{-11}
Temperatures:	
Inlet:	150° C
Detector:	220° C
Carrier Gas:	Helium
Flow Rate:	10 mL/min
Makeup Gas:	Nitrogen
Flow rate:	20 mL/min
Air Flow Rate:	~ 300 mL/min
Hydrogen Flow Rate:	~ 30 mL/min

III. Protocols for the Analysis of Acetone [a., b.]

A. Identity by Infrared Spectrophotometry

1. Place five to six drops of acetone into an infrared gas cell[c.] equipped with sodium chloride windows and allow the sample to volatilize (volatilization may be accomplished by slight warming of the cell with the hands).

2. Obtain a spectrum for the sample from 600 to 4000 cm^{-1} using a suitable infrared spectrophotometer. Adjust the instrument settings or sample concentration to obtain baselines of about 80% transmission, keeping the largest absorbances at greater than or equal to 10% transmission.

a. Fisher Scientific, 585 Alpha Drive, Pittsburg, PA 15238, (800) 766-7000, www.fishersci.com; Column, J & W No. 125-7032, Fisher Cat. No. 05-600-601; Acetone, Cat. No. A949SK

b. Spectrum Quality Products, 755 Jersey Ave., New Brunswick, NJ, 08901-3605, (301) 516-8000, www.spectrumchemical.com.

c. Mini-gas cell available from Analabs, Inc., North Haven, CT 06473, Cat. No. 071-4051 or equivalent.

B. Purity Assessment by Chromatography

1. Sample Preparation

Prepare and analyze two test article (acetone) solutions. Prepare solution A by volumetrically pipetting[d.] 0.5 mL of test article and 0.5 mL of cyclohexanone (internal standard) into a 100-mL volumetric flask. Dilute the contents of the flask to volume with water and mix by inversion. Prepare solution B by delivering a 0.5 mL portion of cyclohexanone into a 100-mL volumetric flask, diluting the contents of the flask to volume with test article and mixing by inversion. Prepare an internal standard blank solution by pipetting 0.5 mL of cyclohexanone into a 100-mL volumetric flask. Bring the contents of the flask to volume with water and mix by inversion.

d. Drummond micro-cap® , potential source: VWR Scientific, Cat. No. 53440-227

2. Analysis

Analyze the two solutions (A and B), the internal standard blank, a portion of the neat test article, and a water blank. Use the instrument system and parameters described below.

3. System Suitability

Evaluate the analytical system described above for precision, theoretical plates, resolution, and Tailing factor, according to U.S.P. guidelines. [e.] Calculate precision using the average response ratio for the acetone peak obtained from six injections of solution A. Evaluate the tailing factor at 5% base height for the acetone peak of a single injection of solution A. Evaluate resolution for the acetone and cyclohexanone peaks of a single injection of solution A. Calculate theoretical plates for the acetone peak of a single injection of solution A. A comparison between the criteria for the current method and those proposed for the modified method

e. The United States Pharmacopeia, Twenty-third Revision, (USP XXIII), Physical Tests/Chromatography 621, System Suitability (1995), p. 1776.

4. Instrument System and Parameters

Instrument:	Varian 3700 with Varian 8000 autosampler (or equivalent)
Column:	DB-Wax, 30 m x 0.53 mm ID, 1-mm film thickness, fused silica
Temperature Program:	40^{o} C (5-min hold) to 220^{o}C; (5-min hold) at 10^{o}C/min
Injection Volume:	1 mL
Mode:	Direct
Detector:	FID
Attenuation:	32×10^{-11}
Temperatures:	
Inlet:	150^{o} C
Detector:	220^{o} C
Carrier Gas:	Helium
Flow Rate:	~ 10 mL/min
Makeup Gas:	Nitrogen
Flow Rate:	~ 20 mL/min
Air Flow Rate:	~ 300 mL/min
Hydrogen Flow Rate:	~ 30 mL/min
Retention Times:	
Acetone:	~ 1.2 min
Cyclohexanone (internal standard):	~ 9.9 min

Set the attenuation so that a 60% to 80% pen deflection is obtained for the internal standard peak (approximately 32×10^{-10} AFS). Use the chromatograms from injection of the neat acetone sample and solution A to correlate observed peaks with their respective retention times. Also use the chromatograms from the neat acetone sample and the water solvent to determine that there are no interferences on the internal standard peak.

5. Calculations

Calculate the relative response factor (RRF_A) for the acetone peak observed from solution A using the following formula:

$$RRF_A = \frac{\text{Peak area of acetone} \times 200}{\text{Peak area of internal standard}}$$

Calculate the relative response factors for each impurity observed from injection of solution B using the following formula:

$$RRF_i = \frac{\text{Peak area of impurity}}{\text{Peak area of internal standard}}$$

Calculate the relative concentration of each impurity in the sample of ethanol using the following formula:

$$\text{Relative concentration (\%)} = \frac{RRF_i \times 100}{RRF_A}$$

Report the retention times and relative concentrations for any impurities with relative concentrations greater than or equal to 0.1%.

6. Limits

   IR spectrum must match with a library reference spectrum. Material less than 99.9% pure is not acceptable.

IV. Protocols for the Analysis of Methylcellulose (Dosing Vehicle) used in Toxicity Studies

A. Receipt of Bulk Chemical

When the bulk chemical is received, remove 0.5 g portions for each subsequent analysis. Place each sample in an appropriately labeled glass vial equipped with a Teflon®-lined screw cap, then tightly close and seal the vial, and store at –20° C. Use this material in subsequent analyses, at intervals specified by the NTP, as the reference standard. Store the remainder of the bulk material at room temperature (~ -25° C).

B. Identity by Infrared Spectrophotometry

1. Prepare separate potassium bromide discs containing approximately 3% methylcellulose for both the bulk chemical and the reference standard.

2. Obtain a spectrum for the two samples from 600 to 4000 cm^{-1} using a suitable infrared spectrophotometer. Adjust the instrument settings or sample concentration to obtain baselines of about 80% transmission, keeping the largest absorbances at greater than or equal to 10% transmission.

C. Purity Analysis by Methoxy Group Determination

1. Procedure

This analysis (duplicate samples) may be performed by the Contractor or by an independent laboratory. If an independent laboratory does the analysis, it is the responsibility of the Contractor to verify the quality assurance compliance of that laboratory.

2. Calculations

a. Calculate the average determined values (%) for methoxy group content of the bulk chemical and the reference standard to the tenths place.

b. Calculate the relative purity (%) for the bulk chemical to the tenths place by dividing the average determined value (%) for the bulk chemical by the average determined value for the reference standard and multiplying by 100.

3. Limits

IR spectrum must match with a library reference spectrum. Results of analysis for methoxy group content must be 27.5 to 31.5 %.

APPENDIX 3

LABORATORY ANIMAL MANAGEMENT

HEAT TREATED HARDWOOD BEDDING
MAXIMUM LEVELS OF CONTAMINANTS

CHEMICAL CONTAMINANTS *

Pesticide Residues PPM	
Chlorinated Hydrocarbons	
Alpha BHC	< 0.02
Beta BHC	< 0.02
Lindane	< 0.02
Aldrin	< 0.02
Heptachlor Epoxide	< 0.02
Dieldrin	< 0.02
Endrin	< 0.02
DDT	< 0.03
DDD	< 0.02
DDE	< 0.02
Organophosphates	
Diazinon	< 0.10
Ethyl Parathion	< 0.03
Methyl Parathion	< 0.03
Malathion	< 0.05
Ethion	< 0.02
Ronnel	< 0.03
Triothion	< 0.03
Polychlorinated Biphenyls	< 0.2
Pentachlorophenol	< 0.1
Aflatoxins	< 10 PPB
Heavy Metals PPM	
Lead	< 0.5
Mercury	< 0.1
Cadmium	< 0.1
Arsenic	< 0.2

MICROBIOLOGICAL CONTAMINANTS

Standard Plate Count	< 100
Coliform	< 10
Pseudomonads	Neg
Yeast and Molds	< 10
Salmonella/Shigella	Neg

* All values in total organisms/gm of bedding

NTP-2000 DIET - LIMITS OF CONTAMINANT LEVELS

AFLATOXINS
(Maximum PPB)

Total	5
B1	2

NITROSAMINES
(Maximum PPB)

Total	15
(Volatile)	
N-nitroso Dimethylamine	10

HEAVY METALS
(Maximum PPM)

Lead	1.0
Cadmium	0.15
Mercury	0.05
Arsenic	0.5
Selenium	0.5

CHLORINATED HYDROCARBONS
(Maximum PPM)

BHC	
Alpha 0.02	
Beta	0.02
Delta	0.02
Lindane	0.02
Heptachlor	0.02
DDE	0.02
DDD	0.02
DDT	0.03
HCB	0.08
Mirex	0.02
Methoxychlor	0.05
Dieldrin	0.02
Endrin	0.02
Telodrin	0.02
Chlordane	0.05
Toxaphene	0.1

ORGANOPHOSPHATES
(Maximum PPM)

Chloropyrifos-methyl	0.10
Ronnel	0.03
Ethion	0.02
Trithion	0.05
Diazinon	0.2
Methylparathion	0.03
Ethylparathion	0.03
Malathion	0.50
Endosulfan I	0.02
Endosulfan II	0.02
Endosulfan Sulfate	0.03

PCB'S
(Maximum PPM) 0.2

MISCELLANEOUS
(Maximum Limits)

Nitrate (N PPM)	20
Nitrite (N PPM)	5
BHA (PPM)	10
BHT (PPM)	5
Total Bacterial Plate Count (CFU/Gm)	1000
Coliform (MPN/Gm)	10
E. Coli (MPN/Gm)	10
Salmonella (/Gm)	Neg

RATING OF THE FEED FOR CONTAMINANTS

Maximum Points - 100
95 to 100 - use the feed
91 to 94 - May use it but replace with a new batch within four weeks
90 and below - reject the feed

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

2. Aflatoxin - Deduct 1 point for each PPB above the specification. If the aflatoxin level is more than 10 PPB, the feed shall not be used.

3. Nitrosamines - Deduct 1 point for each 2 PPB above the specifications.

4. Heavy Metals:

   Lead, Arsenic, and Selenium - Deduct 1 point for each 0.2 PPM above the specifications.

   Cadmium and Mercury - Deduct 1 point for each 0.02 PPM above the specifications.

5. PCB's - Deduct 1 point for each 0.02 PPM above the limit.

   Pesticides - Deduct 1 point for each increase equivalent to the maximum allowable level.

6. Miscellaneous contaminants - Deduct 1 point for each increase equivalent to the maximum allowable level. If microbiological contaminants are 2X the limits, a new sample is to be tested for possible contamination during sampling. If the repeat sample confirms the original results and the total bacterial count is > 5000 cfu/gm, the product was not irradiated properly and cannot be accepted as an irradiated product.

WATER ANALYSIS

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

Metals (mg/L)

Na	Ba
K	Sr
Ca	B
Mg	P
Al	Cr
Fe	Cu
Mn	Zn

Chlorinated Hydrocarbons (mg/L)

Aldrin
Dieldrin
DDT Related Substances

Organophosphates: (mg/L)

Thimet
Diazinon
Methyl Parathion
Malathion
Parathion
Thiodan
Trithion

COLLECTION OF FECAL SAMPLES FOR HELICOBACTER PCR ANALYSIS

I. SAMPLE COLLECTION CONCEPT

Polymerase chain reaction (PCR) technology allows for amplification and detection of a target DNA sequence. The sensitivity of this technique makes it prone to cross contamination between samples from different individuals or from contaminated equipment surfaces, collection instruments, or personnel. Therefore, every effort must be made to separate the collection of specimens between groups or individuals.

A separate sterile set of surgical instruments will need to be used to collect fecal samples from each group (i.e. rooms, racks, cages, etc). Because animals housed in the same cage come into contact with fecal material from cage mates, it is difficult to obtain a valid individual sample by removing an animal to an isolated situation (e.g. a jar or individual housing). Individual results may best be obtained by collecting postmortem fecal samples using aseptic technique (described below).

Recommended Cold Sterilization Solutions:

A. 0.1N HCL (hydrochloric acid).
B. 10% sodium hypochlorite (bleach).

II. COLLECTING A POSTMORTEM SAMPLE FROM AN INDIVIDUAL ANIMAL

Materials:

- Two sterile 1.5 ml Eppendorf tubes (or equivalent). One tube for test sample and one for backup testing.
- Cleaned and disinfected necropsy table and/or hood and necropsy instruments
- Sterile tissue collection instruments (using a cold sterilization solution)
- Gloves (multiple pair)
- Outer lab garments that reduce contamination
- Isopropyl alcohol

Procedure:

- Collect PCR samples before any other necropsy procedure or tissue collection to reduce contamination.
- Change gloves between collection procedures or if contamination is suspected.
- Moisten abdominal fur of the mouse with isopropyl alcohol.
- Open abdomen with a ventral longitudinal incision, using standard necropsy instruments.
- Change to sterile instruments. Move intestine aside until a section of colon containing two fecal pellets is easily identified. With sterile forceps lift colon, and using sterile scissors, isolate and transect the segment containing two complete fecal pellets. Extrude a pellet from the colon segment into each of the two collection tubes and discard the segment of colon.
- Close each tube and freeze at -20^{o} C or below.
- Identify both tubes adequately.
- Repeat the collection procedure for each animal, using new gloves and a new set of sterile instruments.
- Pack all samples in dry ice and ship overnight (preferably).

PROCEDURE FOR COLLECTION AND SHIPMENT
OF TISSUE (TAIL OR EAR) BIOIPSY
FROM MICE FOR GENETIC MONITORING

Animals randomly selected for genetic monitoring must be uniquely identified for repeat sampling, if necessary. The method of collection of tail or ear biopsy samples is to be according to an individual facility ACUC approved procedure. The mice selected for these biopsies may be used as sentinel animals when appropriate.

Label tubes (1.5 ml micro-centrifuge tubes or equivalent) with the unique individual animal number and include enough 70% aqueous ethanol to immerse the sample. Using an appropriate instrument (Scalpel, scissors, etc.) biopsy 0.5 – 1.5 cm of the tail from the tip of the tail and immediately place the sample in the labeled tube containing the ethanol. Transfer the sample to a refrigerator at 4^{o} C as soon as possible. Samples must be kept at 4^{o} C until shipment to the genetic monitoring laboratory. Repeat sample (if requested by the NTP) may be collected by ear punch biopsy.

Samples shall be placed in ***wet ice*** and shipped prepaid by an overnight delivery service to the genetic monitoring laboratory on a Monday, Tuesday, or Wednesday. The Project Officer will provide the address. The shipping box must contain enough ***wet ice*** to last at least 48 hours. Samples shall be shipped no later than one week after sample collection. Individual tubes must not be loose in the package, but are to be bundled with tape or enclosed in small plastic bags. However, tubes shall not be wrapped with insulating material. The contact person at the genetic monitoring laboratory is to be notified by telephone or e-mail at least one day before shipment. The contact person must acknowledge the notification before the samples are shipped. All samples must be entered on the Custody Transfer Record (CTR), and the number of samples shipped must correspond to the number of entries on the CTR. The CTR shall be prepared in duplicate, with one copy included with the shipment of samples to the genetic monitoring laboratory, and the other retained at the collection site. A blank CTR is provided below and may be reproduced as needed.

For transgenic mice, tissue biopsies are to be collected and stored frozen at the testing laboratory until the end of the study, at which time they will be directed to either discard or ship the samples for analysis.

CUSTODY TRANSFER RECORD (SEE FOLLOWING PAGE)

Before storing samples, complete the Custody Transfer Record entries A through F.

(A) Facility -- Name of the laboratory from which the samples were generated.
(B) Species, strain and tissue -- Specify the species/strain from which the samples were collected, as well as the tissues type collected.
(C) Collected by – Name(s) of people who prepared and shipped the samples.
(D) Date Shipped – Date samples shipped.
(E) Item -- Corresponds to the number of samples included in the shipment.
(F) Requestor's Number -- The individual ID number of each sample that is included in the shipment.

Entries G through K are to be completed by the genetic monitoring laboratory upon receipt/analysis of samples.

(G) Lab code/number -- The code/number given each sample by the lab receiving the samples.
(H) Received by -- Person receiving the shipment.

(I) Date Received -- Date of Receipt.
(J) Lab Notebook -- ID number of notebook containing results of analyses.
(K) Analyst – Person who analyzes the samples.

CUSTODY TRANSFER RECORD

A. Facility:
B. Species/strain & tissue:
C. Collected by:
D. Date shipped:

E. Item:	F. Requestor's Number:	G. Genetic Lab Code/Number:
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		

H. Received by:	I. Date Received:
J. Lab Notebook:	K. Analyst:

MORIBUND SACRIFICE OF RODENTS

The following criteria should be supplemented with professional judgment for euthanasia of moribund animals during the course of a study. Final decision for euthanasia of moribund animals shall be made by the Laboratory Veterinarian or an experienced scientist, and shall not be left to the discretion of the technicians. Major reasons for euthanasia of animals during the course of a study include: a) large masses and other conditions preventing eating and drinking, b) major injuries and lesions such as non-healing ulcers related to husbandry and treatment, c) diseases and conditions indicating severe pain, and d) adequate indication that the animal may not survive until the next observation as judged by an experienced laboratory animal specialist. Other conditions for euthanasia of rodents in long-term studies are listed below.

- Loss of 20 to 25% body weight in less than one week
- Gradual but sustained decline in body weight indicating partial and sustained anorexia
- Prolonged unhealthy appearance such as rough coat, hunched posture and distended abdomen
- Prolonged diarrhea leading to emaciation
- Prolonged or intense diuresis leading to emaciation
- Persistent coughing, wheezing and respiratory distress
- Paralysis and other nervous disorders leading to anorexia and continuous decline in body weight
- Bleeding from natural orifices not due to minor injuries
- Persistent self-induced trauma complicating minor injuries
- Microbial infections interfering with toxic and carcinogenic responses

Adapted from Rao, G.N. and Huff, J (1990), Refinement of long-term toxicity and carcinogenesis studies, Fundamental and Applied Toxicology 15: 33-43.

APPENDIX 4

INDIVIDUAL ANIMAL NECROPSY RECORD

I. INTRODUCTION

The Individual Animal Necropsy Record (IANR) is used to record the gross pathology data during necropsy and trimming for each animal on the study. The Pathology Code Table (PCT) contains the vocabulary used for the completion of the IANR. An IANR Continuation Page is used to record gross observations or notes that do not fit on IANR page 1. A sample form filled in with the type of data to be collected, as well as blank forms, are included below.

II. CORRECTION PROCEDURES

During the course of completing the IANR, incorrect entries may be made. These errors may be discovered by the pathologist during his review of the form prior to signing the Pathologist Signature Field (26), or during a quality control check or audit of the data forms. To correct an entry, a single line is drawn through the entry so as not to obscure the original entry. The correct entry is made and a footnote callout (a circled number is entered near the incorrect data). The reason for the change, the initials of the person making the change, and the date, preceded by the corresponding circled callout number, are entered in Field 30, called “Correction Comments”.

III. DESCRIPTION OF FIELD

Each field on the form has a field number and/or name. Listed below are the fields on the form. The "Description" heading describes the appropriate entries for the fields. Guidelines and comments concerning the requirements for the completion of the applicable fields are provided below.

Note: The forms are designed to be <u>species/sex</u> specific. It is important that the appropriate form is used for each animal.

FIELD NO:	NAME:	DESCRIPTION
1.	FACILITY:	Enter the correct two-character NTP acronym for the test facility.
2.	TEST ARTICLE:	Enter the full name of the test article used on this study. DO NOT use an acronym or generic name to designate the test article. In addition, if diet restricted, stop study or other special case is involved, note this beside the test article name, i.e. Test Article XYZ (Stop Study).
3.	PHASE:	Indicate the phase of the study by marking (X) in the appropriate box.
4.	DAYS ON TEST:	Enter the number of days the animal was on test inclusive of the day of death.
5.	DOSE:	Enter the dose for the animal (i.e., mg/kg, %, units, ppm). If the animal is an untreated control or vehicle control, enter accordingly.
6.	ROUTE:	Enter the route of administration, using the appropriate acronym listed below. Route may have multiple entries. Separate entries with a hyphen. DF = Dosed Food IN = Inhalation GV = Gavage DW = Dosed Water SP = Dermal
7.	STUDY-TEST NO:	Enter the TDMS study-test number.
8.	SPECIES/STRAIN:	The IANR is specific for animal species/strain/sex and these fields have already been completed. However if a species/strain other than the Fischer 344 rat and B6C3F1 mouse are used, the correct species/strain is to be placed in this field.
9.	SEX:	The IANR is specific for species/strain/sex, and these fields have already been completed.
10.	ANIMAL NO:	Enter the unique animal number. This entry is right justified. Fill in any empty boxes with a dash or a zero.
11.	HISTO NO:	Enter the unique histology number assigned to the animal. This entry is left justified. The same number may be used for the histology number and the animal number.
12.	DEATH DATE:	Enter the date the animal was found dead, sacrificed moribund, or a scheduled sacrifice. Enter in the format MMDDYY.
13.	BODY WEIGHT: (G)	Enter the weight of the animal to the nearest tenth of a gram at the time of necropsy. This entry is right justified.
14.	DISPOSITION:	Mark (X) the appropriate box to indicate the disposition of the animal. The long text of valid dispositions are as follows: TSAC = Terminal Sacrifice NATD = Natural Death MSAC = Moribund Sacrifice ACCK = Accidentally Killed SSAC = Scheduled Sacrifice (Interim) DACC = Dosing Accident OTHER*

COMMENT: The disposition of OTHER is to be used to designate missing, mis-sexed, etc. The use of this disposition requires the entry of an explanation in the DEFINE OTHER blank. Animals removed as "OTHER" are to be discarded without necropsy. The disposition of missing or mis-sexed requires that the header of the form be completed and submitted with the study. An animal that is humanely killed for a reason unrelated to the chemical, such as broken back, or fractured skull, is to be designated as ACCK. This is not to be confused with a MSAC animal.

15. CONDITION: Mark (X) the appropriate box to indicate the condition of the animal at the time of receipt for necropsy. The long text of the valid conditions is as follows:

FRESH
AUTO/TT = Autolysis/Tissue Taken
PT CANN = Partially Cannibalized
CANN/NTT = Cannibalized/No Tissue Taken
OTHER*

*DEFINE OTHER: Enter free text in this blank to explain the use of OTHER. If there is not sufficient room, use Field 24, Notes.

COMMENT: All tissues must be taken at necropsy unless extreme conditions exist. Therefore, the use of CANN/NTT is not to be used without the permission of the assigned pathologist. Autolysis shall not preclude saving of the tissues in formalin.

16a. ID INFORMATION: Mark (X) in the "verified" box after confirming the identification of the animal,
OR
1) If the animal's identification is partially or completely unreadable, explain the condition of the identifier under comment and indicate the animal number as read, using dashes for numbers that are unreadable. If the animal number cannot be read at necropsy, then the animal is to be uniquely re-identified and this number entered in the ID number field
2) If the tattoo is different from the animal number in box 10, explain under comment.

16b. WET TISSUE REVIEW: At the time of wet tissue review, check appropriate box – PERFORMED OR NOT REQUIRED - for this animal. Initial and date entry.

17a. ORGAN List of organs to be taken at necropsy. The tissue list required for specific studies is dependent on the study protocol. Blank spaces are provided to write in organs that are not listed.

The following is a discussion of the procedure to be used for specific accountable sites and organs.

Organs - Lesions that occur in large blood vessels are described in Fields 18 through 22, but lesions of small vessels are described within the organs containing the vessel. The prosector and the pathologist are to limit the use of the organ blood vessels to the sites listed in the PCT Table.

"Tissue NOS" is an available term used to cover situations in which the site of a mass is not clearly determined. The identification of the Tissue NOS is determined histopathologically. A mass in the inguinal area could be a neoplasm in the mammary gland, a preputial gland abscess, or lymphadenopathy. If the term Tissue NOS is used, then any involved organs are to be designated as "A" and a free text description of the area in which the mass was observed is to be written in Field 24, Notes. The microscopic evaluation will key the pathologist to the correct organ for the observation. (See 24. Notes: below.)

Gross description of blood pertains to abnormal color or consistency. The prosector is responsible for observing the gross appearance of blood at the time of necropsy for all animals.

Site Accountability - The accountable sites of an organ are indented directly under the appropriate organ. If lymph nodes other than those listed on the form are observed to be abnormal, then the blank designated "Other" under Lymph Node is to be marked "B" and the site designated in Field 19.

17b. NEC: Enter one of the letters listed below to indicate the condition of the organ at the time of necropsy.

A = Normal
B = Observed. If this entry is used, an entry is required in fields 18-22.
C = Missing. If one of a paired organ is missing, enter this fact in the notes section, (i.e., right adrenal missing). Record the appropriate status for the contra-lateral organ in Field 17b.
D = Present, not examined. This term is used for organs which are normally difficult to evaluate grossly due to size or location, ie., spinal cord.

17c. WGT (mg): Enter the weight of the organ in milligrams. If an automated data system is used to collect the weight then this field does not have to be completed. The form must, however, indicate that the data has been recorded elsewhere. If the protocol does not require organ weights, the column is to be left blank.

Fields 18 - 24 represent the prosector's, trimming technician's or pathologist's observations of gross abnormalities at the time of necropsy or trimming. All observations receive a sequential Trace Gross Lesion (TGL) number as defined in Field 23 and require an accountability action at the time the pathologist performs the microscopic evaluation of the animal. For liver and lung, up to five nodules/masses shall be recorded individually (the largest). When there are more than five, a note is to be included in section 24 indicating that there were “greater than five” for that organ.

18a. TRIM ID: If an observation is made by the trimming technician, the trimmer must initial that observation in 18a.

18b. ORGAN: Enter the organ that is considered abnormal. If an entry is made in this field at necropsy, then a corresponding "B" (observed) is to be entered in Field 17b. If the organ is found abnormal at trimming then the trimmer enters the appropriate information in Fields 18-22 and initials each new entry to the immediate left of Field 18a. If an entry is made in this field, an entry must also be made in at least one of the following fields: 20-"MORPHOLOGY", 21-"SIZE", or 22-"DISTRIBUTION and/or COLOR."

19. SITE: Enter the site within the organ that best describes the location of the abnormality. Multiple entries may be made in this field (ie., subcutis/r axilla). If no entry is made in the field, draw a short horizontal line indicating not applicable.

20. MORPHOLOGY: Enter the morphology observed in the organ. If **more than one** (1) morphologic observation is present in an organ, **a separate entry must be made for each.** If no entry is made in the field, draw a short horizontal line indicating not applicable.

COMMENT: Tissue NOS is the exception to the above statement. It is preferred that only one morphology is used. However, it may be necessary to use multiple morphologies to describe a large mass encompassing multiple organs (i.e., mass adhesion).

21. SIZE: Enter a numeric value to indicate the metric measurement of the organ or lesion and the unit of measurement. The measurements are recorded in 2 or 3 dimensions depending on the appropriateness. Quantity is an appropriate entry for fluids. If no entry is made in the field, draw a short horizontal line indicating not applicable.

22. DISTRIBUTION and/or COLOR: Enter the qualifier from the group-distribution and/or group-color that best describes the morphology of the organ. Multiple entries may be made in this field. If no entry is made in the field, draw a short horizontal line indicating not applicable.

23. TGL A: Refers to the gross TGL and is used to correlate the gross description with a microscopic diagnosis.

TGL B: The following are valid entries for TGL B:

1. The slide number(s) containing the microscopic lesion that correlate with the gross description listed in items 18-22 on the same line.
2. NCL - No Corresponding Lesion
3. NST - No Section Taken

NCL is used for those gross lesions in which there is no corresponding microscopic lesion, and no lesion found in the wet tissue or tissue block (ie. lymph node, mesenteric, enlarged, but normal microscopically). NST will be used for fluids, amputated tail, malocclusion of incisors or other lesions where no histologic section is prepared. All gross lesions will have a corresponding TGL slide number, NCL, or NST analog entered in TGL-B.

24. NOTES: Enter free text associated with the animal. The notes section is used for free text entered by the prosector, pathologist, or trimming technician during necropsy or trimming, and entries are to be initialed and dated. This section is not intended to be the only documentation of the presence of a lesion in an organ, but should be used to provide additional descriptive information or clarification of the appearance or location of a lesion.

Comments in the Notes section are to be preceded with the TGL number. Instances in which one topography was identified at necropsy, and another during the microscopic examination, are to be reconciled as in the following example: At necropsy a 2 cm diameter mass was observed in the inguinal skin (TGL-2); however, microscopically, it was determined to be a fibroadenoma of the mammary gland. The

pathologist is to reconcile this by listing the TGL number and the correlate microscopic topography (i.e., TGL2-C-mammary gland, where C = correlates).

For the vast majority of TGLs, there is a corresponding lesion. In instances where the pathologist would have expected a microscopic lesion (ie. 2x3x5 mm skin mass), yet there was none, the block and wet tissues are to be examined for the "missing" lesion(s). If the lesion is located, obviously it is to be trimmed in and examined, and the slide number added to TGL-B. However, if after review of both the wet tissue and block, the lesion is not found, the pathologist is to indicate that the wet tissues and blocks were checked by including his/her initials and date in the Notes section corresponding to that TGL (i.e., TGL4 not observed).

25. PROSECTOR/ NECROPSY DATE: The prosector signs and dates the form at the completion of the necropsy.

26. PATHOLOGIST/ NEC DATE: The pathologist responsible for the necropsy of the animal signs and dates the form at the completion of the necropsy. The pathologist validates that the data are complete as soon after necropsy as possible even if he is not present for the performance of the necropsy.

27. TRIMMER/DATE: The trimming technician signs and dates Field 27 at completion of the trimming procedure.

28. PROBABLE CAUSE OF MORIBUNDITY/ DEATH: The pathologist enters the probable **cause of death** (PCOD) or **cause of moribund condition** at necropsy and initials and dates the entry. The pathologist is to address the PCOD based on the necropsy findings. It is not necessary for the pathologist to be present at the time of necropsy to determine the PCOD at necropsy. PCOD will be used for animals with a disposition of NATD, MSAC, DACC, and ACCK. In the case of moribund sacrifice animals, the cause of death will reflect the underlying cause of the moribund condition of the animal, NOT merely the reason for moribund sacrifice. If the PCOD/PCOM cannot be determined, then enter "undetermined".

29. CLINICAL/ REMOVAL OBSERVATIONS: Enter clinical removal observations, i.e., observations made at the time of removal of the animal from the study for all animals. In addition, enter the last formal clinical observations recorded in TDMS and the date of this last entry. At necropsy, the prosector is to indicate if they observed each of the listed clinical removal observations or not by entering a Y (yes) or N (no) and their initials next to each observation.

This information is to aid the prosector and pathologist in observing, describing, and preserving lesions observed clinically. In addition, it aids the pathologist in interpreting and correlating gross and microscopic lesions.

30. CORRECTION COMMENTS: Enter in this field, the callout number, reason for the correction, initials, and date of correction.

Page 1 of __ : Fill in the total number of pages on the space provided. If more than 1 page is necessary number the pages consecutively.

COMMENT: The pathologist assigned to the necropsy is responsible for the conduct of the animal necropsy, proper collection and identification of tissues, and description of gross lesions using only the nomenclature in the PCT. The responsible pathologist who signs Field 26 should review header information at the top of the IANR for accuracy and make sure the prosector and trimmer complied with the requirements of the Specifications and the protocol. The assigned pathologist is responsible for providing the probable cause of death (PCOD/PCOM) in Field 28.

Individual Animal Necropsy Record - Continuation Page

The continuation page of the IANR is to be used when all of the gross abnormalities do not fit on the first page of the IANR. All header information must be completed. The continuation sheet is to be signed by the prosector and Pathologist (Fields 25 and 26) only if lesions listed on the continuation page were found at necropsy. The trimmer is to sign in Field 27.

This form must not be used without the completion of Page 1 of the IANR. It is not a stand-alone form. Refer to the previous section for a description of each field on this form.

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
	Lab XX	SAMPLE			X CHRONIC
		4. DAYS ON TEST 692	5. DOSE 25 mg/Kg	6. ROUTE GV	SUBCHRONIC
					REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
9 9 9 9 9 - 0 5	RAT F344	FEMALE	– – 2 9 8

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
9 9 _ 9 9 9 9 _ 9 9 9	0 4 1 8 0 5	3 0 2 . 8

14. DISPOSITION	15. CONDITION	16a. IDENTIFICATION INFORMATION
TSAC / OTHER* / DACC	CANN/NTT	VERIFIED ☐ ID NUMBER: 2898
X MSAC / NATD / ACCK	X FRESH / PT CANN	IDENTIFICATION COMMENT:
SSAC	AUTO/TT / OTHER *	Should be 298; tattoed in error as 289; changed to 2898
* DEFINE OTHER:	* DEFINE OTHER:	16b. WET TISSUE REVIEW: X PERFORMED B.D. 9/28/05 / NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.	A		INTEST SMALL			LYMPH NODE			SALIVARY GLS	A	
BLOOD	A		DUODENUM	A		BRONCHIAL	A		SKELETAL MUSCLE	A	
BLOOD VESSEL	A		JEJUNUM	A		MEDIASTINAL	A		SKIN	B	
BONE	A		ILEUM	A		MANDIBULAR	A		SPINAL CORD	D	
BONE MARROW	A		INTEST LARGE			MESENTERIC	A		SPLEEN	A[1] B	
BRAIN	A		CECUM	A		OTHER			STOMACH	A	
CARCASS	A		COLON	A		MAMMARY GL	A		THYMUS	A	
CAVITIES	B		RECTUM	A		MESENTERY	A		THYROID GL	A	
CLITORAL GL	A		KIDNEY	A		NOSE	A		TISSUE NOS		
ESOPHAGUS	A		LACRIMAL GL	A		OVARIES	A		TONGUE	A	
EYE	A		LARYNX	A		OVIDUCT	A		TOOTH	A	
HARDERIAN GL	A		LIVER	B		PARATHYROID GL	A		TRACHEA	A	
HEART	A		LUNG	B		PANCREAS	A		URETER	A	
						PERIPH NERVE	A		URINARY BLADDER	A	
						PHARYNX	A		UTERUS	A	
						PITUITARY GL	A		VAGINA	A	
									ZYMBALS GL	A	

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
	Skin	Inguinal	Mass	80x40x40 mm	-	1	5	LIVER CONTAINED > 5 LESIONS; FIVE WERE ENTERED ON IANR M.D. 4/18/05
	Cavities	Chest	Fluid	1 ml	Red	2	NST	
	Spleen	-	Enlarged	30x20x20 mm	Dark	3	4	TGL1-C-mammary gland PRM 10/7/05
	Lung	Left lobe	~~5x5x~~[2] Nodule	5x5x5 mm	-	4	NCL	TGL4 not observed PRM 10/7/05
	Liver	Median	Mass	15x10x10 mm	Dark	5	12	
	Liver	Median	Focus	3x3 mm	Dark	6	10	
	Liver	Left	Focus	4x4 mm	Dark	7	10	
L.S.	**Small intestine**	**Duodenum**	**Nodule**	**2x2 mm**	**Hard, tan**	8	10	
						9	10	
						10	13	

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
R. Cutup 4/18/05	LIVER TUMOR M.D. 4/18/05		[1] wrong entry RC 4/18/05
26. PATHOLOGIST/NEC DATE	29. CLINICAL/REMOVAL OBSERAVTIONS:	Y/N	[2] wrong place RC 4/18/05
M. DOCTOR 4/18/05	Thin	Y RC	
27. TRIMMER/DATE	Mass torso ventral large one	Y RC	
L. Slice 7/11/05	Lethargic LP 4/2/05 4/18/05	N RC	
A = NORMAL C = MISSING B = OBSERVED D = PRESENT, NOT EXAMINED			
REVISED 6/06	Abnormal breathing LP 4/18/05	N RC	PAGE 1 OF ___1___

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC ☐ REPEATED DOSE
7. TDMS - NUMBER		8. SPECIES/STRAIN		9. SEX	10. ANIMAL NUMBER
		RAT F344		MALE	

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
		.

14. DISPOSITION	15. CONDITION	16a. IDENTIFICATION INFORMATION
☐ TSAC ☐ NATD ☐ DACC ☐ MSAC ☐ OTHER* ☐ ACCK ☐ SSAC	☐ FRESH ☐ CANN/NTT ☐ AUTO/TT ☐ PT CANN ☐ OTHER *	VERIFIED ☐ ID NUMBER: _____ IIDENTIFICATION COMMENT:
* DEFINE OTHER:	* DEFINE OTHER:	16b. WET TISSUE REVIEW ☐ PERFORMED ☐ NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.			INTEST SMALL			LYMPH NODE			SALIVARY GLS		
BLOOD			DUODENUM			BRONCHIAL			SEMINAL VESICLES		
BLOOD VESSEL			JEJUNUM			MEDIASTINAL			SKELETAL MUSCLE		
BONE			ILEUM			MANDIBULAR			SKIN		
BONE MARROW			INTEST LARGE			MESENTERIC			SPINAL CORD		
BRAIN			CECUM			OTHER			SPLEEN		
CARCASS			COLON			MAMMARY GL			STOMACH		
CAVITIES			RECTUM			MESENTERY			TESTES		
COAGULAT GL			KIDNEY			NOSE			THYMUS		
EPIDIDYMIS			LACRIMAL GL			PANCREAS			THYROID GL		
ESOPHAGUS			LARYNX			PARATHYROID GL			TISSUE NOS		
EYE			LIVER			PENIS			TONGUE		
HARDERIAN GL			LUNG			PERIPH NERVE			TOOTH		
HEART						PHARYNX			TRACHEA		
						PITUITARY GL			URETER		
						PREPUTIAL GL			URINARY BLADDER		
						PROSTATE			ZYMBALS GL		

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
						1		
						2		
						3		
						4		
						5		
						6		
						7		
						8		
						9		
						10		

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
26. PATHOLOGIST/NEC DATE	29. CLINICAL/REMOVAL OBSERAVTIONS:	Y/N	
27. TRIMMER/DATE			
A = NORMAL C = MISSING B = OBSERVED D = PRESENT, NOT EXAMINED			
REVISED 6/06			PAGE 1 OF _____

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
-	RAT F344	FEMALE	

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
		.

14. DISPOSITION	15. CONDITION	16a. IDENTIFICATION INFORMATION
☐ TSAC ☐ OTHER* ☐ DACC	☐ CANN/NTT	VERIFIED ☐ ID NUMBER:
☐ MSAC ☐ NATD ☐ ACCK	☐ FRESH ☐ PT CANN	IDENTIFICATION COMMENT:
☐ SSAC	☐ AUTO/TT ☐ OTHER *	
* DEFINE OTHER:	* DEFINE OTHER:	16b. WET TISSUE REVIEW ☐ PERFORMED ☐ NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.			INTEST SMALL			LYMPH NODE			SALIVARY GLS		
BLOOD			DUODENUM			BRONCHIAL			SKELETAL MUSCLE		
BLOOD VESSEL			JEJUNUM			MEDIASTINAL			SKIN		
BONE			ILEUM			MANDIBULAR			SPINAL CORD		
BONE MARROW			INTEST LARGE			MESENTERIC			SPLEEN		
BRAIN			CECUM			OTHER			STOMACH		
CARCASS			COLON			MAMMARY GL			THYMUS		
CAVITIES			RECTUM			MESENTERY			THYROID GL		
CLITORAL GL			KIDNEY			NOSE			TISSUE NOS		
ESOPHAGUS			LACRIMAL GL			OVARIES			TONGUE		
EYE			LARYNX			OVIDUCT			TOOTH		
HARDERIAN GL			LIVER			PARATHYROID GL			TRACHEA		
HEART			LUNG			PANCREAS			URETER		
						PERIPH NERVE			URINARY BLADDER		
						PHARYNX			UTERUS		
						PITUITARY GL			VAGINA		
									ZYMBALS GL		

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
						1		
						2		
						3		
						4		
						5		
						6		
						7		
						8		
						9		
						10		

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
26. PATHOLOGIST/NEC DATE	29. CLINICAL/REMOVAL OBSERAVTIONS:	Y/N	
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					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
-	MOUSE B6C3F1	MALE	

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
		.

14. DISPOSITION	15. CONDITION	16a. IDENTIFICATION INFORMATION
☐ TSAC ☐ OTHER* ☐ DACC	☐ CANN/NTT	VERIFIED ☐ ID NUMBER:
☐ MSAC ☐ NATD ☐ ACCK	☐ FRESH ☐ PT CANN	IDENTIFICATION COMMENT:
☐ SSAC	☐ AUTO/TT ☐ OTHER *	
* DEFINE OTHER:	* DEFINE OTHER:	16b. WET TISSUE REVIEW ☐ PERFORMED ☐ NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.			INTEST SMALL			LYMPH NODE			SALIVARY GLS		
BLOOD			DUODENUM			BRONCHIAL			SEMINAL VESICLES		
BLOOD VESSEL			JEJUNUM			MEDIASTINAL			SKELETAL MUSCLE		
BONE			ILEUM			MANDIBULAR			SKIN		
BONE MARROW			INTEST LARGE			MESENTERIC			SPINAL CORD		
BRAIN			CECUM			OTHER			SPLEEN		
CARCASS			COLON			MAMMARY GL			STOMACH		
CAVITIES			RECTUM			MESENTERY			TESTES		
COAGULAT GL			KIDNEY			NOSE			THYMUS		
EPIDIDYMIS			LACRIMAL GL			PANCREAS			THYROID GL		
ESOPHAGUS			LARYNX			PARATHYROID GL			TISSUE NOS		
EYE			LIVER			PENIS			TONGUE		
GALLBLADDER			LUNG			PERIPH NERVE			TOOTH		
HARDERIAN GL						PHARYNX			TRACHEA		
HEART						PITUITARY GL			URETER		
						PREPUTIAL GL			URINARY BLADDER		
						PROSTATE			ZYMBALS GL		

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
						1		
						2		
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						10		

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
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REVISED 6/06			PAGE 1 OF ____

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
-	MOUSE B6C3F1	FEMALE	

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
		.

14. DISPOSITION	15. CONDITION	16a. IDENTIFICATION INFORMATION
☐ TSAC ☐ OTHER* ☐ DACC	☐ CANN/NTT	VERIFIED ☐ ID NUMBER:
☐ MSAC ☐ NATD ☐ ACCK	☐ FRESH ☐ PT CANN	IDENTIFICATION COMMENT:
☐ SSAC	☐ AUTO/TT ☐ OTHER *	
* DEFINE OTHER:	* DEFINE OTHER:	16b. WET TISSUE REVIEW ☐ PERFORMED ☐ NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.			INTEST SMALL			LYMPH NODE			SALIVARY GLS		
BLOOD			DUODENUM			BRONCHIAL			SKELETAL MUSCLE		
BLOOD VESSEL			JEJUNUM			MEDIASTINAL			SKIN		
BONE			ILEUM			MANDIBULAR			SPINAL CORD		
BONE MARROW			INTEST LARGE			MESENTERIC			SPLEEN		
BRAIN			CECUM			OTHER			STOMACH		
CARCASS			COLON			MAMMARY GL			THYMUS		
CAVITIES			RECTUM			MESENTERY			THYROID GL		
CLITORAL GL			KIDNEY			NOSE			TISSU ENOS		
ESOPHAGUS			LACRIMAL GL			OVARIES			TONGUE		
EYE			LARYNX			OVIDUCT			TOOTH		
GALLBLADDER			LIVER			PANCREAS			TRACHEA		
HARDERIAN GL			LUNG			PARATHYROID GL			URETER		
HEART						PERIPH NERVE			URINARY BLADDER		
						PHARYNX			UTERUS		
						PITUITARY GL			VAGINA		
									ZYMBALS GL		

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
						1		
						2		
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						5		
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						9		
						10		

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
26. PATHOLOGIST/DATE	29. CLINICAL/REMOVAL OBSERAVTIONS:	Y/N	
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INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
-			

11. HISTOLOGY NUMBER

CONTINUATION PAGE

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION /COLOR	23. TGL		24. NOTES
						A	B	
						11		
						12		
						13		
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						20		
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						24		
						25		

25. PROSECTOR/NECROPSY DATE	30. CORRECTION COMMENTS:
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APPENDIX 5

NATIONAL TOXICOLOGY PROGRAM
PROCEDURES FOR
SPERM MOTILITY & COUNTS
AND VAGINAL CYTOLOGY EVALUATION
(SMCVC)

INTRODUCTION

In addition to setting doses for the 2-year chronic toxicity and carcinogenicity studies, the 90-day subchronic studies performed by the National Toxicology Program also serve to identify target organs for toxicants. An important part of this strategy is to evaluate the reproductive system. The SMCVC studies perform this valuable function.

Retrospective evaluations (Chapin et al., 1997) confirm that reproductive organ weights and sperm indices (spermatid count in testis and sperm count in epididymis, epididymal sperm motility) relate to fertility in meaningful ways. While it is possible to have altered fertility concomitant with normal values for several of these necropsy indices (e.g., due to altered sexual activity), we know that if these male measures are changed, there is a good likelihood that the fertility of those animals will be reduced. Thus, these sperm endpoints are useful in detecting male reproductive toxicants that deserve further evaluation in functional (breeding) studies.

Fewer endpoints exist for females. Female reproductive organ weights fluctuate with stage of the estrous cycle, making weights a less-than-useful endpoint for study-day-driven necropsies, since females will be scattered through the cycle, and weight variances will be high. The normalcy of the cycle depends on the integrity of the hypothalamic-pituitary-gonadal axis. Since hormones control more of the reproductive system in females than in males, our efforts at surveillance in females focus on assessing the estrous cycle, and determining cyclicity through the cycle of cells that appear in a vaginal lavage.

This relationship between cycle stage and vaginal cells was first reported by Stockard and Papanicolaou in 1917, and continues to be widely used today. While we acknowledge that this focus on vaginal smears and the estrous cycle leaves many more unsurveyed functions and processes in females than in males, this represents the state-of-the-art in terms of proven measures of female fecundity; prolonged cycles correlate with reduced litter size (Chapin et al., 1997). Ovarian follicle counts, while specific, is a poor use of resources when exposure occurs in adulthood, as a small proportion of compounds has been shown to affect this parameter, and the data are exceedingly tedious and laborious to collect.

Thus, the group of endpoints that is currently assessed represents the best use of time and dollars, and collects data that we know are the most useful in identifying potential reproductive toxicants.

A detailed description of techniques to be used by the NTP-designated toxicology laboratories and the NTP-designated SMCVC laboratory to conduct the male and female toxicity studies properly and uniformly is provided in this document. The dose levels for these studies will be designated by the NTP Project Officer for the toxicology laboratory. Ten male and ten female rats or mice in three of the five dose groups plus the control group(s) will be utilized. Specific selection of the three dose groups will be based on the results obtained during the first 70 days of the subchronic studies. The objective is to select doses which are not causing overt toxic effects, i.e., mortality, depressed weight gain, etc.

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I. MALE ORGAN TOXICITY EVALUATION IN MICE AND RATS

The NTP toxicology laboratories are urged to read the material thoroughly because there are a few key differences between mice and rat studies. Sperm motility is highly dependent on having available energy substrate (a buffer containing lactate and glucose), pH (should be 7.25 ± 0.05) and temperature (with rapid decrease in motility with only a few degrees C drop in temperature). Thus all buffers and equipment should be temperature controlled or be held in an incubator or on a slide warmer at 37°C during sample preparation and evaluation. Moreover, rodent sperm are likely to form aggregates that prohibit appropriate motility analysis (a single cell suspension is required) and for this reason a protein supplement is normally added to the buffer, frequently 1% Bovine Serum Albumin (BSA) or egg yolk in modified Tyrode's buffer.

The conduct of sperm motility assessment and sperm count may be performed using manual or automated procedures. Although the automated procedure is preferred, manual assessment will be accepted. Procedures for both methods are provided in this document.

A. SUGGESTED LIST OF MATERIALS & SUPPLIES FOR SPERM ASSAYS IN MICE AND RATS

To be supplied by the toxicology laboratory

NOTE:
Materials & supplies needed will be based on the Method used – Automated versus Manual.

- Scalpels, scissors, forceps, syringes
- Pasteur pipettes
- Pipettes (1.0 and 5.0 mL), pipettors and tips
- Petri plates (15 x 60 mm)
- Pre-cleaned microscope slides (3" x 1") and cover-slips (24 x 40 mm)
- Parafilm
- Microhematocrit capillary tubes
- Hemacytometers and cover-slips (improved Neubaur, 0.1 mm deep)
- Test tubes (16 x 100 mm), test tube racks
- Scintillation Vials 28 x 61 mm or Sample Vials 28 x 60 mm and Scintillation Vial Racks
- Labels
- Modified Tyrode's Solution
- Test Yolk Buffer
- Phosphate Buffered Saline
- Incubator, water bath, slide warmer
- Hand tally counter (ENM-4290 or Clay Adams 4300)
- Timers
- Stage warmer for the microscope
- pH meter and pH paper
- Thermometers
- Oven
- Vortex
- CASA
- Protective Clothing

B. PREPARATION OF BUFFER/STAIN FOR SPERM ASSAYS

1. **Buffer for Automated Mouse and Rat Method**

The diluent (Modified Tyrode's Solution) can be prepared (See below) or purchased from the NTP SMCVC contractor.

To make 1000 mL of Tyrode's solution, bring the following up to 1000 mL with distilled deionized water:

Ingredients	Buffer Concentration	Amount/Liter
NaCl	99.23 mM	5.801 g/L
KCl	2.68 mM	0.200 g/L
$CaCl_2.2H_20$	1.80 mM	0.265 g/L
$NaH_2PO_4.H_20$	0.36 mM	0.050 g/L
$MgCl_2.6H_20$	0.49 mM	0.100 g/L
$NaHC0_3$	25.00 mM	2.1 g/L
Na lactate	25.00 mM	2.801 g/L
Na pyruvate	0.50 mM	0.055 g/L
Glucose	5.56 mM	1.001 g/L

Mix thoroughly until all chemicals are properly dissolved.

To the buffer, add 10 mL of Bovine Serum Albumin (BSA) to make a 1% solution. Shake well until in solution. Adjust the pH to 7.2 with 1N HCl or 1N NaOH. Aliquot the buffer into 5 or 10 mL volumes.

Store at -20^{o}C. Prior to each usage, the buffer's pH should be taken and adjusted to 7.2 if needed. If the buffer is kept at 37^{o}C (incubator temperature) for long periods of time (1-2 hours) then again the pH should be taken and adjusted if necessary.

2. **Buffer for Manual Mouse Method**

The diluent (Modified Tyrode's Solution) can be prepared (See below) or purchased from the NTP SMCVC contractor.

To make 1000 mL of Tyrode's solution, bring the following up to 1000 mL with distilled deionized water:

Ingredients	Buffer Concentration	Amount/Liter
NaCl	99.23 mM	5.801 g/L
KCl	2.68 mM	0.200 g/L
$CaCl_2.2H_20$	1.80 mM	0.265 g/L
$NaH_2PO_4.H_20$	0.36 mM	0.050 g/L
$MgCl_2.6H_20$	0.49 mM	0.100 g/L
$NaHC0_3$	25.00 mM	2.1 g/L
Na lactate	25.00 mM	2.801 g/L
Na pyruvate	0.50 mM	0.055 g/L
Glucose	5.56 mM	1.001 g/L

Mix thoroughly until all chemicals are properly dissolved.

To the buffer, add 200 mL of egg yolk without the yolk membranes. Shake until yolk is in solution. Thermoprecipitate the solution by boiling for 3 minutes at 92-95^{o}C. Filter through 4 layers of cheesecloth. Centrifuge the filtrate at 10,000 rpm for 20 minutes. Filter through Whatman #1 paper. Add 0.7 g/100 mL of percent of D-anhydrous glucose to the solution to raise the osmolarity. Measure the osmotic pressure and it should be at 325 mOsm/L (accuracy within 5 mOsm/L). Adjust the pH to 7.2 with 1N HCl or 1N NaOH. At this time add penicillin (100 u/mL) and streptomycin (50 ug/mL). Aliquot the buffer into 5 or 10 mL volumes. Since this buffer is easily contaminated, it is recommended that all steps after thermoprecipitation be conducted under aseptic atmosphere when possible.

Store at -20^{o}C. Prior to each usage, the buffer's pH should be taken and adjusted to 7.2 if needed. If the buffer is kept at 37^{o}C (incubator temperature) for long periods of time (1-2 hours) then again the pH should be taken and adjusted if necessary.

3. **Buffer and Stain for Manual Rat Method**

a. Test Yolk Buffer can be purchased from the NTP contractor for SMCVC studies, or prepared using the following ingredients per liter:

TES**	14.5 g
TRIS***	3.5 g
Glucose	2.0 g

**(N-tris(hydroxymethly)methyl-1-aminoethane sulfonic acid)
***Tris(hydroxymethly)aminomethane

Skim Milk Powder	33.0 g
Sodium Citrate	6.0 g

Mix the ingredients in 700 mL of distilled deionized water. Adjust the final volume to 800 mL. Add 200 mL of egg yolk without the yolk membranes. Thermoprecipitate the solution by boiling at 92-95^{o}C for 3 minutes. Filter through 4 layers of cheesecloth. Centrifuge the filtrate at 10,000 rpm for 20 minutes. Filter through Whatman #1 paper. Aliquot into 5 or 10 mL volumes. Store at -20^{o}C until further use. pH will be 7.1-7.2.

4. **Phosphate Buffered Saline (PBS)**

PBS is prepared by using the following ingredients per 1000 mL:

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.2 g
$Na_2HPO_47H_2O$	2.1 g
$CaCl_2$	0.1 g
Mg CI_26H_2	0.1 g

Final pH = 7.2

Mix the contents in 950 mL of distilled deionized water. Adjust the pH to 7.2. Bring the final volume to 1000 mL. Filter sterilization is recommended.

C. PROCEDURES FOR REMOVING AND WEIGHING TISSUES

Before sacrificing the animal, be sure that the buffer solution, microscope slides, cover-slips, pipette tips, petri plates, and microscope stage have been warmed to the appropriate temperature (37°C). Also, be sure that the balance is properly calibrated. Check the pH of the appropriate buffer solution and adjust it to 7.2 with HCL (1N) or NaOH (1N).

NOTE: For the Automated Method, use buffer on page 6 for both mice and rats.
For the Manual Method, use the formula on page 7 for mice and page 8 for rats.

The test animal is weighed, the weight is recorded on the Data Sheet (see page 18 for mice and page 20 for rats) and then sacrificed according to the study protocol at the end of the 90-day study.

1. Procedure for Removing Testis and Epididymis

After sacrificing the animal, conduct the sperm collection as quickly as possible. Use the following procedure to remove the testis and epididymis:

a. Pull the left testis into the abdominal cavity by grasping the fat surrounding the caput epididymis. Excise the left testis by cutting the vas deferens approximately 0.5 cm from the point of attachment of the cauda epididymis. The caput epididymis should be located prior to excision so that it will not be clipped accidentally. See Section F. Figures 1 (mouse), 2 (rat), and 3.

b. The intact left epididymis is removed and weighed to the nearest 0.0001 g. Record the weight on Data Sheet.

c. Clip the left cauda epididymis at the point of origin of the ductus deferens at the distal end and at the boundary between the distal corpus and cauda epididymis of the proximal end. In rats, cauda epididymis can be seen as white tissue consisting of convoluted tubules.

d. Weigh left cauda epididymis to the nearest 0.0001 g. Record the weight on Data Sheet.

2. Weighing, Freezing and Shipping of Testis

a. During the incubation period for the motility assessment, the left testis is to be weighed and frozen for testicular spermatid head-count as described below.

- Label each vial (preferably plastic) with the appropriate laboratory name, test article, route, dose group, animal number, L for left testis (or R for right testis when required), and date of collection.

- Weigh the left testis and record the weight to 0.0001 g on Data Sheet.

- Place the testis in the appropriately labeled vial and freeze on dry ice.

- When convenient, transfer frozen samples to a freezer (-70 to -80°C) for storage until ready for shipment to NTP contractor for SMCVC studies.

b. Instructions for shipping frozen testis to SMCVC laboratory

- The NTP toxicology laboratory conducting the toxicity studies is responsible for packaging and shipping costs.
- The frozen testes will be shipped to the NTP-designated SMCVC laboratory along with the corresponding raw data sheets within two weeks of necropsy.
- All vials will be placed in vial racks and wrapped with bubble packing to avoid breakage during shipment.
- The wrapped vial racks will be placed in 350 lb. styrofoam-lined boxes filled with dry ice for shipment.
- An inventory of the vials will be sent with the cover letter.
- Shipping cartons will be sealed and bound with filament tape prior to shipment and labeled "freeze upon arrival".
- Shipping will be expedited so that the testes do not thaw during transit, i.e. overnight delivery. The laboratory must be notified prior to shipment so that they will anticipate delivery.

D. AUTOMATED METHOD FOR SPERM MOTILITY ASSESSMENT AND COUNT

Most CASA (Computerized Assisted Sperm Analysis) machines (the Hamilton Thorne IVOS Tox is recommended) require that the sperm samples be appropriately diluted in buffer before a sample is taken for analysis to allow conditions for uninhibited sperm motion. This can be determined experimentally but is usually approximately 50 mL per cauda for rats and 10 mL per cauda for mice. Other methods of dilution (e.g. a serial dilution from 10 mL mixed with a rat cauda) are acceptable if they give consistent results and concentrations in the CASA chamber that do not impede normal motion (< 100 but > 50 sperm per field). See manufacturer's instructions for more detail.

1. Processing Tissues

Prepare pre-warmed Petri dishes labeled for the individual animal containing an appropriate volume of buffer containing BSA.

Isolate and weigh the cauda as per method described above, place in a Petri dish containing the appropriate buffer and make 2 to 3 small pin-point incisions with a scalpel blade into the distal tip of the proximal cauda epididymis (see Section F. Figure 3). Replace Petri lid and incubate for 2.5 minutes at 37°C. After incubation gently swirl the Petri dish to mix the sperm suspension. Load slide/chamber for the CASA instrument (by capillary action) and then follow the CASA manufacturer's operating instructions to obtain motility and count data. The machine will enable the collection of an individual animal number (as a file number), the dilution undertaken and the weight of the cauda.

2. Sperm Motility Measurements

Check to assure the dilution is appropriate by manually scanning fields on the machine (there should be no more than ~100 sperm per field) and either collect data or further dilute the sample appropriately. Acquire motility and motion characteristics as per manufacturer's instructions. Acquire images from 5 different fields and save to a suitable disk for the capture of electronic data (motion data should be from at least 250 sperm). For samples with low counts, more fields may have to be taken to obtain the requisite 250 sperm. All the motion characteristics should be saved (as raw data) to a disk. If necessary, these images can be re-analyzed at a future date.

The CASA machine will provide the following data that can be exported and saved in a suitable electronically readable file (e.g. ASCII) for further statistical analysis:

Counts	-	Total sperm, Motile sperm, and Progressively motile sperm
		% Motile and % Progressively motile
		Slow cells and Static cells
Concentration	-	Total, Motile, Progressively motile (in millions/mL)
		Slow cells and Static cells (millions/mL)

Other velocity parameters are available from the CASA and can be useful and sensitive indicators of change indicating an adverse quantitative effect on sperm motility/motion (as opposed to the qualitative manual method). Such measurements are made automatically by the instrument and should be retained as part of the dataset for each animal:

VAP - smooth path velocity, microns/sec
VCL - track velocity, microns/sec (sometimes called curvilinear velocity where computer "smoothes" the sperm track to obtain velocity over the whole period of measurement)
VSL - straight-line velocity, microns/sec

3. Sperm Count

With the "ident" mode on the Hamilton Thorne machine, a sample may be taken, after motility analysis, of the total caudal count by gently mincing the cauda in buffer with fine scissors. Following addition of the manufacturer's stain and suitable dilution, (see manufacturer's instructions) sperm numbers can be obtained from the cauda on a million/gram of tissue basis.

4. Data Submission

Complete the top portion of the Data Sheet (page 18 for mice; page 20 for rats) and check the box to indicate that sperm motility and count were performed using AUTOMATED MEASUREMENTS (leave the bottom portion of the Data Sheet blank since these measurements will be generated by CASA). Submit a hard copy of all of the sperm count, concentration and velocity data for each animal, together with a suitable electronic form of the data on disk for statistical analysis. In addition, raw motion images will be saved electronically to disk for each study. All of this information, along with the Data Sheets, is to be sent to the NTP-designated SMCVC contractor for evaluation.

E. MANUAL METHOD FOR SPERM MOTILITY ASSESSMENT AND COUNT

1. Processing Tissues. - **This step differs for mice and rats. Read instructions carefully.**

Mice

a. Two independent slides are prepared to estimate sperm motility. Place < 100 μL of pre-warmed modified Tyrode's Solution (see page 6) on each microscope slide. Slides shall be placed on a slide warmer set at 37°C and retained at this temperature.

b. Hold the left cauda epididymis with forceps and make a small incision near the center of the cauda with a sharp, clean surgical blade. See Section F. Figure 4.

c. Apply gently pressure on the cauda surface so that a small epididymal tubule is exposed or caudal fluid begins to ooze out into the Tyrode's solution on the pre-warmed slide (Caution: Do not apply too much pressure since there is an ample amount of sperm in a piece of cauda equivalent to 1/2 of a pinhead. Use of excessive amounts of cauda fluid for the sperm motility counts may significantly lower sperm density evaluations and impede your determination of sperm motility). Alternately, the cauda epididymis is placed into a petri plate (15 x 60 mm) containing 2 mL of pre-warmed phosphate buffered saline (PBS). The cauda is then held in place with forceps and a very small piece (not more than 1/20 of the total caudal is sliced off and the cut surface applied briefly to the buffer (see Section F. Figure 4). Note: Surgical blades must be cleaned with saline between animals.

d. If necessary, the cauda fluid may be further chopped on the slides with a pair of needles or sharp blades.

e. Place a warm cover-slip (24 x 40 mm) on the slide.

Maximum sperm motility is obtained if the removal of cauda, weighing, and preparation of sperm suspension is done as quickly as possible (approximately 2-4 minutes).

f. Two slides are prepared simultaneously for estimation of sperm motility.

g. The sperm motility is estimated without any further delay as illustrated in Section 2 below.

h. Place remaining caudal tissue into a petri plate (15 x 60 mm) containing 2 mL of phosphate buffered saline (PBS).

i. The remaining caudal tissue is gently chopped (only after the sperm motility counts have been performed) in the petri plate with two scalpels until its contents are released. The petri plate is swirled several times and incubated for 15 minutes at 37^{o}C. It allows aggregates of sperm, which can be seen as pieces of tissue in the suspension, to break up. Petri plates should remain covered during the incubation period.

Rats

a. The cauda epididymis in rats can be seen as white tissue consisting of convoluted tubules. After clipping the cauda at the point of origin of the ductus deferens (Section F. Figures 2, and 3) from the cut end of the cauda (distal cauda), a very small sperm sample (not greater than the size of a pinhead) is removed with the tip of the scalpel blade and placed on a pre-warmed slide with < 100 uL drops of test yolk buffer (see page 7) at 37°C. Do not allow the sperm to dry on the scalpel blade.

b. A cover-slip (24 x 40 mm) is placed on the slide after gentle mixing of sperm in the buffer solution. Two such slides are prepared for estimation of sperm motility as described in Section 2 below.

c. After motility has been estimated, the remaining cauda is weighed to the nearest 0.0001 g. The weight is recorded on the Data Sheet.

d. This step is performed only after sperm motility has been estimated. The cauda is held in place with a scalpel and gently chopped with another sharp scalpel in 10 mL of 0.9% saline or PBS in a petri plate incubated at 37°C for 15 minutes.

2. Estimation of Sperm Motility - **this step is common for both rats and mice.**

a. To estimate sperm motility, the viewer counts the actual number of motile and non-motile sperm for each field of vision. A small hand counter may be used to count the number of motile and non-motile sperm. No more than 20 sperm should be in the visual field.

b. The final motility is based on ten independent fields and observations made by two independent viewers.

c. Person A evaluates the motility in five independent fields and similarly, Person B evaluates the motility for another five independent fields. Both viewers must use independent slides and when possible, independent microscopes.

d. Sperm are considered motile if they show any movement at all. A 40X objective is recommended to evaluate sperm motility.

e. Percent motility decreases with time; therefore, evaluation is critical and must be accomplished as quickly as possible (2-4 minutes).

f. A microscope stage warmer (37°C) is needed for sperm motility evaluation and to maintain the sperm suspension at optimum temperature.

Sperm are very sensitive to temperature and pH changes. Extreme care must be taken to keep all materials at 37°C on a slide warmer and/or in an incubator. The pH of the Tyrode's solution should be checked routinely and adjusted when and if necessary. These factors are very important to obtain an accurate estimation of sperm motility.

g. Average sperm motility readings should be approximately 75% or higher in control mice and rats. If sperm motility is considerably lower than 75%, there is some

problem and personnel at the NTP contractor for SMCVC studies should be consulted.

3. Sperm Count

After the 15-minute incubation period, a sperm count is performed but the sperm suspension must be further diluted. Due to a significant difference in the cauda weight for rats and mice, parts of the dilution procedures for the two species are different and should be noted.

a. Swirl the petri plate to evenly distribute the sperm. In the event the sperm suspension is not uniform, gently chop the cauda until a uniform suspension is obtained, incubate further if necessary. Transfer 0.5 mL (using a 1.0 mL pipette) to a test tube (16 x 100 mm) containing 2 mL of the phosphate buffered saline. The rat suspension should be gently pipetted 20 times to break up sperm aggregates.

b. After transferring 0.5 mL of sperm suspension into 2 mL of PBS, transfer 2 aliquots of the residual suspension into two 1.5 mL vials (preferably plastic, filled to approximately 80%) and place on dry ice. These samples are to be shipped to the NTP Archives following the shipping instructions provided above for testes.

c. Gently and thoroughly mix the suspension with a Vortex mixer for 2 seconds. If a vortex mixer is not available, gently agitate the sperm suspension then gently pipette (with a Pasteur pipette) approximately 5 times to thoroughly mix the suspension. The importance of thorough mixing cannot be overstated.

d. The volume of PBS may be varied according to the density of sperm suspension. The number of sperm per secondary square (see Step g. below) should not be less than 5 or more than 15.

e. The volume of sperm suspension and PBS solution used for the sperm count should be recorded on the data form if different from the suggested volumes.

f. The sperm used for this assay should be killed by placing the test tube in hot water for one minute, in an ice bath for 5 minutes, or adding formaldehyde or glutaraldehyde to 0.5% final concentration.

g. The sperm suspension is thoroughly mixed by vortexing ≥ 5 seconds, and placed on a hemacytometer (see Section F. Figure 5).

h. The shiny surfaces in the middle of the hemacytometer have microscopic grids consisting of heavy lines which divide the field into 25 large squares. Within each of the large squares are 16 smaller squares. The cover-slip does not touch the grid area when placed over the middle portion.

i. Microhematocrit capillary tubes are used to place suspension on the hemacytomer. The sperm suspension is placed on the hemacytometer by touching the tip of the capillary tube to the angle formed by the cover-slip and counting chamber of the hemacytometer. The area under the cover-slip should not be overfilled.

j. Two counts are performed using separate samples of the diluted suspension. Both slides for sperm count must be prepared after thorough mixing of the sperm

suspension. Consistency in the preparation of the sperm samples for density determinations is essential to obtain accurate results.

k. To perform the counts, the counting area is located under low power (10X objective) and then switched to the 20X objective for counting. It is not necessary to count every cell in every square. The number of sperm in five secondary squares is counted. Sperm lying on the lines on the bottom and right sides of the large squares are included so that they will not be counted twice.

l. In case there are less than five (5) sperm per secondary square and it is not possible to make a new (less diluted) suspension, count sperm in two (2) tertiary squares. This change must be recorded on the Data Sheet and taken into consideration while estimating sperm density.

m. Calculation for determining sperm density also varies for mice and rats as explained below:

- The viewer has counted the sperm in 1/50 mm^3 volume;
- The dilution factor for the caudal sperm is the final dilution of caudal weight (normally for mice it is 10 cc/caudal weight, and for rats 50 cc/caudal weight). These values apply only when the suggested volumes are used.
- The number of cells counted multiplied by the volume fraction (1/50 mm^3) counted multiplied by the dilution factor should equal the number of cells per cubic millimeter.
- Sperm concentration is normally expressed as number of sperm per gram of tissue weight. Sperm density in the control mice is generally between 500 x 10^6 and 1000 x 10^6 sperm per gram caudal tissue.

4. Calculation Procedure For Estimation Of Sperm Density

Mice

Suppose the mean number of sperm in "5" secondary squares in mice = 20. The number of sperm in 2 X 10^{-5} mL of diluted sperm suspension = 20. The number of sperm per mL of diluted suspension =

$$\frac{20}{2 \times 10^{-5}} = 1 \times 10^6/\text{mL}$$

Since the diluted suspension represents one-fifth of the original suspension, if the standard dilution of 0.5 mL in 2 mL was used, the number of sperm per mL of the original suspension =

5 X (1 X 10^6) = 5 X 10^6.

Volume of the original suspension = 2 mL.

Total sperm in the original suspension = 2 X (5 X 10^6) = 1 X 10^7/suspension = number sperm/cauda.

If cauda weight = 0.03000 g, total sperm in 0.03000 g of caudal tissue = 1×10^7.

Total sperm per "g" of caudal tissue =

$$\frac{1 \times 10^7}{0.0300} = 3.33 \times 10^8 \text{ sperm/g cauda.}$$

This value can also be estimated by the following formula:

$$\frac{\text{Mean No. of sperm in 5 secondary squares}}{\text{caudal weight in g}} \times 50{,}000 \times 10 \text{ (dilution factor)}$$

When recording final calculated number of sperm/g cauda, record this to one decimal place.

Rats

Suppose the mean number of sperm in "5" secondary squares in mice = 20. The number of sperm in 2×10^{-5} mL of diluted sperm suspension = 20. The number of sperm per mL of diluted suspension =

$$\frac{20}{2 \times 10^{-5}} = 1 \times 10^6/\text{mL}$$

Since the diluted suspension represents one-fifth of the original suspension, if the standard dilution of 0.5 mL in 2 mL was used, the number of sperm per mL of the original suspension =
$5 \times (1 \times 10^6) = 5 \times 10^6$.

Volume of the original suspension = 10 mL.

Total sperm in the original suspension = $10 \times (5 \times 10^6) = 5 \times 10^7$/suspension = number sperm/cauda.

If cauda weight = 0.1500 g, total sperm in 0.1500 g of caudal tissue = 5×10^7.

Total sperm per "g" of caudal tissue =

$$\frac{5 \times 10^7}{0.1500} = 3.33 \times 10^8 \text{ sperm/g cauda.}$$

This value can also be estimated by the following formula:

$$\frac{\text{Mean No. of sperm in 5 secondary squares}}{\text{caudal weight in g}} \times 50{,}000 \times 50 \text{ (dilution factor)}$$

Record the number of sperm/g cauda to one decimal place.

5. Summary Of Manual Procedure for Sperm Evaluation in Mice and Data Sheet

   a. Place instruments and glassware in oven at 37°C. (TT)

   b. Put modified Tyrode's in water bath at 37°C. (TT)

   c. pH of Tyrode's should be 7.2. (TT)

   d. Weigh animal (NT), record weight (NT), bleed animal (TT), kill animal (NT), and bleed again when required (TT).

   e. Excise left testis and epididymis. Remove intact epididymis, weigh and record to nearest 0.0001 g. (NT)

   f. Cut off left cauda, weigh to 0.0001 g and record. (NT)

   g. Place , < 100 uL warm modified Tyrode's on each of 2 slides. Place sperm the size of half a pinhead on the slides and gently mix with Tyrode's, cover-slip and do motility (40X). (TT)

   h. Weigh left testis to 0.0001 g and record. (NT)

   i. Place remaining cauda in petri dish with 2 mL PBS, gently chop, swirl, cover, and incubate for 15 minutes at 37°C. (TT)

   j. Remove petri dish, ensure homogeneity, and transfer 0.5 mL sample to test tube containing 2 ml PBS. (TT) Transfer 2 aliquots of residual suspension into two 1.5 ml vials (filled to approximately 80%) and place on dry ice for shipment to NTP Archives.

   k. Kill sperm by heat, cold, or aldehyde fixatives. (TT)

   l. Pipette 5 times and place sample on hemacytometer for count (20X), Repipette and place a second sample on another hemacytometer for a second count. (TT) Note: Count sperm in 5 secondary squares and if less than 5 per square, then count sperm in 2 tertiary squares and record change.

      Note: NT = Necropsy Technician TT = Toxicology Technician

DATA SHEET - MOUSE SPERM STUDIES

DATE:_____

LABORATORY _____LAB #:_____

TEST ARTICLE:_____CAS #_____

C # _____ TREATMENT GROUP: _____ANIMAL #:_____

ANIMAL WEIGHT:_____ g * EPIDIDYMAL WEIGHT (LEFT):_____ g *

CAUDA WEIGHT (LEFT):_____ g * TESTICULAR WEIGHT (LEFT):_____ g *

* Enter body weights to 1 decimal place & tissue weights to 4 decimal places

❑ AUTOMATED MEASUREMENTS

SPERM MOTILITY

field	1	2	3	4	5
motile					
non-motile					

Total Motile:___

SIGNATURE:_____ Total Non-motile:____
Observer A Date

field	6	7	8	9	10
motile					
non-motile					

% Motility (1) :_____

SIGNATURE: _____
Observer B Date

Sperm Count

(2) Vol. Saline (cauda in 2 mL PBS) _____mL

Count1	Count2	Mean

(2) Vol. Caudal Susp. (0.5 mL in 2 mL) _____mL

(3) Sperm Concentration: _____ X 10^6
Per g caudal tissue

SIGNATURE: _____
Observer Date

(1) Total motile/total motile + Non-motile X 100%

(2) Leave blank unless other volume is used. Do not dilute if the number of sperm per secondary square is less than 15.

(3) X(50000) Y (to the nearest 0.1 X 10^6) (X = # sperm counted; Y = dilution factor, which is 10 cc/caudal weight in grams)

NOTE: DILUTION FACTOR WILL CHANGE IF DIFFERENT VOLUMES OF DILUENT ARE USED.

6. Summary Of Manual Procedure for Sperm Evaluation in Rats and Data Sheet

   a. Place instruments and glassware in oven at 37°C. (TT)

   b. Put test yolk buffer in water bath at 37°C. (TT)

   c. Weigh animal (NT), record weight (NT), bleed animal (TT), kill animal (NT), and bleed again when required (TT).

   d. Excise left testis and epididymis. Remove intact epididymis, weigh and record to nearest 0.0001 g. (NT).

   e. Cut off left cauda, weigh to 0.0001 g and record. (NT).

   f. Place < 100 uL of warm test yolk buffer on each of 2 slides. Place sperm sample the size of a pinhead on the slides and gently mix with yolk, cover-slip, and do motility (40X). (TT).

   g. Note: There should be 3X as many motile compared to nonmotile sperm.

   h. Weigh left testis to 0.0001 g and record. (NT).

   i. Place remaining cauda in petri dish with 10 mL PBS, gently chop, swirl, cover, and incubate for 15 minutes at 37°C. (TT).

   j. Remove suspension from oven and pipette 20 times and remove connective tissue. (TT).

   k. Place 0.5 mL sample in test tube containing 2 mL PBS. (TT) Transfer 2 aliquots of residual suspension into two 1.5 ml vials (filled to approximately 80%) and place on dry ice for shipment to NTP Archives.

   l. Kill sperm by heat, cold, or aldehyde fixatives. (TT).

   m. Pipette 5 times and place sample on hemacytometer for count (20X). Re-pipette and place a second sample on another hemacytometer for a second count. (TT).

   n. Note: Count sperm in 5 secondary squares and if less than 5 per square, then count sperm in 2 tertiary squares and record change.

   Note: NT = Necropsy Technician TT = Toxicology Technician

DATA SHEET - RAT SPERM STUDIES

DATE:_____

LABORATORY _____LAB #:_____

TEST ARTICLE:_____CAS #_____

C # _____ TREATMENT GROUP: _____ANIMAL #:_____

ANIMAL WEIGHT:_____ g * EPIDIDYMAL WEIGHT (LEFT):_____ g *

CAUDA WEIGHT (LEFT):_____ g * TESTICULAR WEIGHT (LEFT):_____ g *

* Enter body weights to 1 decimal place & tissue weights to 4 decimal places

❑ AUTOMATED MEASUREMENTS

SPERM MOTILITY

field	1	2	3	4	5
motile					
non-motile					

Total Motile:____

SIGNATURE:_____ Total Nonmotile:____
Observer A Date

field	6	7	8	9	10
motile					
non-motile					

% Motility (1) :_____

SIGNATURE: _____
Observer B Date

Sperm Count

(2) Vol. Saline (cauda in 10 mL PBS) _____mL

Count1	Count2	Mean

(2) Vol. Caudal Susp. (0.5 mL in 2 mL) _____mL

(3) Sperm Concentration: _____ X 10^6
Per g caudal tissue

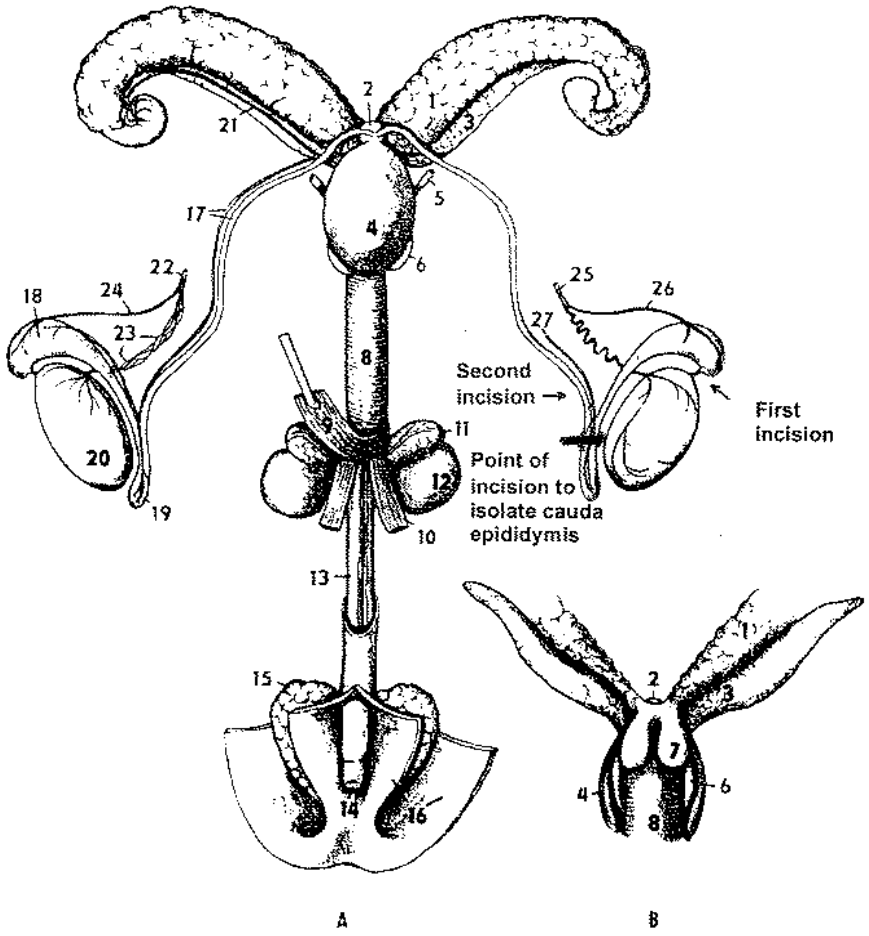
SIGNATURE: _____
Observer Date

(1) Total motile/total motile + non-motile X 100%
(2) Leave blank unless other volume is used
(3) X(50000) Y (to the nearest 0.1 X 10^6) (X = # sperm counted; Y = dilution factor, which is 50 cc/caudal weight in grams)

NOTE: DILUTION FACTOR WILL CHANGE IF DIFFERENT VOLUMES OF DILUENT ARE USED

F. FIGURES

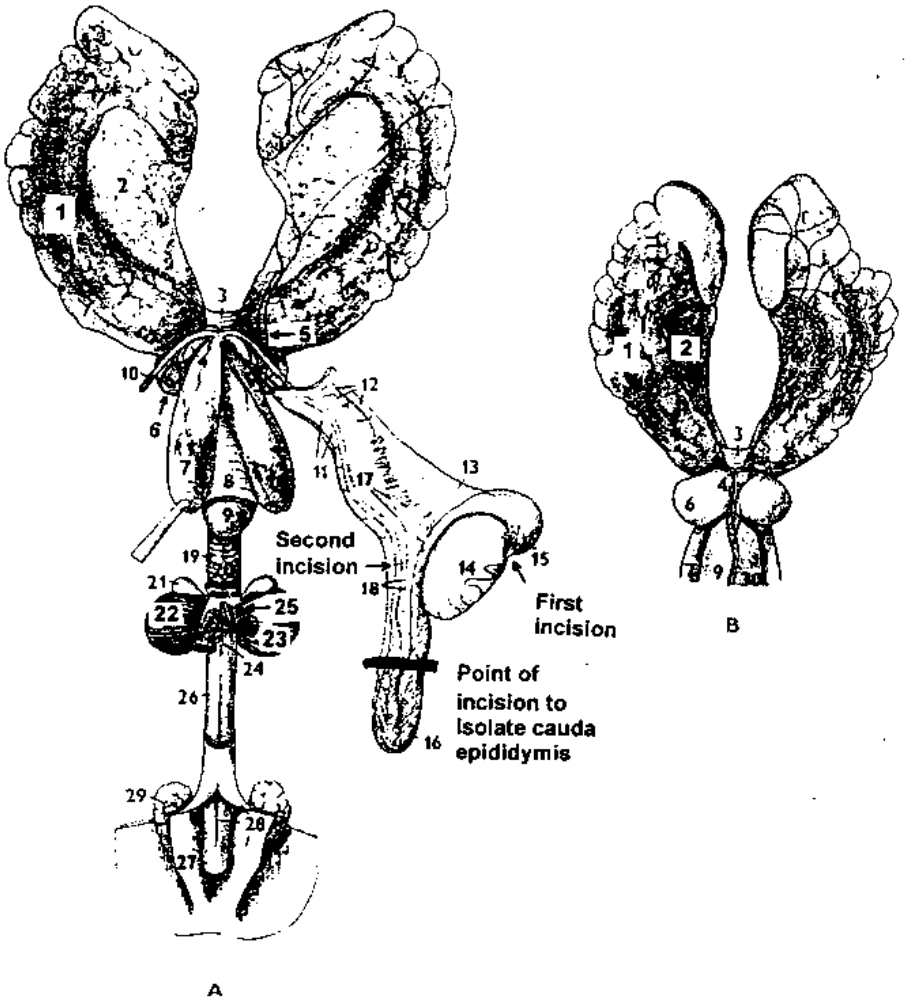
Figure 1. Male **MOUSE** genital organs; view of the ventral (A) and dorsal (B) surfaces.



1	*glandula vesicularis* – vesicular gland
2	*glandula ampullaris* – ampullary gland
3	*glandula coagulationis* – coagulational gland
4	*vesica urinaria* – urinary bladder
5	*ureter* – ureter
6,7	*prostata* – prostate
6	*pars ventralis* – ventral part
7	*pars dorsalis* – dorsal part
8	*pars pelvina urethrae et m. urethralis* – pelvic part of urethra and urethral muscle
9	*m. bulboglandularis* – bulboglandular muscle
10	*m. ischiocavernosus* – ischiocavernous muscle
11	*glandula bulbourethralis* – bulbourethral gland
12	*diverticulum glandulae bulbourethralis* – diverticulum of bulbourethral gland
13	*penis* – penis
14	*glans penis* – glans penis
15	*glandula preputialis* – preputial gland
16	*preputium* – prepuce
17	*ductus deferens v., ductus referentis* – deferent duct, vein of deferent duct
18,19	*epididymis* - epididymis
18	*caput epididymis* – head of epididymis
19	*cauda epididymis* – tail of epididymis
20	*testis* – testis
21	*v. glandulae vesicularis* – vein of vesicular gland
22	*v. testicularis dextra* – right testicular vein
23	*plexus pampiniformis* – pampinifrom plexus
24	*ramus epididymis v. testicularis* – epididymis branch of testicular vein
25	*a. testicularis sinistra* – left testicular artery
26	*ramus epididymidis cranialis a. testicularis* – cranial epididymis branch of testicular artery
27	*a. ductus deferentis* – artery of deferent duct

Popesko, P., Rajtova, V., Horak, J. (1992). *Colour Atlas of the Anatomy of Small Laboratory Animals. Volume 2. Rat, Mouse, Golden Hamster. English translation.* Wolfe Publishing Ltd. ISBN 0 7234 1823 3.

Figure 2. Male **RAT** genital organs *in toto*, showing the ventral surface (A); dorsal view of genital glands, urinary bladder, and dorsal prostate (B).



1 *glandula vesicularis* – vesicular gland
2 *glandula coagulationis* – gland for coagulation
3 *lig. intervesiculare* – intervesicular ligament
4 *glandula ductus deferentis* – gland of deferent duct
5 *rami glandulae vesicularis (a. et v.)* - branches of vesicular gland (artery and vein)
6 *prostata dorsalis* – dorsal prostate
7 *prostata ventralis* – ventral prostate
8 *lig. interprostaticum* – interprostatic ligament
9 *vesica urinaria* – urinary bladder
10 *ductus deferens* – deferent duct
11 *a. et v. ductus deferentis* – artery and vein of deferent duct
12 *a. et v. testicularis, mesorchium proximale* – testicular artery and vein, proximal mesorchium
13 *mesorchium distale* – distal mesorchium
14 *testis* – testis
15,16 *epididymis* - epididymis
15 *caput* – head
16 *cauda* – tail
17 *ramus epididymalis (a.et v.)* – epididymal branch (artery and vein)
18 *ramus ductus deferentis (a. et v.)* – branch for deferent duct (artery and vein)
19 *m. urethralis* – urethral muscle
20 *m. ischiocavernosus* – ischiocavernous muscle
21 *glandula bulbourethralis* – bulbourethral gland
22 *m. bulboglandularis* – bulboglandular muscle
23 *bulbus urethrae (in projectione)* – urethral bulb (in projection)
24 *urethra masculina (in projectione)* – masculine urethra (in projection)
25 *m. bulbospongiosus* – bulbospongious muscle
26 *corpus penis* – body of penis
27 *glans penis* – glans penis
28 *v. dorsalis penis, preputium* – dorsal vein of penis, prepuce
29 *glandula preputialis* – preputial gland
30 *a. et v. vesicalis* – vesical artery and vein

Popesko, P., Rajtova, V., Horak, J. (1992). *Colour Atlas of the Anatomy of Small Laboratory Animals. Volume 2. Rat, Mouse, Golden Hamster. English translation.* Wolfe Publishing Ltd. ISBN 0 7234 1823 3.

Figure 3. Diagram of a rat epididymis showing the site for removal of sperm samples from proximal cauda.

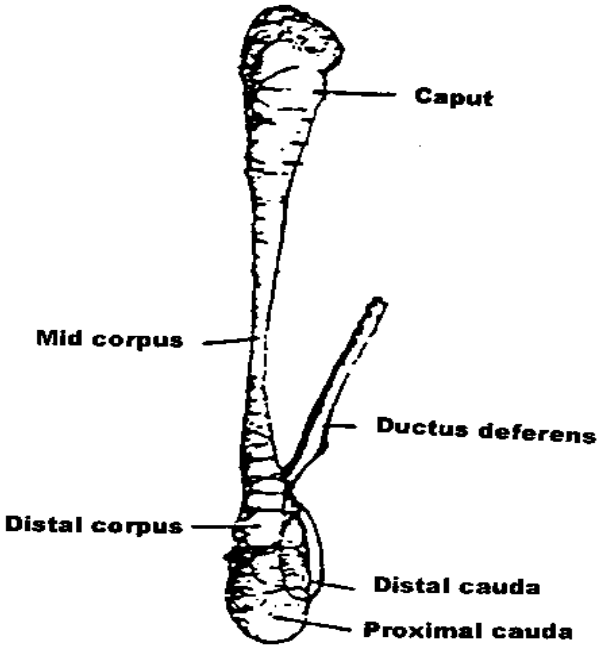
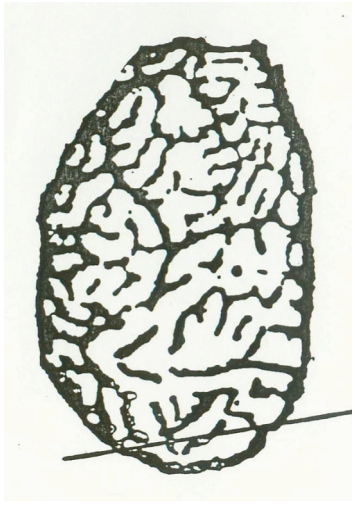
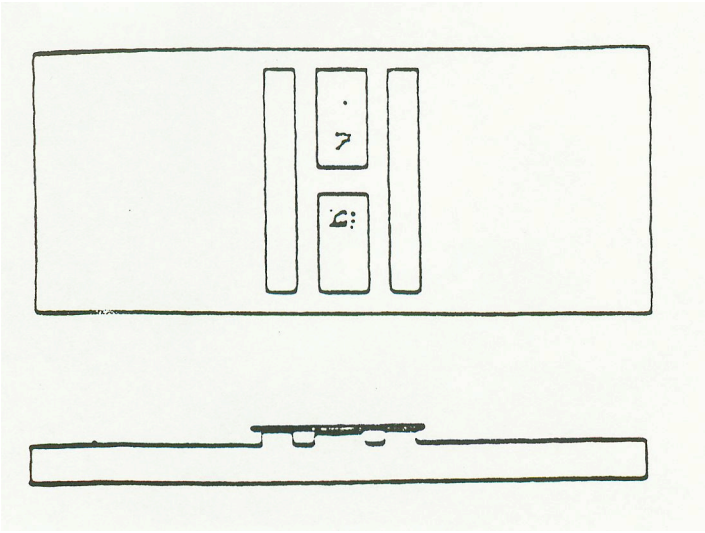


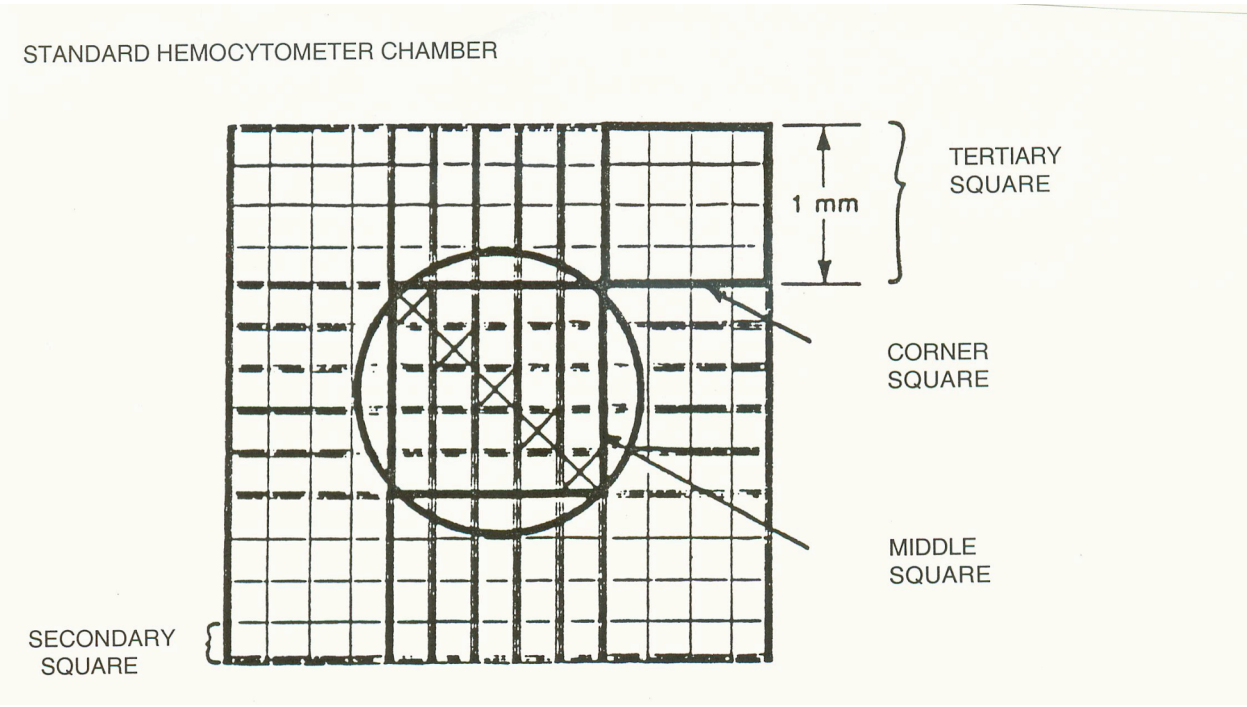
Figure 4: Representation of proximal cauda in mouse.



Recommended portion of cauda adequate for sperm motility evaluations.

Figure 5. Views of hemocytometer.





Volume Occupied by a Primary Square = 2.5×10^{-7} mL

Volume Occupied by a Secondary Square = 4×10^{-6} mL

Volume Occupied by a Tertiary Square = 1×10^{-4} mL

Since sperm count is made in `5` secondary square, it is equivalent to = $5 \times 4 \times 10^{-6}$ mL or 2×10^{-5} mL.

II. VAGINAL CYTOLOGY ASSAY IN MICE AND RATS

A. THE ESTROUS CYCLE

This section provides a quick overview of the estrous cycle and describes the technical protocol for the vaginal cytology assay. Also provided is a list of materials needed.

The existence of a typical estrous cycle in the quinea pig and the associated changes in the vaginal cytology were first reported by Stockard and Papanicolaou in 1917. Shortly thereafter, similar phenomena were reported in the rat and mouse. Vaginal smears obtained from these animals were found to correlate well with changes occurring in the reproductive tract and with the secretion of the ovarian hormones. The most common types of cells present in a vaginal smear are:

- Polymorphonuclear leucocytes
- Nucleated epithelial cells
- Large squamous epithelial cells, also called cornified cells (nucleated or non-nucleated).

The estrous cycle of the mouse and rat is completed in four to five days (polyestrous) although the timing of the cycle may be influenced by exteroceptive factors (light, temperature, etc.). The cycle is roughly divisible into four main phases:

1. Estrus

   This is the period of heat and copulation is possible only at this time. This phase lasts from 9 to 15 hours and is characterized by a high rate of running activity. Many mitoses occur in the vaginal mucosa and as new cells accumulate, the superficial layers become squamous and cornified. The latter cells are exfoliated into the vaginal lumen and their presence in vaginal smears is indicative of estrus. During late estrus there are cheesy masses of cornified cells with degenerate nuclei present in the vaginal lumen; but few, if any, leucocytes are found during estrus. Much of the luminal fluid in the uteri is lost before ovulation.

2. Metestrus

   This occurs shortly after ovulation and is intermediate between estrus and diestrus. This phase lasts for 10 to 24 hours and mating is usually not permitted. Many leucocytes appear in the vaginal lumen along with a few cornified cells.

3. Diestrus

   This phase lasts for 60 to 70 hours, during which functional regression of the corpora lutea occurs. The vaginal mucosa is thin, and leucocytes migrate through it, giving a vaginal smear consisting almost entirely of these cells.

4. Proestrus

   This heralds the next heat and is characterized by functional involution of the corpora lutea and preovulatory swelling of the follicles. Fluid collects in the uteri and they become highly contractile. The vaginal smear is dominated by nucleated epithelial cells that occur singly or in sheets.

B. SUGGESTED LIST OF MATERIALS FOR VAGINAL CYTOLOGY ASSAYS
(To be supplied by the toxicology laboratory)

- Medicine droppers
- Pre-cleaned microscope slides (3 X 1") fully frosted (Dakin)
- Cover-slips (24 X 60 mm)
- 95, 80, 70, 50, and 20% ethanol. These grades of alcohol can be prepared from 95% ethanol stock.
- Spray Cyte II (Clay Adams 7180) or other fixative
- Permount mounting medium and xylene
- 100-slot slide boxes
- 0.9% saline solution or phosphate buffered saline
- Microscope slide labels
- Bibulous paper
- Toluidine Blue O (can be purchased from J.T. Baker Chemical Co., Catalog No. W144; if some other source is utilized, ensure that the stain is certified for use in histology).
- Beakers
- Trays
- 3 X 5" cards or cardboard with small window
- 1 N HCL
- 1N NaOH
- Parafilm

Preparation of Toluidine Blue Stain

2.5 g Toluidine Blue
500 mL 20% ethanol

Mix and allow to sit for one hour before filtering through Whatman Grade filter paper.

C. PROCEDURE FOR OBTAINING A VAGINAL SMEAR FROM MICE AND RATS

1. Two vaginal smears should be prepared from each animal between 8-10 a.m. for the sixteen consecutive last days of the 90-day study. Dose levels at which these are prepared will be designated by the NTP project officer for the toxicology laboratory.

2. Fully frosted slides will be used to make vaginal smears (Dakin-Erie 2958). All smears are made on the frosted front of the slide.

3. Microscope slides are marked with a lead pencil into a grid consisting of six squares per slide on the frosted side of the slide. The squares are labeled 1 through 6, 7 through 12, and 13 through 16 in the upper right hand corner. This is convenient as it allows the incorporation of six days of smears on one slide that can be stained at the same time.

4. A 3-4" medicine dropper is moistened by aspirating 0.9% saline or PBS solution. A very small amount of saline is left in the medicine dropper then placed in the vagina and the vaginal fluids aspirated several times.

5. The contents of the medicine dropper are transferred onto the slide. The use of excessive saline should be avoided to prevent the flow onto surrounding squares.

6. At the end of each day, the smears are fixed by applying fixative according to manufacturer's instructions. In order to fix only the specific square of the slide, use a 2" X 4" file card with a window. A cardboard mask can also be used. The slides are to be stored in closed boxes or in a dust-free atmosphere between collection dates.

7. After the last smear has been made, the slides are allowed to dry completely. The slides can be stained the same day or within the next few days.

8. Slides are then loaded in glass racks and dipped in the following solutions:

   a) 95% Ethanol - 30 minutes
   b) 80% Ethanol - 1 minute with gentle shaking
   c) 70% Ethanol - 1 minute with gentle shaking
   d) 50% Ethanol - 1 minute with gentle shaking
   e) 20% Ethanol - 1 minute with gentle shaking
   f) 0.5% Toluidine Blue in 20% Ethanol - 30 – 45 seconds. Personal judgement should be used because Toluidine Blue improves with age.
   g) Running tap water rinse - until tap water runs clear (at this point if slides aren't dark enough, dip in 20% Ethanol and put back in the stain; this process can be repeated until desired color is obtained).

9. The stained slides are blotted gently under bibulous paper. Wet papers are thrown away because material on a slide can be transferred to other slides. Slides are allowed to dry overnight and then cover-slipped using Permount or suitable mounting medium and xylene. If slides cannot be cover-slipped the following day, store in a dust-free atmosphere and cover-slip as soon as possible.

10. Slide Labeling and Coding

Each laboratory participating in this project will be assigned an identification number by the NTP-designated SMCVC laboratory. (Contact the SMCVC laboratory to find out your assigned identification number.) Each slide shall contain the following information:
(See example of slides and decoding form provided below).
Line 1: C #.
Line 2: Lab Code - Species - Animal Number & Slide: Example 01-M-001A
01 designates the lab code; M indicates mouse (R indicates rat);
001A designates the randomly assigned number to an animal plus
A indicates first of three slides for that animal (B = second slide, C = third slide)
Line 3: The date the first smear was made
Line 4: The date the last smear was made

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

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

Example: Preparation of slides for observation of the estrous cycle by means of vaginal changes.

11. All decoding information is to remain with the individual testing laboratory and a copy sent to the SMCVC laboratory to be opened only after completing all evaluations and in the presence of the Quality Assurance Officer who will date and sign when opened. Decoding information to be provided is shown below. All slides will be shipped according to instructions provided in Section D below.

LABORATORY: XYZ LAB # 01
TEST ARTICLE: Emodin ROUTE/VEHICLE: Dosed Feed/NTP2000
CAS #: 518-82-1 C #: C62408 STRAIN: B6C3F1 Mice
DATES SMEARS TAKEN: 4/6/06 - 4/21/06 TSAC DATE: 4/22/06

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

D. INSTRUCTIONS FOR SHIPPING SLIDES TO THE SMCVC LABORATORY

- Shipping and packaging cost will be incurred by the NTP testing laboratory conducting the toxicity studies.
- Stained and cover-slipped slides will be randomized by animal and shipped to the NTP SMCVC laboratory within 2 weeks of necropsy. When randomizing by animal number, keep all three slides from each animal together.
- A copy of the code for all slides will be placed in a sealed envelope and sent to the SMCVC laboratory along with the coded slides.
- All vaginal cytology slides will be placed in plastic slide boxes, keeping slides from mice and rats separate.
- These slide boxes will be placed in 350 lb. test cardboard boxes, separated by abundant packaging material for shipment.
- An appropriate listing of slides will be packed in the slide boxes.
- Slide boxes will be packed to avoid any breakage.
- Shipping cartons will be sealed and bound with filament tape prior to shipment.
- Slides sent separately in slide set will be counted as present in the inventory; a copy of the slide set inventory will accompany the major inventory document.
- Each plastic shipping box will be marked with the name and address of the laboratory, the test article, CAS #, C #, Strain, and the range of animal numbers included in that box.
- Shipping cartons containing slides and the appropriate information will be directly mailed to the NTP-designated SMCVC laboratory.

E. SUGGESTIONS FOR IMPROVING VAGINAL CYTOLOGY QUALITY

General instructions to make vaginal smears are described in detail in the protocol. A few simple precautions, however, can significantly increase the quality of the slides. These are discussed in this section.

Under each category there are suggestions and/or possible explanations for correcting the potential problems that may be encountered during the preparation of a vaginal cytology smear.

a. Crystallization

This is a frequent problem since most staining solutions easily crystallize upon drying. 0.5% Toluidine Blue in 20% ethanol solution is optimal for staining vaginal smears. We have not noticed any excessive crystallization with this concentration. Mucous crystallizes easily in the presence of stain. Since the mucous is darkly stained, that make the smears hard to evaluate and, furthermore, the presence of crystals distorts the morphology of the cells. Also, debris on the slide will provide crystallization sites. Smears must be made on clean slides and slides should always be stored in a dust-free atmosphere.

b. Debris

Presence of debris in vaginal smears will interfere during the evaluation. Debris usually comes from dirty slides, tissue fragments, or dust in the air. Therefore, all slides should be precleaned by wiping them with a clean qauze or Kim Wipe. Most of the tissue fragments can be eliminated by carefully aspirating vaginal fluids. It is also important to place all uncover-slipped smears in a dust-free environment so that dust settling on the slides is minimized. Cover-slips, if dusty, should be cleaned prior to use.

c. Staining

The major problem encountered in this category is the non-uniformity of the stain. This results from the different densities of cells in each smear. By scanning the slide, it is possible to determine if all the smears are adequately stained. If they are not, place the slides back in the alcohol series and then in Toluidine Blue to re-stain them. Be careful not to stain cells too dark because it severely interferes with evaluation.

d. Clumping and Cell Density

The clumping of cells is mainly due to an excess number of cells on the slide. This can be remedied by not placing too many cells on the slide. If the smear is too dense, it will stain heavily and clump. If too much saline is used in making the smear, the cell density of the smear will be very low. This causes the smear to have too few cells to allow accurate evaluation. It must be added that some of the stages of the estrous cycle have a higher density of cells than others. This adds to the problem of cell density.

e. Air Bubbles

Air bubbles are a major problem especially when they occupy a large portion of the slide. They are mainly a technical problem caused by faulty technique and/or carelessness. More mounting medium may be needed to cover the entire slide or better pressing of the cover-slip to squeeze out air bubbles is required. The use of Flo-Tex or similar medium may also alleviate this problem.

F. VAGINAL CYTOLOGY QUALITY CODES

(Codes used by SMCVC Laboratory for the Evaluation of Slides)

Code		Subcode[1]	
00	Excellent	- 1	Refers to Day 1 only
01	Good	- 2	Refers to Day 2 only
02	Moderate crystallization	- 3	Refers to Day 3 only
03	Heavy crystallization	- 4	Refers to Day 4 only
04	Moderate debris	- 5	Refers to Day 5 only
05	Heavy debris	- 6	Refers to Day 6 only
06	Debris surrounding cells	- 7	Refers to Day 7 only
07	Staining too light	- 8	Refers to Day 8 only
08	Cornified cells stained lightly	- 9	Refers to Day 9 only
09	Staining too dark	-10	Refers to Day 10 only
10	Non-uniform staining	-11	Refers to Day 11 only
11	Cells heavily clumped	-12	Refers to Day 12 only
12	Few cells	-13	Refers to Day 13 only
13	No cells	-14	Refers to Day 14 only
14	Air bubbles (>5% < 20% slide)	-15	Refers to Day 15 only
15	Air bubbles (>20% slide)	-16	Refers to Day 16 only
16	Slides prepared on non-frosted side of slide		

[1]Subcodes are used in conjunction with code when that remark applies to a particular day (e.g., 13-2 means no cells on Day 2).

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