APPENDIX AVAILABLE ON THE HEI WEB SITE

Research Report 166

Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity

Part 1. Biologic Responses in Rats and Mice to Subchronic Inhalation of Diesel Exhaust from U.S. 2007-Compliant Engines: Report on 1-, 3-, and 12-Month Exposures in the ACES Bioassay

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Appendix H. ACES Phase 3B Protocol

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ADVANCED COLLABORATIVE EMISSSIONS STUDY (ACES): PHASE 3B

LRRI Protocol Number: FY09-111

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I. PURPOSE

The purpose of this study is to evaluate certain biological responses of rats and mice to repeated inhalation exposure to combined tailpipe exhaust and crankcase emissions from a heavy-duty diesel engine meeting U.S. 2007 on-road emission standards. This study is being conducted at the Lovelace Respiratory Research Institute (LRRI) by contract for the Health Effects Institute (HEI), which has responsibility for sponsor oversight of the bioassay component of the ACES (Advanced Collaborative Emissions Study) Program. The contract was developed in response to the May 2006 HEI RFP 06-1, "Development of a Diesel Exhaust Exposure Facility and Conduct of a Chronic Inhalation Bioassay in Rats and Mice", in accordance with the general research strategy described in the accompanying May 2006 "Project Plan for Emission Characterization and Health Effects Assessment". Details of the experimental design were developed through iterative discussions following award of the contract, and vary somewhat from the original Project Plan. The single greatest change was substitution of a 3-month study for a lifetime bioassay of mice.

Mobile source emissions are a significant source of exposure of people to air pollution. There has been longstanding interest in the health effects of diesel exhaust (DE), largely because of its historic content of higher concentrations of particulate matter (PM) than emissions from gasoline engines. The presence of trace amounts of known carcinogens and mutagens in DE, and especially on diesel PM (DPM), raised concern for potential cancer risk from occupational and environmental exposures. Numerous epidemiological studies of occupational groups having presumed high exposure to DE have demonstrated an increased risk for lung cancer, although uncertainties in estimating exposure made ascribing effects to DE and estimating unit risks correspondingly uncertain.

Several near-lifetime (chronic) inhalation studies of DE in rodents during the 1980s demonstrated that extreme exposures of rats caused progressive chronic lung disease and increased rates of lung tumors. Similar exposures of mice and Syrian hamsters caused non-cancer disease, but little or no increase in lung tumors. Further study revealed that the lung tumor response of rats could be largely ascribed to a chemically nonspecific, species-specific, response to loading of the lung with poorly-soluble particles. This response has questionable parallel in humans exposed heavily to PM. Regardless, the rat response is frequently cited as supporting evidence for a cancer hazard from DE.

Emissions from on-road diesel engines have been markedly reduced in the U.S. and other developed nations in response to progressively stringent fuel quality and emissions standards. The 2007 standards for diesel fuel composition and tailpipe emissions of DPM and nitrogen oxides (NOx) represent a major benchmark in this progression in the U.S. Emissions from 2007-compliant on-road diesel engines are markedly lower than those used in the earlier rodent inhalation studies, and the relative ratios of different physical-chemical components are also changed. It is reasonable to determine whether repeated exposure to 2007-compliant DE causes effects in rodents similar to those caused by older emissions.

The RFP states that the primary (null) hypothesis underlying the ACES Program is that *"Emissions from combined new heavy-duty diesel engine, after treatment, lubrication, and fuel technologies designed to meet the 2007 NOx and PM emission standards will have very low*

pollutant levels and will not cause an increase in tumor formation or substantial toxic health effects in rats and mice at the highest concentration of exhaust that can be used (based on temperature and NO_2 or CO levels) compared to animals exposed to clean air, although some biological effects may occur." The purpose of this protocol is to conduct a study to directly test that hypothesis.

A. General Research Plan

The ACES Program involves multiple phases. In the first phase, emissions from four candidate 395-455 hp engines from different manufacturers were evaluated in detail at Southwest Research Institute (SwRI, San Antonio, TX). One engine was selected by HEI to be used for the animal exposures. The identity of the selected engine is confidential until otherwise directed by HEI. Among the four, it is referred to as "engine B´". Concurrent with that effort, a facility capable of exposing rodents to multiple dilutions of emissions from an engine of this class was developed at LRRI. In a subsequent phase, the selected engine was installed at LRRI, its proper operation on the prescribed 16-hr duty cycle was confirmed, emissions data were collected for comparison to SwRI data, the lowest dilution (highest concentration) acceptable for animal welfare in the exposure chambers was determined, and the detailed composition of the exposure atmosphere was determined in an exposure chamber without animals. The dilutions to be used in this protocol were selected by HEI.

This protocol describes a study in which HsdRccHan:Wist (Wistar) rats and C57BL/6 mice will be exposed by inhalation 16 hr/day, 5 days/wk to one of three dilutions of whole DE or to clean air as controls. Rats will be exposed for 24 mo, with the option to extend exposures to 30 mo if survival permits. Portions of the rats will be sacrificed after 1, 3, 12, and 24 mo of exposure for interim evaluations. These evaluations will include pulmonary function, necropsy and organ weights, bronchoalveolar lavage, hematology, serum chemistry, lung cell proliferation, and histopathology of lesions and standard organ sections. The majority of rats will be evaluated at spontaneous death or terminal sacrifice for lung tumors. Mice will be similarly exposed for up to 13 weeks (3 mo), with one-half sacrificed after 1 or 3 mo of exposure. All interim evaluations used for rats except lung function and histopathology will be applied to mice. Tissues for potential histopathology will be collected from mice, but only the evaluation of lungs is included in this protocol. HEI may elect later to also evaluate other tissues. These procedures will be performed by the LRRI team. In addition, LRRI will collect other blood and tissue samples and provide them to external investigators for ancillary studies funded by HEI under a separate RFA.

B. Value of the Research

This research will evaluate certain health outcomes in animals exposed repeatedly to contemporary (2007-compliant) diesel emissions. No toxicological studies have yet been done on this generation of diesel emissions. This information on health hazards will facilitate estimates of the health risks from human exposures, and thus the classification of diesel exhaust by health and regulatory agencies. In that sense, this study is similar in nature to studies sponsored by the National Toxicology Program and other agencies to evaluate the health hazards of other materials to which people are, or may be, exposed.

This research will also help determine whether or not changes in diesel engine emissions are accompanied by reductions of adverse health effects. This study will examine that issue indirectly, by evaluating selected health outcomes in animals exposed to contemporary

emissions. Because the Program does not include groups exposed to "old" emissions, the relative effects of new and old emissions must necessarily be inferred by comparison to results of previous studies using similar methods.

This research will also serve to benchmark the extent and nature of measurable effects of exposure to contemporary diesel emissions, as an aid to evaluating the utility of conducting similar studies of even cleaner, emissions in the future. Such a benchmark for diesel emissions has not been established for many years. This benchmark will also serve for comparison to new results from future studies of emissions from alternative fuels and engine technologies.

C. Laboratory Practice Standards

This research will be conducted in a manner that is consistent with many of the standards developed for Good Laboratory Practices (GLP), although full compliance with GLP requirements is neither intended by this protocol nor required by the sponsor. The levels of quality control (QC) and quality assurance (QA) stringency will parallel those employed in the National Environmental Respiratory Center Program at LRRI (www.nercenter.org). The institutional policies to be followed are outlined in the *LRRI Guidelines for Conducting Research*. In addition, the LRRI Quality Assurance Unit (QAU) will independently monitor the quality of operations and scientific data. Although interim results may be communicated with HEI's permission, final conclusions will be derived only from analyses of data that have been fully audited by the QAU. The responsibilities and duties of the LRRI QAU are outlined in the *NERC Quality Assurance Plan* and *LRRI Quality Assurance Plan*. QAU duties will include evaluating study-start parameters, auditing exposure system functions, auditing study-related animal care functions, auditing the processes of scientific data collection and determining that data are recorded and stored according to NERC/LRRI QA standards and LRRI Policy 602. Data will be stored in a secure electronic database.

D. Animal Welfare Standards

The LRRI animal facilities are directed by an American College of Laboratory Animal Medicine (ACLAM)-certified Attending Veterinarian who supervises animal health. LRRI is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (File #000200). LRRI has a current approved Animal Welfare Assurance filed with NIH, Office of Protection from Research Risks (Assurance #A3083-01) and is a USDA-registered facility (Registration #85-R-0003) in good standing that is regularly inspected by USDA veterinarians. This protocol will be approved by the LRRI Institutional Animal Care and Use Committee (IACUC) prior to study start.

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III. STUDY FACILITY

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V. GENERATION OF TEST ATMOSPHERES

A. Fuel Identity, Source, and Storage

Diesel fuel meeting current on-road specifications will be delivered to LRRI by tanker truck from a local commercial source (Chevron-branded D-2 legal for on-road use, Ever-Ready Oil Co., Albuquerque, NM) and stored in an above-ground storage tank. Fuel will be transferred on-site directly into the 5,000 gallon 8 x 14 ft. Fireguard storage tank (SN 19911, Brown-Minneapolis Tank Co.).

A sample from each batch of delivered fuel will be submitted to an independent laboratory for analysis of sulfur and aromatic content and cetane index. The fuel must contain no more than 15 ppm sulfur in order to be legal for on-road use, and any delivery testing higher than this sulfur level will be removed from the tank as soon as possible and replaced with legal fuel. Cetane index and aromatic content are prescribed by ASTM guidelines (currently ASTM D975 – 08a), which will be used as target limits for this study. It is intended that fuel for this study will have a minimum cetane index of 40 or a maximum of 35 vol% aromatics. More detailed analytical data will be obtained from the fuel supplier as available, and by independent analysis on a case-by-case basis as requested by HEI. All fuel data will be retained as a part of

the study records and will be summarized in quarterly reports. If analytical values consistently fall at the extremes of federal requirements or ASTM guidelines the implications for interpreting the results of the study and the potential for shifting to a different fuel supply will be discussed with HEI.

B. Fuel Handling

Fuel from the storage tank will be delivered to an intermediate day-tank in the test cell. This process occurs automatically when the DyneSystems programmable logic controller (PLC) receives a fill signal from the fuel level sensor (Flowline Inc., Los Alamitos, CA 90720). The pump will stop filling once a full level signal is received. The temperature of fuel in the day tank will be maintained at 70º F by an electric heater within the tank and will be gravity fed through a fuel conditioning delivery system to the engine. The excess fuel will be returned from the engine to the fuel conditioning delivery system where it will be filtered and cooled by a heat exchanger to a temperature of 104º F before being returned to the engine. Engine fuel filters will be changed on a schedule set by the engine manufacturer.

C. Engine Operation

The engine will be mated to a DyneSytems, 550 hp, 660 A, AC dynamometer and controlled remotely using a DyneSystems, Inter-Loc V, Digital Multi-Loop Controller interfaced to a personal computer running DyneSystems, Cell Assistant for Windows software. The 16 hr ACES test cycle will be controlled by the Cell Assistant software. Each daily exposure will begin with a room temperature "cold" start. After-treatment regeneration events will be allowed to occur as controlled by the engine's electronic control unit. Engine cooling (jacket water temperature) will be controlled by an ethylene glycol closed loop heat exchanger that is supplied by the house chilled water supply. The jacket water cooling system will be maintained at a set point of 190º F using feedback from the DyneSystems PLC.

The engine intake air (charge air) supply will be conditioned to a set point of 77 ºF, filtered and delivered to the turbo charger. The charge air will then be compressed by the turbo charger and cooled as it passes through the intercooler. The intercooler will be cooled through a closed loop heat exchanger to maintain 77 ºF using feedback from the DyneSystems PLC. Lubricating oil will be changed on a schedule recommended by the engine manufacturer. The crankcase lubricating oil will be a proprietary blend approved by HEI and the Coordinating Research Council (CRC) and provided by Lubrizol. Aliquots of used oil will be obtained at each oil change for potential analysis. The oil filter (Power Guard sn 23530573) will be changed with the change in lubrication oil.

D. Dilution System

Exhaust will be passed through a stock after-treatment system prior to injection into a 35.6 cm ID dilution tunnel. The crankcase ventilation effluent will join the exhaust stream downstream of the particle trap. The exhaust will be diluted with filtered air under turbulent conditions at the point of injection. The dilution tunnel supply air flow will be approximately 3000 cubic feet per minute. The primary dilution tunnel is a constant pressure, rather than a constant volume, tunnel. When exhaust flow increases, the increased pressure causes the dilution air to be dumped into a by-pass leg in the test cell. At a distance of 5.5 m from the injection point (in the exposure room), a portion of the diluted exhaust will be drawn through an in-line extraction probe. The exhaust mixture will be withdrawn from the primary dilution plenum

through individual probes and transit lines for each exposure chamber. Subsequent to this extraction, the exhaust will be diluted by pre-cleaned (HEPA/charcoal) compressed air provided through a rotary dilution/dilution by-pass system. Diluting flows will be adjusted as needed to reach the final dilution and concentration targets. All dilution and transit lines will be constructed of stainless steel, and will be of near equal lengths for each of the exposure levels. Exhaust will be diluted to achieve target exposure concentrations of 4.2, 0.8 and 0.1 ppm NO_2. This will require dilution ratios of approximately 40:1, 210:1, and 1680:1.

E. Daily System Operation and Atmospheric Monitoring

Exposures will be conducted 16 hr/day, 5 days per wk. Exposures will be conducted from approximately 1600–0800 hr Sunday through Thursday. Because the animals are to be exposed at least 1 day immediately prior to sacrifice, the Sunday-Thursday schedule allows sacrifices during the regular Monday-Friday work week. Exposure on other days may occur to compensate for days lost during the regular week. The daily schedule for operating the exposure system will start with all required performance verifications or calibrations for monitoring and/or control equipment. Once the operator conducts and documents daily calibrations, the operator will start the exposure system at approximately 1600. Exposures are conducted for 16 hr + T_{90} (time to reach 90 % of target atmosphere). The system will be operated without continuous operator presence between 1800–0800. The system will be programmed to automatically terminate the engine cycle and switch the exposure chambers to clean dilution air at 0800. Experience has shown that once the dilutions are adjusted for each day, the system should be stable enough so that adjustments between 1800 and 0800 are not needed.

Regular system operating personnel will be present during the daily start-up and shut-down periods. A member of the facility surveillance team will be on the premises continuously during the exposure hours, and will check the system periodically throughout the time that regular operators are not present. These specialized individuals are trained to monitor experiments and special equipment as well as to cover routine facility and security issues, and they will be thoroughly familiarized with the engine/exposure system. Surveillance personnel will be trained to shut down the exposure manually, if so instructed.

The system will include alarms for several key operating parameters, and will automatically shut down exposure if key parameters reach pre-set limits (Table 1). Out-of-limit and shut-down alarms will automatically trigger a dialing system that notifies surveillance. Surveillance will respond immediately to make an initial assessment of the problem. A call list of operators and supervisors will be maintained daily, to ensure that an appropriate individual (and a back-up) will be in town to respond to a call from surveillance at any hour. Depending on the nature of the alarm, the individual on call will either direct the surveillance person to take corrective action, will go to the laboratory to assess and correct the situation, or will contact other individuals as appropriate. Correction of a minor alarm may allow the exposure to be continued. The default for any alarm condition that is not readily corrected will be to shut down the exposure. The concentration of NO_2 at the highest exposure level will serve as the alarm for the exposure atmosphere. NO_2 at the high level will be monitored continuously and a rolling 20-minute average will be calculated. An alarm will occur if the 20-minute average reaches 10 ppm, and automatic shut-down will occur if the 20-minute average reaches 15 ppm.

Table 1. Key System Shut-Down Parameters

Parameter	Alarm Limits	Action
Animal chamber temperature	26.7°C (high)	alarm at 26.7 °C, shut down at 29°C
NO_2 in high level chamber (20 min avg.)	10-15 ppm (high)	alarm at 10 ppm, shut down at 15 ppm
Engine oil pressure	14 psi (low)	shut down engine
Engine fuel supply overpressure	76 psi (high)	shut down engine
Dynamometer coolant exit temperature	115°C (high)	shut down engine
Test cell ambient temperature	54°C (high)	shut down engine

Concentrations of nitrogen monoxide (NO) and nitrogen dioxide (NO_2) will be measured directly from each exposure level on each exposure day. Nitrogen oxides (NOx) will also be measured from the primary dilution tunnel to enable measurements of the secondary dilution rate (ratio of concentration between tunnel and chambers). Carbon monoxide (CO), carbon dioxide (CO_2), total hydrocarbons (THC), particle mass by the dekati mass monitor (DMM) and black carbon by the soot sensor will be measured daily from the high exposure level. During periodic intensive characterizations, these measurements will be taken at the other exposure levels, and on those days the measurements will not be made at the high level. Particle size will be measured as part of the DMM analysis. A more detailed measurement of particle size will be conducted once per week at each exposure level by the fast mobility particle sizer (FMPS). Particle mass concentration by gravimetric analysis of Teflon membrane filters will be conducted once/week at each exposure level. These measurements will be conducted at both the midpoint of the exposure chamber and at the chamber inlet. More detailed atmospheric monitoring is conducted periodically as described below.

F. Exposure System Verification

The exposure system performance was characterized in detail prior to the study according to Protocol FY08-147. That protocol characterized the T_{90}, chamber homogeneity, stability, and verification of exposure concentration in non-exposure hours at three dilutions. Additional chamber homogeneity and stability at the final dilution conditions will be conducted as described in Protocol FY08-147 once the animals are in the chambers.

G. Contingency Plan for Major Trouble-shooting, Repair, and Engine Swap

The procedure for addressing system alarms is described above. In the event of a shut-down due to an engine or particle trap malfunction, exposure will not be resumed until the engine manufacturer is consulted, troubleshooting occurs as appropriate, and the engine-trap system is determined to be operating satisfactorily. Confirmation of satisfactory engine operation will include running an engine map and approval by the engine manufacturer of the results and resumption of exposure.

The engine manufacturer will maintain a point of contact (with back-up) for technical issues. That contact will be consulted before any non-trivial troubleshooting or repair is performed. The engine manufacturer will also designate a manufacturer-approved local shop as a contact for parts, on-site troubleshooting, and repair. All repairs by the local shop will be at the guidance of the engine manufacturer, as will follow-up procedures to verify satisfactory repair.

A decision to switch to the back-up engine will be made in consultation with the engine manufacturer. This would occur in the case of a critical failure, when the time required to repair the engine in current use is estimated to be longer than the time required to switch to the back-up engine (including verification of proper operation). The engines may also be switched for other reasons, such as unresolved difficulties with the engine in use. LRRI personnel are trained to perform the engine swap and have multiple experiences. An engine manufacturer representative or designee may also participate as deemed appropriate by the engine manufacturer. Following an engine swap, satisfactory performance of the back-up engine will be confirmed by verification of normal values for an engine map, and verification of normal operating values in the steady-state test modes. Animals will not be exposed to exhaust generated during these tests. Exposures will not resume until the engine manufacturer determines that the engine is performing satisfactorily.

Lost exposure time of more than 30 minutes duration will be made up as soon as feasible, preferably during the next exposure day. Regular exposures will occur five days/week. The outcomes to be measured in this project are expected to result from the integrated effects of exposures over periods of weeks, rather than hours or days. The first measurements will occur after one month of exposure and subsequent measurements will occur at longer intervals. Small losses of exposure time may be made up by adding to the length of remaining exposure days in the same week, if the amount of time is not sufficient to markedly disrupt the daily chamber maintenance and animal observation routine. Larger losses of exposure time will be made up by conducting exposures during the next subsequent non-exposure day. The strategy for making up losses of more than two days will be developed on a case-by-case basis, in consultation with the sponsor.

H. Decision Criteria for Swapping the Engine or Particle Trap

The principal criterion for controlling exposure will be maintenance of target exposure atmospheres. Secondary dilution rates will be adjusted as necessary to maintain target exposures throughout the study, regardless of short- or long-term drifts in engine performance or other operating variables. Nitrogen oxides, PM (DMM and photoacoustic black carbon), CO and CO_2 will be monitored daily at the high exposure level. The key criterion for adjusting secondary dilutions will be the maintenance of target concentrations of NO_2. Along with other monitored measures of engine and dynamometer performance, chamber atmosphere data will serve secondarily as an indicator of engine and particle trap (DPF) performance.

The study is intended to evaluate the potential health effects of 2007-compliant heavy-duty diesel engine emissions. The exposures would be valid therefore, if generated from an engine/after-treatment system meeting the 2007 standards of 0.01 g/hp-hr PM, 1.2 g/hp-hr NOx, 0.14 g/hp-hr NMHC, and 15.5 g/hp-hr CO. The engine family from which engines B and B' were selected was certified within those standards, and PM emissions especially were well below the standard. Although engine B´ might be repaired or substituted with engine B because of changes in performance that might not cause emissions to exceed the 2007 standards under certification conditions, all exposures from an engine performing within those standards would be technically acceptable for the purposes of the study. Within the limits of the standards however, it is also the intent to conduct exposures using an engine/after-treatment system that is performing normally for the selected engine family. For that reason, guidelines for consideration of repairing or exchanging the engine or DPF are stated below.

All decisions regarding repair or exchange of the engine or DPF will be made jointly with the engine manufacturer, in consideration of the situation at hand. It is not possible to specify in advance all potential operating issues or remedies. The following criteria for repairing or exchanging the engine or DPF will serve as guidelines. HEI will be contacted immediately if exchange of either the engine or DPF is recommended, and will have the opportunity to comment on the decision in a timely manner.

The need to minimize lost exposure time will be one criterion for exchanging the engine (the DPF can be exchanged more quickly, and is thus less time-critical). The engine may be exchanged in the event of obvious mechanical or electronic failure to operate properly, or an engine-related inability to generate acceptable emissions, if it is estimated that resolution of the problem will require missing more than three consecutive scheduled exposure days. Although the engine can be exchanged mechanically in one day, exchange and verification of proper operation would most likely result in a downtime of two days. Considering the estimated time required to correct the problem and the point within the 5-day exposure week, a decision to exchange the engine may be made to avoid missing more than one additional scheduled exposure day. Loss of 1-3 exposure days will be made up by adding a sixth day of exposure to subsequent weeks. A decision regarding compensation for longer losses of exposure time will be made jointly with HEI.

The following criteria will trigger review of the need to repair or exchange the engine if other solutions are not readily identified. Multiple performance parameters will be reviewed and considered in decisions regarding the need for repair or exchange.

- Exceeding a manufacturer-defined maximum crankcase pressure during weekly performance checks;
- Greater than 20% decrease from baseline power or torque or 20% increase in BSFC during weekly performance checks;
- A 100% or greater increase in NOx emissions as indicated by concentrations in the primary dilution tunnel averaged for the cycle or observed during weekly performance checks;
- A substantial increase in PM emissions as indicated by excessive pressure drop across the DPF. An engine exchange criterion based on PM emissions is impractical to define precisely because it could vary considerably from the extremely low baseline values and still reflect performance meeting the 2007 standard;

The following criteria will trigger review of the need to repair or exchange the DPF:

- An abrupt, substantial increase in black carbon or PM emissions as indicated by abnormal exposure chamber concentrations under satisfactory NOx dilution conditions. A numerical criterion for black carbon is difficult to specify because it (and other particulate species) could vary considerably from the extremely low baseline values and still reflect performance meeting the 2007 standard;
- Exceeding a backpressure defined by the manufacturer at maximum steady-state power during weekly performance checks;
- Reaching the manufacturer's recommended DPF reconditioning interval based on hours of usage;
- Failure of an engine component or regeneration control system that causes a high temperature event considered hazardous to the integrity of the DPF (monitored by the electronic control system); or
- An engine failure considered likely to have caused secondary damage to the DPF.

- Black carbon concentrations that exceed 15 µg/m^3 average concentration over the 16 hr duration.
- Particle mass concentrations that exceed 100 µg/m^3 average concentration over the 16 hr duration.

VI. ANIMALS

HsdRccHan:Wist rats and C57BL/6 mice will be allocated among the four exposure groups as indicated in Table 2. All groups will consist of equal numbers of males and females. Additional animals will be ordered to allow for the small mortality typically associated with shipping and quarantine, to allow culling to ensure that only healthy animals are entered into the study, and to provide unexposed sentinel groups for biological surveillance.

Table 2. Study Animals[a]

Treatment Group	Purpose	Species/Strain	Age of Animals at Study Start	Total # of Animals
RATS				
Core and Ancillary Studies	Chronic Inhalation Carcinogenesis	Harlan Wistar HsdRccHan:Wist	Young Adult (6 weeks)	200
1 Month Sacrifice	Pulmonary Toxicity	Harlan Wistar HsdRccHan:Wist	Young Adult (6 weeks)	20
3 Month Sacrifice	Pulmonary Toxicity	Harlan Wistar HsdRccHan:Wist	Young Adult (6 weeks)	20
12 Month Sacrifice	Pulmonary Toxicity	Harlan Wistar HsdRccHan:Wist	Young Adult (6 weeks)	20
24 Month Sacrifice	Pulmonary Toxicity	Harlan Wistar HsdRccHan:Wist	Young Adult (6 weeks)	20
			Total for Each of Four Exposure Groups	280
			Total Study Rats[c]	1120
MICE				
1 Month Sacrifice	Pulmonary Toxicity	C57Bl/6 Mice	Young Adult (6 weeks)	20
3 Month Sacrifice	Pulmonary Toxicity	C57Bl/6 Mice	Young Adult (6 weeks)	24[b]
Ancillary Studies 1 Month	Collections for Ancillary Studies	C57Bl/6 Mice	Young Adult (6 weeks)	40
Ancillary Studies 3 Months	Collections for Ancillary Studies	C57Bl/6 Mice	Young Adult (6 weeks)	48[b]
			Total for Each Exposure Group	132
			Total Study Mice[c]	528

[a]Additional rats and mice will be purchased to allow for shipping mortality & culling before assignment

[b]Four and eight mice are included for the 3-month core and ancillary sacrifices, respectively, to ensure availability of full group sizes

[c]Additional rats and mice will serve as sentinels for microbiological surveillance

A. Animal Receipt, Housing, Quarantine

Animals will be quarantined in inhalation exposure chambers. During quarantine, rodents will be acclimatized to inhalation exposure chamber housing conditions for a period of no less than 14 days before being assigned to groups (see section on group assignment and randomization). Three weeks will be allowed for receipt, quarantine, chamber conditioning, randomization to treatment groups, identification, and movement to exposure chambers. Five percent more animals than required for treatment groups will be obtained to compensate for the limited culling of animals in poor physical condition that is typically required prior to study start.

Assigned animals will be housed individually in stainless steel wire mesh cages in stainless steel 1 or 2 m^3 whole-body inhalation exposure chambers (Hazleton H1000 and H2000, respectively, Lab Products) throughout the quarantine/conditioning and exposure periods. Chamber housing records will be kept as described per LRRI SOPs for animal tracking and experimental purposes. These records consist of chamber maps identifying each animal by chamber location. Animal care personnel will verify the identity of each animal weekly during animal movement to clean chambers, and document verification of identification on a specified form. Animal chambers will be maintained daily and water availability will be verified daily by bleeding out the water lines. Chambers will be cleaned weekly and the automatic watering system will be sanitized and checked at that time. The chamber cage units (three for H1000 chambers and six for H2000 chambers) will be rotated one position each week, so that each cage unit is rotated through all positions within its chamber as the study progresses.

In order to verify the specific pathogen-free status of the rodents in the study, blood for serological analysis will be obtained on each order of animals (5 mice and 5 rats per gender) prior to their entry into exposure chambers (after release from quarantine). Five unexposed sentinel mice per gender will be sampled for serology at the 3 month (final) sacrifice time. Five unexposed sentinel rats per gender will be sampled for serology quarterly throughout the duration of the study. Serum separated from the cell fraction of each blood sample will be frozen and shipped to BioReliance (Rockville, MD) for analysis of the presence of antibodies to common rodent pathogens. The BioReliance 80-221 panel will be used for rats and includes: Parvovirus, H-1, Kilham Rat Virus (KRV), Carbacillus, Pneumonia virus of mice (PVM), Rat Coronavirus/Sialodacryoadenitis Virus (RCV,SDA), and Sendai Virus. The 80-220 panel will be used for mice and includes: Enteric Diarrhea of Infant Mice (EDIM), Theiler's Encephalomyelitis virus (GDVII), Mouse Hepatitis virus (MHV), Mouse Minute virus (MMV), MPV, *Mycoplasma pulmonis*, PVM, and Sendai. Positive results for any agent will result in additional testing for the agent identified if not already included in the panels listed.

VII. ENVIRONMENTAL CONDITIONS

Exposure chamber flow rates will be adjusted to maintain a minimum of 12 air changes per hour. Chambers will be held at approximately 2-5 cm of water negative pressure with respect to the room. Animals will be housed in separate compartments within the chamber.

Daily average temperatures will be maintained within a target range of 18-26°C. The National Research Council "Guide for the Care and Use of Laboratory Animals" recommends a temperature range of 18-26°C for both rats and mice. The National Toxicology Program guidelines are based on the thermal-neutral temperature range for mice. The thermal-neutral

range is larger for rats, but NTP recommends the narrower range as a conservative measure. Procedures for this study will follow LRRI SOP ACS-0592.5, which calls for an alarm at 26.7^{o}C and shut-down of exposure if the temperature is above that limit for more than one hour, or at any time it reaches 29°C.

Relative humidity will be monitored, but not controlled or alarmed. The decision of the sponsor was to not control humidity of the engine supply or diluting air, and the air supply for the animal chambers comes from the same source.

A 12-hour per day light cycle from approximately 0600 to 1800 hours will be used.

Chamber environmental conditions will be monitored by a computer 24 hours per day, each parameter will sampled regularly, and summary data (running averages, max, min) will be recorded every 30 minutes. For each chamber, these data will consist of measurements of air flow (by orifice meter), pressure (by electronic pressure transducer), temperature (by thermistor probe), and relative humidity (by electronic transducer). Excursions of any of the first three environmental parameters will trigger alarms, and temperature will also be linked to automatic shut-down. Parameters out of specifications for longer than 3 hours will be evaluated for impacts on the study. Noise levels will be measured at the beginning of the study.

All animals will be supplied tap water and 2016C Harlan Global Certified Rodent Chow available *ad libitum*.

VIII. ATMOSPHERE CHARACTERIZATION

The exposure chambers contain multiple sample ports that allow samples to be taken directly from the breathing zone of the animals (immediately above the wire cages at different levels in the chamber). A limitation that has been observed with the use of these sampling ports is that a sample volume above 10 L per minute will disturb the homogeneity of material inside the chamber. For some of the analyses, a larger sample flow is required to obtain the mass of material necessary for trace analysis. To address this, some of the samples will be collected immediately downstream from the exposure chamber from a stainless steel sampling plenum. The sampling strategy is outlined in Table 3. The frequencies of measurements are listed in the table, as either daily, weekly, or during periodic detailed characterizations (“detailed”).

Daily measurements will include analytes that will be used to control and monitor dilution as well as to serve as diagnostics for engine and dilution system performance. Details of sample collections and measurements beyond those presented in Table 3 will be defined by study specific procedures as appropriate. Detailed organic speciation will include all of the detectable analytes that were measured during Phase 1 of the ACES program, will use the same analytical protocols that were used for Phase 1.

Filter mass concentrations will be measured once each week at each exposure level. Samples for these measurements will be collected simultaneously at the chamber inlet and at the midpoint of the chamber. The first detailed characterization will occur at the midpoint (after 1.5 months) of the mouse exposures. Anticipating continuation of rat exposures for 30 months, three subsequent detailed characterizations will occur after 2.5, 11.5, and 23.5 months of the rat exposures. The timing of the last detailed characterization may be changed if the rat exposures are allowed to continue past 24 months.

Table 3. Description of Methodology for Sampling and Analysis of Exposure Atmospheres

Analysis	Collection Device	Collection Media	Collection Point	Sample Flow Rate (L/m)	Analytical Instrument	Analytical Laboratory	Sampling Frequency
gravimetric mass	aluminum in-line filter holder	Teflon filter	Chamber/ Chamber Inlet	10	MB	LRRI	weekly
continuous mass/black carbon	Dekati Mass Monitor /Photoacoustic	NA	Chamber	2	NA	LRRI	daily
Number size distribution (5-500 nm)	Fast Mobility Particle Sizer	NA	Chamber	10	FMPS	LRRI	daily
Size distribution (0.5-20 µm)	Aerodynamic Particle Sizer	NA	Chamber	5	APS	LRRI	detailed
nitric oxides	chemiluminescence analyzer	NA	Chamber	0.4	NA	LRRI	daily
CO/CO_2/THC	Infrared	NA	Chamber	1	NA	LRRI	daily
THC	flame ionization detector	NA	Chamber	1	NA	LRRI	daily
organic/black carbon	aluminum in-line filter holder	quartz filter (1)	Plenum	10–50	TOR	DRI	detailed
ions (sulfate/nitrate/ammonium)	aluminum in-line filter holder	quartz filter (2)	Plenum	10–50	IC, AC	DRI	detailed
SO_2	aluminum in-line filter holder	K_2CO_3-impregnated filter	Chamber	2–5	IC	DRI	detailed
NH_3	URG denuder (20 cm, 4 channel)	citric acid-coated	Chamber	5	AC	DRI	detailed
metals and other elements	Teflon in-line filter holder	Teflon filter (2)	Plenum	20–50	ICPMS	DRI	detailed
volatile hydrocarbons (C_1-C_{12})	volatile organic sampler	electropolished canister	Chamber	0.1	GCMS/GCFID	DRI	detailed
volatile carbonyls	volatile organic sampler	DNPH cartridge	Chamber	0.3	LC/ /MS	DRI	detailed
semivolatile/fine particle organics	Tisch environmental sampler	quartz filter/ XAD-4	Plenum	80	GCMS	DRI	detailed

List of Abbreviations

Chemical species: CO = carbon monoxide; CO_2 = carbon dioxide; SO_2 = sulfur dioxide; NH_3 = ammonia; THC = total hydrocarbons

Instruments/collection devices: MB = microbalance; NA = not applicable; TOR = thermal/optical reflectance; IC = ion chromatography; ICPMS = inductively coupled plasma mass spectrometry; GCMS = gas chromatography mass spectrometry; GCFID = gas chromatography flame ionization detector; DNPH = dinitrophenylhydrazine; LC/DMS = liquid chromatography/diode array/mass spectrometry; GCMS/LCMS = gas chromatography mass spectrometry/liquid chromatography mass spectrometry; FMPS = fast mobility particle sizer; CPC = condensation particle counter. TIGF = Teflon impregnated glass fiber filter. AC= automated colorimetry

IX. RANDOMIZATION AND GROUP ASSIGNMENT

A. Randomization

In order to ensure that results are not biased due to imbalanced placement of animals on study, care will be taken to determine that the animals are healthy and distributed randomly to the four exposure groups. At receipt and during the quarantine/conditioning period, the animals will be examined visually. Only animals judged to be of acceptable health will be placed on study. Animals will be assigned randomly to treatment groups by weight. A computerized data acquisition system (Provantis Animal Management, Instem Provantis) or Microsoft Excel will be used to generate group assignments and to track animals on study. After assignment to treatment groups, the range of initial individual body weights will be within ±20% of each group mean.

B. Animal Identification, Block Designation and Experimental Sampling

Each animal will have a unique alphanumeric code indicating the species, entry block, gender, and individual (Table 4). The animals will be entered into the study in six sequential blocks; three blocks of mice (blocks A, B, and C), followed by three blocks of rats (blocks D, E, and F). Within each block, numbers 001-499 will be reserved for males and numbers 500-999 will be reserved for females (not all of these individual numbers will be required). These unique animal codes will be used to track each animal and all tissues, samples, and data resulting from that animal. Each animal will be implanted with a coded magnetic transponder (Trovan Electronic Identification Systems). The alphanumeric identification system will be entered into the Provantis animal management system and all animals will be tracked from study entry to necropsy. The transponder will be read and animals will be positively identified using a Lid 570-C Pocket Reader (Midfingerprint). Pocket readers will be programmed so that each transponder code corresponds to the proper unique animal identifier. Pocket readers will be used by both the animal care technicians and necropsy personnel.

Table 4. Block Design and Animal Numbering Scheme

Entry Block	Species	Males	Females
A	Mouse	A001-A499	A501-A999
B	Mouse	B001-B499	B501-B999
C	Mouse	C001-C499	C501-C999
D	Rat	D001-D499	D501-D999
E	Rat	E001-E499	E501-E999
F	Rat	F001-F499	F501-F999

As described in greater detail in Appendix C, blocks A, B, and C will be entered at 1-week intervals to begin the study, in order to complete the mouse study before the H1000 chambers are required for rats. Blocks D, E, and F will also be entered at 1-week intervals at a later time. The three blocks of each species will be evaluated (weighings, sacrifices, etc.) at the same time after entry into the study, and those evaluations will be spread over a 3-week period for each species. This design facilitates study logistics and is common for large studies. Sacrifices will occur the week following completion of the assigned exposure time, and all

animals will have been exposed at least one day immediately prior to sacrifice. Sacrifices will be spread over a 2- to 3-day period.

X. STUDY DESIGN, OBSERVATIONS AND MEASUREMENTS

A. Chronic Bioassay of Rats

Physical observations of the rats and body weights will be recorded throughout the exposure period. The tissues listed below will be examined by light microscopy for histopathology. Routine examination of all treatment groups will include the liver, preputial or clitoral glands, kidney, brain, lung, stomach/gastrointestinal tract, larynx/trachea/parathyroid/esophagus, pituitary, head/nose, pancreas, salivary glands/lymph nodes, adrenals, spleen, testes with epididymides, prostate and seminal vesicle, ovaries and uterus, femur, eyes, urinary bladder, heart and aorta, peripheral nerve (sciatic), and thymus. Lungs will be examined for small lesions using trans-illumination and a dissecting microscope. All gross lesions will be sampled. Complete details will be included in a Study Specific Procedure (SSP) that will be approved by the Study Director, Study Pathologists, and Necropsy Supervisor prior to the necropsy. Tissues will be cut to approximately 5 microns in thickness and slides prepared and stained with hematoxylin & eosin for analysis.

Exposures: 16 hours/day, 5 days/week for 24-30 months

Subjects: Young HsdRccHan:Wist (Wistar) rats from Harlan

Animal Numbers: 100 males and 100 females/exposure level (x 4 = 800 total)

1. Mortality and Morbidity

Animals will be examined by laboratory animal technicians during non-exposure hours on each day of the study. On days when there are no exposures, animals will be examined twice per day, once in the morning and once in the afternoon. Examination will be oriented toward 1) identifying dead, weak or moribund animals, and 2) documenting the onset and progression of any abnormal clinical signs. A Laboratory Animal Veterinarian or the Study Director will make decisions regarding the euthanasia of weak or moribund animals. Moribund or dead animals will be necropsied as soon as possible after being found. LRRI SOPs for removal and transport of animals to the necropsy laboratory will be followed.

2. Body Weights

All animals will be weighed prior to study start for randomization procedures and monthly thereafter. Terminal body weight will be measured at necropsy.

3. Clinical Observations

A detailed clinical examination will be performed during weighing sessions. All observations will be recorded in the automated Provantis system or manually recorded utilizing standardized terminology. These observations will include, but not be limited to, the following: reactivity to general stimuli, description and severity of any convulsions, tremors, or abnormal motor movements (including posture or gait abnormalities), and description of any abnormal behaviors, emaciation, dehydration, or any other abnormal masses, lesions, or appearances.

4. Necropsy and Histopathology

Animals will be sacrificed using a lethal intraperitoneal injection of Euthasol®. Animals will undergo detailed necropsy procedures as described in Appendices A and B.

B. Rats Sacrificed for Pulmonary Toxicity Assays

Twenty rats per treatment group (10 males and 10 females) will be selected randomly from the living population (using standard software) for evaluation after 1, 3, 12, and 24 months of exposure. Pulmonary toxicity assays at 1 month will include bronchoalveolar lavage, lung cell proliferation, and lung histopathology. Assays at 3, 12, and 24 months will include those assays and also pulmonary function, hematology, and serum chemistry. The procedures are described below; more detailed procedures will be described in Study specific Procedures (SSPs).

Exposures: 16 hr/day, 5 days/wk for 1, 3, 12, or 24 mo

Subjects: Young HsdRccHan:Wist (Wistar) rats from Harlan

Animal Numbers: 80 males and 80 females/exposure level (x 4 = 640 total)

1. Pulmonary Function.

Pulmonary function measurements will be performed on all 80 rats during the week prior to sacrifices at the 3, 12, and 24 month scheduled necropsies. Rats will be anesthetized using an inhalation anesthetic (Isoflurane) for careful control of anesthetic depth, intubated with a thin-walled orotracheal catheter, placed in a heated whole-body flow plethysmograph, and intubated with a saline-filled esophageal catheter. The depth of the esophageal catheter will be adjusted for maximal tidal deflections in transpulmonary pressure (Ptp). Respiration will be monitored and anesthetic depth will be stabilized at a light surgical plane. Respiratory frequency, tidal volume, and Ptp will be monitored during steady spontaneous breathing. Flow and Ptp signals from differential pressure transducers will be acquired digitally by a PC-based software system (AcKnowledge V.3.7.2, Biopac Systems), and displayed on the screen in real time. The transducers will be calibrated using a NIST-traceable mercury manometer. Combinations of worksheets and programmed computations will be used to derive the final values.

Quasistatic lung compliance (Cqs), carbon monoxide diffusing capacity (DLco), and forced expiratory flow-volume curves, including forced expiratory volume in 0.1 sec ($FEV_{0.1}$) will be measured during simulated voluntary inhalations and exhalations by first inducing transient apnea by a few breaths from a syringe, and then inflating and deflating the lung using either a syringe or an automated system of valves and pressure reservoirs calibrated for the purpose. Stable spontaneous respiration will be re-established between each single-breath maneuver. Periodically during spontaneous breathing and before each measurement, the lungs will be inflated briefly to a Ptp of +30 cm H_2O to standardize lung volume history and prevent anesthetic-related progressive reduction of lung volume. The Cqs maneuver will be performed by inflating the lungs at 5 mL/sec to total lung capacity, which will be defined as the lung volume at a Ptp of +30 cm H_2O, and then deflating the lung at 3 mL/sec until flow stops (defined as residual volume). Volume and Ptp will be recorded during the deflation. The difference between maximum and minimum lung volume (total lung capacity and residual volume) will be

recorded as the quasistatic vital capacity (VCqs), and the slope of the pressure-volume curve between 0 and +10 cm H_2O Ptp will be measured as Cqs.

The single-breath method will be used to measure DLco. Apnea will be induced, and the inflation volume required to reach +20 cm H_2O Ptp will be determined. The rat will regain stable spontaneous respiration while a syringe is flushed and filled with that volume of test gas (mixture of CO and Ne in air). During apnea, the lung will be inflated with the test gas, a standardized period of "breath-hold" at end inspiration will be allowed, and the test gas will be withdrawn. The final portion of the exhaled gas will be captured in a small gas-tight syringe, and its composition will be analyzed using a gas chromatograph. The inhaled and exhaled gas concentrations, inflation volume, and inflation time will be used to calculate DLco, which is the pressure-adjusted uptake rate of CO across the alveolar-capillary membrane.

The forced expiratory maneuver will be induced during apnea by inflating the lung to total lung capacity and then deflating without intentional flow restriction to a vacuum reservoir maintained at -50 cm H_2O. That negative pressure induces an adequately diagnostic flow-volume curve, although much greater pressures are generated in human lungs by voluntary exhalations. The conservative use of pressure has proven satisfactory to prevent noticeable artifacts in subsequent evaluations of BAL parameters and histopathology. The maneuver generates values for forced vital capacity (FVC), forced expired volume on 0.1 sec ($FEV_{0.1}$, which parallels FEV_1 in humans), and flow rates at various lung volumes and volume intervals during exhalation.

2. Necropsy and Histopathology

The necropsy and histopathology of this group of animals will be evaluated largely as described above for the chronic bioassay animals in Section X-A-4 above – with some exceptions. First, histological sections will only be taken from the left lung. The right lung lobes will be used for other purposes, including lavage, provision of tissue for core pulmonary toxicity assays, and provision of frozen tissue for ancillary studies. Second, blood and other tissues not in the chronic bioassay protocol will be taken for both the core assays and the ancillary studies. In brief, the following collections or preparation of samples will be performed in addition to collection of standard tissues:

- Blood for hematology and serum chemistry (3, 12, and 24 months) and micronuclei analysis (all sacrifice times)
- Plasma and sectioned aorta (unstained) for inflammatory markers
- Plasma, frozen right accessory lung lobe, and frozen ½ brain (with olfactory bulb) for oxidative markers
- Sectioned (unstained) heart and a portion of the caudal thoracic aorta coated with OCT and frozen, mid-jejunal mesenteric arbor frozen, and the cremaster muscle frozen for oxidative markers
- Unstained section from left lung, frozen right middle lung lobe for gene array
- Right caudal lung lobe to homogenize for core HO-1, glutathione, and 8-OHdG assays
- Right cranial lung lobe to freeze for archive

Complete details are presented in Appendices A and B.

3. Hematology and Serum Chemistry

Standard clinical pathology will be performed at the 3, 12, and 24 month necropsies.

Whole blood for hematology will be collected using a sample holder containing K_3EDTA as an anticoagulant. Hematology samples will be analyzed using an automated system (ADVIA™ 120 Hematology System, Bayer Corporation, Tarrytown, NY).

Hematology variables are listed in Table 5. Additional variables may be measured at the discretion of the Study Director.

For clotting variables, whole blood will be collected into tubes containing sodium citrate as an anticoagulant, centrifuged, and plasma separated for analysis. Samples will be analyzed by an automated analyzer (KC4A™ Micro Coagulation Analyzer, Trinity Biotech, Jamestown, NY).

For serum chemistry analyses, additional blood will be collected in serum separator Vacutainer™ or clot tubes for centrifugation and separation into cellular and serum fractions. Serum samples will be analyzed using a Hitachi Modular Analytics Clinical Chemistry System (Roche Diagnostics, Indianapolis, IN). The clinical chemistry variables to be measured or calculated are listed in Table 6. Additional variables may be measured at the discretion of the Study Director.

Table 5. Hematology

Variable	Abbreviation[a]	Units
Red Blood Cell Count	RBC	10^6/µL
Hemoglobin	HGB	g/dL
Hematocrit	HCT	%
Mean Corpuscular Volume	MCV	fL
Mean Corpuscular Hemoglobin Concentration	MCHC	g/dL
Mean Corpuscular Hemoglobin	MCH	pg
Platelet Count	PLT	10^3/µL
Percent Reticulocytes	RETIC	% RBC
White Blood Cell Count and Absolute Differential		
White Blood Cell Count	WBC	10^3/µL
Neutrophils	PMN	10^3/µL
Lymphocytes	LYM	10^3/µL
Monocytes	MONO	10^3/µL
Eosinophils	EOS	10^3/µL
Basophils	BASO	10^3/µL
Large Unstained Cells	LUC	10^3/µL
Coagulation		
Partial Thromboplastin Time	PTT	seconds
Prothrombin Time	PT	seconds

[a]Abbreviations produced by the hematology analyzer may different from those listed above; however, the final results will be reported using the above terms.

If the required blood volumes are not obtained or if evaluations are not performed for a given animal, a reason and notation will be included in the raw data and in the study file.

Table 6. Serum Chemistry

Analyte	Abbreviation[a]	Units
Alanine Aminotransferase (Alanine Transaminase)-Serum	ALT	IU/L
Albumin	ALB	g/dL
Aspartate Aminotransferase (Aspartate Transaminase)-Serum	AST	IU/L
Bilirubin (Total)	BILI-T	mg/dL
Blood Urea Nitrogen	BUN	mg/dL
Calcium	CA	mg/dL
Chloride (Serum)	CL-S	mmol/L
Cholesterol (Total)	CHOL	mg/dL
Creatinine (Serum)	CRE-S	mg/dL
Glucose	GLU	mg/dL
Gamma Glutamyltransferase	GGT	IU/L
Alkaline Phosphatase	ALP	IU/L
Phosphates	PHOS	mg/dL
Potassium (Serum)	K-S	mmol/L
Protein (Total)	TP	g/dL
Sodium (Serum)	NA-S	mmol/L
Triglycerides	TRIG	mg/dL
Calculated Variables and Ratios		
Albumin/Globulin	A/G	None
Blood Urea Nitrogen/Creatinine	BUN/CRE	None
Globulin	GLOBN	g/dL

[a]Abbreviations from the chemistry system may differ from those listed above, however, the final results will be reported using the above terms.

4. Bronchoalveolar Lavage (BAL) and Lung Tissue Collection

After completion of the pulmonary function tests, the anesthetized rats will be euthanized by intraperitoneal injection of a pentobarbital-based euthanasia solution (Euthasol). The heart-lung block will be carefully dissected from the thorax with special care to avoid letting blood enter the trachea, and the heart will be removed. A blunt cannula with a Luer lock fitting will be inserted into the trachea and ligated in place. The left bronchus will be closed using a vascular micro-clamp. A syringe filled with the appropriate volume (20 µL/gram body weight for mice, 12 µL/gram body weight for rats) of Dulbecco's phosphate buffered saline (without calcium or magnesium) will be attached to the hub of the tracheal cannula. The saline will be gradually injected into the right lung with gentle pressure to force it into the most distal airspaces, then withdrawn into the same syringe and transferred to a container on ice. The

procedure will be repeated twice, for a total of three washes. Recovered volumes will be gradually injected into the right lung with gentle pressure to force it into the most distal

airspaces, then withdrawn into the same syringe and transferred to a container on ice. The procedure will be repeated twice, for a total of three washes. Recovered volumes will be recorded. Following completion of lavage, the right bronchus the right bronchus will be ligated, the right lung lobes will be removed for analyses of tissue endpoints, and the micro-clamp on the left bronchus will be released. The left lung will be fixed with 10% NBF at constant pressure as per procedures described for other necropsies, and retained for histology and immunohistochemistry. The left lung will be embedded to allow sectioning in a frontal plane to include the hilus, major airways and broncho-alveolar structures.

BAL Indicators of Cytotoxicity

BAL fluid will be measured for lactate dehydroganse activity (LDH), total protein/albumin/hemoglobin, and alkaline phosphatase (ALP). These indicators will be measured using commercially available kits and reagents by a clinical chemistry autoanalyzer (Hitachi 911, Roche Diagnostics).

BAL Indicators of Inflammation

BAL fluid will be measured for total and differential cell counts (macrophages, neutrophils, and lymphocytes by hemocytology). Homogenized lung tissue will be analyzed for pro-inflammatory cytokines via ELISA (Biosource/Invitrogen, Carlsbad, CA). Cytokines will include IL-1β, TNFα, MIP-2, KC, and IL-6.

Indicators of Oxidative Stress

BAL fluid and lung tissue homogenate in metaphosphoric acid will be analyzed for oxidized (GSSG) and reduced (GSH) glutathione levels using a kit (EMD Biosciences) based on the dithionitrobenzoic acid/glutathione reductase recycling assay of Tietze. GSSG will be measured in duplicate aliquots that have been treated with an agent that removes GSH from the pool of total glutathione that is a substrate for the reductase. GSH then will be quantified by the difference.

The total antioxidant capacity of the BAL fluid will be quantified by comparing the ability of the fluid to scavenge a stable free radical of 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) with that of Trolox (a water soluble vitamin E analog).

Tissue Heme Oxygenase-1 will be measured in homogenized lung tissue using a commercial ELISA kit. 8-Hydroxy-Guanosine (8-OHdG) will be measured in homogenized lung tissue to quantify damage to the nucleotides in DNA due to reactive oxygen species. The latter assay will be performed by Dr. Jim Swenberg, University of North Carolina, using a mass spectrometric method developed in his laboratory.

5. Cell Proliferation

Sections will be prepared from the paraffin-embedded, fixed, left lung as described elsewhere for histopathology. Antigen will be recovered by heating to 100°C for 20 min in 100 mM Tris buffer (pH 9.0). Endogenous peroxidases will be quenched by treatment with 1% hydrogen peroxide in methanol, twice for 20 min, and the sections will be blocked by incubation in 10% goat serum in Tris-buffered saline for 1 hr. The primary antibody

(monoclonal anti-Ki67, Invitrogen) will be diluted to 0.4 µg/mL in the same buffer with 1% goat serum and 2% bovine serum albumin and the slides will be incubated with this solution for 1 hr at room temperature in a humidified chamber. After washing the slides, the secondary antibody (biotinylated goat anti-mouse) will be diluted in the same buffer and incubated with the samples for 1 hr. Finally, the antigen will be visualized using the avidin-biotin peroxidase complex with $NiCl_2$-enhanced diaminobenzidine as the substrate. Sections will be counterstained with hematoxylin and eosin. Each experimental series will include a negative control in which an isotype-matched (IgG1) nonspecific antibody will be substituted for the primary antibody. A tracheobronchial lymph node will be collected from a sham-exposed control animal during each necropsy to serve as a positive control, and will be processed identically.

Sections will be digitally photographed and evaluated by a well-trained technologist. The images will be recorded to allow confirmation by the Study Pathologist of any questionable cells. For each animal, at least 300 parenchymal cells will be evaluated and scored as positive or negative for nuclear staining. Results will be reported as % positive cells.

C. Subchronic Inhalation Study of Mice

Groups of C57Bl/6 mice will be sacrificed and evaluated after 1 or 3 months (4 or 13 weeks) of exposure. Ten male and 10 female mice per each treatment group sacrificed at each time (block A) will comprise the core mouse study performed by LRRI. Two blocks of 10 male and 10 female mice per each treatment group sacrificed at each time (blocks B and C) will be used for collection of fluid and tissue samples for ancillary investigations to be performed by non-LRRI investigators. The exposures, sample collections, and shipping of samples for the ancillary studies are encompassed by this protocol. The analyses of the ancillary samples are not encompassed by this protocol.

Exposures: 16 hours/day, 5 days/week for 1 or 3 months (4 or 13 weeks)

Subjects: Young C57Bl/6 mice from Taconic

Animal Numbers: 60 males and 60 females/exposure level (x 4 = 480 total)

1. Mortality and Morbidity

Animals will be examined by laboratory animal technicians during non-exposure hours on each day of the study. On days when there are no exposures, animals will be examined twice per day, once in the morning and once in the afternoon. Examination will be oriented toward 1) identifying dead, weak or moribund animals, and 2) documenting the onset and progression of any abnormal clinical signs. A Laboratory Animal Veterinarian or the Study Director will make decisions regarding the euthanasia of weak or moribund animals. Moribund or dead animals will be necropsied as soon as possible after being found. LRRI SOPs for removal and transport of animals to the necropsy laboratory will be followed.

2. Body Weights

All animals will be weighed prior to study start for randomization procedures and monthly thereafter. Terminal body weight will be measured at necropsy.

3. Clinical Observations

A detailed clinical examination will be performed during weighing sessions. All observations will be recorded in the automated Provantis system or manually recorded utilizing standardized terminology. These observations will include, but not be limited to, the following: reactivity to general stimuli, description and severity of any convulsions, tremors, or abnormal motor movements (including posture or gait abnormalities), and description of any abnormal behaviors, emaciation, dehydration, or any other abnormal masses, lesions, or appearances.

4. Necropsy and Histopathology

Animals will be sacrificed using a lethal intraperitoneal injection of Euthasol®. Animals will undergo detailed necropsy procedures as described in Appendix A.

5. Hematology and Serum Chemistry

Standard clinical pathology will be performed on all animals at 3 months as described above in the Chronic Rat Bioassay section, with the exception that coagulation assays will not be performed for mice. If there should be insufficient blood from a mouse to complete both the hematology and serum chemistry assays, priority will be given to hematology.

6. Bronchoalveolar Lavage

BALF from all mice will be evaluated at 1 and 3 months as described above in the Chronic Rat Bioassay section.

7. Lung Cell Proliferation

Sections from lungs at 1 and 3 months will be prepared as described above in the Chronic Rat Bioassay section for histopathology and lung cell proliferation.

D. Data Management and Statistical Analyses

1. Data Management

Data will be managed by a strategy involving three tiers of records having different levels of quality control and protection.

a. *Tier I: Raw Data*

This tier consists of day-to-day experimental records, including "raw," or freshly-generated, data that have not yet undergone quality control checks, and data logged electronically into interim databases on stand-alone or intranet-linked computers. These records include samples, worksheets, notebooks, and electronic records. These data will not be entered into the database prior to having undergone quality control and quality assurance audits.

b. *Tier II: Database*

Study results will be submitted to the database after standardized formatting and quality assurance review. The data file will be placed into an interim database and audited by a trained auditor that is independent of the data generation. Once audit findings are corrected, the data will be moved into the final database. Once data are entered into the final

database, they will be accessible only on a read-only basis, and could only be altered with the Principal Investigator's approval and documentation of the full audit trail. Each datum will be coded with a string of uniquely-identifying descriptors. The final database will be structured using Microsoft SQL Server 2000 software. Data and summary reports of data can be generated from the final database in a read-only manner, and can be exported in Excel, comma delineated, or XML files. Final statistical analyses (i.e., those for publication) will be performed only on data from the final database.

2. Statistical Analyses

Appropriate statistical analysis of carcinogenicity, mortality, pulmonary inflammation and oxidative damage, pulmonary function, and respiratory tract cell proliferation data will be conducted using strategies defined below for each sets of endpoints.

a. *Neoplasm and Non-Neoplastic Lesion Incidences*

The "Poly-k test", which addresses and mitigates the issues of intercurrent mortality and tumor lethality, will be used to evaluate the incidences of neoplasms and non-neoplastic lesions. This method uses a modified Cochran-Armitage test statistic that is based on "survival adjusted" tumor rates. The adjustment is accomplished by dividing the tumor count for a group of animals by the sum of tumor risk weights for these animals. For this method, the risk weight is **one** if the animal had a lesion or survived until terminal sacrifice. If the animal died before the terminal sacrifice, its risk weight is the fraction of the study length that it survived raised to the **k**th power. The power **k** reflects the assumed polynomial degree of the tumor onset function.

b. *Tumor Multiplicity and Lesion Severity Scores*

A weighted least squares ANOVA approach will be used for analysis of lesion severity scores that are obtained by histopathology examinations. Comparisons between treated and control groups, with an adjustment for multiple comparisons, may be performed using the T3 procedure of Dunnett.

c. *Survival*

The product-limit method of Kaplan and Meier, which provides a means to include survival information until the time of censoring, will be used to quantify survival throughout the study. These estimates will be computed at each point in time at which a death has occurred and plotted as decreasing step functions over the time of study. Cox's proportional hazards model will be used to assess the significance of differences between individual dosed groups and controls, and Tarone's extension of the method will be applied to test for a dose-related trend.

d. *Analysis of Continuous Variables*

Data collected by the pulmonary function, hematology, clinical chemistry, bronchoalveolar lavage, and cell proliferations assays will be analyzed individually at each of the time points.

Multiple comparison procedures will be used to identify exposure groups in which responses differ significantly from those of controls. The following tests will be used: Williams' and Dunnett's tests for data that follow a normal (Gaussian) distribution; and Dunn's and Shirley's tests. Jonckheere's trend test will be used to determine whether there is strong evidence of a monotonic trend ($p < 0.01$). If this occurs, Williams' (or Shirley's) test will be applied. Alternatively, Dunnett's (or Dunn's) test will be applied.

In addition, where the data distributions are clearly asymmetric or long-tailed, and not amenable to variance stabilizing transformations, nonparametric methods will be used in conjunction with descriptive statistics that are insensitive to extreme values (e.g., medians and their nonparametrically derived confidence intervals.)

XI. RECORDS TO BE MAINTAINED

All tissues and specimens collected in this study shall be identified by protocol number, animal number, experiment number, and test system. All raw data and records that would be required to reconstruct the study will be maintained in the LRRI archives and/or as described in the data management and statistical analyses section.

In the event that changes to the protocol are merited or deviations from the protocol take place, the change and the reason for it will be recorded promptly in the form of a protocol amendment. The amendment will be signed by the Study Director and Principal Investigator.

XII. STUDY SCHEDULE

Proposed Start Date:	January 2010[a]
Proposed Completion of In-life Phase:	Mice: April 2010
	Rats: October 2012

[a]Depending on timing of HEI approval

XIII. REFERENCES

A. Study Specific Procedures, LRRI SSPs

[list to be generated]

APPENDIX A

Detailed Necropsy and Histopathology Procedures for Rats and Mice

A. Detailed Necropsy Procedures

Multi-chambered plastic tissue trays will be used to ensure complete collection. These will be pre-labeled with the organs and tissues to be collected and filled with 10% NBF. As organs and tissues are removed from the animal, they will be immediately placed into the appropriate chamber in the tissue tray. After all protocol-specified tissue samples are collected, tissues will be removed from the tray one by one, inventoried, and placed into the labeled container of fixative for that animal.

The animals will be euthanized by injection of standard solution. Each animal will be positively identified using a microchip reader. If the chip is missing (which is rare, but has happened), identification will be based on the information on the individual animal necropsy report (IANR) form and/or cage cards and chamber maps. Initial verification of animal identity will be documented on the IANR. Terminal body weight will be measured and recorded on the IANR.

External surfaces, eyes, and orifices will be examined. Evidence of diarrhea, bleeding from any orifices, or broken bones will be recorded. Suspected fractures or bone lesions may be radiographed.

The pelt will be reflected and the head skinned. The left mammary chain (glands 4–5) with skin will be sampled and placed flat on a paper towel. Mandibular lymph nodes and salivary glands will be removed and placed in a labeled embedding bag. The preputial/clitoral gland (female) and penis (male) will be removed.

The abdominal cavity will be opened. The spleen will be removed, leaving the pancreas attached. Mesenteric lymph nodes will be removed, and bagged. The gastrointestinal tract will be positioned to the side. The liver will be removed, weighed, and sliced at 5-mm intervals. A portion will be saved for freezing, and the remainder will be placed in fixative.

The right kidney and adrenal gland will be removed together. The adrenal gland will then be removed from the kidney and placed in a labeled embedding bag. The right kidney will be sliced crosswise. The left kidney and adrenal gland will be removed. The left adrenal gland will be placed in the embedding bag with the right adrenal gland. The left kidney will be sliced longitudinally.

The pelvis will be dissected to expose the rectum and anus. Sex organs and the urinary bladder will be removed *in toto*. The urinary bladder will be separated and weighed, and then perfused with 10% NBF. Ovaries and testes will be weighed and divided into portions for freezing and fixation.

The entire gastrointestinal (GI) tract will be removed. The stomach will be transected at the junction of the pylorus and duodenum and injected with 10% NBF. The rest of the GI tract will be opened and examined. Areas with lesions will be identified and pinned flat on paper or cardboard for fixation. Cross sections of duodenum, jejunum, ileum, cecum, colon, and rectum, and suspect lesions will be taken. Peyer’s patch will be included with these sections when possible.

The left femur will be removed, disjointing at the hip and knee joints.

The lower mandible will be removed to expose the larynx. The tongue, pharynx, and oral cavity will be examined. The thoracic cavity will be opened to expose the thymus and heart-lung block. The sternum will be placed in fixative. The thymus and mediastinal lymph nodes will be removed. A portion of the lymph nodes will be saved for freezing. Each day of necropsy, a tracheobronchial lymph node from a control animal will be collected as a positive control for the cell proliferation assay. The heart and aorta will be removed, separated, the heart will be weighed, and a portion of heart will be saved for freezing. After fixation, a section of the heart will be sliced from the base through the apex so that all four chambers are visualized.

The lungs will be removed from the thorax and the larynx, trachea, and esophagus will be dissected free. Lungs will be examined for small lesions using trans-illumination and a dissecting microscope. The anterior one-half of trachea with the larynx, thyroid, parathyroid, and esophagus will be placed in an embedding bag. The remaining esophagus will be opened and examined. The lungs will be weighed. A minimum of one right lung lobe will be saved for freezing. The bronchus to that lobe will be ligated, the lobe will be removed, and the remaining lung will be fixed for a minimum of 2 hr under constant 25 cm 10% neutral buffered formalin pressure.

The harderian glands will be removed and fixed. The head will be removed and the nasal cavity gently instilled with 10% NBF through the nasopharynx until drops appear at the external nares. The brain will be removed and weighed, a portion will be saved for freezing, and the remainder will be fixed. The pituitary gland will be left in the skull for fixation in situ.

The spinal cord and sciatic nerve will be removed, examined grossly, and fixed.

Gross lesions will be described by the pathologist and recorded using the terminology in the TDMS Pathology Code Table (PCT) inclusive of morphologic lesion, anatomic site, quantity, size, number, shape, color, and consistency. Each gross observation will ultimately be correlated with a microscopic evaluation. Masses less than 0.5 cm in diameter may be fixed in their entirety. Lesions will be photographed, and each photograph will be identified. If multiple nodules are present in organs, up to five are recorded. After five nodules have been counted, the record shows the number as “greater than five.”

Target organs will be weighed before sectioning, as indicated above, including brain, heart, liver, lung and mainstem bronchi, kidneys, ovaries, testicles, thymus, and urinary bladder. An electronic balance calibrated with NIST-traceable standards will be used. The organs will be weighed to the nearest 10 mg except for testicle and thymus, which will be weighed to the nearest 1.0 mg. Organ-to-body weight ratios will be calculated.

After necropsy, carcasses will be placed in properly labeled containers and fixed in 10% NBF. Carcasses will be discarded only after the histopathologic evaluation and the audit of residual tissues have been completed and it is determined that no lesions were missed at necropsy. Disposal of the rat carcasses will require approval of the Principal Investigator, Study Director, and Quality Assurance Manager. Carcasses of mice will be placed in the container with the tissues and fixed in 10% NBF. Carcasses of mice will be submitted to the sponsor with the residual formalin-fixed tissues at the end of the study.

B. Histopathology

1. Tissue Trimming

Tissue trimming will be supervised by the Associate Study Pathologist/Necropsy Manager, although the continued presence of this individual is not required. Tissues will be trimmed not less than 48 hr and not greater than 12 wk following necropsy. Tissues will be placed in blocks in a consistent manner so that the same tissues are in the same numbered blocks for all animals.

At the time of tissue trimming, the original IANR for each animal will be available to the technician. The technician will refer to the IANR as each tissue is trimmed to ensure that no lesions observed grossly are overlooked. Any additional gross observations are recorded during the trimming procedure.

a. Tumors or Masses

Multiple portions will be submitted for histopathological evaluation. When possible, some adjacent normal tissue will be included.

b. Liver

The liver, free of adjacent tissues, will be trimmed to allow the larges cross section surface area possible for microscopic evaluation. One section of each nodule/tumor (up to five; the five largest lesions will be taken if greater than five are present) with adjacent normal tissue will be. Two sections of normal liver through the median and left lateral lobes will be prepared. Sections are transverse sections taken midway along the greatest dimension. In mice, a section of the gall bladder will be taken separately and apart from the liver sections.

c. Preputial or Clitoral Glands

Longitudinal sections will be submitted. For mice and smaller female rats, whole organs may be embedded.

d. Kidney

A mid-longitudinal section (left kidney) and cross section (right kidney) through the entire cortex and medulla of each kidney will be submitted.

e. Brain

Three cross sections of the brain will include the frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons. If any lesions are observed after sectioning, these will be noted on the IANR form.

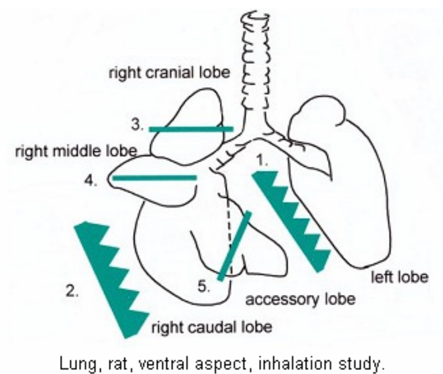
f. Lung

1) Lungs from Chronic Bioassay Rats

The lungs will be embedded and sectioned to obtain one section per lobe (five sections total) according to the strategy recommended by the Society of Toxicologic Pathology (*Exp.Tox.Pathol*. 55; 91-106, 2003, “Revised guides for organ sampling and trimming in rats and mice – Part 1-3”). The sectioning strategy is illustrated in Figure 2. With the lung lying on its dorsal surface, the left lung and right caudal lobe will be sectioned along the airways (frontal/coronal plane) in a horizontal manner. The right cranial and accessory lobes will be sectioned in a transverse, or vertical, manner. The right middle lobe will be sectioned in a

longitudinal, or vertical, manner. In addition, lesions identified grossly and by dissecting microscope but not sampled by the standard cuts will be sectioned separately.

Figure 2. Strategy for sectioning lungs of chronic bioassay rats. The lung is viewed as removed from the thorax, and from the ventral aspect. Serrated bars indicate horizontal cuts; narrow bars indicate vertical cuts.



2) Lungs from Rats and Mice Sacrificed at 1, 3, 12, or 24 Months

The left lung of core sacrifice rats and mice will be fixed and sectioned for histopathology as described for chronic bioassay rats. The right lung lobes will be frozen for other core and ancillary assays. Lungs of mice for ancillary studies will not be fixed and sectioned; all tissue will be used for other assays.

g. Stomach/Gastrointestinal Tract

The stomach will be opened and examined for gross lesions at trimming. The stomach will be cut through the midsagittal plane thereby dividing it into two equal halves. The section taken for histology will encompass the entire greater curvature of the stomach to include glandular stomach, forestomach, and pyloric regions. Cross sections will be trimmed from duodenum, jejunum, ileum, colon, cecum, and rectum to include from mucosa to serosa.

h. Larynx/Trachea/Thyroid/Parathyroid/Esophagus

The larynx will be embedded whole and a transverse section obtained at the base of the epiglottis just anterior to the laryngeal saccule. A cross section of the trachea that includes the thyroids/parathyroids and esophagus, if attached, will be taken.

i. Pituitary

The pituitary will be removed and trimmed to allow a coronal section.

j. Head/Nose

After decalcification of the head, three sections will be taken: at the level of the incisor teeth; midway between incisors and first molar; and middle of second molar.

k. Pancreas

The pancreas will be trimmed to allow the largest surface area possible for microscopic examination. A portion of the pancreas (about 1 cm^2) will be placed flat to provide a section through the frontal plane.

l. Salivary Glands and Lymph Nodes

The left mandibular and left major sublingual salivary gland and the mandibular lymph node will be embedded as a single unit and sectioned through the frontal plane to include all three organs. The whole bronchial and mediastinal lymph nodes are submitted.

m. Adrenals

One cross section from each adrenal gland (two) will be taken to include the cortex and medulla. The whole organ may be embedded for mice.

n. Spleen

A single cross section will be taken at the point of greatest width. If the spleen is diffusely enlarged due to leukemia or lymphoma, the transverse section may be trimmed on one side to allow placement on the slide. For mice, the spleen will be cut in half and one piece submitted.

o. Testes with Epididymides

A single transverse section at the midpoint of both testes will be taken. The epididymides from both testes will be bisected along the midsaggital plane to include the head, body, and tail.

p. Prostate and Seminal Vesicle

A mid-transverse section of the prostate will be taken to include dorsal, lateral, and ventral lobes of the prostate and ampullary gland. A mid-transverse section of the seminal vesicle and coagulating gland will be taken bilaterally.

q. Ovaries and Uterus

A transverse section through each uterine horn, approximately 0.5 cm from the body of the uterus will be taken. One cross section will be prepared from each ovary.

r. Femur

The distal 1.5 cm (rat) or 1 cm (mouse) of the femur will 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 the bone will include the joint surface and marrow.

s. Eyes

Eyes will be embedded intact, in a block by themselves, and step-sectioned until a section includes both the lens and optic nerve. Alternatively, the eyes and Harderian glands may be embedded in the same block if consistent sections can be taken of both tissues and the sections of the eyes include the required elements.

t. Urinary Bladder

The urinary bladder will be trimmed and embedded to allow a cross section.

u. Heart and Aorta

A section of the heart will be trimmed from the base to the apex so that all four chambers can be visualized. Care will be taken to include coronary vessels. A cross section of the thoracic aorta will be submitted.

v. Peripheral Nerve, Sciatic

A 5-mm length of nerve will be taken from the mid thigh.

w. Thymus

A cross section will be taken through the center of organ, or if small, the thymus may be embedded whole.

2. Slide Preparation

All tissues collected from the rats will be trimmed and processed as described below. Only the lung and lymph node will be trimmed and processed for mice.

All trimmed tissues will be processed (dehydrated and infiltrated with paraffin) by an automatic tissue processor, sectioned, and stained as described below. LRRI has an SOP in place for tissue processing. Slide label format will comply with requirements noted in NTP specifications.

Tissues will be cut to approximately 5 microns in thickness. Slides will be deparaffinized and hydrated using the following procedures: three 5-min xylene washes, two 5-min washes with 100% ethanol, one 5-min wash with 95% ethanol, one 5-min wash with 80% ethanol, and a final wash in running tap water until clear of alcohol.

Slides to be sent to ancillary investigators for special staining will be provided without further processing.

Slides that will be evaluated at LRRI will be stained in filtered hematoxylin (pH 2.5) for 7 min. Slides will be occasionally agitated during the staining process. Slides will then be washed in running tap water until clear of surplus dye (30–60 sec). Excess stain will be removed (differentiation) with 0.5% hydrochloric acid in 70% ethanol by briefly dipping the slides in the acid-alcohol solution (3–7 dips or rinses; time: 4–5 sec). Slides will be placed in a dish of tap water until clear of acid-alcohol solution, and transferred to ammonia water for 5–15 dips until the slides are uniformly blue. The ammonia water rinse will contain 1.5 mL of 58% ammonium hydroxide in 500 mL of deionized water (pH 9.5). Slides will be rinsed in deionized water for 5–6 rinses until excess ammonia is removed. Slides will then be stained with eosin solution for 1 min with occasional agitation, and rinsed clear of eosin in two dishes of deionized water to remove excess dye (2–4 rinses). Slides will be dehydrated as follows: three rapid dips in 80% ethanol; three rinses in 95% ethanol; a second set of three rinses in 95% ethanol; 4–5 rinses in 100% ethanol (about 1 min); 3-min rinse in 100% ethanol; and three changes of xylene, each 3 min in duration. Finally, the slides will be mounted with Permount®. Demineralized tissues such as bone and bone marrow sections will be stained in hematoxylin for 8 min, and eosin staining time may be reduced to 30 sec.

APPENDIX B

Sample Collections for Rats and Mice at Scheduled Sacrifice Times

This pertains only to rats and mice scheduled for sacrifices at 1, 3, 12, and 24 months. It does not pertain to deaths or terminal sacrifices of other chronic bioassay rats)

A. Rat Procedures

1. 20 Rats/Group at 1 Month:

Body weight
Blood:
  20 rats - save all extra blood frozen at LRRI for archive after collecting:
  10 rats - 100 µL blood in special anticoagulant, frozen for Bemis
  14 rats - 500 µL plasma frozen for Conklin
    Blood cell pellet after removing plasma, frozen for Conklin
  10 rats - 50 µL plasma frozen for Hallberg
Gross necropsy (LRRI)
Weigh 8 target organs (LRRI, provide heart weight to Sun)
Lungs:
  20 rats - Bronchoalveolar lavage right lung lobes (LRRI)
  20 rats - Fix left lung (10% NBF, 25 cm fixative constant pressure)
    20 rats - Section & stain H&E for LRRI histopathology
    20 rats - Cut 2 sections and mount unstained for LRRI cell proliferation
    20 rats - Cut 6 sections and mount unstained for Veranth
  20 rats - Freeze right lung lobes
    20 rats - Caudal lobe for LRRI glutathione, HO-1, and 8-OhdG
    20 rats - Middle lobe for Veranth
    20 rats - Cranial lobe for archive
    10 rats - right accessory lobe for Hallberg
    10 rats - right accessory lobe for archive
Brain:
  20 rats - Fix ½ brain (incl. olfactory bulb) for LRRI histopathology
    Freeze ½ brain (incl. olfactory bulb)
      10 rats - send to Hallberg
      10 rats - save extra frozen brain for archive
Heart:
  20 rats - Fix in 10% NBF and section to include 4 chambers
      20 rats - H&E slides for LRRI histopathology
      10 rats - Cut 2 sections & mount unstained for Sun
Aorta:
  20 rats - Portion of caudal thoracic aorta coated with OCT & frozen for Sun
    Fix remainder in 10% NBF, section linear portion of descending thoracic
      20 rats - H&E slides for LRRI histopathology
      14 rats - cut 7 unstained slides (2-3 cross-sections each) for Conklin
Other Tissues:
  20 rats - Collect RFP list and fix in 10% NBF for LRRI histopathology
  20 rats - Mid-jejunal mesenteric arbor coated with OCT & frozen for Sun
  20 rats - Cremaster muscle coated with OCT & frozen for Sun

2. 20 rats/Group at 3, 12, & 24 months:

Pulmonary Function (LRRI)
Body weight
Blood:
- 20 rats - Hematology (LRRI)
  - Serum chemistry (LRRI)
- 10 rats - 100 µL blood in special anticoagulant, frozen for Bemis
- 14 rats - 500 µL plasma frozen for Conklin
  - Blood cell pellet after removing plasma, frozen for Conklin
- 10 rats - 50 µL plasma frozen for Hallberg

Gross necropsy (LRRI)
Weigh 8 target organs (LRRI, provide heart weight to Sun)
Lungs:
- 20 rats - Bronchoalveolar lavage right lung lobes (LRRI)
- 20 rats - Fix left lung (10% NBF, 25 cm fixative constant pressure)
  - 20 rats - Section & stain H&E for LRRI histopathology
  - 20 rats - Cut 2 sections and mount unstained for LRRI cell proliferation
  - 20 rats - Cut 6 sections and mount unstained for Veranth
- 20 rats - Freeze right lung lobes
  - 20 rats - Caudal lobe for LRRI glutathione, HO-1, and 8-OhdG
  - 20 rats - Middle lobe for Veranth
  - 20 rats - Cranial lobe for archive
  - 10 rats - right accessory lobe for Hallberg
  - 10 rats - right accessory lobe for archive

Brain:
- 20 rats - Fix ½ brain (incl. olfactory bulb) for LRRI histopathology
  - Freeze ½ brain (incl. olfactory bulb)
    - 10 rats - send to Hallberg
    - 10 rats - save extra frozen brain for archive

Heart:
- 20 rats - Fix in 10% NBF and section to include 4 chambers
  - 20 rats - H&E slides for LRRI histopathology
  - 10 rats - Cut 2 sections & mount unstained for Sun

Aorta:
- 20 rats - Portion of caudal thoracic aorta coated with OCT & frozen for Sun
  - Fix remainder in 10% NBF, section linear portion of descending thoracic
    - 20 rats - H&E slides for LRRI histopathology
    - 14 rats - cut 7 unstained slides (2-3 cross-sections each) for Conklin

Other Tissues:
- 20 rats - Collect RFP list and fix in 10% NBF for LRRI histopathology
- 20 rats - Mid-jejunal mesenteric arbor coated with OCT & frozen for Sun
- 20 rats - Cremaster muscle coated with OCT & frozen for Sun

B. Mouse Procedures:

1. 60 mice/group at 1 month:

20 mice - LRRI (only)
- Body weight
- Blood:
  - Archive frozen blood and serum
- Gross necropsy and tissue collection as for rats
- Weigh target organs
- Lungs:
  - Bronchoalveolar lavage of right lung lobes
  - Fix left lung (10% NBF, 25 cm fixative constant pressure)
    - Section as for rats
      - Stain section with H&E for histopathology
      - Cut 2 sections unstained for cell proliferation
  - Freeze right middle, caudal, and accessory lung lobes for glutathione, HO-1, and 8-OHdG

10 mice - Bemis (only)
- 100 μL blood in special anticoagulant, frozen

10 mice - Hallberg (only)
- 50 μL plasma, frozen (collect as much as feasible)
- Rt. accessory lung lobe, frozen
- Brain (with olfactory bulbs), frozen

20 mice - Conklin, Sun, and Veranth (shared)
- 100 μL plasma, frozen for Conklin (collect as much as feasible)
  - Blood cell pellet after removing plasma, frozen for Conklin
- Lung:
  - Left lung fixed in 10% NBF, 6 mounted, unstained slides for Veranth
  - Right lung lobes frozen for Veranth
- Heart:
  - Weigh for Sun
  - Fix in 10% NBF, section as for rats, cut 2 unstained slides for Sun
- Aorta:
  - Fix arch in 10% NBF for Conklin
  - Portion of caudal thoracic aorta coated with OCT & frozen for Sun
  - Remainder of linear portion of thoracic aorta frozen for Conklin
- Other tissues:
  - Mid-jejunal mesenteric arbor coated with OCT & frozen for Sun
  - Cremaster muscle coated with OCT & frozen for Sun

2. 60 mice/group at 3 months:

20 mice - LRRI (only)
- Body weight
- Blood:
  - Hematology
  - Serum chemistry
- Gross necropsy and tissue collection as for rats
- Weigh target organs
- Lungs:
  - Bronchoalveolar lavage of right lung lobes
  - Fix left lung (10% NBF, 25 cm fixative constant pressure)
    - Section as for rats
      - Stain section with H&E for histopathology
      - Cut 2 sections unstained for cell proliferation
  - Freeze right middle, caudal, and accessory lung lobes for glutathione, HO-1, and 8-OHdG

10 mice - Bemis (only)
- 100 µL blood in special anticoagulant, frozen

10 mice - Hallberg (only)
- 50 µL plasma, frozen (collect as much as feasible)
- Rt. accessory lung lobe, frozen
- Brain (with olfactory bulbs), frozen

20 mice - Conklin, Sun, and Veranth (shared)
- 100 µL plasma, frozen for Conklin (collect as much as feasible)
  - Blood cell pellet after removing plasma, frozen for Conklin
- Lung:
  - Left lung fixed in 10% NBF, 6 mounted, unstained slides for Veranth
  - Right lung lobes frozen for Veranth
- Heart:
  - Weigh for Sun
  - Fix in 10% NBF, section as for rats, cut 2 unstained slides for Sun
- Aorta:
  - Fix arch in 10% NBF for Conklin
  - Portion of caudal thoracic aorta coated with OCT & frozen for Sun
  - Remainder of linear portion of thoracic aorta frozen for Conklin
- Other tissues:
  - Mid-jejunal mesenteric arbor coated with OCT & frozen for Sun
  - Cremaster muscle coated with OCT & frozen for Sun

APPENDIX C

Study Schedule and Animal Caging Logistics

Study Schedule

The schedule of key events by study week is presented in Table 1. The animals will be entered into the study in blocks to meet both pre-study and necropsy logistical limitations. Three blocks of mice (A-C) and three blocks of rats (D-F) will be entered into the study in that order. The week during which block A mice begin pre-exposure conditioning is considered study week 1. Three study weeks are allotted for each entry block. Although a minimum of only two weeks of conditioning to chamber housing is required, a period of three weeks is allotted to encompass receipt of animals, conditioning, identification, assignment to treatment groups, and placement in the exposure chambers.

Exposures of mice will begin before exposures of rats for three reasons: 1) chamber space and necropsy logistics mandate that the exposures of rats and mice be staggered; 2) the H1000 chambers used for mice will be required for the rats early in the rat exposure period; and 3) a lapse of a few weeks between entry of the rats will serve to verify reliability of the generation-exposure system before beginning exposures for the chronic rat bioassay.

Exposure chamber space is not a limiting factor for mice; the numbers of mice required for the short-term study design can readily be accommodated. Exposure chamber space is, however, limiting for rats. The study design maximizes the number of rats available for the chronic cancer bioassay, given that 80 rats per treatment group will be sacrificed and considering expected mortality and the increasing cage size required as the rats gain weight. Accordingly, the design is based on the best available survival (Figure 1) and body weight (Figure 2) data provided by the supplier (Harlan).

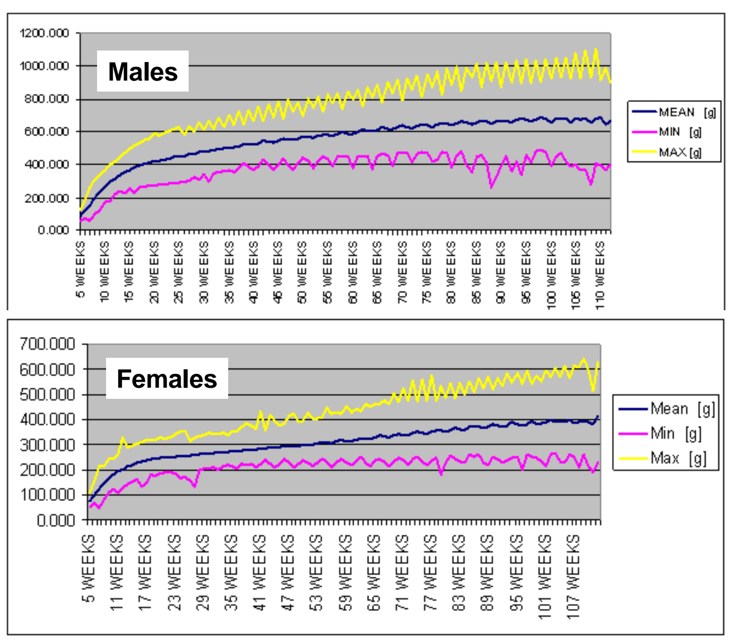


Figure 1. Body weight curves for male and female HsdRccHan:Wist rats, from the Harlan web site.

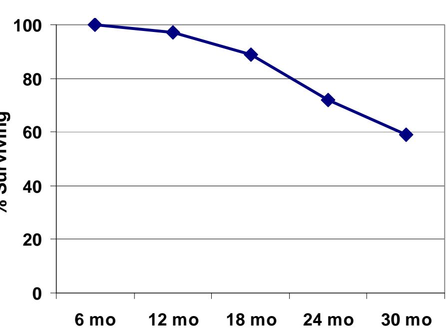


Figure 2. Expected survival of HsdRccHan:Wist rats, from the Harlan web site.

Table 1. Sequence of Study Events by Study Week

(Sacrifices occur during the week following completion of the exposure interval)
(Animal numbers are numbers per necropsy day, taken equally from each of 4 treatment groups)

Study Week	Event
1	Start conditioning block A (core mice)
2	Start conditioning block B (ancillary mice)
3	Start conditioning block C (ancillary mice)
4	Start exposure block A (core mice)
5	Start exposure block B (ancillary mice)
6	Start exposure block C (ancillary mice)
8	4 wk sacrifice block A (core mice: 28, 28, 24 = 80)
9	4 wk sacrifice block B (ancillary mice: 28, 28, 24 = 80)
10	4 wk sacrifice block C (ancillary mice: 28, 28, 24 = 80)
13	Start conditioning block D (rats)
14	Start conditioning block E (rats)
15	Start conditioning block F (rats)
16	Start exposure block D (rats)
17	Start exposure block E (rats), 13 wk sacrifice block A (core mice: 28, 28, 24 = 80)
18	Start exposure block F (rats), 13 wk sacrifice block B (ancillary mice: 28, 28, 24 = 80)
19	13 wk sacrifice block C (ancillary mice: 28, 28, 24 = 80) *[H1000 chambers empty by end of wk]*
20	4 wk sacrifice block D (rats: 16, 16 = 32)
21	4 wk sacrifice block E (rats: 12, 12 = 24)
22	4 wk sacrifice block F (rats: 12, 12 = 24), *male rats reach 400g*
28	Pulmonary function block D
29	Pulmonary function block E, 13 wk sacrifice block D (16, 16 = 32)
30	Pulmonary function block F, 13 wk sacrifice block E (12, 12 = 24)
31	13 wk sacrifice block F (12,12 = 24)
32	*Male rats reach 500g*
59	*Reach 97% survival*
65	*Reach 90% survival*
67	Pulmonary function block D
68	Pulmonary function block E, 52 wk sacrifice block D (16, 16 = 32)
69	Pulmonary function block F, 52 wk sacrifice block E (12, 12 = 24)
70	52 wk sacrifice block F (12, 12 = 24)
71	*Reach 72% survival*
72	*Female rats reach 400g*
119	Pulmonary function block D
120	Pulmonary function block E, 104 wk sacrifice block D (16, 16 = 32)
121	Pulmonary function block F, 104 wk sacrifice block E (12, 12 = 24)
122	104 wk sacrifice block F (12, 12 = 24)
137	*Reach 60% survival*
146	Terminal (30 mo) sacrifice block D
147	Terminal (30 mo) sacrifice block E
148	Terminal (30 mo) sacrifice block F

The three blocks of each species are staggered at 1-week intervals to ensure that the necropsies can be conducted as scheduled. The necropsy procedures are complex, and the total necropsy resources (facilities and personnel) can not be dedicated solely to this study for an entire week. Limiting necropsies to no more than three days in each sacrifice week facilitates meeting the requirement for at least one day of exposure immediately prior to the sacrifice day, and ensures that the LRRI necropsy resources are not solely committed to this study during any week.

The three blocks of mice are designated for three different necropsy procedures. At each of two sacrifice times (after 4 and 13 wk of exposure) 80 block A mice (20 x 4 treatment groups) are designated for core (LRRI) sacrifices, and two blocks of 80 (blocks B and C) are designated for two different sample collection protocols for ancillary studies. The limited volumes of blood and tissue available from mice preclude accommodating all core and ancillary studies with a single group of mice. In contrast, the three blocks of rats will all undergo the same necropsy procedures with measurements and sample collections taken from all sacrifice rats for both core and ancillary study purposes. The number of rats sacrificed at each of the four sacrifice intervals (after 4, 13, 52, and 104 wk of exposure) will total 80, of which 32, 24, and 24 will be taken from blocks D, E, and F, respectively. These numbers are divisible by 4 so that an equal number of male and female rats from each of the four treatment groups can be included in each necropsy day.

Key factors determining the maximum size of the rat treatment groups are presented in Table 2. Rats will be obtained at 6 wk of age. The maximum number of rats that can be housed in the available chamber space can be derived from the ages (and thus the study weeks) at which cage size must be increased based on body weight, together with reductions of populations by planned sacrifices and expected natural mortality. Based on previous studies involving much higher concentrations of diesel exhaust, the exposures in this study are not expected to increase mortality.

Table 2. Key Determinants of Rat Caging Requirements and Maximum Group Size

A. Critical body weight points *(to be conservative, all blocks are assumed to reach these weights when the first block reaches the critical age)*:

Males reach 400g @ 15 wk of age = study wk 22
“ “ 500g @ 25 “ “ “ “ “ 32

Females reach 400g @ 70 wk of age = study wk 77

B. Expected Survival of 200 chronic rats *(same survival for males & females)*:

mo of age	mo exposure	survival	living	loss in interval
0-12	0-10	.97	194	6
12-18	10-16	.90	180	14
18-24	16-22	.72	144	36
24-30	22-28	.60	120	24
	Total subtracted for natural mortality			80

(To be conservative, mortality losses are subtracted *at the end of each survival interval, and distributed among blocks.)*

C. Available rat caging units *(maximum capacity is 15 units per treatment group)*

R24 houses 24 rats < 400g
R16 houses 16 rats 400-499g
R12 houses 12 rats ≥ 500g

A back-calculation from the cage load choke-point (Table 3, red line at wk 22 at which 15 total cage units are required per treatment group) dictates maximum treatment group sizes of 280 rats, of which 80 are required for sacrifices and 200 will be available for chronic observation for tumor incidence. Of those 200, 80 are expected to die from natural mortality by 30 months of age, leaving an estimated 120 rats per treatment group for observation to 30 months of exposure. As the 24-month point is approached, survival to that point will be evaluated and a decision made by the sponsor as to whether to terminate the exposures at 24 months or continue to 30 months. The study will be terminated at the selected time by sacrifice of all remaining rats. The design and initial budgeting of the study anticipate that the exposures will continue to 30 months.

Additional mice and rats will be obtained, conditioned, and assigned to sentinel groups that are housed in the same type of chambers and exposed to only clean (control) air. These animals will be sacrificed for serology at times indicated in the protocol, as surveillance for introduction of rodent pathogens into the population. The numbers of animals required for sentinels can be readily housed in a single mouse and a single rat chamber that are separate from the study exposure chambers. At each surveillance interval, animals will be selected randomly from the sentinel population for serology.

Table 3. Rat Exposure Chamber Caging Logistics (per each treatment group)

Type style indicates weight range: italics = < 400g, regular = 400-499g, bold = 500+g

Study		Male Blocks			Female Blocks			Total	Total	
Wk	Event	D	E	F	D	E	F	males	females	Caging
16	enter blk D	*48*			*48*			*48*	*48*	4 R24
16	“ “ E	*48*	*46*		*48*	*46*		*94*	*94*	8 R24
17	“ “ F	*48*	*46*	*46*	*48*	*46*	*46*	*140*	*140*	12 R24
20	4 wk sac D	*-4*			*-4*					
		44	*46*	*46*	*44*	*46*	*46*	*136*	*136*	“ “
21	“ “ “ E		*-3*			*-3*				
		44	*43*	*46*	*44*	*43*	*46*	*133*	*133*	“ “
22	“ “ “ F			*-3*			*-3*			
	Males = 400g	44	43	43	*44*	*43*	*43*	130	*130*	9 R16, 6 R24
29	13 wk sac D	-4			*-4*					
		40	43	43	40	*43*	*43*	126	*126*	“ “ “ “
30	“ “ “ E		-3			*-3*				
		40	40	43	40	*40*	*43*	123	*123*	“ “ “ “
31	“ “ “ F			-3			*-3*			
		40	40	40	40	*40*	*40*	120	*120*	“ “ “ “
32	Males = 500g	**40**	**40**	**40**	40	*40*	*40*	**120**	*120*	10 R12, 5 R24
59	97% survival	**-1**	**-1**	**-1**	*-1*	*-1*	*-1*			
		39	**39**	**39**	*39*	*39*	*39*	**117**	*117*	“ “ “ “
65	90% survival	**-3**	**-2**	**-2**	*-3*	*-2*	*-2*			
		36	**37**	**37**	*36*	*37*	*37*	**110**	*110*	“ “ “ “
68	52 wk sac D	**-4**			*-4*					
		32	**37**	**37**	*32*	*37*	*37*	**106**	*106*	9 R12, 5 R24
69	“ “ “ E		**-3**			*-3*				
		32	**34**	**37**	*32*	*34*	*37*	**103**	*103*	“ “ “ “
70	“ “ “ F			**-3**			*-3*			
		32	**34**	**34**	*32*	*34*	*34*	**100**	*100*	“ “ “ “
71	72% survival	**-6**	**-6**	**-6**	*-6*	*-6*	*-6*			
		26	**28**	**28**	*26*	*28*	*28*	**82**	*82*	7 R12, 4 R24
72	Females = 400g	**26**	**28**	**28**	26	28	28	**82**	82	7 R12, 6 R16
120	104 wk sac D	**-4**			-4					
		22	**28**	**28**	22	28	28	**78**	78	7 R12, 5 R16
121	“ “ “ E		**-3**			3-				
		22	**25**	**28**	22	25	28	**75**	75	“ “ “ “
122	“ “ “ F			**-3**			-3			
		22	**25**	**25**	22	25	25	**72**	72	6 R12, 5 R16
137	60% survival	**-4**	**-4**	**-4**	-4	-4	-4			
		18	**21**	**21**	18	21	21	**60**	60	5 R12, 4 R16
146	Term sac D	**0**	**21**	**21**	0	21	21	**42**	42	“ “ “ “
147	Term sac E	0	0	**21**	0	0	21	**21**	21	“ “ “ “
148	Term sac F	0	0	0	0	0	0	0	0	