§79.6 Requirement for testing.

Provisions regarding testing that is required for registration of a designated fuel or fuel additive are contained in subpart F of this part.

[59 FR 33092, June 27, 1994]

§79.7 Samples for test purposes.

When the Administrator requires for test purposes a fuel or additive which is not readily available in the open market, he may request the manufacturer of such fuel or additive to furnish a sample in a reasonable quantity. The fuel or additive manufacturer shall comply with such request within 30 days.

§79.8 Penalties.

Any person who violates section 211(a) of the Act or who fails to furnish any information or conduct any tests required under this part shall be liable to the United States for a civil penalty of not more than the sum of $25,000 for every day of such violation and the amount of economic benefit or savings resulting from the violation. Civil penalties shall be assessed in accordance with paragraphs (b) and (c) of section 205 of the Act.

[58 FR 65554, Dec. 15, 1993]

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§79.60 Good laboratory practices (GLP) standards for inhalation exposure health effects testing.

(a) General Provisions—(1) Scope. (i) This section prescribes good laboratory practices (GLPs) for conducting inhalation exposure studies relating to motor vehicle emissions health effects testing under this part. These directions are intended to ensure the quality and integrity of health effects data submitted pursuant to registration regulations issued under sections 211(b) or 211(e) of the Clean Air Act (CAA) (42 U.S.C. 7545).

(ii) This section applies to any study described by paragraph (a)(1)(i) of this section which any person conducts, initiates, or supports on or after May 27, 1994.

(iii) It is EPA's policy that all health effects data developed under sections 211(b) and (e) of CAA be in accordance with provisions of this section. If data are not developed in accordance with the provisions of this section, EPA may consider such data insufficient to evaluate the health effects of a motor vehicle's fuel or fuel additive emissions, unless the submitter provides additional information demonstrating that the data are reliable and adequate and EPA determines that the data are sufficient.

(2) Definitions. As used in this section, the following terms shall have the meanings specified:

Batch means a specific quantity or lot of a test fuel, additive/base fuel mixture, or reference substance that has been characterized according to §79.60(f)(1)(i).

CAA means the Clean Air Act.

Carrier means any material which is combined with engine/motor vehicle emissions or a reference substance for administration to a test system. “Carrier” includes, but is not limited to, clean, filtered air, water, feed, and nutrient media.

Control atmosphere means clean, filtered air which is administered to the test system in the course of a study for the purpose of establishing a basis for comparison with the test atmosphere for chemical or biological measurements.

Experimental start date means the first date the test atmosphere is applied to the test system.

Experimental termination date means the last date on which data are collected directly from the study.

Person includes an individual, partnership, corporation, association, scientific or academic establishment, government agency, or organizational unit thereof, and any other legal entity.

Quality assurance unit means any person or organizational element, except the study director, designated by testing facility management to perform the duties relating to quality assurance of the studies.

Raw data means any laboratory worksheets, records, memoranda, notes, or exact copies thereof, that are the result of original observations and activities of a study and are necessary for the reconstruction and evaluation of the report of that study. In the event that exact transcripts of raw data have been prepared (e.g., tapes which have been transcribed verbatim, dated, and verified accurate by signature), the exact copy or exact transcript may be substituted for the original source as raw data. “Raw data” may include photographs, videotape, microfilm or microfiche copies, computer printouts, magnetic media, including dictated observations, and recorded data from automated instruments.

Reference substance means any chemical substance or mixture, analytical standard, or material other than engine/motor vehicle emissions and/or its carrier, that is administered to or used in analyzing the test system in the course of a study. A “reference substance” is used to establish a basis for comparison with the test atmosphere for known chemical or biological measurements, i.e., positive or negative control substance.

Specimen means any material derived from a test system for examination or analysis.

Sponsor means person who initiates and supports, by provision of financial or other resources, a study or a person who submits a study to EPA in response to the CAA Section 211(b) or 211(e) Fuels and Fuel Additives Registration Rule or a testing facility, if it both initiates and actually conducts the study.

Study means any experiment, at one or more test sites, in which a test system is exposed to a test atmosphere under laboratory conditions to determine or help predict the health effects of that exposure in humans, other living organisms, or media.

Study completion date means the date the final report is signed by the study director.

Study director means the individual responsible for the overall conduct of a study.

Study initiation date means the date the protocol is signed by the study director.

Test substance means a vapor and/or aerosol mixture composed of engine/motor vehicle emissions and clean, filtered air which is administered directly, or indirectly, by the inhalation route to a test system in a study which develops data to meet the registration requirements of CAA section 211(b) or (e).

Test system means any animal, microorganism, chemical or physical matrix, to which the test, control, or reference substance is administered or added for study. This definition also includes appropriate groups or components of the system not treated with the test, control, or reference substance.

Testing facility means a person who actually conducts a study, i.e., actually uses the test substance in a test system. “Testing facility” encompasses only those operational units that are being or have been used to conduct studies.

TSCA means the Toxic Substances Control Act (15 U.S.C. 2601 et seq.).

(3) Applicability to studies performed under grants and contracts. When a sponsor or other person utilizes the services of a consulting laboratory, contractor, or grantee to perform all or a part of a study to which this section applies, it shall notify the consulting laboratory, contractor, or grantee that the service is, or is part of, a study that must be conducted in compliance with the provisions of this section.

(4) Statement of compliance or non-compliance. Any person who submits to EPA a test in compliance with registration regulations issued under CAA section 211(b) or section 211(e) shall include in the submission a true and correct statement, signed by the sponsor and the study director, of one of the following types:

(i) A statement that the study was conducted in accordance with this section; or

(ii) A statement describing in detail all differences between the practices used in the study and those required by this section; or

(iii) A statement that the person was not a sponsor of the study, did not conduct the study, and does not know whether the study was conducted in accordance with this section.

(5) Inspection of a testing facility. (i) A testing facility shall permit an authorized employee or duly designated representative of EPA, at reasonable times and in a reasonable manner, to inspect the facility and to inspect (and in the case of records also to copy) all records and specimens required to be maintained regarding studies to which this section applies. The records inspection and copying requirements shall not apply to quality assurance unit records of findings and problems, or to actions recommended and taken, except the EPA may seek production of these records in litigation or formal adjudicatory hearings.

(ii) EPA will not consider reliable for purposes of showing that a test substance does or does not present a risk of injury to health or the environment any data developed by a testing facility or sponsor that refuses to permit inspection in accordance with this section. The determination that a study will not be considered reliable does not, however, relieve the sponsor of a required test of any obligation under any applicable statute or regulation to submit the results of the study to EPA.

(6) Effects of non-compliance. (i) Pursuant to sections 114, 208, and 211(d) of the CAA, it shall be a violation of this section and a violation of this rule (40 CFR part 79, subpart F) if:

(A) The test is not being or was not conducted in accordance with any requirement of this part; or

(B) Data or information submitted to EPA under part 79, including the statement required by §79.60(a)(4), include information or data that are false or misleading, contain significant omissions, or otherwise do not fulfill the requirements of this part; or

(C) Entry in accordance with §79.60(a)(5) for the purpose of auditing test data is denied.

(ii) EPA, at its discretion, may not consider reliable for purposes of showing that a chemical substance or mixture does not present a risk of injury to health any study which was not conducted in accordance with this part. EPA, at its discretion, may rely upon such studies for purposes of showing adverse effects. The determination that a study will not be considered reliable does not, however, relieve the sponsor of a required test of the obligation under any applicable statute or regulation to submit the results of the study to EPA.

(iii) If data submitted in compliance with registration regulations issued under CAA section 211(b) or section 211(e) are not developed in accordance with this section, EPA may determine that the sponsor has not fulfilled its obligations under 40 CFR part 79 and may require the sponsor to develop data in accordance with the requirements of this section in order to satisfy such obligations.

(b) Organization and Personnel—(1) Personnel. (i) Each individual engaged in the conduct of or responsible for the supervision of a study shall have education, training, and experience, or combination thereof, to enable that individual to perform the assigned functions.

(ii) Each testing facility shall maintain a current summary of training and experience and job description for each individual engaged in or supervising the conduct of a study.

(iii) There shall be a sufficient number of personnel for the timely and proper conduct of the study according to the protocol.

(iv) Personnel shall take necessary personal sanitation and health precautions designed to avoid contamination of test fuel and additive/base fuel mixtures, test and reference substances, and test systems.

(v) Personnel engaged in a study shall wear clothing appropriate for the duties they perform. Such clothing shall be changed as often as necessary to prevent microbiological, radiological, or chemical contamination of test systems and test, control, and reference substances.

(vi) Any individual found at any time to have an illness that may adversely affect the quality and integrity of the study shall be excluded from direct contact with test systems, fuel and fuel/additive mixtures, test and reference substances and any other operation or function that may adversely affect the study until the condition is corrected. All personnel shall be instructed to report to their immediate supervisors any health or medical conditions that may reasonably be considered to have an adverse effect on a study.

(2) Testing facility management. For each study, testing facility management shall:

(i) Designate a study director as described in §79.60(b)(3) before the study is initiated.

(ii) Replace the study director promptly if it becomes necessary to do so during the conduct of a study.

(iii) Assure that there is a quality assurance unit as described in §79.60(b)(4).

(iv) Assure that test fuels and fuel/additive mixtures and test and reference substances have been identified as to content, strength, purity, stability, and uniformity, as applicable.

(v) Assure that personnel, resources, facilities, equipment, materials and methodologies are available as scheduled.

(vi) Assure that personnel clearly understand the functions they are to perform.

(vii) Assure that any deviations from these regulations reported by the quality assurance unit are communicated to the study director and corrective actions are taken and documented.

(3) Study director. For each study, a scientist or other professional person with a doctorate degree or equivalent in toxicology or other appropriate discipline shall be identified as the study director. The study director has overall responsibility for the technical conduct of the study, as well as for the interpretation, analysis, documentation, and reporting of results, and represents the single point of study control. The study director shall assure that:

(i) The protocol, including any changes, is approved as provided by §79.60(g)(1)(i) and is followed;

(ii) All experimental data, including observations of unanticipated responses of the test system are accurately recorded and verified;

(iii) Unforeseen circumstances that may affect the quality and integrity of the study are noted when they occur, and corrective action is taken and documented;

(iv) Test systems are as specified in the protocol;

(v) All applicable good laboratory practice regulations are followed; and

(vi) All raw data, documentation, protocols, specimens, and final reports are archived properly during or at the close of the study.

(4) Quality assurance unit. A testing facility shall have a quality assurance unit which shall be responsible for monitoring each study to assure management that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with the regulations in this section. For any given study, the quality assurance unit shall be entirely separate from and independent of the personnel engaged in the direction and conduct of that study. The quality assurance unit shall conduct inspections and maintain records appropriate to the study.

(i) Quality assurance unit duties. (A) Maintain a copy of a master schedule sheet of all studies conducted at the testing facility indexed by test substance and containing the test system, nature of study, date study was initiated, current status of each study, identity of the sponsor, and name of the study director.

(B) Maintain copies of all protocols pertaining to all studies for which the unit is responsible.

(C) Inspect each study at intervals adequate to ensure the integrity of the study and maintain written and properly signed records of each periodic inspection showing the date of the inspection, the study inspected, the phase or segment of the study inspected, the person performing the inspection, findings and problems, action recommended and taken to resolve existing problems, and any scheduled date for re-inspection. Any problems which are likely to affect study integrity found during the course of an inspection shall be brought to the attention of the study director and management immediately.

(D) Periodically submit to management and the study director written status reports on each study, noting any problems and the corrective actions taken.

(E) Determine that no deviations from approved protocols or standard operating procedures were made without proper authorization and documentation.

(F) Review the final study report to assure that such report accurately describes the methods and standard operating procedures, and that the reported results accurately reflect the raw data of the study.

(G) Prepare and sign a statement to be included with the final study report which shall specify the dates inspections were made and findings reported to management and to the study director.

(ii) The responsibilities and procedures applicable to the quality assurance unit, the records maintained by the quality assurance unit, and the method of indexing such records shall be in writing and shall be maintained. These items including inspection dates, the study inspected, the phase or segment of the study inspected, and the name of the individual performing the inspection shall be made available for inspection to authorized employees or duly designated representatives of EPA.

(iii) An authorized employee or a duly designated representative of EPA shall have access to the written procedures established for the inspection and may request test facility management to certify that inspections are being implemented, performed, documented, and followed up in accordance with this paragraph.

(c) Facilities—(1) General. Each testing facility shall be of suitable size and construction to facilitate the proper conduct of studies. Testing facilities which are not completely located within an indoor controlled environment shall be of suitable location/proximity to facilitate the proper conduct of studies. Testing facilities shall be designed so that there is a degree of separation that will prevent any function or activity from having an adverse effect on the study.

(2) Test system care facilities. (i) A testing facility shall have a sufficient number of animal rooms or other test system areas, as needed, to ensure proper separation of species or test systems, quarantine or isolation of animals or other test systems, and routine or specialized housing of animals or other test systems.

(ii) A testing facility shall have a number of animal rooms or other test system areas separate from those described in paragraph (a) of this section to ensure isolation of studies being done with test systems or test, control, and reference substances known to be biohazardous, including volatile atmospheres and aerosols, radioactive materials, and infectious agents. The animal handling facility must operate under the supervision of a veterinarian.

(iii) Separate areas shall be provided, as appropriate, for the diagnosis, treatment, and control of laboratory test system diseases. These areas shall provide effective isolation for the housing of test systems either known or suspected of being diseased, or of being carriers of disease, from other test systems.

(iv) Facilities shall have proper provisions for collection and disposal of contaminated air, water, or other spent materials. When animals are housed, facilities shall exist for the collection and disposal of all animal waste and refuse or for safe sanitary storage of waste before removal from the testing facility. Disposal facilities shall be so provided and operated as to minimize vermin infestation, odors, disease hazards, and environmental contamination.

(v) Facilities shall have provisions to regulate environmental conditions (e.g., temperature, humidity, day length, etc.) as specified in the protocol.

(3) Test system supply/operation areas. (i) There shall be storage areas, as needed, for feed, bedding, supplies, and equipment. Storage areas for feed and bedding shall be separated from areas where the test systems are located and shall be protected against infestation or contamination. Perishable supplies shall be preserved by appropriate means.

(ii) Separate laboratory space and other space shall be provided, as needed, for the performance of the routine and specialized procedures required by studies.

(4) Facilities for handling test fuels and fuel/additive mixtures and reference substances. (i) As necessary to prevent contamination or mixups, there shall be separate areas for:

(A) Receipt and storage of the test fuels and fuel/additive mixtures and reference substances;

(B) Mixing of the test fuels, fuel/additive mixtures, and reference substances with a carrier, i.e., liquid hydrocarbon; and

(C) Storage of the test fuels, fuel/additive mixtures, and reference substance/carrier mixtures.

(ii) Storage areas for test fuels and fuel/additive mixtures and reference substances and for reference mixtures shall be separate from areas housing the test systems and shall be adequate to preserve the identity, strength, purity, and stability of the substances and mixtures.

(5) Specimen and data storage facilities. Space shall be secured for archives for the storage and retrieval of all raw data and specimens from completed studies.

(d) Equipment—(1) Equipment design. Equipment used in the generation, measurement, or assessment of data and equipment used for facility environmental control shall be of appropriate design and adequate capacity to function according to the protocol and shall be suitably located for operation, inspection, cleaning, and maintenance.

(2) Maintenance and calibration of equipment. (i) Equipment shall be adequately inspected, cleaned, and maintained. Equipment used for the generation, measurement, or assessment of data shall be adequately tested, calibrated, and/or standardized.

(ii) The written standard operating procedures required under §79.60(e)(1)(ii)(K) shall set forth in sufficient detail the methods, materials, and schedules to be used in the routine inspection, cleaning, maintenance, testing, calibration, and/or standardization of equipment, and shall specify, when appropriate, remedial action to be taken in the event of failure or malfunction of equipment. The written standard operating procedures shall designate the person responsible for the performance of each operation.

(iii) Written records shall be maintained of all inspection, maintenance, testing, calibrating, and/or standardizing operations. These records, containing the date of the operation, shall describe whether the maintenance operations were routine and followed the written standard operating procedures. Written records shall be kept of non-routine repairs performed on equipment as a result of failure and malfunction. Such records shall document the nature of the defect, how and when the defect was discovered, and any remedial action taken in response to the defect.

(e) Testing Facilities Operation—(1) Standard operating procedures. (i) A testing facility shall have standard operating procedures in writing, setting forth study methods that management is satisfied are adequate to insure the quality and integrity of the data generated in the course of a study. All deviations in a study from standard operating procedures shall be authorized by the study director and shall be documented in the raw data. Significant changes in established standard operating procedures shall be properly authorized in writing by management.

(ii) Standard operating procedures shall be established for, but not limited to, the following:

(A) Test system room preparation;

(B) Test system care;

(C) Receipt, identification, storage, handling, mixing, and method of sampling of test fuels and fuel/additive mixtures and reference substances;

(D) Test system observations;

(E) Laboratory or other tests;

(F) Handling of test animals found moribund or dead during study;

(G) Necropsy or postmortem examination of test animals;

(H) Collection and identification of specimens;

(I) Histopathology

(J) Data handling, storage and retrieval.

(K) Maintenance and calibration of equipment.

(L) Transfer, proper placement, and identification of test systems.

(iii) Each laboratory or other study area shall have immediately available manuals and standard operating procedures relative to the laboratory procedures being performed. Published literature may be used as a supplement to standard operating procedures.

(iv) A historical file of standard operating procedures, and all revisions thereof, including the dates of such revisions, shall be maintained.

(2) Reagents and solutions. All reagents and solutions in the laboratory areas shall be labeled to indicate identity, titer or concentration, storage requirements, and expiration date. Deteriorated or outdated reagents and solutions shall not be used.

(3) Animal and other test system care. (i) There shall be standard operating procedures for the housing, feeding, handling, and care of animals and other test systems.

(ii) All newly received test systems from outside sources shall be isolated and their health status or appropriateness for the study shall be evaluated. This evaluation shall be in accordance with acceptable veterinary medical practice or scientific methods.

(iii) At the initiation of a study, test systems shall be free of any disease or condition that might interfere with the purpose or conduct of the study. If during the course of the study, the test systems contract such a disease or condition, the diseased test systems shall be isolated, if necessary. These test systems may be treated for disease or signs of disease provided that such treatment does not interfere with the study. The diagnosis, authorization of treatment, description of treatment, and each date of treatment shall be documented and shall be retained.

(iv) When laboratory procedures require test animals to be manipulated and observed over an extended period of time or when studies require test animals to be removed from and returned to their housing units for any reason (e.g., cage cleaning, treatment, etc.), these test systems shall receive appropriate identification (e.g., tattoo, color code, etc.). Test system identification shall conform with current laboratory animal handling practice. All information needed to specifically identify each test system within the test system-housing unit shall appear on the outside of that unit. Suckling animals are excluded from the requirement of individual identification unless otherwise specified in the protocol.

(v) Except as specified in paragraph (e)(3)(v)(A) of this section, test animals of different species shall be housed in separate rooms when necessary. Test animals of the same species, but used in different studies, shall not ordinarily be housed in the same room when inadvertent exposure to the test or reference substances or test system mixup could affect the outcome of either study. If such mixed housing is necessary, adequate differentiation by space and identification shall be made.

(A) Test systems that may be used in multispecies tests need not be housed in separate rooms, provided that they are adequately segregated to avoid mixup and cross-contamination.

(B) [Reserved]

(vi) Cages, racks, pens, enclosures, and other holding, rearing, and breeding areas, and accessory equipment, shall be cleaned and sanitized at appropriate intervals.

(vii) Feed and water used for the test animals shall be analyzed periodically to ensure that contaminants known to be capable of interfering with the study and reasonably expected to be present in such feed or water are not present at greater than trace levels. Documentation of such analyses shall be maintained as raw data.

(viii) Bedding used in animal cages or pens shall not interfere with the purpose or conduct of the study and shall be changed as often as necessary to keep the animals dry and clean.

(ix) If any pest control materials are used, the use shall be documented. Cleaning and pest control materials that interfere with the study shall not be used.

(x) All test systems shall be acclimatized to the environmental conditions of the test, prior to their use in a study.

(f) Test fuels, additive/base fuel mixtures, and reference substances—(1) Test fuel, fuel/additive mixture, and reference substance identity. (i) The product brand name/service mark, strength, purity, content, or other characteristics which appropriately define the test fuel, fuel/additive mixture, or reference substance shall be reported for each batch and shall be documented before its use in a study. Methods of synthesis, fabrication, or derivation, as appropriate, of the test fuel, fuel/additive mixture, or reference substance shall be documented by the sponsor or the testing facility, and such location of documentation shall be specified.

(ii) The stability of test fuel, fuel/additive mixture, and reference substances under storage conditions at the test site shall be known for all studies.

(2) Test fuel, additive/base fuel mixture, and reference substance handling. Procedures shall be established for a system for the handling of the test fuel, fuel/additive mixture, and reference substance(s) to ensure that:

(i) There is proper storage.

(ii) Distribution is made in a manner designed to preclude the possibility of contamination, deterioration, or damage.

(iii) Proper identification is maintained throughout the distribution process.

(iv) The receipt and distribution of each batch is documented. Such documentation shall include the date and quantity of each batch distributed or returned.

(3) Mixtures of test emissions or reference solutions with carriers.

(i) For test emissions or each reference substance mixed with a carrier, tests by appropriate analytical methods shall be conducted:

(A) To determine the uniformity of the test substance and to determine, periodically, the concentration of the test emissions or reference substance in the mixture;

(B) When relevant to the conduct of the experiment, to determine the solubility of each reference substance in the carrier mixture before the experimental start date; and

(C) To determine the stability of test emissions or a reference solution in the test substance before the experimental start date or concomitantly according to written standard operating procedures, which provide for periodic analysis of each batch.

(ii) Where any of the components of the reference substance/carrier mixture has an expiration date, that date shall be clearly shown on the container. If more than one component has an expiration date, the earliest date shall be shown.

(iii) If a chemical or physical agent is used to facilitate the mixing of a test substance with a carrier, assurance shall be provided that the agent does not interfere with the integrity of the test.

(g) Protocol for and conduct of a study—(1) Protocol. (i) Each study shall have a written protocol that clearly indicates the objectives and all methods for the conduct of the study. The protocol shall contain but shall not be limited to the following information:

(A) A descriptive title and statement of the purpose of the study.

(B) Identification of the test fuel, fuel/additive mixture, and reference substance by name, chemical abstracts service (CAS) number or code number, as applicable.

(C) The name and address of the sponsor and the name and address of the testing facility at which the study is being conducted.

(D) The proposed experimental start and termination dates.

(E) Justification for selection of the test system, as necessary.

(F) Where applicable, the number, body weight, sex, source of supply, species, strain, substrain, and age of the test system.

(G) The procedure for identification of the test system.

(H) A description of the experimental design, including methods for the control of bias.

(I) Where applicable, a description and/or identification of the diet used in the study. The description shall include specifications for acceptable levels of contaminants that are reasonably expected to be present in the dietary materials and are known to be capable of interfering with the purpose or conduct of the study if present at levels greater than established by the specifications.

(J) Each concentration level, expressed in milligrams per cubic meter of air or other appropriate units, of the test or reference substance to be administered and the frequency of administration.

(K) The type and frequency of tests, analyses, and measurements to be made.

(L) The records to be maintained.

(M) The date of approval of the protocol by the sponsor and the dated signature of the study director.

(N) A statement of the proposed statistical method.

(ii) All changes in or revisions of an approved protocol and the reasons therefor shall be documented, signed by the study director, dated, and maintained with the protocol.

(2) Conduct of a study. (i) The study shall be conducted in accordance with the protocol.

(ii) The test systems shall be monitored in conformity with the protocol.

(iii) Specimens shall be identified by test system, study, nature, and date of collection. This information shall be located on the specimen container or shall accompany the specimen in a manner that precludes error in the recording and storage of data.

(iv) In animal studies where histopathology is required, records of gross findings for a specimen from postmortem observations shall be available to a pathologist when examining that specimen histopathologically.

(v) All data generated during the conduct of a study, except those that are generated by automated data collection systems, shall be recorded directly, promptly, and legibly in ink. All data entries shall be dated on the day of entry and signed or initialed by the person entering the data. Any change in entries shall be made so as not to obscure the original entry, shall indicate the reason for such change, and shall be dated and signed or identified at the time of the change. In automated data collection systems, the individual responsible for direct data input shall be identified at the time of data input. Any change in automated data entries shall be made so as not to obscure the original entry, shall indicate the reason for change, shall be dated, and the responsible individual shall be identified.

(h) Records and Reports—(1) Reporting of study results. (i) A final report shall be prepared for each study and shall include, but not necessarily be limited to, the following:

(A) Name and address of the facility performing the study and the dates on which the study was initiated and was completed, terminated, or discontinued.

(B) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(C) Statistical methods employed for analyzing the data.

(D) The test fuel, additive/base fuel mixture, and test and reference substances identified by name, chemical abstracts service (CAS) number or code number, strength, purity, content, or other appropriate characteristics.

(E) Stability, and when relevant to the conduct of the study, the solubility of the test emissions and reference substances under the conditions of administration.

(F) A description of the methods used.

(G) A description of the test system used. Where applicable, the final report shall include the number of animals or other test organisms used, sex, body weight range, source of supply, species, strain and substrain, age, and procedure used for identification.

(H) A description of the concentration regimen as daily exposure period, i.e., number of hours, and exposure duration, i.e., number of days.

(I) A description of all circumstances that may have affected the quality or integrity of the data.

(J) The name of the study director, the names of other scientists or professionals and the names of all supervisory personnel, involved in the study.

(K) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

(L) The signed and dated reports of each of the individual scientists or other professionals involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(M) The locations where all specimens, raw data, and the final report are to be kept or stored.

(N) The statement, prepared and signed by the quality assurance unit, as described in §79.60(b)(4)(i)(G).

(ii) The final report shall be signed and dated by the study director.

(iii) Corrections or additions to a final report shall be in the form of an amendment by the study director. The amendment shall clearly identify that part of the final report that is being added to or corrected and the reasons for the correction or addition, and shall be signed and dated by the person responsible. Modification of a final report to comply with the submission requirements of EPA does not constitute a correction, addition, or amendment to a final report.

(iv) A copy of the final report and of any amendment to it shall be maintained by the sponsor and the test facility.

(2) Storage and retrieval of records and data. (i) All raw data, documentation, records, protocols, specimens, and final reports generated as a result of a study shall be retained. Specimens obtained from mutagenicity tests, wet specimens of blood, urine, feces, and biological fluids, do not need to be retained after quality assurance verification. Correspondence and other documents relating to interpretation and evaluation of data, other than those documents contained in the final report, also shall be retained.

(ii) All raw data, documentation, protocols, specimens, and interim and final reports shall be archived for orderly storage and expedient retrieval. Conditions of storage shall minimize deterioration of the documents or specimens in accordance with the requirements for the time period of their retention and the nature of the documents of specimens. A testing facility may contract with commercial archives to provide a repository for all material to be retained. Raw data and specimens may be retained elsewhere provided that the archives have specific reference to those other locations.

(iii) An individual shall be identified as responsible for the archiving of records.

(iv) Access to archived material shall require authorization and documentation.

(v) Archived material shall be indexed to permit expedient retrieval.

(3) Retention of records. (i) Record retention requirements set forth in this section do not supersede the record retention requirements of any other regulations in this subchapter.

(ii) Except as provided in paragraph (h)(3)(iii) of this section, documentation records, raw data, and specimens pertaining to a study and required to be retained by this part shall be archived for a period of at least ten years following the completion of the study.

(iii) Wet specimens, samples of test fuel, additive/base fuel mixtures, or reference substances, and specially prepared material which are relatively fragile and differ markedly in stability and quality during storage, shall be retained only as long as the quality of the preparation affords evaluation. Specimens obtained from mutagenicity tests, wet specimens of blood, urine, feces, biological fluids, do not need to be retained after quality assurance verification. In no case shall retention be required for a longer period than that set forth in paragraph (h)(3)(ii) of this section.

(iv) The master schedule sheet, copies of protocols, and records of quality assurance inspections, as required by §79.60(b)(4)(iii) shall be maintained by the quality assurance unit as an easily accessible system of records for the period of time specified in paragraph (h)(3)(ii) of this section.

(v) Summaries of training and experience and job descriptions required to be maintained by §79.60(b)(1)(ii) may be retained along with all other testing facility employment records for the length of time specified in paragraph (h)(3)(ii) of this section.

(vi) Records and reports of the maintenance and calibration and inspection of equipment, as required by §79.60(d)(2) (ii) and (iii), shall be retained for the length of time specified in paragraph (h)(3)(ii) of this section.

(vii) If a facility conducting testing or an archive contracting facility goes out of business, all raw data, documentation, and other material specified in this section shall be transferred to the sponsor of the study for archival.

(viii) Records required by this section may be retained either as original records or as true copies such as photocopies, microfilm, microfiche, or other accurate reproductions of the original records.

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§79.61 Vehicle emissions inhalation exposure guideline.

(a) Purpose. This guideline provides additional information on methodologies required to conduct health effects tests involving inhalation exposures to vehicle combustion emissions from fuels or fuel/additive mixtures. Where this guideline and the other health effects testing guidelines in 40 CFR 79.62 through 79.68 specify differing values for the same test parameter, the specifications in the individual health test guideline shall prevail for that health effect endpoint.

(b) Definitions. For the purposes of this section the following definitions apply.

Acute inhalation study means a short-term toxicity test characterized by a single exposure by inhalation over a short period of time (at least 4 hours and less than 24 hours), followed by at least 14 days of observation.

Aerodynamic diameter means the diameter of a sphere of unit density that has the same settling velocity as the particle of the test substance. It is used to compare particles of different sizes, densities and shapes, and to predict where in the respiratory tract such particles may be deposited. It applies to the size of aerosol particles.

Chronic inhalation study means a prolonged and repeated exposure by inhalation for the life span of the test animal; technically, two years in the rat.

Concentration means an exposure level. Exposure is expressed as weight or volume of test aerosol/substance per volume of air, usually mg/m3 or as parts per million (ppm) over a given time period. Micrograms per cubic meter (µg/m3) or parts per billion may be appropriate, as well.

Cumulative toxicity means the adverse effects of repeated exposures occurring as a result of prolonged action or increased concentration of the administered test substance or its metabolites in the susceptible tissues.

Inhalable diameter means that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the alveoli.

Mass median aerodynamic diameter (MMAD) means the calculated aerodynamic diameter, which divides the particles of an aerosol in half based on the mass of the particles. Fifty percent of the particles in mass will be larger than the median diameter, and fifty percent will be smaller than the median diameter. MMAD describes the particle distribution of any aerosol based on the weight and size of the particles. MMAD and the geometric standard deviation describe the particle-size distribution.

Material safety data sheet (MSDS) means documentation or information on the physical, chemical, and hazardous characteristics of a given chemical, usually provided by the product's manufacturer.

Reynolds number means a dimensionless number that is proportional to the ratio of inertial forces to frictional forces acting on a fluid. It quantitatively provides a measure of whether flow is laminar or turbulent. A fluid traveling through a pipe is fully developed into a laminar flow for a Reynolds number less than 2000, and fully developed into a turbulent flow for a Reynolds number greater than 4000.

Subacute inhalation toxicity means the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by inhalation for part (less than 10 percent) of a lifespan; generally, less than 90 days.

Subchronic inhalation study means a repeated exposure by inhalation for part (approximately 10 percent) of a life span of the exposed test animal.

Toxic effect means an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(c) Principles and design criteria of inhalation exposure systems. Proper conduct of inhalation toxicity studies of the emissions of fuels and additive/fuel mixtures requires that the exposure system be designed to ensure the controlled generation of the exposure atmosphere, the

adequate dilution of the test emissions, delivery of the diluted exposure atmosphere to the test animals, and use of appropriate exposure chamber systems selected to meet criteria for a given exposure study.

(1) Emissions generation. Emissions shall be generated according to the specifications in 40 CFR 79.57.

(2) Dilution and delivery systems. (i) The delivery system is the means used to transport the emissions from the generation system to the exposure system. The dilution system is generally a component of the delivery system.

(ii) Dilution provides control of the emissions concentration delivered to the exposure system, serving the function of diluting the associated combustion gases, such as carbon monoxide, carbon dioxide, nitrogen oxides, sulfur dioxide and other noxious gases and vapors, to levels that will ensure that there are no significant or measurable responses in the test animals as a result of exposure to the combustion gases. The formation of particle species is strongly dependent on the dilution rate, as well.

(iii) The engine exhaust system shall connect to the first-stage-dilution section at 90° to the axis of the dilution section. This is then connected to a right angle elbow on the center line of the dilution section. Engine emissions are injected through the elbow so that exhaust flow is concurrent to dilution flow.

(iv) Materials. In designing the dilution and delivery systems, the use of plastic, e.g., PVC and similar materials, copper, brass, and aluminum pipe and tubing shall be avoided if there exists a possibility of chemical reaction occurring between emissions and tubing. Stainless steel pipe and tubing is recommended as the best choice for most emission dilution and delivery applications, although glass and teflon may be appropriate, as well.

(v) Flow requirements. (A) Conduit for dilute raw emissions shall be of such dimensions as to provide residence times for the emissions on the order of less than one second to several seconds before the emissions are further diluted and introduced to the test chambers. With the high flow rates in the dilute raw emissions conduit, it will be necessary to sample various portions of the dilute emissions for delivering differing concentrations to the test chambers. The unused portions of the emissions stream are normally exhausted to the atmosphere outside of the exposure facility.

(B) Dimensions of the dilute raw exhaust conduit shall be such that, at a minimum, the flow Reynolds number is 70,000 or greater (see Mokler, et al., 1984 in paragraph (f)(13) of this section). This will maintain highly turbulent flow conditions so that there is more complete mixing of the exhaust emissions.

(C) Wall losses. The delivery system shall be designed to minimize wall losses. This can be done by sizing the tubing or pipe to maintain laminar flow of the diluted emissions to the exposure chamber. A flow Reynolds number of 1000-3000 will ensure minimal wall losses. Also, the length of and number and degree of bends in the delivery lines to the exposure chamber system shall be minimized.

(D) Whole-body exposure vs. nose-only exposure delivery systems. Flow rates through whole-body chamber systems are of the order of 100 liters per minute to 500 liters per minute. Nose-only systems are on the order of less than 50 liters per minute. To maintain laminar flow conditions, the principles described in paragraph (c)(2)(v)(C) of this section apply to both systems.

(vi) Dilution requirements. (A) To maintain the water vapor, and dissolved organic compounds, in the raw exhaust emissions stream, a manufacturer/tester will initially dilute one part emissions with a minimum of five parts clean, filtered air (see Hinners, et al., 1979 in paragraph (f)(11) of this section). Depending on the water vapor content of a particular fuel/additive mixture's combustion emissions and the humidity of the dilution air, initial exhaust dilutions as high as 1:15 or 1:20 may be necessary to maintain the general character of the exhaust as it cools, e.g., M100. At this point, it is expected that the exhaust stream would be further diluted to more appropriate levels for rodent health effects testing.

(B) A maximum concentration (minimum dilution) of the raw exhaust going into the test animal cages is anticipated to lie in the range between 1:5 and 1:50 exhaust emissions to clean, filtered air. The minimum concentration (maximum dilution) of raw exhaust for health effects testing is anticipated to be in range between 1:100 and 1:150. Individual manufacturers will treat these ranges as approximations only and will determine the optimum range of emission concentrations to elicit effects in Tier 2 health testing for their particular fuel/fuel additive mixture.

(3) Exposure chamber systems—(i) Referenced Guidelines. (A) The U.S. Department of Health and Human Services “Guide for the Care and Use of Laboratory Animals” (Guide), 1985 cited in paragraph (c)(3)(ii)(A)(4), and in paragraphs (d)(2)(i), (d)(2)(ii), (d)(2)(iii), (d)(4)(ii), and (d)(4)(iii) of this section, has been incorporated by reference.

(B) This incorporation by reference was approved by the Director of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies may be purchased from the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402. Copies may be inspected at U.S. EPA, OAR, 401 M Street SW, Washington, DC 20460 or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.

(ii) Exposure chambers. There are two basic types of dynamic inhalation exposure chambers, whole-body chambers and nose-/head-only exposure chambers (see Cheng and Moss, 1989 in paragraph (f)(8) of this section).

(A) Whole-body chambers. (1) The flow rate through a chamber shall be maintained at 15 air changes per hour.

(2) The chambers are usually maintained at a slightly negative pressure (0.5 to 1.5 inch of water) to prevent leakage of test substance into the exposure room.

(3) The exposure chamber shall be designed in such a way as to provide uniform distribution of exposure concentrations in all compartments (see Cheng et al., 1989 in paragraph (f)(7) of this section).

(4) Animals are housed in separate compartments inside the chamber, where the whole surface area of an animal is exposed to the test material. The spaces required for different animal species shall follow the Guide. In general, the volume of animal bodies occupy less than 5 percent of the chamber volume.

(B) Head/nose-only exposure chambers. (1) In head/nose-only exposure chambers, only the head (oronasal) portion of the animal is exposed to the test material.

(2) The chamber volume and flow rates are much less than in the whole-body exposure chambers because the subjects are usually restrained in a tube holder where the animal's breathing can be easily monitored. The head/nose-only exposure chamber is suitable for short-term exposures or when use of a small amount of test material is required.

(iii) Since whole-body exposure appears to be the least stressful mode of exposure, it is the preferred method. In general, head/nose only exposure, which is sometimes used to avoid concurrent exposure by the dermal or oral routes, i.e., grooming, is not recommended because of the stress accompanying the restraining of the animals. However, there may be specific instances where it may be more appropriate than whole-body exposure. The tester shall provide justification for its selection.

(d) Inhalation exposure procedures—(1) Animal selection. (i) The rat is the preferred species for vehicle emission inhalation health effects testing. Commonly used laboratory strains shall be used. Any rodent species may be used, but the tester shall provide justification for the choice of that species.

(ii) Young adult animals, approximately ten weeks of age for the rat, shall be used. At the commencement of the study, the weight variation of animals used shall not exceed ±20 percent of the mean weight for each sex. Animals shall be randomly assigned to treatment and control groups according to their weight.

(iii) An equal number of male and female rodents shall be used at each concentration level. Situations may arise where use of a single sex may be appropriate. Females, in general, shall be nulliparous and nonpregnant.

(iv) The number of animals used at each concentration level and in the control group(s) depends on the type of study, number of biological end points used in the toxicity evaluation, the pre-determined sensitivity of detection and power of significance of the study, and the animal species. For an acute study, at least five animals of each sex shall be used in each test group. For both the subacute and subchronic studies, at least 10 rodents of each sex shall be used in each test group. For a chronic study, at least 20 male and 20 female rodents shall be used in each test group.

(A) If interim sacrifices are planned, the number of animals shall be increased by the number of animals scheduled to be sacrificed during the course of the study.

(B) For a chronic study, the number of animals at the termination of the study must be adequate for a meaningful and valid statistical evaluation of chronic effects.

(v) A concurrent control group is required. This group shall be exposed to clean, filtered air under conditions identical to those used for the group exposed to the test atmosphere.

(vi) The same species/strain shall be used to make comparisons between fuel-only and fuel/additive mixture studies. If another species/strain is used, the tester shall provide justification for its selection.

(2) Animal handling and care. (i) A key element in the conduct of inhalation exposure studies is the proper handling and care of the test animal population. Therefore, the exposure conditions must conform strictly with the conditions for housing and animal care and use set forth in the Guide.

(ii) In whole-body exposure chambers, animals shall be housed in individual caging. The minimum cage size per animal will be in accordance with instructions set forth in the Guide.

(iii) Chambers shall be cleaned and maintained in accordance with recommendations and schedules set forth in the Guide.

(A) Observations shall be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of animals found dead and isolation or sacrifice of weak or moribund animals). Exposure systems using head/nose-only exposure chambers require no special daily chamber maintenance. Chambers shall be inspected to ensure that they are clean, and that there are no obstructions in the chamber which would restrict air flow to the animals. Whole-body exposure chambers will be inspected on a minimum of twice daily, once before exposures and once after exposures.

(B) Signs of toxicity shall be recorded as they are observed, including the time of onset, degree, and duration.

(C) Cage-side observations shall include, but are not limited to: changes in skin, fur, eye and mucous membranes, respiratory, autonomic, and central nervous systems, somatomotor activity, and behavioral patterns. Particular attention shall be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma.

(iv) Food and water will be withheld from animals for head/nose-only exposure systems. For whole-body-exposure systems, water only may be provided. When the exposure generation system is not operating, food will be available ad libitum. During operation of the generation system, food will be withheld to avoid possible contamination by emissions.

(v) At the end of the study period, all survivors in the main study population shall be sacrificed. Moribund animals shall be removed and sacrificed when observed.

(3) Concentration levels and selection. (i) In acute and subacute toxicity tests, at least three exposure concentrations and a control group shall be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data shall be sufficient to produce a concentration-response curve and permit an acceptable estimation of the median lethal concentration.

(ii) In subchronic and chronic toxicity tests, testers shall use at least three different concentration levels, with a control exposure group, to determine a concentration-response relationship. Concentrations shall be spaced appropriately to produce test groups with a range of toxic effects. The concentration-response data may also be sufficient to determine a NOAEL, unless the result of a limit test precludes such findings. The criteria for selecting concentration levels has been published (40 CFR 798.2450 and 798.3260).

(A) The highest concentration shall result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation of the study.

(B) The lowest concentration shall not produce toxic effects which are directly attributable to the test exposure. Where there is a useful estimation of human exposure, the lowest concentration shall exceed this.

(C) The intermediate concentration level(s) shall produce minimal observable toxic effects. If more than one intermediate concentration level is used, the concentrations shall be spaced to produce a gradation of toxic effects.

(D) In the low, intermediate, and control exposure groups, the incidence of fatalities shall be low to absent, so as not to preclude a meaningful evaluation of the results.

(4) Exposure chamber environmental conditions. The following environmental conditions in the exposure chamber are critical to the maintenance of the test animals: flow; temperature; relative humidity; lighting; and noise.

(i) Filtered and conditioned air shall be used during exposure, to dilute the exhaust emissions, and during non- exposure periods to maintain environmental conditions that are free of trace gases, dusts, and microorganisms on the test animals. Twelve to fifteen air changes per hour will be provided at all times to whole-body-exposure chambers. The minimum air flow rate for head/nose-only exposure chambers will be a function of the number of animals and the average minute volume of the animals:

$Q_{minimum}(L/min) = 2 \times \text{number of animals} \times \text{average minute volume}$

(see Cheng and Moss, 1989 in paragraph (f)(8) of this section).

(ii) Recommended ranges of temperature for various species are given in the Guide. The recommended temperature ranges will be used for establishing temperature conditions of whole-body- exposure chambers. For rodents in whole-body-exposure chambers, the recommended temperature is 22 °C ±2 °C and for rabbits, it is 20 °C ±3 °C. Temperature ranges have not been established for head/nose-only tubes; however, recommended maximum temperature limits have been established at the Inhalation Toxicology Research Institute (see Barr, 1988 in paragraph (f)(1) of this section). Maximum temperature for rats and mice in head/nose-only tubes is 23 °C.

(iii) Relative humidity. The relative humidity in the chamber air is important for heat balance and shall be maintained between 40 percent and 60 percent, but in certain instances, this may not be practicable. Testers shall follow Guide recommends for a 30 percent to 70 percent relative humidity range for rodents in exposure chambers.

(iv) Lighting. Light intensity of 30 foot candles at 3 ft. from the floor of the exposure facility is recommended (see Rao, 1986 in paragraph (f)(16) of this section).

(5) Exposure conditions. Unless precluded by the requirements of a particular test protocol, animal subjects shall be exposed to the test atmosphere based on a nominal 5-day-per-week regimen, subject to the following rules:

(i) Each daily exposure must be at least 6 hours plus the time necessary to build the chamber atmosphere to 90 percent of the target exposure atmosphere. Interruptions of daily exposures caused by technical difficulties, if infrequent in occurrence and limited in duration, may be made up the same day by adding equivalent exposure time after the technical problem has been corrected and the exposure atmosphere restored to the required level.

(ii) Normally, no more than two non-exposure days may occur consecutively during the test period. However, if a third consecutive non-exposure day should occur due to circumstances beyond the tester's control, it may be remedied by adding a supplementary exposure day. Federal and other holidays do not constitute such circumstances. Whenever possible, a make-up day should be taken at the first opportunity, i.e., on the next day which would otherwise have been an intentional non-exposure day. If a compensatory day must be scheduled at the end of the standard test period, then it may occur either:

(A) Immediately following the last standard exposure day, with no intervening non-exposure days; or

(B) With up to two intervening non-exposure days, provided that no fewer than two consecutive compensatory exposure days are completed before the test is terminated and the animals sacrificed.

(iii) Except as allowed in paragraph (d)(5)(ii)(B) of this section, in no case shall there be fewer than four exposure days per week at any time during the test period.

(iv) A nominal 90-day (13-week) subchronic test period shall include no fewer than 63 total exposure days.

(6) Exposure atmosphere. (i) The exposure atmosphere shall be held as constant as is practicable and must be monitored continuously or intermittently, depending on the method of analysis, to ensure that exposure levels are at the target values or within stated limits during the exposure period. Sampling methodology will be determined based on the type of generation system and the type of exposure chamber system specified for the exposure study.

(A) Integrated samples of test atmosphere aerosol shall be taken daily during the exposure period from a single representative sample port in the chamber near the breathing zone of the animals. Gas samples shall be taken daily to determine concentrations (ppm) of the major vapor components of the test atmosphere including CO, CO2, NOX, SO2, and total hydrocarbons.

(B) To ensure that animals in different locations of the chamber receive a similar exposure atmosphere, distribution of an aerosol or vapor concentration in exposure chambers can be determined without animals during the developmental phase of the study, or it can be determined with animals early in the study. For head/nose-only exposure chambers, it may not be possible to monitor the chamber distribution during the exposure, because the exposure port contains the animal.

(C) During the development of the emissions generation system, particle size analysis shall be performed to establish the stability of an aerosol concentration with respect to particle size. Over the course of the exposure, analysis shall be conducted as often as is necessary to determine the consistency of particle size distribution.

(D) Chamber rise and fall times. The rise time required for the exposure concentration to reach 90 percent of the stable concentration after the generator is turned on, and the fall time when the chamber concentration decreases to 10 percent of the stable concentration after the generation system is stopped shall be determined in the developmental phase of the study. Time-integrated samples collected for calculating exposure concentrations shall be taken after the rise time. The daily exposure time is exclusive of the rise or the fall time.

(ii) Instrumentation used for a given study will be determined based on the type of generation system and the type of exposure chamber system specified for the exposure study.

(A) For exhaust studies, combustion gases shall be sampled by collecting exposure air in bags and then analyzing the collected air sample to determine major components of the combustion gas using gas analyzers. Exposure chambers can also be connected to gas analyzers directly by using sampling lines and switching valves. Samples can be taken more frequently using the latter method. Aerosol instruments, such as photometers, or time-integrated gravimetric determination may be used to determine the stability of any aerosol concentration in the chamber.

(B) For evaporative emission studies, concentration of fuel vapors can usually be determined by using a gas chromatograph (GC) and/or infrared (IR) spectrometry. Grab samples for intermittent sampling can be taken from the chamber by using bubble samplers with the appropriate solvent to collect the vapors, or by collecting a small volume of air in a syringe. Intermediate or continuous monitoring of the chamber concentration is also possible by connecting the chamber with a GC or IR detector.

(7) Monitoring chamber environmental conditions may be performed by a computer system or by exposure system operating personnel.

(i) The flow-metering device used for the exposure chambers must be a continuous monitoring device, and actual flow measurements must be recorded at least every 30 minutes. Accuracy must be ±5 percent of full scale range. Measurement of air flow through the exposure chamber may be accomplished using any device that has sufficient range to accurately measure the air flow for the given chamber. Types of flow metering devices include rotameters, orifice meters, venturi meters, critical orifices, and turbinemeters (see Benedict, 1984 in paragraph (f)(4) and Spitzer, 1984 in paragraph (f)(17) of this section).

(ii) Pressure. Pressure measurement may be accomplished using manometers, electronic pressure transducers, magnehelics, or similar devices (see Gillum, 1982 in paragraph (f)(10) of this section). Accuracy of the pressure device must be ±5 percent of full scale range. Pressure measurements must be continuous and recorded at least every 30 minutes.

(iii) Temperature. The temperature of exposure chambers must be monitored continuously and recorded at least every 30 minutes. Temperature may be measured using thermometers, RTD's, thermocouples, thermistors, or other devices (see Benedict, 1984 in paragraph (f)(4) of this section). It is necessary to incorporate an alarm system into the temperature monitoring system.

The exposure operators must be notified by the alarm system when the chamber temperature exceeds 26.7 °C (80 °F). The exposure must be discontinued and emergency procedures enacted to immediately reduce temperatures or remove test animals from high temperature environment when chamber temperatures exceed 29 °C. Accuracy of the temperature monitoring device will be ±1 °C for the temperature range of 20-30 °C.

(iv) Relative humidity. The relative humidity of exposure chambers must be monitored continuously and recorded at least every 30 minutes. Relative humidity may be measured using various devices (see Chaddock, 1985 in paragraph (f)(6) of this section).

(v) Lighting shall be measured quarterly, or once at the beginning, middle, and end of the study for shorter studies.

(vi) Noise level in the exposure chamber(s) shall be measured quarterly, or once at the beginning, middle, and end of the study for shorter studies.

(vii) Oxygen content is critical, especially in nose-only chamber systems, and shall be greater than or equal to 19 percent in the test cages. An oxygen sensor shall be located at a single position in the test chamber and a lower alarm limit of 18 percent shall be used to activate an alarm system.

(8) Safety procedures and requirements. In the case of potentially explosive test substance concentrations, care shall be taken to avoid generating explosive atmospheres.

(i) It is mandatory that the upper explosive limit (UEL) and lower explosive limit (LEL) for the fuel and/or fuel additive(s) that are being tested be determined. These limits can be found in the material safety data sheets (MSDS) for each substance and in various reference texts. The air concentration of the fuel or additive-base fuel mixture in the generation system, dilution/delivery system, and the exposure chamber system shall be calculated to ensure that explosive limits are not present.

(ii) Storage, handling, and use of fuels or fuel/additive mixtures shall follow guidelines given in 29 CFR 1910.106.

(iii) Monitoring for carbon monoxide (CO) levels is mandatory for combustion systems. CO shall be continuously monitored in the immediate area of the engine/vehicle system and in the exposure chamber(s).

(iv) Air samples shall be taken quarterly in the immediate area of the vapor generation system and the exposure chamber system, or once at the beginning, middle, and end of the study for shorter studies. These samples shall be analyzed by methods described in paragraph (d)(6)(ii)(B) of this section.

(v) With the presence of fuels and/or fuel additives, all electrical and electronic equipment must be grounded. Also, the dilution/delivery system and chamber exposure system must be grounded. Guidelines for grounding are given in 29 CFR 1910.304.

(9) Quality control and quality assurance procedures—(i) Standard operating procedures (SOPs). SOPs for exposure operations, sampling instruments, animal handling, and analytical methods shall be written during the developmental phase of the study.

(ii) Technicians/operators shall be trained in exposure operation, maintenance, and documentation, as appropriate, and their training shall be documented.

(iii) Flow meters, sampling instruments, and balances used in the inhalation experiments shall be calibrated with standards during the developmental phase to determine their sensitivity, detection limits, and linearity. During the exposure period, instruments shall be checked for calibration and documented to ensure that each instrument still functions properly.

(iv) The mean exposure concentration shall be within 10 percent of the target concentration on 90 percent or more of exposure days. The coefficient of variation shall be within 25 percent of target on 90 percent or more of exposure days. For example, a manufacturer might determine a mean exposure concentration of its product's exposure emissions by identifying “marker” compound(s) typical of the emissions of the fuel or fuel/additive mixture under study as a surrogate for the total of individual compounds in those exposure emissions. The manufacturer would note any concentration changes in the level of the “marker” compound(s) in the sample's daily emissions for biological testing.

(v) The spatial variation of the chamber concentration shall be 10 percent, or less. If a higher spatial variation is observed during the developmental phase, then air mixing in the chamber shall

be increased. In any case, animals shall be rotated among the various cages in the exposure chamber(s) to insure each animal's uniform exposure during the study.

(e) Data and reporting. Data shall be summarized in tabular form, showing for each group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion.

(1) Treatment of results. All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods shall be selected during the design of the study.

(2) Evaluation of results. The findings of an inhalation toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the concentration of the test atmosphere and the duration of exposure, and the severity of abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects.

(3) Test conditions. (i) The exposure apparatus shall be described, including:

(A) The vehicle/engine design and type, the dynamometer, the cooling system, if any, the computer control system, and the dilution system for exhaust emission generation;

(B) The evaporative emissions generator model, type, or design and its dilution system; and

(C) Other test conditions, such as the source and quality of mixing air, fuel or fuel/additive mixture used, treatment of exhaust air, design of exposure chamber and the method of housing animals in a test chamber shall be described.

(ii) The equipment for measuring temperature, humidity, particulate aerosol concentrations and size distribution, gas analyzers, fuel vapor concentrations, chamber distribution, and rise and fall time shall be described.

(iii) Daily exposure results. The daily record shall document the date, the start and stop times of the exposure, number of samples taken during the day, daily concentrations determined, calibration of instruments, and problems encountered during the exposure. The daily exposure data shall be signed by the exposure operator and reviewed and signed by the exposure supervisor responsible for the study.

(4) Exposure data shall be tabulated and presented with mean values and a measure of variability (e.g., standard deviation), and shall include:

(i) Airflow rates through the inhalation equipment;

(ii) Temperature and humidity of air;

(iii) Chamber concentrations in the chamber breathing zone;

(iv) Concentration of combustion exhaust gases in the chamber breathing zone;

(v) Particle size distribution (e.g., mass median aerodynamic diameter and geometric standard deviation from the mean);

(vi) Rise and fall time;

(vii) Chamber concentrations during the non-exposure period; and

(viii) Distribution of test substance in the chamber.

(5) Animal data. Tabulation of toxic response data by species, strain, sex and exposure level for:

(i) Number of animals exposed;

(ii) Number of animals showing signs of toxicity; and

(iii) Number of animals dying.

(f) References. For additional background information on this exposure guideline, the following references should be consulted.

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[59 FR 33093, June 27, 1994, as amended at 61 FR 58746, Nov. 18, 1996; 61 FR 36512, July 11, 1996]

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§79.62 Subchronic toxicity study with specific health effect assessments.

(a) Purpose—(1) General toxicity. This subchronic inhalation study is designed to determine a concentration-response relationship for potential toxic effects in rats resulting from continuous or repeated inhalation exposure to vehicle/engine emissions over a period of 90 days. A subgroup of perfusion-fixed animals is required, in addition to the main study population, for more exacting organ and tissue histology. This test will provide screening information on target organ toxicities and on concentration levels useful for running chronic studies and establishing exposure criteria. Initial information on effective concentrations/exposures of the test atmosphere may be determined from the literature of previous studies or through concentration range-finding trials prior to starting this study. This health effects screening test is not capable of directly determining those effects which have a long latency period for development (e.g., carcinogenicity and life-shortening), though it may permit the detremination of a no-observed-adverse-effect level, or NOAEL.

(2) Specific health effects assessments (HEAs). These supplemental studies are designed to determine the potential for reproductive/teratologic, carcinogenic, mutagenic, and neurotoxic health effect outcomes from vehicle/engine emission exposures. They are done in combination with the subchronic toxicity study and paragraph (c) of this section or may be done separately as outlined by the appropriate test guideline.

(i) Fertility assessment/teratology. The fertility assessment is an in vivo study designed to provide information on potential health hazards to the fetus arising from the mother's repeated exposure to vehicle/engine emissions before and during her pregnancy. By including a mating of test animals, the study provides preliminary data on the effects of repeated vehicle/engine emissions exposure on gonadal function, conception, and fertility. The fertility assessment/teratology guideline is found in §79.63.

(ii) Micronucleus (MN) Assay. The MN assay is an in vivo cytogenetic test which gives information on potential carcinogenic and/or mutagenic effects of exposure to vehicle/engine emissions. The MN assay detects damage to the chromosomes or mitotic apparatus of cells in the tissues of a test subject exposed repeatedly to vehicle/engine emissions. The assay is based on an increase in the frequency of micronucleated erythrocytes found in bone marrow from treated animals compared to that of control animals. The guideline for the MN assay is found in §79.64.

(iii) Sister Chromatid Exchange (SCE) Assay. The SCE assay is an in vivo analysis which gives information on potential mutagenic and/or carcinogenic effects of exposure to vehicle/engine emissions. The assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. This assay uses peripheral blood lymphocytes isolated from an exposed rodent test species and grown to confluence in cell culture. The guideline for the SCE assay is found in §79.65.

(iv) Neurotoxicity (NTX) measures. NTX measures include (A) histopathology of specified central and peripheral nervous system tissues taken from emission-exposed rodents, and (B) an assay of brain tissue levels of glial fibrillary acidic protein (GFAP), a major filament protein of astrocytes, from emission-exposed rodents. The guidelines for the neurohistopathology and GFAP studies are found in §79.66 and §79.67, respectively.

(b) Definitions. For the purposes of this section, the following definitions apply:

No-observed-adverse-effect-level (NOAEL) means the maximum concentration used in a test which produces no observed adverse effects. A NOAEL is expressed in terms of weight or volume of test substance given daily per unit volume of air (µg/L or ppm).

Subchronic inhalation toxicity means the adverse effects occurring as a result of the continuous or repeated daily exposure of experimental animals to a chemical by inhalation for part (approximately 10 percent) of a life span.

(c) Principle of the test method. As long as none of the requirements of any study are violated by the combination, one or more HEAs may be combined with the general toxicity study through concurrent exposures of their study populations and/or by sharing the analysis of the same animal subjects. Requirements duplicated in combined studies need not be repeated. Guidelines for combining HEAs with the general toxicity study are as follows.

(1) Fertility assessment. (i) The number of study animals in the test population is increased when the fertility assessment is run concurrently with the 90-day toxicity study. A minimum of 40 females per test group shall undergo vaginal lavage daily for two weeks before the start of the exposure period. The resulting wet smears are examined to cull those animals which are acyclic. Twenty-five females shall be randomly assigned to a for-breeding group with the balance of females assigned to a group for histopathologic examination.

(ii) All test groups are exposed over a period of 90 days to various concentrations of the test atmosphere for a minimum of six hours per day. After seven weeks of exposures, analysis of vaginal cell smears shall resume on a daily basis for the 25 for-breeding females and shall continue for a period of four weeks or until each female in the group is confirmed pregnant. Following the ninth week of exposures, each for-breeding female is housed overnight with a single study male. Matings shall continue for as long as two weeks, or until pregnancy is confirmed (pregnancy day 0). Pregnant females are only exposed through day 15 of their pregnancy while daily exposures continue throughout the course of the study for non-pregnant females and study males.

(iii) On pregnancy day 20, pregnant females are sacrificed and their uteri are examined. Pregnancy status and fetal effects are recorded as described in §79.63. At the end of the exposure period, all males and non-pregnant females are sacrificed and necropsied. Testes and epididymal tissue samples are taken from five perfusion-fixed test subjects and histopathological examinations are carried out on the remainder of the non-pregnant females and study males.

(2) Carcinogenicity/mutagenicity(C/M) assessment. When combined with the subchronic toxicity study, the main study population is used to perform both the in vivo MN and SCE assays. Because of the constant turnover of the cells to be analyzed in these assays, a separate study population may be used for this assessment. A study population needs only to be exposed a minimum of four weeks. At exposure's end, ten animals per exposure and control groups are anaesthetized and heart punctures are performed on all members. After separating blood components, individual lymphocyte cell cultures are set up for SCE analysis. One femur from each study subject is also removed and the marrow extracted. The marrow is smeared onto a glass slide, and stained for analysis of micronuclei in erythrocytes.

(3) Neurotoxicity (NTX) measures. (i) When combined with this subchronic toxicity study, test animals designated for whole-body perfusion fixation/lung histology and exposed as part of the main animal population are used to perform the neurohistology portion of these measures. After the last exposure period, a minimum of ten animals from each exposure group shall be preserved in situ with fixative. Sections of brain, spinal cord, and proximal sciatic or tibial nerve are then cut, processed further in formalin, and mounted for viewing under a light microscope. Fibers from the sciatic or tibial nerve sample are teased apart for further analysis under the microscope.

(ii) GFAP assay. After the last exposure period, a minimum of ten rodents from each exposure group shall be sacrificed, and their brains excised and divided into regions. The tissue samples are then applied to filter paper, washed with anti-GFAP antibody, and visualized with a radio-labelled Protein A. The filters are quantified for degree of immunoreactivity between the antibody and GFAP in the tissue samples. A non-radioactive ELISA format is also referenced in the GFAP guideline cited in paragraph (a)(2)(iv) of this section. Note: Because the GFAP assay requires fresh, i.e., non-preserved, brain tissue, the number of test animals may need to be increased to provide an adequate number of test subjects to complete the histopathology requirements of both the GFAP and the general toxicity portion of the 90-day inhalation study.

(iii) The start of the exposure period for the NTX measures study population may be staggered from that of the main study group to more evenly distribute the analytical work required in both study populations. The exposures would remain the same in all other respects.

(d) Test procedures—(1) Animal selection—(i) Species and sex. The rat is the recommended species. If another rodent species is used, the tester shall provide justification for its selection. Both sexes shall be used in any assessment unless it is demonstrated that one sex is refractory to the effects of exposure.

(ii) Age and number. Rats shall be at least ten weeks of age at the beginning of the study exposure. The number of animals necessary for individual health effect outcomes is as follows:

(A) Thirty rodents per concentration level/group, fifteen of each sex, shall be used to satisfy the reporting requirements of the 90-day toxicity study. Ten animals per concentration level/group shall

be designated for whole body perfusion with fixative (by gravity) for lung studies, and neurohistology and testes studies, as appropriate.

(B) Thirty-five rodents, 25 females and ten males, shall be added for each test concentration or control group when combining a 90-day toxicity study with a fertility assessment.

(C) The tester shall provide a group of 10 animals (five animals per sex per experimental/control groups) in addition to the main test population when performing the GFAP neurotoxicity HEA.

(2) Recovery group. The manufacturer shall include a group of 20 animals (10 animals per sex) in the test population, exposing them to the highest concentration level for the entire length of the study's exposure period. This group shall then be observed for reversibility, persistence, or delayed occurrence of toxic effects during a post-exposure period of not less than 28 days.

(3) Inhalation exposure. (i) All data developed within this study shall be in accordance with good laboratory practice provisions under §79.60.

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in §79.61.

(4) Observation of animals. (i) All toxicological (e.g., weight loss) and neurological signs (e.g., motor disturbance) shall be recorded frequently enough to observe any abnormality, and not less than weekly for all study animals. Animals shall be weighed weekly.

(ii) The following is a minimal list of measures that shall be noted:

(A) Body weight;

(B) Subject's reactivity to general stimuli such as removal from the cage or handling;

(C) Description, incidence, and severity of any convulsions, tremors, or abnormal motor movements in the home cage;

(D) Descriptions and incidence of posture and gait abnormalities observed in the home cage;

(E) Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may facilitate interpretation of the data.

(iii) Any animal which dies during the test is necropsied as soon as possible after discovery.

(5) Clinical examinations. (i) The following examinations shall be performed on the twenty animals designated as the 90-day study population, exclusive of pregnant dams and those study animals targeted for perfusion by gravity:

(A) The following hematology determinations shall be carried out at least two times during the test period (after 30 days of exposure and just prior to terminal sacrifice at the end of the exposure period): hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as prothrombin time, thromboplastin time, or platelet count.

(B) Clinical biochemistry determinations on blood shall be carried out at least two times during the test period, after 30 days of exposure and just prior to terminal sacrifice at the end of the exposure period, on all groups of animals including concurrent controls. Clinical biochemical testing shall include assessment of electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. In the absence of more specific tests, the following determinations may be made: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum alanine aminotransferase, serum aspartate aminotransferase, sorbitol dehydrogenase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, methemoglobin, bile acids, total bilirubin, and total serum protein measurements. Additional clinical biochemistry shall be employed, where necessary, to extend the investigation of observed effects, e.g., analyses of lipids, hormones, acid/base balance, and cholinesterase activity.

(ii) The following examinations shall initially be performed on the high concentration and control groups only:

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to exposure to the test substance and at the termination of the study. If changes in the eyes are detected, all animals shall be examined.

(B) Urinalysis is not required on a routine basis, but shall be done when there is an indication based on expected and/or observed toxicity.

(iii) Preservation by whole-body perfusion of fixative into the anaesthetized animal for lung histology of ten animals from the 90-day study population for each experimental and control group.

(6) Gross pathology. With the exception of the whole body perfusion-fixed test animals cited in paragraph (d)(1)(ii)(A) of this section, all rodents shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices and the cranial, thoracic, and abdominal cavities and their contents. Gross pathology shall be performed on the following organs and tissues:

(i) The liver, kidneys, lungs, adrenals, brain, and gonads, including uterus, ovaries, testes, epididymides, seminal vesicles (with coagulating glands), and prostate, constitute the group of target organs for histology and shall be weighed as soon as possible after dissection to avoid drying. In addition, for other than rodent test species, the thyroid with parathyroids, when present, shall also be weighed as soon as possible after dissection to avoid drying.

(ii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; lungs—which shall be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; reproductive organs: uterus; cervix; ovaries; vagina; testes; epididymides; prostate; and, if present, seminal vesicles; aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve/tissue; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zymbal and exorbital lachrymal glands).

(7) Histopathology. Histopathology shall be performed on the following organs and tissues from all rodents:

(i) All gross lesions.

(ii) Respiratory tract and other organs and tissues, listed in paragraph (d)(6)(ii) of this section (except organs/tissues in parentheses), of all animals in the control and high dose groups.

(iii) The tissues mentioned in parentheses, listed in paragraph (d)(6)(ii) of this section, if indicated by signs of toxicity or target organ involvement.

(iv) Lungs of animals in the low and intermediate dose groups shall also be subjected to histopathological examination, primarily for evidence of infection since this provides a convenient assessment of the state of health of the animals.

(v) Lungs and trachea of the whole-body perfusion-fixed test animals cited in paragraph (d)(1)(ii)(A) of this section are examined for inhaled particle distribution.

(e) Interpretation of results. All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. The specific methods, including consideration of statistical power, shall be selected during the design of the study.

(f) Test report. In addition to the reporting requirements as specified under §§79.60 and 79.61(e), the following individual animal data information shall be reported:

(1) Date of death during the study or whether animals survived to termination.

(2) Date of observation of each abnormal sign and its subsequent course.

(3) Individual body weight data, and group average body weight data vs. time.

(4) Feed consumption data, when collected.

(5) Hematological tests employed and all results.

(6) Clinical biochemistry tests employed and all results.

(7) Necropsy findings.

(8) Type of stain/fixative and procedures used in preparing tissue samples.

(9) Detailed description of all histopathological findings.

(10) Statistical treatment of the study results, where appropriate.

(g) References. For additional background information on this test guideline, the following references should be consulted.

(1) 40 CFR 798.2450, Inhalation toxicity.

(2) 40 CFR 798.2675, Oral Toxicity with Satellite Reproduction and Fertility Study.

(3) General Statement of Work for the Conduct of Toxicity and Carcinogenicity Studies in Laboratory Animals (revised April, 1987/modifications through January, 1990) appendix G, National Toxicology Program—U.S. Dept. of Health and Human Services (Public Health Service), P.O. Box 12233, Research Triangle Park, NC 27709.

[59 FR 33093, June 27, 1994, as amended at 63 FR 63793, Nov. 17, 1998]

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§79.63 Fertility assessment/teratology.

(a) Purpose. Fertility assessment/teratology is an in vivo study designed to provide information on potential health hazards to the fetus arising from the mother's repeated inhalation exposure to vehicle/engine emissions before and during her pregnancy. By including a mating of test animals, the study provides preliminary data on the effects of repeated vehicle/engine emissions exposure on gonadal function, conception, and fertility. Since this is a one-generation test that ends with examination of full-term fetuses, but not of live pups, it is not capable of determining effects on reproductive development which would only be detected in viable offspring of treated parents.

(b) Definitions. For the purposes of this section, the following definitions apply:

Developmental toxicity means the ability of an agent to induce in utero death, structural or functional abnormalities, or growth retardation after contact with the pregnant animal.

Estrous cycle means the periodic recurrence of the biological phases of the female reproductive system which prepare the animal for conception and the development of offspring. The phases of the estrous cycle for a particular animal can be characterized by the general condition of the cells present in the vagina and the presence or absence of various cell types.

Vaginal cytology evaluation means the use of wet vaginal cell smears to determine the phase of a test animal's estrous cycle and the potential for adverse exposure effects on the regularity of the animal's cycle. In the rat, common cell types found in the smears correlate well with the various stages of the estrous cycle and to changes occurring in the reproductive tract.

(c) Principle of the test method. (1) For a two week period before exposures start, daily vaginal cell smears are examined from a surplus of female test animals to identify and cull those females which are acyclic. After culling, testers shall randomly assign at each exposure concentration (including unexposed) a minimum of twenty-five females for breeding and fifteen non-bred females for later histologic evaluation. Test animals shall be exposed by inhalation to graduated concentrations of the test atmosphere for a minimum of six hours per day over the next 13 weeks. Males and females in both test and control groups are mated after nine weeks of exposure. Exposures for pregnant females continue through gestation day 15, while exposures for males and all non-pregnant females shall continue for the full exposure period.

(2) Beginning two weeks before the start of the mating period, daily vaginal smears resume for all to-be-bred females to characterize their estrous cycles. This will continue for four weeks or until a rat's pregnancy is confirmed, i.e., day 0, by the presence of sperm in the cell smear. On pregnancy day 20, shortly before the expected date of delivery, each pregnant female is sacrificed, her uterus removed, and the contents examined for embryonic or fetal deaths, and live fetuses. At the end of the exposure period, males and all non-pregnant females shall be weighed, and various organs

and tissues, as appropriate, shall be removed and weighed, fixed with stain, and sectioned for viewing under a light microscope.

(3) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions in §79.62.

(d) Limit test. If a test at one dose level of the highest concentration that can be achieved while maintaining a particle size distribution with a mass median aerodynamic diameter (MMAD) of 4 micrometers (µm) or less, using the procedures described in section 79.60 of this part produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary. Expected human exposure though may indicate the need for a higher dose level.

(e) Test procedures—(1) Animal selection—(i) Species and strain. The rat is the preferred species. Strains with low fecundity shall not be used and the candidate species shall be characterized for its sensitivity to developmental toxins. If another rodent species is used, the tester shall provide justification for its selection.

(ii) Animals shall be a minimum of 10 weeks old at the start of the exposure period.

(iii) Number and sex. Each test and control group shall have a minimum of 25 males and 40 females. In order to ensure that sufficient pups are produced to permit meaningful evaluation of the potential developmental toxicity of the test substance, twenty pregnant test animals are required for each exposure and control level.

(2) Observation period. The observation period shall be 13 weeks, at a minimum.

(3) Concentration levels and concentration selection. (i) To select the appropriate concentration levels, a pilot or trial study may be advisable. Since pregnant animals have an increased minute ventilation as compared to non-pregnant animals, it is recommended that the trial study be conducted in pregnant animals. Similarly, since presumably the minute ventilation will vary with progression of pregnancy, the animals should be exposed during the same period of gestation as in the main study. It is not always necessary, though, to carry out a trial study in pregnant animals. Comparisons between the results of a trial study in non-pregnant animals, and the main study in pregnant animals will demonstrate whether or not the test substance is more toxic in pregnant animals. In the trial study, the concentration producing embryonic or fetal lethalities or maternal toxicity should be determined.

(ii) The highest concentration level shall induce some overt maternal toxicity such as reduced body weight or body weight gain, but not more than 10 percent maternal deaths.

(iii) The lowest concentration level shall not produce any grossly observable evidence of either maternal or developmental toxicity.

(4) Inhalation exposure. (i) All data developed within this study shall be in accordance with good laboratory practice provisions under §79.60.

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in §79.61.

(iii) Pregnant females shall be exposed to the test atmosphere on each and every day between (and including) the first and fifteenth day of gestation.

(f) Test performance—(1) Study conduct. Directions specific to this study are:

(i) The duration of exposure shall be at least six hours daily, allowing appropriate additional time for chamber equilibrium.

(ii) Where an exposure chamber is used, its design shall minimize crowding of the test animals. This is best accomplished by individual caging.

(iii) Pregnant animals shall not be subjected to beyond the minimum amount of stress. Since whole-body exposure appears to be the least stressful mode of exposure, it is the preferred method. In general oronasal or head-only exposure, which is sometimes used to avoid concurrent exposure by the dermal or oral routes, is not recommended because of the associated stress accompanying the restraining of the animals. However, there may be specific instances where it may be more appropriate than whole-body exposure. The tester shall provide justification/reasoning for its selection.

(iv) Measurements shall be made at least every other day of food consumption for all animals in the study. Males and females shall be weighed on the first day of exposure and 2-3 times per week thereafter, except for pregnant dams.

(v) The test animal housing, mating, and exposure chambers shall be operated on a twenty-four hour lighting schedule, with twelve hours of light and twelve hours of darkness. Test animal exposure shall only occur during the light portion of the cycle.

(vi) Signs of toxicity shall be recorded as they are observed including the time of onset, degree, and duration.

(vii) Females showing signs of abortion or premature delivery shall be sacrificed and subjected to a thorough macroscopic examination.

(viii) Animals that die or are euthanized because of morbidity will be necropsied promptly.

(2) Vaginal cytology. (i) For a two week period before the mating period starts, each female in the to-be-bred population shall undergo a daily saline vaginal lavage. Two wet cell smears from this lavage shall be examined daily for each subject to determine a baseline pattern of estrus. Testers shall avoid excessive handling and roughness in obtaining the vaginal cell samples, as this may induce a condition of pseudo-pregnancy in the test animals.

(ii) This will continue for four weeks or until day 0 of a rat's pregnancy is confirmed by the presence of sperm in the cell smear.

(3) Mating and fertility assessment. (i) Beginning nine weeks after the start of exposure, each exposed and control group female (exclusive of the histology group females) shall be paired during non-exposure hours with a male from the same exposure concentration group. Matings shall continue for a period of two weeks, or until all mated females are determined to be pregnant. Mating pairs shall be clearly identified.

(ii) Each morning, including weekends, cages shall be examined for the presence of a sperm plug. When found, this shall mark gestation day 0 and pregnancy shall be confirmed by the presence of sperm in the day's wet vaginal cell smears.

(iii) Two weeks after mating is begun, or as females are determined to be pregnant, bred animals are returned to pre-mating housing. Daily exposures continues through gestation day 15 for all pregnant females or through the balance of the exposure period for non-pregnant females and all males.

(iv) Those pairs which fail to mate shall be evaluated in the course of the study to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with a proven fertile partner, histological examination of the reproductive organs, and, in males, examination of the spermatogenic cycles. The stage of estrus for each non-pregnant female in the breeding group will be determined at the end of the exposure period.

(4) All animals in the histology group shall be subject to histopathologic examination at the end of the study's exposure period.

(g) Treatment of results. (1) All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. The specific methods, including consideration of statistical power, shall be selected during the design of the study.

(2) Data and reporting. In addition to the reporting requirements specified under §§79.60 and 79.61, the final test report must include the following information:

(i) Gross necropsy. (A) All animals shall be subjected to a full necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents. Special attention shall be directed to the organs of the reproductive system.

(B) The liver, kidneys, adrenals, pituitary, uterus, vagina, ovaries, testes, epididymides and seminal vesicles (with coagulating glands), and prostate shall be weighed wet, as soon as possible after dissection, to avoid drying.

(i) At the time of sacrifice on gestation day 20 or at death during the study, each dam shall be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

(ii) The contents of the uterus shall be examined for embryonic or fetal deaths and the number of viable fetuses. Gravid uterine weights need not be obtained from dead animals where decomposition has occurred. The degree of resorption shall be described in order to help estimate the relative time of death.

(iii) The number of corpora lutea shall be determined in each pregnant dam.

(iv) Each fetus shall be weighed, all weights recorded, and mean fetal weights determined.

(v) Each fetus shall be examined externally and the sex determined.

(vi) One-half of the rat fetuses in each litter shall be examined for skeletal anomalies, and the remaining half shall be examined for soft tissue anomalies, using appropriate methods.

(ii) Histopathology. (A) Histopathology on vagina, uterus, ovaries, testes, epididymides, seminal vesicles, and prostate as appropriate for all males and histology group females in the control and high concentration groups and for all animals that died or were euthanized during the study. If abnormalities or equivocal results are seen in any of these organs/tissues, the same organ/tissue from test animals in lower concentration groups shall be examined.

Note: Testes, seminal vesicles, epididymides, and ovaries, at a minimum, shall be examined in perfusion-fixed (pressure or gravity method) test subjects, when available.

(B) All gross lesions in all study animals shall be examined.

(C) As noted under mating procedures, reproductive organs of animals suspected of infertility shall be subject to microscopic examination.

(D) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for future histopathological examination: all gross lesions; vagina; uterus; ovaries; testes; epididymides; seminal vesicles; prostate; liver; and kidneys/adrenals.

(3) Evaluation of results. (i) The findings of a developmental toxicity study shall be evaluated in terms of the observed effects and the exposure levels producing effects. It is necessary to consider the historical developmental toxicity data on the species/strain tested.

(ii) There are several criteria for determining a positive result for reproductive/teratologic effects; a statistically significant dose-related decrease in the weight of the testes for treated subjects over control subjects, a decrease in neonatal viability, a significant change in the presence of soft tissue or skeletal abnormalities, or an increased rate of embryonic or fetal resorption or death. Other criteria, e.g., lengthening of the estrous cycle or the time spent in any one stage of estrus, changes in the proportion of viable male vs female fetuses or offspring, the number and type of cells in vaginal smears, or pathologic changes found during gross or microscopic examination of male or female reproductive organs may be based upon detection of a reproducible and statistically significant positive response for that evaluation parameter. A positive result indicates that, under the test conditions, the test substance does induce reproductive organ or fetal toxicity in the test species.

(iii) A test substance which does not produce either a statistically significant dose-related change in the reproductive organs or cycle or a statistically significant and reproducible positive response at any one of the test points may not induce reproductive organ toxicity in this test species, but further investigation , e.g., to establish absorption and bioavailability of the test substance, should be considered.

(h) Test report. In addition to the reporting requirements as specified under 40 CFR 79.60 and the vehicle emissions inhalation toxicity guideline as published in 40 CFR 79.61, the following specific information shall be reported:

(1) Individual animal data. (i) Time of death during the study or whether animals survived to termination.

(ii) Date of onset and duration of each abnormal sign and its subsequent course.

(iii) Feed and body weight data.

(iv) Necropsy findings.

(v) Male test subjects.

(A) Testicle weight, and body weight: testicle weight ratio.

(B) Detailed description of all histopathological findings, especially for the testes and the epididymides.

(vi) Female test subjects.

(A) Uterine weight data.

(B) Beginning and ending collection dates for vaginal cell smears.

(C) Estrous cycle length compared within and between groups including mean cycle length for groups.

(D) Percentage of time spent in each stage of cycle.

(E) Stage of estrus at time of mating/sacrifice and proportion of females in estrus between concentration groups.

(F) Detailed description of all histopathological findings, especially for uterine/ovary samples.

(vii) Pregnancy and litter data. Toxic response data by exposure level, including but not limited to, indices of fertility and time-to-mating, including the number of days until mating and the number of full or partial estrous cycles until mating.

(A) Number of pregnant animals,

(B) Number and percentage of live fetuses, resorptions.

(viii) Fetal data. (A) Numbers of each sex.

(B) Number of fetuses with any soft tissue or skeletal abnormalities.

(2) Type of stain/fixative and procedures used in preparing tissue samples.

(3) Statistical treatment of the study results.

(i) References. For additional background information on this test guideline, the following references should be consulted.

(1) 40 CFR 798.2675, Oral Toxicity with Satellite Reproduction and Fertility Study.

(2) 40 CFR 798.4350, Inhalation Developmental Toxicity Study.

(3) Chapin, R.E. and J.J. Heindel (1993) Methods in Toxicology, Vol. 3, Parts A and B: Reproductive Toxicology, Academic Press, Orlando, FL.

(4) Gray, L.E., et al. (1989) “A Dose-Response Analysis of Methoxychlor-Induced Alterations of Reproductive Development and Function in the Rat” Fund. App. Tox. 12, 92-108.

(5) Leblond, C.P. and Y. Clermont (1952) “Definition of the Stages of the Cycle of the Seminiferous Epithelium of the Rat.” Ann. N. Y. Acad. Sci. 55:548-73.

(6) Morrissey, R.E., et al. (1988) “Evaluation of Rodent Sperm, Vaginal Cytology, and Reproductive Organ Weight Data from National Toxicology Program 13-week Studies.” Fundam. Appl. Toxicol. 11:343-358.

(7) Russell, L.D., Ettlin, R.A., Sinhattikim, A.P., and Clegg, E.D (1990) Histological and Histopathological Evaluation of the Testes, Cache River Press, Clearwater, FL.

[59 FR 33093, June 27, 1994, as amended at 61 FR 36513, July 11, 1996]

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§79.64 In vivo micronucleus assay.

(a) Purpose. The micronucleus assay is an in vivo cytogenetic test which uses erythrocytes in the bone marrow of rodents to detect chemical damage to the chromosomes or mitotic apparatus of mammalian cells. As the erythroblast develops into an erythrocyte (red blood cell), its main nucleus is extruded and may leave a micronucleus in the cell body; a few micronuclei form under normal conditions in blood elements. This assay is based on an increase in the frequency of micronucleated erythrocytes found in bone marrow from treated animals compared to that of

control animals. The visualization of micronuclei is facilitated in these cells because they lack a main nucleus.

(b) Definitions. For the purposes of this section the following definitions apply:

Micronuclei mean small particles consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm.

Polychromatic erythrocyte (PCE) means an immature red blood cell that, because it contains RNA, can be differentiated by appropriate staining techniques from a normochromatic erythrocyte (NCE), which lacks RNA. In one to two days, a PCE matures into a NCE.

(c) Test method—(1) Principle of the test method. (i) Groups of rodents are exposed by the inhalation route for a minimum of 6 hours/day over a period of not less than 28 days to three or more concentrations of a test substance in air. Groups of animals are sacrificed at the end of the exposure period and femoral bone marrow is extracted. The bone marrow is then smeared onto glass slides, stained, and PCEs are scored for micronuclei. Researchers may need to run a trial at the highest tolerated concentration of the test atmosphere to optimize the sample collection time for micronucleated cells.

(ii) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions in §79.62.

(2) Species and strain. (i) The rat is the recommended test animal. Other rodent species may be used in this assay, but use of that species will be justified by the tester.

(ii) If a strain of mouse is used in this assay, the tester shall sample peripheral blood from an appropriate site on the test animal, e.g., the tail vein, as a source of normochromatic erythrocytes. Results shall be reported as outlined later in this guideline with “normochromatic” interchanged for “polychromatic”, where specified.

(3) Animal number and sex. At least five female and five male animals per experimental/sample and control group shall be used. The use of a single sex or a smaller number of animals shall be justified.

(4) Positive control group. A single concentration of a compound known to produce micronuclei in vivo is adequate as a positive control if it shows a significant response at any one time point; additional concentration levels may be used. To select an appropriate concentration level, a pilot or trial study may be advisable. Initially, one concentration of the test substance may be used, the maximum tolerated dose or that producing some indication of toxicity, e.g., a drop in the ratio of polychromatic to normochromatic erythrocytes. Intraperitoneal injection of 1,2-dimethyl-benz-anthracene or benzene are examples of positive control exposures. A concentration of 50-80 percent of an LD50 may be a suitable guide.

(d) Test performance—(1) Inhalation exposure. (i) All data developed within this study shall be in accordance with good laboratory practice provisions under §79.60.

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in §79.61.

(2) Preparation of slides and sampling times. Within twenty-four hours of the last exposure, test animals will be sacrificed. One femur from each test animal will be removed and placed in fetal bovine serum. The bone marrow is removed, cells processed, and two bone marrow smears are made for each animal on glass microscope slides. The slides are stained with acridine- orange (AO) or another appropriate stain (Giemsa + Wright's, etc.) and examined under a microscope.

(3) Analysis. Slides shall be coded for study before microscopic analysis. At least 1,000 first-division erythrocytes per animal shall be scored for the incidence of micronuclei. Sexes will be analyzed separately.

(e) Data and report—(1) Treatment of results. In addition to the reporting requirements specified under §§79.60 and 79.61, the final test report must include the criteria for scoring micronuclei. Individual data shall be presented in a tabular form including both positive and negative controls and experimental groups. The number of polychromatic erythrocytes scored, the number of micronucleated erythrocytes, the percentage of micronucleated cells, and, where applicable, the

percentage of micronucleated erythrocytes shall be listed separately for each experimental and control animal. Absolute numbers shall be included if percentages are reported.

(2) Interpretation of data. (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(3) Test evaluation. (i) Positive results in the micronucleus test provide information on the ability of a chemical to induce micronuclei in erythrocytes of the test species under the conditions of the test. This damage may have been the result of chromosomal damage or damage to the mitotic apparatus.

(ii) Negative results indicate that under the test conditions the test substance does not produce micronuclei in the bone marrow of the test species.

(f) Test report. In addition to the reporting recommendations as specified under §79.60, the following specific information shall be reported:

(1) Test atmosphere concentration(s) used and rationale for concentration selection.

(2) Rationale for and description of treatment and sampling schedules, toxicity data, negative and positive controls.

(3) Historical control data (negative and positive), if available.

(4) Details of the protocol used for slide preparation.

(5) Criteria for identifying micronucleated erythrocytes.

(6) Micronucleus analysis by animal and by group for each concentration (sexes analyzed separately).

(i) Ratio of polychromatic to normochromatic erythrocytes.

(ii) Number of polychromatic erythrocytes with micronuclei.

(iii) Number of polychromatic erythrocytes scored.

(7) Statistical methodology chosen for test analysis.

(g) References. For additional background information on this test guideline, the following references should be consulted.

(1) 40 CFR 798.5395, In Vivo, Mammalian Bone Marrow Cytogenetics Tests: Micronucleus Assay.

(2) Cihak, R. “Evaluation of Benzidine by the Micronucleus Test.” Mutation Research, 67: 383-384 (1979).

(3) Evans, H.J. “Cytological Methods for Detecting Chemical Mutagens.” Chemical Mutagens: Principles and Methods for Their Detection, Vol. 4. Ed. A. Hollaender (New York and London: Plenum Press, 1976) pp. 1-29.

(4) Heddle, J.A., et al. “The Induction of Micronuclei as a Measure of Genotoxicity. A Report of the U.S. Environmental Protection Agency Gene-Tox Program.” Mutation Research, 123:61-118 (1983).

(5) Preston, J.R. et al. “Mammalian In Vivo and In Vitro Cytogenetics Assays: Report of the Gene-Tox Program.” Mutation Research, 87:143-188 (1981).

(6) Schmid, W. “The micronucleus test for cytogenetic analysis”, Chemical Mutagens, Principles and Methods for their Detection. Vol. 4 Hollaender A, (Ed. A ed. (New York and London: Plenum Press, (1976) pp. 31-53.

(7) Tice, R.E., and Al Pellom “User's guide: Micronucleus assay data management and analysis system”, NTIS Order no. PB-90-212-598AS.

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§79.65 In vivo sister chromatid exchange assay.

(a) Purpose. The in vivo sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. The most commonly used assays employ mammalian bone marrow cells or peripheral blood lymphocytes, often from rodent species.

(b) Definitions. For the purposes of this section, the following definitions apply:

C-metaphase means a state of arrested cell growth typically seen after treatment with a spindle inhibitor, i.e., colchicine.

Sister chromatid exchange means a reciprocal interchange of the two chromatid arms within a single chromosome. This exchange is visualized during the metaphase portion of the cell cycle and presumably requires the enzymatic incision, translocation and ligation of at least two DNA helices.

(c) Test method—(1) Principle of the test method. (i) Groups of rodents are exposed by the inhalation route for a minimum of 6 hours/day over a period of not less than 28 days to three or more concentrations of a test substance in air. Groups of animals are sacrificed at the end of the exposure period and blood lymphocyte cell cultures are prepared from study animals. Cell growth is suspended after a time and cells are harvested, fixed and stained before scoring for SCEs. Researchers may need to run a trial at the highest tolerated concentration of the test atmosphere to optimize the sample collection time for second division metaphase cells.

(ii) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions in §79.62.

(2) Description. (i) The method described here employs peripheral blood lymphocytes (PBL) of laboratory rodents exposed to the test atmosphere.

(ii) Within twenty-four hours of the last exposure, test animal lymphocytes are obtained by heart puncture and duplicate cell cultures are started for each animal. Cultures are grown in bromo-deoxyuridine (BrdU), and then a spindle inhibitor (e.g., colchicine) is added to arrest cell growth. Cells are harvested, fixed, and stained and their chromosomes are scored for SCEs.

(3) Species and strain. The rat is the recommended test animal. Other rodent species may be used in this assay, but use of that species will be justified by the tester.

(4) Animal number and sex. At least five female and five male animals per experimental and control group shall be used. The use of a single sex or different number of animals shall be justified.

(5) Positive control group. A single concentration of a compound known to produce SCEs in vivo is adequate as a positive control if it shows a significant response at any one time point; additional concentration levels may be used. To select an appropriate concentration level, a pilot or trial study may be advisable. Initially, one concentration of the test substance may be used, the maximum tolerated dose or that producing some indication of toxicity as evidenced by animal morbidity (including death) or target cell toxicity. Intraperitoneal injection of 1,2-dimethyl-benz-anthracene or benzene are examples of positive control exposures. A concentration of 50-80 percent of an LD50 would also be a suitable guide.

(6) Inhalation exposure. (i) All data developed within this study shall be in accordance with good laboratory practice provisions under §79.60.

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in §79.61.

(d) Test performance—(1) Treatment. At the conclusion of the exposure period, all test animals are anaesthetized and heart punctures are performed. Lymphocytes are isolated over a Ficoll gradient and replicate cell cultures are started for each animal. After some 21 hours, the cells are treated with BrdU and returned to incubation. The following day, a spindle inhibitor (e.g., colchicine) is added to arrest cell growth in c-metaphase. Cells are harvested 4 hours later and second-division metaphase cells are washed and fixed in methanol:acetic acid, stained, and chromosome preparations are scored for SCEs.

(2) Staining method. Staining of slides to reveal SCEs can be performed according to any of several protocols. However, the fluorescence plus Giemsa method is recommended.

(3) Number of cells scored. (i) A minimum of 25 well-stained, second-division metaphase cells shall be scored for each animal for each cell type.

(ii) At least 100 consecutive metaphase cells shall be scored for the number of first, second, and third division metaphases for each animal for each cell type.

(iii) At least 1000 consecutive PBL's shall be scored for the number of metaphase cells present.

(iv) The number of cells to be analyzed per animal shall be based upon the number of animals used, the negative control frequency, the pre-determined sensitivity and the power chosen for the test. Slides shall be coded before microscopic analysis.

(e) Data and report—(1) Treatment of results. In addition to the reporting requirements specified under §§79.60 and 61, data shall be presented in tabular form, providing scores for both the number of SCE for each metaphase. Differences among animals within each group shall be considered before making comparisons between treated and control groups.

(2) Statistical evaluation. Data shall be evaluated by appropriate statistical methods.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of SCE. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of SCE or a statistically significant and reproducible positive response at any one of the test concentrations is considered not to induce rearrangements of DNA segments in this system.

(iii) Both biological and statistical significance shall be considered together in the evaluation.

(4) Test evaluation. (i) A positive result in the in vivo SCE assay for either, or both, the lung or lymphocyte cultures indicates that under the test conditions the test substance induces reciprocal interchanges of DNA in duplicating chromosomes from lung or lymphocyte cells of the test species.

(ii) Negative results indicate that under the test conditions the test substance does not induce reciprocal interchanges in lung or lymphocyte cells of the test species.

(5) Test report. In addition to the reporting recommendations as specified under §§79.60 and 79.61, the following specific information shall be reported:

(i) Test concentrations used, rationale for concentration selection, negative and positive controls;

(ii) Toxic response data by concentration;

(iii) Schedule of administration of test atmosphere, BrdU, and spindle inhibitor;

(iv) Time of harvest after administration of BrdU;

(v) Identity of spindle inhibitor, its concentration and timing of treatment;

(vi) Details of the protocol used for cell culture and slide preparation;

(vii) Criteria for scoring SCE;

(viii) Replicative index, i.e., [percent 1st division + (2 × percent 2nd division) + (3 × percent 3rd division) metaphases]/100; and

(ix) Mitotic activity, i.e., # of metaphases/1000 cells.

(f) References. For additional background information on this test guideline, the following references should be consulted.

(1) 40 CFR 798.5915, In vivo Sister Chromatid Exchange Assay.

(2) Kato, H. “Spontaneous Sister Chromatid Exchanges Detected by a BudR-Labeling Method.” Nature, 251:70-72 (1974).

(4) Kligerman, A. D., et al. “Sister Chromatid Exchange Analysis in Lung and Peripheral Blood Lymphocytes of Mice Exposed to Methyl Isocyanate by Inhalation.” Environmental Mutagenesis 9:29-36 (1987).

(5) Kligerman, A.D., et al., “Cytogenetic Studies of Rodents Exposed to Styrene by Inhalation”, IARC Monographs no. 127 “Butadiene and Styrene: Assesment of Health Hazards” (Sorsa, et al., eds), pp 217-224, 1993.

(6) Kligerman, A., et al., “Cytogenetic Studies of Mice Exposed to Styrene by Inhalation.”, Mutation Research, 280:35-43, 1992.

(7) Wolff, S., and P. Perry. “Differential Giemsa Staining of Sister Chromatids and the Study of Sister Chromatid Exchanges Without Autoradiography.” Chromosoma 48: 341-53 (1974).

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§79.66 Neuropathology assessment.

(a) Purpose. (1) The histopathological and biochemical techniques in this guideline are designed to develop data in animals on morphologic changes in the nervous system associated with repeated inhalation exposures to motor vehicle emissions. These tests are not intended to provide a detailed evaluation of neurotoxicity. Neuropathological evaluation should be complemented by other neurotoxicity studies, e.g. behavioral and neurophysiological studies and/or general toxicity testing, to more completely assess the neurotoxic potential of an exposure.

(2) [Reserved]

(b) Definition. Neurotoxicity (NTX) or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical substance.

(c) Principle of the test method. (1) Laboratory rodents are exposed to one of several concentration levels of a test atmosphere for at least six hours daily over a period of 90 days. At the end of the exposure period, the animals are anaesthetized, perfused in situ with fixative, and tissues in the nervous system are examined grossly and prepared for microscopic examination. Starting with the highest dosage level, tissues are examined under the light microscope for morphologic changes, until a no-observed-adverse-effect level is determined. In cases where light microscopy has revealed neuropathology, the NOAEL may be confirmed by electron microscopy.

(2) The tests described herein may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination. Specifically, this assay may be combined with a subchronic toxicity study, pursuant to provisions in §79.62.

(d) Limit test. If a test at one dose level of the highest concentration that can be achieved while maintaining a particle size distribution with a mass median aerodynamic diameter (MMAD) of 4 micrometers (µm) or less, using the procedures described in paragraph (a) of this section, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary. Expected human exposure though may indicate the need for a higher dose level.

(e) Test procedures—(1) Animal selection—(i) Species and strain. Testing shall be performed in the species being used in other NTX tests. A standard strain of laboratory rat is recommended. The choice of species shall take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, the potential for combined studies, and the availability of other toxicity data for the species.

(ii) Age. Animals shall be at least ten weeks of age at the start of exposure.

(iii) Sex. Both sexes shall be used unless it is demonstrated that one sex is refractory to the effects of exposure.

(2) Number of Animals. A minimum of ten animals per group shall be used. The tissues from each animal shall be examined separately.

(3) Control Groups. (i) A concurrent control group, exposed to clean, filtered air only, is required.

(ii) The laboratory performing the testing shall provide positive control data, e.g., results from repeated acrylamide exposure, as evidence of the ability of their histology procedures to detect neurotoxic endpoints. Positive control data shall be collected at the time of the test study unless the laboratory can demonstrate the adequacy of historical data for the planned study.

(iii) A satellite group of 10 female and 10 male test subjects shall be treated with the highest concentration level for the duration of the exposure and observed thereafter for reversibility, persistence, or delayed occurrence of toxic effects during a post-treatment period of not less than 28 days.

(4) Inhalation exposure. (i) All data developed within this study shall be in accordance with good laboratory practice provisions under §79.60.

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in §79.61.

(5) Study conduct—(i) Observation of animals. All toxicological (e.g., weight loss) and neurological signs (e.g., motor disturbance) shall be recorded frequently enough to observe any abnormality, and not less than weekly.

(ii) The following is a minimal list of measures that shall be noted:

(A) Body weight;

(B) Subject's reactivity to general stimuli such as removal from the cage or handling;

(C) Description, incidence, and severity of any convulsions, tremors, or abnormal motor movements in the home cage;

(D) Descriptions and incidence of posture and gait abnormalities observed in the home cage; and

(E) Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may facilitate interpretation of the data.

(iii) Sacrifice of animals—(A) General. The goal of the techniques outlined for sacrifice of animals and preparation of tissues is preservation of tissue morphology to simulate the living state of the cell.

(B) Perfusion technique. Animals shall be perfused in situ by a generally recognized technique. For fixation suitable for light or electronic microscopy, saline solution followed by buffered 2.5 percent glutaraldehyde or buffered 4.0 percent paraformaldehyde, is recommended. While some minor modifications or variations in procedures are used in different laboratories, a detailed and standard procedure for vascular perfusion may be found in the text by Zeman and Innes (1963), Hayat (1970), and Spencer and Schaumburg (1980) under paragraph (g) of this section. A more sophisticated technique is described by Palay and Chan-Palay (1974) under paragraph (g) of this section. In addition, the lungs shall be instilled with fixative via the trachea during the fixation process in order to preserve the lungs and achieve whole-body fixation.

(C) Removal of brain and cord. After perfusion, the bony structure (cranium and vertebral column) shall be exposed. Animals shall then be stored in fixative-filled bags at 4 °C for 8-12 hours. The cranium and vertebral column shall be removed carefully by trained technicians without physical damage of the brain and cord. Detailed dissection procedures may be found in the text by Palay and Chan-Palay (1974) under paragraph (g) of this section. After removal, simple measurement of the size (length and width) and weight of the whole brain (cerebrum, cerebellum, pons-medulla) shall be made. Any abnormal coloration or discoloration of the brain and cord shall also be noted and recorded.

(D) Sampling. Cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum, the midbrain, the cerebellum, and the medulla oblongata; the spinal cord at the cervical swelling (C3-C6), and proximal sciatic nerve (mid-thigh and sciatic notch) or tibial nerve (at knee). Other sites and tissue elements (e.g., gastrocnemius muscle) shall be examined if deemed necessary. Any observable gross changes shall be recorded.

(iv) Specimen storage. Tissue samples from both the central and peripheral nervous system shall be further immersion fixed and stored in appropriate fixative (e.g., 10 percent buffered formalin for light microscopy; 2.5 percent buffered gluteraldehyde or 4.0 percent buffered paraformaldehyde for electron microscopy) for future examination. The volume of fixative versus the volume of tissues in

a specimen jar shall be no less than 25:1. All stored tissues shall be washed with buffer for at least 2 hours prior to further tissue processing.

(v) Histopathology examination—(A) Fixation. Tissue specimens stored in 10 percent buffered formalin may be used for this purpose. All tissues must be immersion fixed in fixative for at least 48 hours prior to further tissue processing.

(B) Dehydration. All tissue specimens shall be washed for at least 1 hour with water or buffer, prior to dehydration. (A longer washing time is needed if the specimens have been stored in fixative for a prolonged period of time.) Dehydration can be performed with increasing concentration of graded ethanols up to absolute alcohol.

(C) Clearing and embedding. After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast. Multiple tissue specimens (e.g. brain, cord, ganglia) may be embedded together in one single block for sectioning. All tissue blocks shall be labelled showing at least the experiment number, animal number, and specimens embedded.

(D) Sectioning. Tissue sections, 5 to 6 microns in thickness, shall be prepared from the tissue blocks and mounted on standard glass slides. It is recommended that several additional sections be made from each block at this time for possible future needs for special stainings. All tissue blocks and slides shall be filed and stored in properly labeled files or boxes.

(E) Histopathological techniques. The following general testing sequence is proposed for gathering histopathological data:

(1) General staining. A general staining procedure shall be performed on all tissue specimens in the highest treatment group. Hematoxylin and eosin (H&E) shall be used for this purpose. The staining shall be differentiated properly to achieve bluish nuclei with pinkish background.

(2) Peripheral nerve teasing. Peripheral nerve fiber teasing shall be used. Detailed staining methodology is available in standard histotechnological manuals such as AFIP (1968), Ralis et al. (1973), and Chang (1979) under paragraph (g) of this section. The nerve fiber teasing technique is discussed in Spencer and Schaumberg (1980) under paragraph (g) of this section. A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If a lesion(s) is observed, the special techniques shall be repeated in the next lower treatment group until no further lesion is detectable.

(F) Examination. All stained microscopic slides shall be examined with a standard research microscope. Examples of cellular alterations (e.g., neuronal vacuolation, degeneration, and necrosis) and tissue changes (e.g., gliosis, leukocytic infiltration, and cystic formation) shall be recorded and photographed.

(f) Data collection, reporting, and evaluation. In addition to information meeting the requirements stated under 40 CFR 79.60 and 79.61, the following specific information shall be reported:

(1) Description of test system and test methods. (i) A description of the general design of the experiment shall be provided. This shall include a short justification explaining any decisions where professional judgment is involved such as fixation technique and choice of stains; and

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. Historical data may be used if all essential aspects of the experimental protocol are the same.

(2) Results. All observations shall be recorded and arranged by test groups. This data may be presented in the following recommended format:

(i) Description of signs and lesions for each animal. For each animal, data must be submitted showing its identification (animal number, treatment, dose, duration), neurologic signs, location(s) nature of, frequency, and severity of lesion(s). A commonly-used scale such as 1 + , 2 + , 3 + , and 4 + for degree of severity ranging from very slight to extensive may be used. Any diagnoses derived from neurologic signs and lesions including naturally occurring diseases or conditions, shall also be recorded;

(ii) Counts and incidence of lesions, by test group. Data shall be tabulated to show:

(A) The number of animals used in each group, the number of animals displaying specific neurologic signs, and the number of animals in which any lesion was found; and

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, and the frequency of each different type and/or location of lesion.

(iii) Evaluation of data. (A) An evaluation of the data based on gross necropsy findings and microscopic pathology observations shall be made and supplied. The evaluation shall include the relationship, if any, between the animal's exposure to the test atmosphere and the frequency and severity of any lesions observed; and

(B) The evaluation of dose-response, if existent, for various groups shall be given, and a description of statistical method must be presented. The evaluation of neuropathology data shall include, where applicable, an assessment in conjunction with any other neurotoxicity studies, electrophysiological, behavioral, or neurochemical, which may be relevant to this study.

(g) References. For additional background information on this test guideline, the following references should be consulted.

(1) 40 CFR 798.6400, Neuropathology.

(2) AFIP Manual of Histologic Staining Methods. (New York: McGraw-Hill (1968).

(3) Chang, L.W. A Color Atlas and Manual for Applied Histochemistry. (Springfield, IL: Charles C. Thomas, 1979).

(4) Dunnick, J.K., et.al. Thirteen-week Toxicity Study of N-Hexane in B6C3F1 Mice After Inhalation Exposure (1989) Toxicology, 57, 163-172.

(5) Hayat, M.A. “Vol. 1. Biological applications,” Principles and techniques of electron microscopy. (New York: Van Nostrand Reinhold, 1970).

(6) Palay S.L., Chan-Palay, V. Cerebellar Cortex: Cytology and Organization. (New York: Springer-Verlag, 1974).

(7) Ralis, H.M., Beesley, R.A., Ralis, Z.A. Techniques in Neurohistology. (London: Butterworths, 1973).

(8) Sette, W. “Pesticide Assessment Guidelines, Subdivision F, Neurotoxicity Test Guidelines.” Report No. 540/09-91-123 U.S. Environmental Protection Agency 1991 (NTIS # PB91-154617).

(9) Spencer, P.S., Schaumburg, H.H. (eds). Experimental and Clinical Neurotoxicology. (Baltimore: Williams and Wilkins, 1980).

(10) Zeman, W., Innes, J.R.M. Craigie's Neuroanatomy of the Rat. (New York: Academic, 1963).

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§79.67 Glial fibrillary acidic protein assay.

(a) Purpose. Chemical-induced injury of the nervous system, i.e., the brain, is associated with astrocytic hypertrophy at the site of damage (see O'Callaghan, 1988 in paragraph (e)(3) in this section). Assays of glial fibrillary acidic protein (GFAP), the major intermediate filament protein of astrocytes, can be used to document this response. To date, a diverse variety of chemical insults known to be injurious to the central nervous system have been shown to increase GFAP. Moreover, increases in GFAP can be seen at concentrations below those necessary to produce cytopathology as determined by routine Nissl stains (standard neuropathology). Thus it appears that assays of GFAP represent a sensitive approach for documenting the existence and location of chemical-induced injury of the central nervous system. Additional functional, histopathological, and biochemical tests are necessary to assess completely the neurotoxic potential of any chemical. This biochemical test is intended to be used in conjunction with neurohistopathological studies.

(b) Principle of the test method. (1) This guideline describes the conduct of a radioimmunoassay for measurement of the amount of GFAP in the brain of vehicle emission-exposed and unexposed control animals. It is based on modifications (O'Callaghan & Miller 1985 in paragraph (e)(5), O'Callaghan 1987 in paragraph (e)(1) of this section) of the dot-immunobinding procedure described by Jahn et al. (1984) in paragraph (e)(2) of this section. Briefly, brain tissue samples from study animals are assayed for total protein, diluted in dot-immunobinding buffer, and applied to nitrocellulose sheets. The spotted sheets are then fixed, blocked, washed and incubated in anti-

GFAP antibody and [I125] Protein A. Bound protein A is then quantified by gamma spectrometry. In lieu of purified protein standards, standard curves are constructed from dilution of a single control sample. By comparing the immunoreactivity of individual samples (both control and exposed groups) with that of the sample used to generate the standard curve, the relative immunoreactivity of each sample is obtained. The immunoreactivity of the control groups is normalized to 100 percent and all data are expressed as a percentage of control. A variation on this radioimmunoassay procedure has been proposed (O'Callaghan 1991 in paragraph (e)(4) of this section) which uses a “sandwich” of GFAP, anti-GFAP, and a chromophore in a microtiter plate format enzyme-link immunosorbent assay (ELISA). The use of this variation shall be justified.

(2) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions of §79.62.

(c) Test procedure—(1) Animal selection—(i) Species and strain. Test shall be performed on the species being used in concurrent testing for neurotoxic or other health effect endpoints. This will generally be a species of laboratory rat. The use of other rodent or non-rodent species shall be justified.

(ii) Age. Based on other concurrent testing, young adult rats shall be used. Study rodents shall not be older than ten weeks at the start of exposures.

(iii) Number of animals. A minimum of ten animals per group shall be used. The tissues from each animal shall be examined separately.

(iv) Sex. Both sexes shall be used unless it is demonstrated that one sex is refractory to the effects.

(2) Materials. The materials necessary to perform this study are [I125] Protein A (2-10 µCi/µg), Anti-sera to GFAP, nitrocellulose paper (0.1 or 0.2 µm pore size), sample application template (optional; e.g., “Minifold II”, Schleicher & Schuell, Keene, NH), plastic sheet incubation trays.

(3) Study conduct. (i) All data developed within this study shall be in accordance with good laboratory practice provisions under §79.60.

(ii) Tissue Preparation. Animals are euthanized 24 hours after the last exposure and the brain is excised from the skull. On a cold dissecting platform, the following six regions are dissected freehand: cerebellum; cerebral cortex; hippocampus; striatum; thalamus/hypothalamus; and the rest of the brain. Each region is then weighed and homogenized in 10 volumes of hot (70-90 °C) 1 percent (w/v) sodium dodecyl sulfate (SDS). Homogenization is best achieved through sonic disruption. A motor driven pestle inserted into a tissue grinding vessel is a suitable alternative. The homogenized samples can then be stored frozen at −70 °C for at least 4 years without loss of GFAP content.

(iii) Total Protein Assay. Aliquots of the tissue samples are assayed for total protein using the method of Smith et al. (1985) in paragraph (e)(7) of this section. This assay may be purchased in kit form (e.g., Pierce Chemical Company, Rockford, IL).

(iv) Sample Preparation. Dilute tissue samples in sample buffer (120 mM KCl, 20 mM NaCl, 2 mM MgCl2), 5 mM Hepes, pH 7.4, 0.7 percent Triton X-100) to a final concentration of 0.25 mg total protein per ml (5 µg/20 µl).

(v) Preparation of Standard Curve. Dilute a single control sample in sample buffer to give at least five standards, between 1 and 10 µg total protein per 20 µl. The suggested values of total protein per 20 µl sample buffer are 1.25, 2.50, 3.25, 5.0, 6.25, 7.5, 8.75, and 10.0 µg.

(vi) Preparation of Nitrocellulose Sheets. Nitrocellulose sheets of 0.1 or 0.2 micron pore size are rinsed by immersion in distilled water for 5 minutes and then air dried.

(vii) Sample Application. Samples can be spotted onto the nitrocellulose sheets free-hand or with the aid of a template. For free-hand application, draw a grid of squares approximately 2 centimeters by 2 centimeters (cm) on the nitrocellulose sheets using a soft pencil. Spot 5-10 µl portions to the center of each square for a total sample volume of 20 µl. For template aided sample application a washerless microliter capacity sample application manifold is used. Position the nitrocellulose sheet in the sample application device as recommended by the manufacturer and spot a 20 µl sample in one application. Do not wet the nitrocellulose or any support elements prior to sample application. Do not apply vacuum during or after sample application. After spotting samples (using either method), let the sheets air dry. The sheets can be stored at room temperature for several days after sample application.

(viii) Standard Incubation Conditions. These conditions have been described by Jahn et al. (1984) in paragraph (e)(2) of this section. All steps are carried out at room temperature on a flat shaking platform (one complete excursion every 2-3 seconds). For best results, do not use rocking or orbital shakers. Perform the following steps in enough solution to cover the nitrocellulose sheets to a depth of 1 cm.

(A) Incubate 20 minutes in fixer (25 percent (v/v) isopropanol, 10 percent (v/v) acetic acid).

(B) Discard fixer, wash several times in deionized water to eliminate the fixer, and then incubate for 5 minutes in Tris-buffered saline (TBS): 200 mM NaCL, 60 mM Tris-HCl to pH 7.4.

(C) Discard TBS and incubate 1 hour in blocking solution (0.5 percent gelatin (w/v)) in TBS.

(D) Discard blocking solution and incubate for 2 hours in antibody solution (anti-GFAP antiserum diluted to the desired dilution in blocking solution containing 0.1 percent Triton X-100). Serum anti-bovine GFAP, which cross reacts with GFAP from rodents and humans, can be obtained commercially (e.g., Dako Corp.) and used at a dilution of 1:500.

(E) Discard antibody solution, and wash in 4 changes of TBS for 5 minutes each time. Then wash in TBS for 10 minutes.

(F) Discard TBS and incubate in blocking solution for 30 minutes.

(G) Discard blocking solution and incubate for 1 hour in Protein A solution ([I125]-labeled Protein A diluted in blocking solution containing 0.1 percent Triton X-100, sufficient to produce 2000 counts per minute (cpm) per 10 µl of Protein A solution).

(H) Remove Protein A solution (it may be reused once). Wash in 0.1 percent Triton X-100 in TBS (TBSTX) for 5 minutes, 4 times. Then wash in TBSTX for 2-3 hours for 4 additional times. An overnight wash in a larger volume can be used to replace the last 4 washes.

(I) Hang sheets to air-dry. Cut out squares or spots and count radioactivity in a gamma counter.

(ix) Expression of data. Compare radioactivity counts for samples obtained from control and treated animals with counts obtained from the standard curve. By comparing the immunoreactivity (counts) of each sample with that of the standard curve, the relative amount of GFAP in each sample can be determined and expressed as a percent of control.

(d) Data Reporting and Evaluation—(1) Test Report. In addition to information meeting the requirements stated under 40 CFR 79.60, the following specific information shall be reported:

(i) Body weight and brain region weights at time of sacrifice for each subject tested;

(ii) Indication of whether each subject survived to sacrifice or time of death;

(iii) Data from control animals and blank samples; and

(iv) Statistical evaluation of results;

(2) Evaluation of Results. (i) Results shall be evaluated in terms of the extent of change in the amount of GFAP as a function of treatment and dose. GFAP assays (of any brain region) from a minimum of 6 samples typically will result in a standard error of the mean of ±5 percent. In this case, a chemically-induced increase in GFAP of 115 percent of control is likely to be statistically significant.

(ii) The results of this assay shall be compared to and evaluated with any relevant behavioral and histopathological data.

(e) References. For additional background information on this test guideline the following references should be consulted.

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§79.68 Salmonella typhimurium reverse mutation assay.

(a) Purpose. The Salmonella typhimurium histidine (his) reversion system is a microbial assay which measures his− → his + reversion induced by chemicals which cause base changes or frameshift mutations in the genome of the microorganism Salmonella typhimurium.

(b) Definitions. For the purposes of this section, the following definitions apply:

Base pair mutagen means an agent which causes a base change in DNA. In a reversion assay, this change may occur at the site of the original mutation or at a second site in the chromosome.

Frameshift mutagen is an agent which causes the addition or deletion of single or multiple base pairs in the DNA molecule.

Salmonella typhimurium reverse mutation assay detects mutation in a gene of a histidine-requiring strain to produce a histidine independent strain of this organism.

(c) Reference substances. These may include, but need not be limited to, sodium azide, 2-nitrofluorene, 9-aminoacridine, 2-aminoanthracene, congo red, benzopurpurin 4B, trypan blue or direct blue 1.

(d) Test method—(1) Principle. Motor vehicle combustion emissions from fuel or additive/base fuel mixtures are, first, filtered to trap particulate matter and, then, passed through a sorbent resin to trap semi-volatile gases. Bacteria are separately exposed to the extract from both the filtered particulates and the resin-trapped organics. Assays are conducted using both test mixtures with and without a metabolic activation system and exposed cells are plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted in test cultures and compared to the number of spontaneous revertants in unexposed control cultures.

(2) Description. Several methods for performing the test have been described. The procedures described here are for the direct plate incorporation method and the azo-reduction method. Among those used are:

(i) Direct plate incorporation method;

(ii) Preincubation method;

(iii) Azo-reduction method;

(iv) Microsuspension method; and

(v) Spiral assay.

(3) Strain selection—(i) Designation. Five tester strains shall be used in the assay. At the present time, TA1535, TA1537, TA98, and TA100 are designated as tester strains. The fifth strain will be chosen from the pool of Salmonella strains commonly used to determine the degree to which nitrated organic compounds, i.e., nitroarenes, contribute to the overall mutagenic activity of a test substance. TA98/1,8-DNP6 or other suitable Rosenkranz nitro-reductase resistant strains will be

considered acceptable. The choice of the particular strain is left to the discretion of the researcher. However, the researcher shall justify the use of the selected bacterial tester strains.

(ii) Preparation and storage of bacterial tester strains. Recognized methods of stock culture preparation and storage shall be used. The requirement of histidine for growth shall be demonstrated for each strain. Other phenotypic characteristics shall be checked using such methods as crystal violet sensitivity and resistance to ampicillin. Spontaneous reversion frequency shall be in the range expected as reported in the literature and as established in the laboratory by historical control values.

(iii) Bacterial growth. Fresh cultures of bacteria shall be grown up to the late exponential or early stationary phase of growth (approximately 108-109 cells per ml).

(4) Exogenous metabolic activation. Bacteria shall be exposed to the test substance both in the presence and absence of an appropriate exogenous metabolic activation system. For the direct plate incorporation method, the most commonly used system is a cofactor-supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme-inducing agents, such as Aroclor 1254. For the azo-reduction method, a cofactor- supplemented postmitochondrial fraction (S-9) prepared from the livers of untreated hamsters is preferred. For this method, the cofactor supplement shall contain flavin mononucleotide, exogenous glucose 6-phosphate dehydrogenase, NADH and excess of glucose-6-phosphate.

(5) Control groups—(i) Concurrent controls. Concurrent positive and negative (untreated) controls shall be included in each experiment. Positive controls shall ensure both strain responsiveness and efficacy of the metabolic activation system.

(ii) Strain specific positive controls shall be included in the assay. Examples of strain specific positive controls are as follows:

(A) Strain TA1535, TA100: sodium azide;

(B) TA98: 2-nitrofluorene (without activation), 2-anthramine (with activation);

(C) TA1537: 9-aminoacridine; and

(D) TA98/1,8-DNP6: benzo(a)pyrene (with activation).

The papers by Claxton et al., 1991 and 1992 in paragraph (g) in this section will provide helpful information for the selection of positive controls.

(iii) Positive controls to ensure the efficacy of the activation system. The positive control reference substances for tests including a metabolic activation system shall be selected on the basis of the type of activation system used in the test. 2-Aminoanthracene is an example of a positive control compound in plate-incorporation tests using postmitochondrial fractions from the livers of rodents treated with enzyme-inducing agents such as Aroclor-1254. Congo red is an example of a positive control compound in the azo-reduction method. Other positive control reference substances may be used.

(iv) Class-specific positive controls. The azo-reduction method shall include positive controls from the same class of compounds as the test agent wherever possible.

(6) Sampling the test atmosphere. (i) Extracts of test emissions are collected on Teflon®-coated glass fiber filters using an exhaust dilution setup. The particulates are extracted with dichloromethane (DCM) using Soxhlet extraction techniques. Extracts in DCM can be stored at dry ice temperatures until use.

(ii) Gaseous hydrocarbons passing through the filter are trapped by a porous, polymer resin, like XAD-2/styrene-divinylbenzene, or an equivalent product. Methylene chloride is used to extract the resin and the sample is evaporated to dryness before storage or use.

(iii) Samples taken from this material are then used to expose the cells in this assay. Final concentration of extracts in solvent/vehicle, or after solvent exchange, shall not interfere with cell viability or growth rate. The paper by Stump (1982) in paragraph (g) of this section is useful for preparing extracts of particulate and semi-volatile organic compounds from diesel and gasoline exhaust stream.

(iv) Exposure concentrations. (A) The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test substance concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may

be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble samples shall be tested up to the limits of solubility. The upper test chemical concentration shall be determined on a case by case basis.

(B) Generally, a maximum of 5 mg/plate for pure substances is considered acceptable. At least 5 different concentrations of test substance shall be used with adequate intervals between test points.

(C) When appropriate, a single positive response shall be confirmed by testing over a narrow range of concentrations.

(e) Test performance. All data developed within this study shall be in accordance with good laboratory practice provisions under §79.60.

(1) Direct plate incorporation method. When testing with metabolic activation, test solution, bacteria, and 0.5 ml of activation mixture containing an adequate amount of postmitochondrial fraction shall be added to the liquid overlay agar and mixed. This mixture is poured over the surface of a selective agar plate. Overlay agar shall be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate shall be counted. When testing without metabolic activation, the test sample and 0.1 ml of a fresh bacterial culture shall be added to 2.0 ml of overlay agar.

(2) Azo-reduction method. When testing with metabolic activation, 0.5 ml of activation mixture containing 150 µl of postmitochondrial fraction and 0.1 ml of bacterial culture shall be added to a test tube kept on ice. 0.1 ml of test solution shall be added, and the tubes shall be incubated with shaking at 30 °C for 30 minutes. At the end of the incubation period, 2.0 ml of agar shall be added to each tube, the contents mixed and poured over the surface of a selective agar plate. Overlay agar shall be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate shall be counted. For tests without metabolic activation, 0.5 ml of buffer shall be used in place of the 0.5 ml of activation mixture. All other procedures shall be the same as those used for the test with metabolic activation.

(3) Other methods/modifications may also be appropriate.

(4) Media. An appropriate selective medium with an adequate overlay agar shall be used.

(5) Incubation conditions. All plates within a given experiment shall be incubated for the same time period. This incubation period shall be for 48-72 hours at 37 °C.

(6) Number of cultures. All plating shall be done at least in triplicate.

(f) Data and report—(1) Treatment of results. Data shall be presented as number of revertant colonies per plate, revertants per kilogram (or liter) of fuel, and as revertants per kilometer (or mile, or brake-horsepower/hour, as appropriate) for each replicate and dose. These same measures shall be recorded on both the negative and positive control plates. The mean number of revertant colonies per plate, revertants per kilogram (or liter) of fuel, and revertants per kilometer (or mile, or brake-horsepower/hour), as well as individual plate counts and standard deviations shall be presented for the test substance, positive control, and negative control plates.

(2) Statistical evaluation. Data shall be evaluated by appropriate statistical methods. Those methods shall include, at a minimum, means and standard deviations of the reversion data.

(3) Interpretation of results. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of revertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of revertants or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance shall be considered together in the evaluation.

(4) Test evaluation. (i) Positive results from the Salmonella typhimurium reverse mutation assay indicate that, under the test conditions, the test substance induces point mutations by base changes or frameshifts in the genome of this organism.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in Salmonella typhimurium.

(5) Test report. In addition to the reporting recommendations as specified under 40 CFR 79.60, the following specific information shall be reported:

(i) Sampling method(s) used and manner in which cells are exposed to sample solution;

(ii) Bacterial strains used;

(iii) Metabolic activation system used (source, amount and cofactor); details of preparation of postmitochondrial fraction;

(iv) Concentration levels and rationale for selection of concentration range;

(v) Description of positive and negative controls, and concentrations used, if appropriate;

(vi) Individual plate counts, mean number of revertant colonies per plate, number of revertants per kilometer (or mile, or brake-horsepower/hour), and standard deviation; and

(vii) Dose-response relationship, if applicable.

(g) References. For additional background information on this test guideline, the following references should be consulted.

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(14) Prival, M.J., and Mitchell, V.D. “Analysis of a method for testing azo dyes for mutagenic activity in Salmonella typhimurium in the presence of flavin mononucleotide and hamster liver S-9,” Mutation Research 97:103-116 (1982).

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