
This content is from the eCFR and is authoritative but unofficial.

Title 40 —Protection of Environment
Chapter I —Environmental Protection Agency
Subchapter C —Air Programs

Part 79 Registration of Fuels and Fuel Additives

Subpart A General Provisions

- § 79.1 Applicability.
- § 79.2 Definitions.
- § 79.3 Availability of information.
- § 79.4 Requirement of registration.
- § 79.5 Periodic reporting requirements.
- § 79.6 Requirement for testing.
- § 79.7 Samples for test purposes.
- § 79.8 Penalties.

Subpart B Fuel Registration Procedures

- § 79.10 Application for registration by fuel manufacturer.
- § 79.11 Information and assurances to be provided by the fuel manufacturer.
- § 79.12 Determination of noncompliance.
- § 79.13 Registration.
- § 79.14 Termination of registration of fuels.

Subpart C Additive Registration Procedures

- § 79.20 Application for registration by additive manufacturer.
- § 79.21 Information and assurances to be provided by the additive manufacturer.
- § 79.22 Determination of noncompliance.
- § 79.23 Registration.
- § 79.24 Termination of registration of additives.

Subpart D Designation of Fuels and Additives

- § 79.30 Scope.
- § 79.31 Additives.
- § 79.32 Motor vehicle gasoline.
- § 79.33 Motor vehicle diesel fuel.

Subpart E [Reserved]

Subpart F Testing Requirements for Registration

- § 79.50 Definitions.
- § 79.51 General requirements and provisions.
- § 79.52 Tier 1.
- § 79.53 Tier 2.

- § 79.54 Tier 3.
- § 79.55 Base fuel specifications.
- § 79.56 Fuel and fuel additive grouping system.
- § 79.57 Emission generation.
- § 79.58 Special provisions.
- § 79.59 Reporting requirements.
- § 79.60 Good laboratory practices (GLP) standards for inhalation exposure health effects testing.
- § 79.61 Vehicle emissions inhalation exposure guideline.
- § 79.62 Subchronic toxicity study with specific health effect assessments.
- § 79.63 Fertility assessment/teratology.
- § 79.64 *In vivo* micronucleus assay.
- § 79.65 *In vivo* sister chromatid exchange assay.
- § 79.66 Neuropathology assessment.
- § 79.67 Glial fibrillary acidic protein assay.
- § 79.68 *Salmonella typhimurium* reverse mutation assay.

PART 79—REGISTRATION OF FUELS AND FUEL ADDITIVES

Authority: 42 U.S.C. 7414, 7524, 7545 and 7601.

Source: 40 FR 52011, Nov. 7, 1975, unless otherwise noted.

Subpart A—General Provisions

§ 79.1 Applicability.

The regulations of this part apply to the registration of fuels and fuel additives designated by the Administrator, pursuant to section 211 of the Clean Air Act (42 U.S.C. 1857f-6c, as amended by section 9, Pub. L. 91-604).

§ 79.2 Definitions.

As used in this part, all terms not defined herein shall have the meaning given them in the Act:

- (a) **Act** means the Clean Air Act (42 U.S.C. 1857 *et seq.*, as amended by Pub. L. 91-604).
- (b) **Administrator** means the Administrator of the Environmental Protection Agency.
- (c) **Fuel** means any material which is capable of releasing energy or power by combustion or other chemical or physical reaction.
- (d) **Fuel manufacturer** means any person who, for sale or introduction into commerce, produces, manufactures, or imports a fuel or causes or directs the alteration of the chemical composition of a bulk fuel, or the mixture of chemical compounds in a bulk fuel, by adding to it an additive, except:

- (1) A party (other than a fuel refiner or importer) who adds a quantity of additive(s) amounting to less than 1.0 percent by volume of the resultant additive(s)/fuel mixture is not thereby considered a fuel manufacturer.
- (2) A party (other than a fuel refiner or importer) who adds an oxygenate compound to fuel in any otherwise allowable amount is not thereby considered a fuel manufacturer.
- (e) **Additive** means any substance, other than one composed solely of carbon and/or hydrogen, that is intentionally added to a fuel named in the designation (including any added to a motor vehicle's fuel system) and that is not intentionally removed prior to sale or use.
- (f) **Additive manufacturer** means any person who produces, manufactures, or imports an additive for use as an additive and/or sells or imports for sale such additive under the person's own name.
- (g) **Range of concentration** means the highest concentration, the lowest concentration, and the average concentration of an additive in a fuel.
- (h) **Chemical composition** means the name and percentage by weight of each compound in an additive and the name and percentage by weight of each element in an additive.
- (i) **Chemical structure** means the molecular structure of a compound in an additive.
- (j) **Impurity** means any chemical element present in an additive that is not included in the chemical formula or identified in the breakdown by element in the chemical composition of such additive.
- (k) **Oxygenate compound** means an oxygen-containing, ashless organic compound, such as an alcohol or ether, which may be used as a fuel or fuel additive.

[40 FR 52011, Nov. 7, 1975, as amended at 59 FR 33092, June 27, 1994; 62 FR 12571, Mar. 17, 1997]

§ 79.3 Availability of information.

The availability to the public of information provided to, or otherwise obtained by, the Administrator under this part shall be governed by part 2 of this chapter except as expressly noted in subpart F of this part.

[59 FR 33092, June 27, 1994]

§ 79.4 Requirement of registration.

- (a) **Fuels.**
 - (1) No manufacturer of any fuel designated under this part shall, after the date prescribed for such fuel in this part, sell, offer for sale, or introduce into commerce such fuel unless the Administrator has registered such fuel.
 - (2) No manufacturer of a registered fuel shall add or direct the addition to it of an additive which he has not previously reported unless he has notified the Administrator of such intended use, including the expected or estimated range of concentration. If necessary to meet an unforeseen production problem, however, a fuel manufacturer may use an additive that he has not previously reported provided that
 - (i) the additive is on the current list of registered additives and

- (ii) the fuel manufacturer notifies the Administrator within 30 days regarding such unforeseen use and his plans regarding continued use, including the expected or estimated range of concentration.
- (3) Any designated fuel that is
 - (i) in a research, development, or test status;
 - (ii) sold to automobile, engine, or component manufacturers for research, development, or test purposes; or
 - (iii) sold to automobile manufacturers for factory fill, and is not in any case offered for commercial sale to the public, shall be exempt from registration.
- (4) A domestic fuel manufacturer may purchase and offer for commercial sale foreign-produced fuel containing unidentified additives provided that within 30 days of his offer for sale he notifies the Administrator of the purchase, the source of purchase, the quantity purchased, and summarized results of any tests performed to determine the acceptability of the purchased fuel to the fuel manufacturer.

(b) **Additives.**

- (1) No manufacturer of any fuel additive designated under this part shall, after the date by which the additive must be registered under this part, sell, offer for sale, or introduce into commerce such additive for use in any type of fuel designated under this part unless the Administrator has registered that additive for use in that type of fuel.
- (2) Any designated additive that is either
 - (i) in a research, development, or test status or
 - (ii) sold to petroleum, automobile, engine, or component manufacturers for research, development, or test purposes, and in either case is not offered for commercial sale to the public, shall be exempt from registration.
- (3) Process chemicals used by refineries during the refinery process are exempted from the requirement for registration.
- (4) If an additive manufacturer prepares for sale only to fuel manufacturers
 - (i) a blend or mixture of two or more registered additives or
 - (ii) a blend or mixture of one or more registered additives with one or more substances containing only carbon and/or hydrogen, he will not be required to register such blend or mixture provided he will, upon request, furnish the Administrator with the names and percentages by weight of all components of such blend or mixture.

[40 FR 52011, Nov. 7, 1975, as amended at 41 FR 21324, May 25, 1976; 59 FR 33092, June 27, 1994]

§ 79.5 Periodic reporting requirements.

(a) **Fuel manufacturers.**

- (1) For each calendar year (January 1 through December 31) commencing after the date prescribed for any fuel in subpart D of this part, fuel manufacturers must submit to the Administrator a report for each registered fuel showing the range of concentration of each additive reported under § 79.11(a) and the volume of such fuel produced in the year. Reports must be submitted by March 31 for the preceding year, or part thereof, on forms supplied by the Administrator. If the date prescribed for a particular fuel in subpart D of this part, or the later registration of a fuel is between October 1 and December 31, no report will be required for the period to the end of that year.

TABLE 1 TO § 79.5—QUARTERLY REPORTING DEADLINES

Calendar quarter	Time period covered	Quartely report deadline
Quarter 1	January 1-March 31	June 1.
Quarter 2	April 1-June 30	September 1.
Quarter 3	July 1-September 30	December 1.
Quarter 4	October 1-December 31	March 31.

- (2) Fuel manufacturers shall submit to the Administrator a report annually for each registered fuel providing additional data and information as specified in §§ 79.32(c) and (d) and 79.33(c) and (d) in the designation of the fuel in subpart D of this part. Reports shall be submitted by March 31 for the preceding year, or part thereof, on forms supplied by the Administrator upon request. If the date prescribed for a particular fuel in subpart D of this part, or the later registration of a fuel is between October 1 and December 31, no report will be required for the period to the end of that year.
- (b) **Additive manufacturers.** Additive manufacturers shall submit to the Administrator a report annually for each registered additive providing additional data and information as specified in § 79.31(c) and (d) in the designation of the additive in subpart D of this part. Additive manufacturers shall also report annually the volume of each additive produced. Reports shall be submitted by March 31 for the preceding year, or part thereof, on forms supplied by the Administrator upon request. If the date prescribed for a particular additive in subpart D of this part, or the later registration of an additive is between October 1 and December 31, no report will be required for the period to the end of that year. These periodic reports shall not, however, be required for any additive that is:
- (1) An additive registered under another name,
 - (2) A blend or mixture of two or more registered additives, or
 - (3) A blend or mixture of one or more registered additives with one or more substances containing only carbon and/or hydrogen.

[40 FR 52011, Nov. 7, 1975, as amended at 79 FR 23630, Apr. 28, 2014; 85 FR 78463, Dec. 4, 2020]

§ 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. The civil monetary penalty amount listed in this section may not reflect recent inflation adjustments EPA is required to make. The current maximum and minimum statutory civil penalty amounts are located in § 19.4.

[58 FR 65554, Dec. 15, 1993, as amended at 89 FR 88655, Nov. 8, 2024]

Subpart B—Fuel Registration Procedures

§ 79.10 Application for registration by fuel manufacturer.

Any manufacturer of a designated fuel who wishes to register that fuel shall submit an application for registration including all of the information set forth in § 79.11. If the manufacturer produces more than one grade or brand of a designated fuel, a manufacturer may include more than one grade or brand in a single application, provided that the application includes all information required for registration of each such grade or brand by this part. Each application shall be signed by the fuel manufacturer and shall be submitted on such forms as the Administrator will supply on request.

[59 FR 33092, June 27, 1994]

§ 79.11 Information and assurances to be provided by the fuel manufacturer.

Each application for registration submitted by the manufacturer of a designated fuel shall include the following:

- (a) The commercial identifying name of each additive that will or may be used in a designated fuel subsequent to the date prescribed for such fuel in subpart D;
- (b) The name of the additive manufacturer of each additive named;
- (c) The range of concentration of each additive named, as follows:
 - (1) In the case of an additive which has been or is being used in the designated fuel, the range during any 3-month or longer period prior to the date of submission;
 - (2) In the case of an additive which has not been used in the designated fuel, the expected or estimated range;
- (d) The purpose-in-use of each additive named;

- (e) The description (or identification, in the case of a generally accepted method) of a suitable analytical technique (if one is known) that can be used to detect the presence of each named additive in the designated fuel and/or to measure its concentration therein;
- (f) Such other data and information as are specified in the designation of the fuel in subpart D;
- (g) Assurances that the fuel manufacturer will notify the Administrator in writing and within a reasonable time of any change in:
 - (1) The name of any additive previously reported;
 - (2) The name of the manufacturer of any additive being used;
 - (3) The purpose-in-use of any additive;
 - (4) Information submitted pursuant to paragraph (e) of this section;
- (h) Assurances that the fuel manufacturer will not represent, directly or indirectly, in any notice, circular, letter, or other written communication, or any written, oral, or pictorial notice or other announcement in any publication or by radio or television, that registration of the fuel constitutes endorsement, certification, or approval by any agency of the United States;
- (i) The manufacturer of any fuel which will be sold, offered for sale, or introduced into commerce for use in motor vehicles manufactured after model year 1974 shall demonstrate that the fuel is substantially similar to any fuel utilized in the certification of any 1975 or subsequent model year vehicle or engine, or that the manufacturer has obtained a waiver under 42 U.S.C. 7545(f)(4); and
- (j) The manufacturer shall submit, or shall reference prior submissions, including all of the test data and other information required prior to registration of the fuel by the provisions of subpart F of this part.

[40 FR 52011, Nov. 7, 1975, as amended at 59 FR 33092, June 27, 1994]

§ 79.12 Determination of noncompliance.

If the Administrator determines that an applicant for registration of a designated fuel has failed to submit all of the information required by § 79.11, or determines within the applicable period provided for Agency review that the applicant has not satisfactorily completed any testing which is required prior to registration of the fuel by any provision of subpart F of this part, he shall return the application to the manufacturer, along with an explanation of all deficiencies in the required information.

[59 FR 33093, June 27, 1994]

§ 79.13 Registration.

- (a) If the Administrator determines that a manufacturer has submitted an application for registration of a designated fuel which includes all of the information and assurances required by § 79.11 and has satisfactorily completed all of the testing required by subpart F of this part, the Administrator shall promptly register the fuel and notify the fuel manufacturer of such registration.
- (b) The Administrator shall maintain a list of registered fuels, which shall be available to the public upon request.

[40 FR 52011, Nov. 7, 1975, as amended at 41 FR 21324, May 25, 1976; 59 FR 33093, June 27, 1994]

§ 79.14 Termination of registration of fuels.

Registration may be terminated by the Administrator if the fuel manufacturer requests such termination in writing.

Subpart C—Additive Registration Procedures

§ 79.20 Application for registration by additive manufacturer.

Any manufacturer of a designated fuel additive who wishes to register that additive shall submit an application for registration including all of the information set forth in § 79.21. Each application shall be signed by the fuel additive manufacturer and shall be submitted on such forms as the Administrator will supply on request.

[59 FR 33093, June 27, 1994]

§ 79.21 Information and assurances to be provided by the additive manufacturer.

Each application for registration submitted by the manufacturer of a designated fuel additive shall include the following:

- (a) The chemical composition of the additive with the methods of analysis identified, except that
 - (1) If the chemical composition is not known, full disclosure of the chemical process of manufacture will be accepted in lieu thereof;
 - (2) In the case of an additive for engine oil, only the name, percentage by weight, and method of analysis of each element in the additive are required provided, however, that a percentage figure combining the percentages of carbon, hydrogen, and/or oxygen may be provided unless the breakdown into percentages for these individual elements is already known to the registrant.
 - (3) In the case of a purchased component, only the name, manufacturer, and percent by weight of such purchased component are required if the manufacturer of the component will, upon request, furnish the Administrator with the chemical composition thereof.
- (b) The chemical structure of each compound in the additive if such structure is known and is not adequately specified by the name given under “chemical composition.” Nominal identification is adequate if mixed isomers are present.
- (c) The description (or identification, in the case of a generally accepted method) of a suitable analytical technique (if one is known) that can be used to detect the presence of the additive in any fuel named in the designation and/or to measure its concentration therein.
- (d) The specific types of fuels designated under § 79.32 for which the fuel additive will be sold, offered for sale, or introduced into commerce, and the fuel additive manufacturer's recommended range of concentration and purpose-in-use for each such type of fuel.
- (e) Such other data and information as are specified in the designation of the additive in subpart D.
- (f) Assurances that any change in information submitted pursuant to:
 - (1) Paragraphs (a), (b), (c), (d), and (j) of this section will be provided to the Administrator in writing within 30 days of such change; and
 - (2) Paragraph (e) of this section as provided in § 79.5(b).

(g)

- (1) Assurances that the additive manufacturer will not represent, directly or indirectly, in any notice, circular, letter, or other written communication or any written, oral, or pictorial notice or other announcement in any publication or by radio or television, that registration of the additive constitutes endorsement, certification, or approval by any agency of the United States, except as specified in paragraph (g)(2) of this section.
 - (2) In the case of an additive that has its purpose-in-use identified as a deposit control additive for use in gasoline pursuant to the requirements of paragraph (d) of this section, the additive manufacturer may publicly represent that the additive meets the EPA's gasoline deposit control requirements, provided that the additive manufacturer is in compliance with the requirements of 40 CFR 1090.260.
- (h) The manufacturer of any fuel additive which will be sold, offered for sale, or introduced into commerce for use in any type of fuel intended for use in motor vehicles manufactured after model year 1974 shall demonstrate that the fuel additive, when used at the recommended range of concentration, is substantially similar to any fuel additive included in a fuel utilized in the certification of any 1975 or subsequent model year vehicle or engine, or that the manufacturer has obtained a waiver under 42 U.S.C. 7545(f)(4).
- (i) The manufacturer shall submit, or shall reference prior submissions, including all of the test data and other information required prior to registration of the fuel additive by the provisions of subpart F of this part.
- (j) If the purpose-in-use of the additive identified pursuant to the requirements of paragraph (d) of this section is a deposit control additive for use in gasoline, the manufacturer must submit the following in addition to the other information specified in this section:
- (1) The lowest additive concentration (LAC) that is compliant with the gasoline deposit control requirements of 40 CFR 1090.260.
 - (2) The deposit control test method in 40 CFR 1090.1395 that the additive is compliant with.
 - (3) A complete listing of the additive's components and the weight or volume percent (as applicable) of each component.
 - (i) **Nomenclature.** When possible, standard chemical nomenclature must be used or the chemical structure of the component must be given. Polymeric components may be reported as the product of other chemical reactants, provided that the supporting data specified in paragraph (j)(3) of this section is also reported.
 - (ii) **Designation.** Each detergent-active component of the package must be classified into one of the following designations:
 - (A) Polyalkyl amine.
 - (B) Polyether amine.
 - (C) Polyalkylsuccinimide.
 - (D) Polyalkylaminophenol.
 - (E) Detergent-active petroleum-based carrier oil.
 - (F) Detergent-active synthetic carrier oil.

(G) Other detergent-active component (identify category, if feasible).

(iii) *Composition variability.*

(A) The composition of a detergent additive reported in a single additive registration (and the detergent additive product sold under a single additive registration) may not include the following:

(1) Detergent-active components that differ in identity from those contained in the detergent additive package at the time of deposit control testing.

(2) A range of concentrations for any detergent-active component such that, if the component were present in the detergent additive package at the lower bound of the reported range, the deposit control effectiveness of the additive package would be reduced as compared with the level of effectiveness demonstrated pursuant to the requirements of 40 CFR 1090.260. Subject to the foregoing constraint, a gasoline detergent additive sold under a particular additive registration may contain a higher concentration of the detergent-active component(s) than the concentration(s) of such component(s) reported in the registration for the additive.

(B) The identity or concentration of non-detergent-active components of the detergent additive package may vary under a single registration provided that such variability does not reduce the deposit control effectiveness of the additive package as compared with the level of effectiveness demonstrated pursuant to the requirements of 40 CFR 1090.260.

(C) Unless the additive manufacturer provides EPA with data to substantiate that a carrier oil does not act to enhance the detergent additive's ability to control deposits, any carrier oil contained in the detergent additive, whether petroleum-based or synthetic, must be treated as a detergent-active component in accordance with the requirements in paragraph (j)(3)(ii) of this section.

(D) Except as provided in paragraph (j)(3)(iii)(E) of this section, detergent additive packages that do not satisfy the requirements in paragraphs (j)(3)(iii)(A) through (C) must be separately registered. EPA may disqualify an additive for use in satisfying the requirements of this subpart if EPA determines that the variability included within a given detergent additive registration may reduce the deposit control effectiveness of the detergent package such that it may invalidate the lowest additive concentration reported in accordance with the requirements of paragraph (j)(1) of this section and 40 CFR 1090.260.

(E) A change in minimum concentration requirements resulting from a modification of detergent additive composition does not require a new detergent additive registration or a change in existing registration if the modification is affected by a detergent blender pursuant to the requirements of 40 CFR 1090.1240.

(4) For detergent-active polymers and detergent-active carrier oils that are reported as the product of other chemical reactants:

- (i) Identification of the reactant materials and the manufacturer's acceptance criteria for determining that these materials are suitable for use in synthesizing detergent components. The manufacturer must maintain documentation, and submit it to EPA upon request, demonstrating that the acceptance criteria reported to EPA are the same criteria which the manufacturer specifies to the suppliers of the reactant materials.
 - (ii) A Gel Permeation Chromatograph (GPC), providing the molecular weight distribution of the polymer or detergent-active carrier oil components and the concentration of each chromatographic peak representing more than one percent of the total mass. For these results to be acceptable, the GPC test procedure must include equipment calibration with a polystyrene standard or other readily attainable and generally accepted calibration standard. The identity of the calibration standard must be provided, together with the GPC characterization of the standard.
- (5) For non-detergent-active carrier oils, the following parameters:
 - (i) T10, T50, and T90 distillation points, and end boiling point, measured according to applicable test procedures cited in 40 CFR 1090.1350.
 - (ii) API gravity and viscosity.
 - (iii) Concentration of oxygen, sulfur, and nitrogen, if greater than or equal to 0.5 percent (by weight) of the carrier oil.
- (6) Description of an FTIR-based method appropriate for identifying the detergent additive package and its detergent-active components (polymers, carrier oils, and others) both qualitatively and quantitatively, together with the actual infrared spectra of the detergent additive package and each detergent-active component obtained by this test method. The FTIR infrared spectra submitted in connection with the registration of a detergent additive package must reflect the results of a test conducted on a sample of the additive containing the detergent-active component(s) at a concentration no lower than the concentration(s) (or the lower bound of a range of concentration) reported in the registration pursuant to paragraph (j)(1) of this section.
- (7) Specific physical parameters must be identified which the manufacturer considers adequate and appropriate, in combination with other information in this section, for identifying the detergent additive package and monitoring its production quality control.
 - (i) Such parameters must include (but need not be limited to) viscosity, density, and basic nitrogen content, unless the additive manufacturer specifically requests, and EPA approves, the substitution of other parameter(s) which the manufacturer considers to be more appropriate for a particular additive package. The request must be made in writing and must include an explanation of how the requested physical parameter(s) are helpful as indicator(s) of detergent production quality control. EPA will respond to such requests in writing; the additional parameters are not approved until the manufacturer receives EPA's written approval.
 - (ii) The manufacturer must identify a standardized measurement method, consistent with the chemical and physical nature of the detergent product, which will be used to measure each parameter. The documented ASTM repeatability for the method must also be cited. The manufacturer's target value for each parameter in the additive, and the expected range of production values for each parameter, must be specified.

- (iii) The expected range of variability must differ from the target value by an amount no greater than five times the standard repeatability of the test procedure, or by no more than 10 percent of the target value, whichever is less. However, in the case of nitrogen analysis or other procedures for measuring concentrations of specific chemical compounds or elements, when the target value is less than 10 parts per million, a range of variability up to 50 percent of the target value will be considered acceptable.
- (iv) If a manufacturer wishes to rely on measurement methods or production variability ranges which do not conform to the above limitations, then the manufacturer must receive prior written approval from EPA. A request for such allowance must be made in writing. It must fully justify the adequacy of the test procedure, explain why a broader range of variability is required, and provide evidence that the production detergent will perform adequately throughout the requested range of variability pursuant to the requirements of 40 CFR 1090.1395.

[40 FR 52011, Nov. 7, 1975, as amended at 41 FR 21324, May 25, 1976; 59 FR 33093, June 27, 1994; 85 FR 78463, Dec. 4, 2020]

§ 79.22 Determination of noncompliance.

If the Administrator determines that an applicant for registration of a designated fuel additive has failed to submit all of the information required by § 79.21, or determines within the applicable period provided for Agency review that the applicant has not satisfactorily completed any testing which is required prior to registration of the fuel additive by any provision of subpart F of this part, he shall return the application to the manufacturer, along with an explanation of all deficiencies in the required information.

[59 FR 33093, June 27, 1994]

§ 79.23 Registration.

- (a) If the Administrator determines that a manufacturer has submitted an application for registration of a designated fuel additive which includes all of the information and assurances required by § 79.21 and has satisfactorily completed all of the testing required by subpart F of this part, the Administrator shall promptly register the fuel additive and notify the fuel manufacturer of such registration.
- (b) The Administrator shall maintain a list of registered additives, which shall be available to the public upon request.

[40 FR 52011, Nov. 7, 1975, as amended at 41 FR 21324, May 25, 1976; 59 FR 33093, June 27, 1994]

§ 79.24 Termination of registration of additives.

- (a) Registration may be terminated by the Administrator if the additive manufacturer requests such termination in writing.
- (b) Registration for an additive that has its purpose-in-use identified as a deposit control additive for use in gasoline pursuant to the requirements of § 79.21(d) may be terminated by the Administrator if the EPA determines that the detergent additive is not compliant with the gasoline deposit control requirements of 40 CFR 1090.260.

[85 FR 78465, Dec. 4, 2020]

Subpart D—Designation of Fuels and Additives

§ 79.30 Scope.

Fuels and additives designated and dates prescribed by the Administrator for the registration of such fuels and additives, pursuant to section 211 of the Act, are listed in this subpart. In addition, specific informational requirements under §§ 79.11(f) and 79.21(e) are set forth for each designated fuel or additive. Additional fuels and/or additives may be designated and pertinent dates and additional specific informational requirements prescribed as the Administrator deems advisable.

§ 79.31 Additives.

- (a) All additives produced or sold for use in motor vehicle gasoline and/or motor vehicle diesel fuel are hereby designated. The Act defines the term “motor vehicle” to mean any self-propelled vehicle designed for transporting persons or property on a street or highway. For purposes of this registration, however, additives specifically manufactured and marketed for use in motorcycle fuels are excluded.
- (b) All designated additives must be registered by July 7, 1976.
- (c) In accordance with §§ 79.5(b) and 79.21(e), and to the extent such information is known to the additive manufacturer as a result of testing conducted for reasons other than additive registration or reporting purposes, the additive manufacturer shall furnish the highest, lowest, and average values of the impurities in each designated additive, if greater than 0.1 percent by weight. The methods of analysis in making the determinations shall also be given.
- (d) In accordance with §§ 79.5(b) and 79.21(e), and to the extent such information is known to the additive manufacturer, he shall furnish summaries of any information developed by or specifically for him concerning the following items:
 - (1) Mechanisms of action of the additive;
 - (2) Reactions between the additive and the fuels listed in paragraph (a) of this section;
 - (3) Identification and measurement of the emission products of the additive when used in the fuels listed in paragraph (a) of this section;
 - (4) Effects of the additive on all emissions;
 - (5) Toxicity and any other public health or welfare effects of the emission products of the additive;
 - (6) Effects of the emission products of the additive on the performance of emission control devices/systems. Such submissions shall be accompanied by a description of the test procedures used in obtaining the information. Information will be considered to be known to the additive manufacturer if a report thereon has been prepared and circulated or distributed outside the research department or division.

(Secs. 211, 301(a), Clean Air Act as amended (40 U.S.C. 7545, 7601(a)))

[40 FR 52011, Nov. 7, 1975, as amended at 41 FR 21324, May 25, 1976; 43 FR 28490, June 30, 1978; 59 FR 33093, June 27, 1994]

§ 79.32 Motor vehicle gasoline.

- (a) The following fuels commonly or commercially known or sold as motor vehicle gasoline are hereby individually designated:

- (1) Motor vehicle gasoline, unleaded—motor vehicle gasoline that contains no more than 0.05 gram of lead per gallon;
- (2) Motor vehicle gasoline, leaded, premium—motor vehicle gasoline that contains more than 0.05 gram of lead per gallon and is sold as “premium;”
- (3) Motor vehicle gasoline, leaded, non-premium—motor vehicle gasoline that contains more than 0.05 gram of lead per gallon but is not sold as “premium.”

The Act defines the term “motor vehicle” to mean any self-propelled vehicle designed for transporting persons or property on a street or highway. For purposes of this registration, however, gasoline specifically blended and marketed for motorcycles is excluded.

- (b) All designated motor vehicle gasolines must be registered by September 7, 1976.
- (c) Fuel manufacturers must submit the reports specified in 40 CFR part 1090, subpart J.
- (d) In accordance with §§ 79.5(a)(2) and 79.11(f), and to the extent such information is known to the fuel manufacturer, he shall furnish summaries of any information developed by or specifically for him concerning the following items:
 - (1) Mechanisms of action of each additive he reports;
 - (2) Reactions between such additives and motor vehicle gasoline;
 - (3) Identification and measurement of the emission products of such additives when used in motor vehicle gasoline;
 - (4) Effects of such additives on all emissions;
 - (5) Toxicity and any other public health or welfare effects of the emission products of such additives;
 - (6) Effects of the emission products of such additives on the performance of emission control devices/systems. Such submissions shall be accompanied by a description of the test procedures used in obtaining the information. Information will be considered to be known to the fuel manufacturer if a report thereon has been prepared and circulated or distributed outside the research department or division.

[40 FR 52011, Nov. 7, 1975, as amended at 41 FR 21324, May 25, 1976; 85 FR 78465, Dec. 4, 2020]

§ 79.33 Motor vehicle diesel fuel.

- (a) The following fuels commonly or commercially known or sold as motor vehicle diesel fuel are hereby individually designated:
 - (1) Motor vehicle diesel fuel, grade 1-D;
 - (2) Motor vehicle diesel fuel, grade 2-D.

The Act defines the term “motor vehicle” to mean any self-propelled vehicle designed for transporting persons or property on a street or highway.

- (b) All designated motor vehicle diesel fuels must be registered within 12 months after promulgation of this part.
- (c) Fuel manufacturers must submit the reports specified in 40 CFR part 1090, subpart J.

- (d) In accordance with §§ 79.5(a)(2) and 79.11(f), and to the extent such information is known to the fuel manufacturer, he shall furnish summaries of any information developed by or specifically for him concerning the following items:
- (1) Mechanisms of action of each additive he reports;
 - (2) Reactions between such additives and motor vehicle diesel fuel;
 - (3) Identification and measurement of the emission products of such additives when used in motor vehicle diesel fuel;
 - (4) Effects of such additives on all emissions;
 - (5) Toxicity and any other public health or welfare effects of the emission products of such additives.

Such submission shall be accompanied by a description of the test procedures used in obtaining the information. Information will be considered to be known to the fuel manufacturer if a report thereon has been prepared and circulated or distributed outside the research department or division.

[40 FR 52011, Nov. 7, 1975, as amended at 85 FR 78465, Dec. 4, 2020]

Subpart E [Reserved]

Subpart F—Testing Requirements for Registration

Source: 59 FR 33093, June 27, 1994, unless otherwise noted.

§ 79.50 Definitions.

The definitions listed in this section apply only to subpart F of this part.

Additive/base fuel mixture means the mixture resulting when a fuel additive is added in specified proportion to the base fuel of the fuel family to which the additive belongs.

Aerosol additive means a chemical mixture in aerosol form generally used as a motor vehicle engine starting aid or carburetor cleaner and not recommended to be placed in the fuel tank.

Aftermarket fuel additive means a product which is added by the end-user directly to fuel in a motor vehicle or engine to modify the performance or other characteristics of the fuel, the engine, or its emissions.

Atypical element means any chemical element found in a fuel or additive product which is not allowed in the baseline category of the associated fuel family, and an “atypical fuel or fuel additive” is a product which contains such an atypical element.

Base fuel means a generic fuel formulated from a set of specifications to be representative of a particular fuel family.

Basic emissions means the total hydrocarbons, carbon monoxide, oxides of nitrogen, and particulates occurring in motor vehicle or engine emissions.

Bulk fuel additive means a product which is added to fuel at the refinery as part of the original blending stream or after the fuel is transported from the refinery but before the fuel is purchased for introduction into the fuel tank of a motor vehicle.

Emission characterization means the determination of the chemical composition of emissions.

Emission generation means the operation of a vehicle or engine or the vaporization of a fuel or additive/fuel mixture under controlled conditions for the purpose of creating emissions to be used for testing purposes.

Emission sampling means the removal of a fraction of collected emissions for testing purposes.

Emission speciation means the analysis of vehicle or engine emissions to determine the individual chemical compounds which comprise those emissions.

Engine Dynamometer Schedule (EDS) means the transient engine speed versus torque time sequence commonly used in heavy-duty engine evaluation. The EDS for heavy-duty diesel engines is specified in 40 CFR part 86, appendix I(f)(2).

Evaporative Emission Generator (EEG) means a fuel tank or vessel to which heat is applied to cause a portion of the fuel to evaporate at a desired rate.

Evaporative emissions means chemical compounds emitted into the atmosphere by vaporization of contents of a fuel or additive/fuel mixture.

Evaporative fuel means a fuel which has a Reid Vapor Pressure (RVP, pursuant to 40 CFR part 80, appendix "E") of 2.0 pounds per square inch or greater and is not supplied to motor vehicle engines by way of sealed containment and delivery systems.

Evaporative fuel additive means a fuel additive which, when mixed with its specified base fuel, causes an increase in the RVP of the base fuel by 0.4 psi or more relative to the RVP of the base fuel alone and results in an additive/base fuel mixture whose RVP is 2.0 psi, or greater. Excluded from this definition are fuel additives used with fuels which are supplied to motor vehicle engines by way of sealed containment and delivery systems.

Federal Test Procedure (FTP) means the body of exhaust and evaporative emissions test procedures described in 40 CFR 86 for the certification of new motor vehicles to Federal motor vehicle emissions standards.

Fuel family means a set of fuels and fuel additives which share basic chemical and physical formulation characteristics and can be used in the same engine or vehicle.

Manufacturer means a person who is a fuel manufacturer or additive manufacturer as defined in § 79.2 (d) and (f).

Nitrated polycyclic aromatic hydrocarbons (NPAH) means the class of compounds whose molecular structure includes two or more aromatic rings and contains one or more nitrogen substitutions.

Non-catalyzed emissions means exhaust emissions not subject to an effective aftertreatment device such as a functional catalyst or particulate trap.

Oxygenate compound means an oxygen-containing, ashless organic compound, such as an alcohol or ether, which may be used as a fuel or fuel additive.

Polycyclic aromatic hydrocarbons (PAH) means the class of hydrocarbon compounds whose molecular structure includes two or more aromatic rings.

Relabeled additive means a fuel additive which is registered by its original manufacturer with EPA and is also registered and sold, unchanged in composition, under a different label and/or by a different entity.

Semi-volatile organic compounds means that fraction of gaseous combustion emissions which consists of compounds with greater than twelve carbon atoms and can be trapped in sorbent polymer resins.

Urban Dynamometer Driving Schedule (UDDS) means the 1372 second transient speed driving sequence used by EPA to simulate typical urban driving. The UDDS for light-duty vehicles is described in 40 CFR part 86, appendix I(a).

Vapor phase means the gaseous fraction of combustion emissions.

Vehicle classes/subclasses means the divisions of vehicle groups within a vehicle type, including light-duty vehicles, light-duty trucks, and heavy-duty vehicles as specified in 40 CFR part 86.

Vehicle type means the divisions of motor vehicles according to combustion cycle and intended fuel class, including, but not necessarily limited to, Otto cycle gasoline-fueled vehicles, Otto cycle methanol-fueled vehicles, diesel cycle diesel-fueled vehicles, and diesel cycle methanol-fueled vehicles.

Whole emissions means all components of unfiltered combustion emissions or evaporative emissions.

§ 79.51 General requirements and provisions.

(a) Overview of requirements.

(1) All manufacturers of fuels and fuel additives that are designated for registration under this part are required to comply with the requirements of subpart F of this part either on an individual basis or as a participant in a group of manufacturers of the same or similar fuels and fuel additives, as defined in § 79.56. If manufacturers elect to comply by participation in a group, each manufacturer continues to be individually subject to the requirements of subpart F of this part, and responsible for testing under this subpart. Each manufacturer, subject to the provisions for group applications in § 79.51(b) and the special provisions in § 79.58, shall submit all Tier 1 and Tier 2 information required by §§ 79.52, 79.53 and 79.59 for each fuel or additive, except that the Tier 1 emission characterization requirements in § 79.52(b) and/or the Tier 2 testing requirements in § 79.53 may be satisfied by adequate existing information pursuant to the Tier 1 literature search requirements in § 79.52(d). The adequacy of existing information to serve in compliance with specific Tier 1 and/or Tier 2 requirements shall be determined according to the criteria and procedures specified in §§ 79.52(b) and 79.53 (c) and (d). In all cases, EPA reserves the right to require, based upon the information contained in the application or any other information available to the Agency, that manufacturers conduct additional testing of any fuel or additive (or fuel/additive group) if EPA determines that there is inadequate information upon which to base regulatory decisions for such product(s). In any case where EPA determines that the requirements of Tiers 1 and 2 have been satisfied but that further testing is required, the provisions of Tier 3 (§ 79.54) shall apply.

(2) Laboratory facilities shall perform testing in compliance with Good Laboratory Practice (GLP) requirements as those requirements apply to inhalation toxicology studies. All studies shall be monitored by the facilities' Quality Assurance units (as specified in § 79.60).

(b) **Group Applications.** Subject to the provisions of § 79.56 (a) through (c), EPA will consider any testing requirements of this subpart to have been met for any fuel or fuel additive when a fuel or fuel additive which meets the criteria for inclusion in the same group as the subject fuel or fuel additive has met that testing requirement, provided that all fuels and additives must be individually registered as described in §

79.59(b). For purposes of this subpart, a determination of which group contains a particular fuel or additive will be made pursuant to the provisions of § 79.56 (d) and (e). Nothing in this subsection (b) shall be deemed to require a manufacturer to rely on another manufacturer's testing.

- (c) **Application Procedures and Dates.** Each application submitted in compliance with this subpart shall be signed by the manufacturer of the designated fuel or additive, or by the manufacturer's agent, and shall be submitted to the address and in the format prescribed in § 79.59. A manufacturer who chooses to comply as part of a group pursuant to § 79.56 shall be covered by the group's joint application. Subject to any modifications pursuant to the special provisions in §§ 79.51(f) or 79.58, the schedule for compliance with the requirements of this subpart is as follows:

(1) **Fuels and fuel additives with existing registrations.**

- (i) The manufacturer of a fuel or fuel additive product which, pursuant to subpart B or C of this part, is registered as of May 27, 1994 must submit the additional basic registration data specified in § 79.59(b) before November 28, 1994.
- (ii) Except as provided in paragraphs (c)(1)(vi) and (vii) of this section, the manufacturer of such products must also satisfy the requirements and time schedules in either of the following paragraphs (c)(1)(ii) (A) or (B) of this section:
 - (A) No later than May 27, 1997, all applicable Tier 1 and Tier 2 requirements must be submitted to EPA, pursuant to §§ 79.52, 79.53, and 79.59; or
 - (B) No later than May 27, 1997, all applicable Tier 1 requirements (pursuant to §§ 79.52 and 79.59), plus evidence of a contract with a qualified laboratory (or other suitable arrangement) for completion of all applicable Tier 2 requirements, must be submitted to EPA. For this purpose, a qualified laboratory is one which can demonstrate the capabilities and credentials specified in § 79.53(c)(1). In addition, by May 26, 2000, all applicable Tier 2 requirements (pursuant to §§ 79.53 and 79.59) must be submitted to EPA.
- (iii) In the case of such fuels and fuel additives which, pursuant to applicable special provisions in § 79.58, are not subject to Tier 2 requirements, all other requirements (except Tier 3) must be submitted to EPA before May 27, 1997.
- (iv) In the event that Tier 3 testing is also required (under § 79.54), EPA shall determine an appropriate timeline for completion of the additional requirements and shall communicate this schedule to the manufacturer according to the provisions of § 79.54(b).
- (v) The manufacturer may at any time modify an existing fuel registration by submitting a request to EPA to add or delete a bulk additive to the existing registration information for such fuel product, provided that any additional additive must be registered by EPA for use in the specific fuel family to which the fuel product belongs. However, the addition or deletion of a bulk additive to a fuel registration may effect the grouping of such registered fuel under the criteria of § 79.56, and thus may effect the testing responsibilities of the fuel manufacturer under this subpart.
- (vi) In regard to atypical fuels or additives in the gasoline and diesel fuel families (pursuant to the specifications in § 79.56(e)(4)(iii)(A) (1) and (2)):
 - (A) All applicable Tier 1 requirements, pursuant to §§ 79.52 and 79.59, must be submitted to EPA by May 27, 1997.

- (B) Tier 2 requirements, pursuant to §§ 79.53 and 79.59, must be satisfied according to the deadlines in either of the following paragraphs (c)(1)(vi)(B) (1) or (2) of this section:
 - (1) All applicable Tier 2 requirements shall be submitted to EPA by November 27, 1998; or
 - (2) Evidence of a contract with a qualified laboratory (or other suitable arrangement) for completion of all applicable Tier 2 requirements shall be submitted to EPA by November 27, 1998. For this purpose, a qualified laboratory is one which can demonstrate the capabilities and credentials specified in § 79.53(c)(1). In addition, all applicable Tier 2 requirements must be submitted to EPA by November 27, 2001.
- (vii) In regard to nonbaseline diesel products formulated with mixed alkyl esters of plant and/or animal origin (i.e., "biodiesel" fuels, pursuant to § 79.56(e)(4)(ii)(B)(2)):
 - (A) All applicable Tier 1 requirements, pursuant to §§ 79.52 and 79.59, must be submitted to EPA by March 17, 1998.
 - (B) Tier 2 requirements, pursuant to §§ 79.53 and 79.59, must be satisfied according to the deadlines in either of the following paragraphs (c)(1)(vii)(B) (1) or (2) of this section:
 - (1) All applicable Tier 2 requirements shall be submitted to EPA by March 17, 1998; or
 - (2) Evidence of a contract with a qualified laboratory (or other suitable arrangement) for completion of all applicable Tier 2 requirements shall be submitted to EPA by March 17, 1998. For this purpose, a qualified laboratory is one which can demonstrate the capabilities and credentials specified in § 79.53(c)(1). In addition, all applicable Tier 2 requirements must be submitted to EPA by May 27, 2000.
- (2) **Registrable fuels and fuel additives.**
 - (i) A fuel product which is not registered pursuant to subpart B of this part as of May 27, 1994 shall be considered registrable if, under the criteria established by § 79.56, the fuel can be enrolled in the same fuel/additive group with one or more currently registered fuels. A fuel additive product which is not registered for a specific type of fuel pursuant to subpart C of this part as of May 27, 1994 shall be considered registrable for that type of fuel if, under the criteria established by § 79.56, the fuel/additive mixture resulting from use of the additive product in the specific type of fuel can be enrolled in the same fuel/additive group with one or more currently registered fuels or bulk fuel additives. For the purpose of this determination, currently registered fuels and bulk additives are those with existing registrations as of the date on which EPA receives the basic registration data (pursuant to § 79.59(b)) for the product in question.
 - (ii) A manufacturer seeking to register under subpart B of this part a fuel product which is deemed registrable under this section, or to register under subpart C of this part a fuel additive product for a specific type of fuel for which it is deemed registrable under this section, shall submit the basic registration data (pursuant to § 79.59(b)) for that product as part of the application for registration. If the Administrator determines that the product is registrable under this section, then the Administrator shall promptly register the product, provided that the applicant has satisfied all of the other requirements for registration under subpart B or subpart C of this part, and contingent upon satisfactory submission of required information under paragraph (c)(2)(iii) of this section.

(iii) Registration of a registrable fuel or additive shall be subject to the same requirements and compliance schedule as specified in paragraph (c)(1) of this section for existing fuels and fuel additives. Accordingly, manufacturers of registrable fuels or additives may be granted and may retain registration for such products only if any applicable and due Tier 1, 2, and 3 requirements have also been satisfied by either the manufacturer of the product or the fuel/additive group to which the product belongs.

(3) **New fuels and fuel additives.** A fuel product shall be considered new if it is not registered pursuant to subpart B of this part as of May 27, 1994 and if, under the criteria established by § 79.56, it cannot be enrolled in the same fuel/additive group with one or more currently registered fuels. A fuel additive product shall be considered new with respect to a specific type of fuel if it is not expressly registered for that type of fuel pursuant to subpart C of this part as of May 27, 1994 and if, under the criteria established by § 79.56, the fuel/additive mixture resulting from use of the additive product in the specific type of fuel cannot be enrolled in the same fuel/additive group with one or more currently registered fuels or bulk fuel additives. For the purpose of this determination, currently registered fuels and bulk additives are those with existing registrations as of the date on which EPA receives the basic registration data (pursuant to § 79.59(b)) for the product in question. For such new product, the manufacturer must satisfactorily complete all applicable Tier 1 and Tier 2 requirements, followed by any Tier 3 testing which the Administrator may require, before registration will be granted.

(d) **Notifications.** Upon receipt of a manufacturer's (or group's) submittal in compliance with the requirements of this subpart, EPA will notify such manufacturer (or group) that the application has been received and what, if any, information, testing, or retesting is necessary to bring the application into compliance with the requirements of this subpart. EPA intends to provide such notification of receipt in a timely manner for each such application.

(1) **Registered fuel and fuel additive notification.**

- (i) The manufacturer of a registered fuel or fuel additive product who is notified that the submittal for such product contains adequate information pursuant to the Tier 1 and Tier 2 testing and reporting requirements (§§ 79.52, 79.53, and 79.59 (a) through (c)) may continue to sell, offer for sale, or introduce into commerce the registered product as permitted by the existing registration for the product under § 79.4.
- (ii) If the manufacturer of a registered fuel or fuel additive product is notified that testing or retesting is necessary to bring the Tier 1 and/or Tier 2 submittal into compliance, the continued sale or importation of the product shall be conditional upon satisfactorily completing the requirements within the time frame specified in paragraph (c)(1) of this section.
- (iii) EPA intends to notify the manufacturer of the adequacy of the submitted data within two years of EPA's receipt of such data. However, EPA retains the right to require that adequate data be submitted to EPA if, upon subsequent review, EPA finds that the original Tier 1 and/or Tier 2 submittal is not consistent with the requirements of this subpart. If EPA does not notify the manufacturer of the adequacy of the Tier 1 and/or Tier 2 data within two years, EPA will not hold the manufacturer liable for penalties for violating this rule for the period beginning when the data was due until the time EPA notifies the manufacturer of the violation.
- (iv) If the manufacturer of a registered fuel or fuel additive product is notified (pursuant to § 79.54(b)) that Tier 3 testing is required for its product, then the manufacturer may continue to sell, offer for sale, introduce into commerce the registered product as permitted by the existing

registration for the product under § 79.4. However, if the manufacturer fails to complete the specified Tier 3 requirements within the specified time, the registration of the product will be subject to cancellation under § 79.51(f)(6).

(v) EPA retains the right to require additional Tier 3 testing pursuant to the procedures in § 79.54.

(2) ***New fuel and fuel additive notification.***

(i) Within six months following its receipt of the Tier 1 and Tier 2 submittal for a new product (as defined in paragraph (c)(3) of this section), EPA shall notify the manufacturer of the adequacy of such submittal in compliance with the requirements of §§ 79.52, 79.53, and 79.59 (a) through (c).

(A) If EPA notifies the manufacturer that testing, retesting, or additional information is necessary to bring the Tier 1 and Tier 2 submittal into compliance, the manufacturer shall remedy all inadequacies and provide Tier 3 data, if required, before EPA shall consider the requirements for registration to have been met for the product in question.

(B) If EPA does not notify the manufacturer of the adequacy of the Tier 1 and Tier 2 submittal within six months following the submittal, the manufacturer shall be deemed to have satisfactorily completed Tiers 1 and 2.

(ii) Within six months of the date on which EPA notifies the manufacturer of satisfactory completion of Tiers 1 and 2 for a new product, or within one year of the submittal of the Tier 1 and Tier 2 data (whichever is earlier), EPA shall determine whether additional testing is currently needed under the provisions of Tier 3 and, pursuant to § 79.54(b), shall notify the manufacturer of its determination.

(A) If the manufacturer of a new fuel or fuel additive product is notified that Tier 3 testing is required for such product, then EPA shall have the authority to withhold registration until the specified Tier 3 requirements have been satisfactorily completed. EPA shall determine whether the Tier 3 requirements have been met, and shall notify the manufacturer of this determination, within one year of receiving the manufacturer's Tier 3 submittal.

(B) If EPA does not notify the manufacturer of potential Tier 3 requirements within the prescribed timeframe, then additional testing at the Tier 3 level is deemed currently unnecessary and the manufacturer shall be considered to have complied with all current registration requirements for the new fuel or additive product.

(iii) Upon completion of all current Tier 1, Tier 2, and Tier 3 requirements, and submission of an application for registration which includes all of the information and assurances required by § 79.11 or § 79.21, the registration of the new fuel or additive shall be granted, and the registrant may then sell, offer for sale, or introduce into commerce the registered product as permitted by § 79.4.

(iv) Once the new product becomes registered, EPA reserves the right to require additional Tier 3 testing pursuant to the procedures specified in § 79.54.

(e) ***Inspection of a testing facility.***

(1) A testing facility, whether engaged in emissions analysis or health and/or welfare effects testing under the regulations in this subpart, 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 subpart 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 informal hearings.

- (2) 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.
- (3) Effects of non-compliance. Pursuant to sections 114, 208, and 211(d) of the CAA, it shall be a violation of this section and a violation of 40 CFR part 79, subpart F to deny entry to an authorized employee or duly designated representative of EPA for the purpose of auditing a testing facility or test data.

(f) ***Penalties and Injunctive Relief.***

- (1) Any person who violates these regulations shall be subject to a civil penalty of up to \$25,000 for each and every day of the continuance of the violation and the economic benefit or savings resulting from the violation. Action to collect such civil penalties shall be commenced in accordance with paragraph (b) of section 205 of the Clean Air Act or assessed in accordance with paragraph (c) of section 205 of the Clean Air Act, 42 U.S.C. 7524 (b) and (c).
- (2) Under section 205(b) of the CAA, the Administrator may commence a civil action for violation of this subpart in the district court of the United States for the district in which the violation is alleged to have occurred or in which the defendant resides or has a principal place of business.
- (3) Under section 205(c) of the CAA, the Administrator may assess a civil penalty of \$25,000 for each and every day of the continuance of the violation and the economic benefit or savings resulting from the violation, except that the maximum penalty assessment shall not exceed \$200,000, unless the Administrator and the Attorney General jointly determine that a matter involving a larger penalty amount is appropriate for administrative penalty assessment. Any such determination by the Administrator and the Attorney General shall not be subject to judicial review.
- (4) The Administrator may, upon application by the person against whom any such penalty has been assessed, remit or mitigate, with or without conditions, any such penalty.
- (5) The district courts of the United States shall have jurisdiction to compel the furnishing of information and the conduct of tests required by the Administrator under these regulations and to award other appropriate relief. Actions to compel such actions shall be brought by and in the name of the United States. In any such action, subpoenas for witnesses who are required to attend a district court in any district may run into any other district.
- (6) ***Cancellation.***
 - (i) The Administrator of EPA may issue a notice of intent to cancel a fuel or fuel additive registration if the Administrator determines that the registrant has failed to submit in a timely manner any data required to maintain registration under this part or under section 211(b) or 211(e) of the Clean Air Act.

- (ii) Upon issuance of a notice of intent to cancel, EPA will forward a copy of the notice to the registrant by certified mail, return receipt requested, at the address of record given in the registration, along with an explanation of the reasons for the proposed cancellation.
 - (iii) The registrant will be afforded 60 days from the date of receipt of the notice of intent to cancel to submit written comments concerning the notice, and to demonstrate or achieve compliance with the specific data requirements which provide the basis for the proposed cancellation. If the registrant does not respond in writing within 60 days from the date of receipt of the notice of intent to cancel, the cancellation of the registration shall become final by operation of law and the Administrator shall notify the registrant of such cancellation. If the registrant responds in writing within 60 days from the date of receipt of the notice of intent to cancel, the Administrator shall review and consider all comments submitted by the registrant before taking final action concerning the proposed cancellation. The registrants' communications should be sent to the following address: Attn: Fuel/Additives Registration, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave. NW, Mail Code 6405A, Washington, DC 20460.
 - (iv) As part of a written response to a notice of intent to cancel, a registrant may request an informal hearing concerning the notice. Any such request shall state with specificity the information the registrant wishes to present at such a hearing. If an informal hearing is requested, EPA shall schedule such a hearing within 60 days from the date of receipt of the request. If an informal hearing is held, the subject matter of the hearing shall be confined solely to whether or not the registrant has complied with the specific data requirements which provide the basis for the proposed cancellation. If an informal hearing is held, the designated presiding officer may be any EPA employee, the hearing procedures shall be informal, and the hearing shall not be subject to or governed by 40 CFR part 22 or by 5 U.S.C. 554, 556, or 557. A verbatim transcript of each informal hearing shall be kept and the Administrator shall consider all relevant evidence and arguments presented at the hearing in making a final decision concerning a proposed cancellation.
 - (v) If a registrant who has received a notice of intent to cancel submits a timely written response, and the Administrator decides after reviewing the response and the transcript of any informal hearing to cancel the registration, the Administrator shall issue a final cancellation order, forward a copy of the cancellation order to the registrant by certified mail, and promptly publish the cancellation order in the FEDERAL REGISTER. Any cancellation order issued after receipt of a timely written response by the registrant shall become legally effective five days after it is published in the FEDERAL REGISTER.
- (7) The civil monetary penalty amounts listed in this section may not reflect recent inflation adjustments EPA is required to make. The current maximum and minimum statutory civil penalty amounts are located in § 19.4.

(g) *Modification of Regulation.*

- (1) In special circumstances, a manufacturer subject to the registration requirements of this rule may petition the Administrator to modify the mandatory testing requirements in the test standard for any test required by this rule by application to Director, Field Operations and Support Division, at the address in paragraph (f)(6)(iii) of this section.
 - (i) Such request shall be made as soon as the test sponsor is aware that the modification is necessary, but in no event shall the request be made after 30 days following the event which precipitated the request.

- (ii) Upon such request, the Administrator may, in circumstances which are outside the control of the manufacturer(s) or his/their agent and which could not have been reasonably foreseen or avoided, modify the mandatory testing requirements in the rule if such requirements are infeasible.
- (iii) If the Administrator determines that such modifications would not significantly alter the scope of the test, EPA will not ask for public comment before approving the modification. The Administrator will notify the test sponsor by certified mail of the response to the request. EPA will place copies of each application and EPA response in the public docket. EPA will publish a notice in the FEDERAL REGISTER annually describing such changes which have occurred during the previous year. Until such FEDERAL REGISTER notice is published, any modification approved by EPA shall apply only to the person or group who requested the modification; EPA shall state the applicability of each modification in such notice.
- (iv) Where, in EPA's judgment, the requested modification of a test standard would significantly change the scope of the test, EPA will publish a notice in the FEDERAL REGISTER requesting comment on the request and proposed modification. However, EPA may approve a requested modification of a test standard without first seeking public comment if necessary to preserve the validity of an ongoing test undertaken in good faith.

(2) [Reserved]

(h) ***Special Requirements for Additives.*** When an additive is the test subject, the following rules apply:

- (1) All required emission characterization and health effects testing procedures shall be performed on the mixture which results when the additive is combined with the base fuel for the appropriate fuel family (as specified in § 79.55) at the maximum concentration recommended by the additive manufacturer pursuant to § 79.21(d). This combination shall be known as the additive/base fuel mixture.
 - (i) The appropriate fuel family to be utilized for the additive/base fuel mixture is the fuel family which contains the specific type(s) of fuel for which the additive is presently registered or for which the manufacturer of the additive is seeking registration.
 - (ii) Additives belonging to more than one fuel family.
 - (A) If an additive product is registered in two or more fuel families as of May 27, 1994, then the manufacturer of that additive is responsible for testing (or participating in group testing of) the respective additive/base fuel mixtures in compliance with the requirements of this subpart for each fuel family in which the manufacturer wishes to maintain a registration for its additive.
 - (B) If a manufacturer is seeking to register such additive in two or more fuel families then, for testing and registration purposes, the additive shall be considered to be a member of each fuel family in which the manufacturer is seeking registration. The manufacturer is responsible for testing (or participating in group testing of) the respective additive/base fuel mixture in compliance with the requirements of this subpart for each fuel family in which the manufacturer wishes to obtain a product registration for its additive.

- (iii) In the case of the methanol fuel family, which contains two base fuels (M100 and M85 base fuels, pursuant to § 79.55(d)), the applicable base fuel is the one which represents the fuel/additive group (specified in § 79.56(e)(4)(i)(C)) containing fuels of which the most gallons are sold annually.
 - (iv) Aftermarket additives which are intended by the manufacturer to be added to the fuel tank only at infrequent intervals shall be applied according to the manufacturer's specifications during mileage accumulation, pursuant to § 79.57(c). However, during emission generation and testing, each tankful of fuel used must contain the fuel additive at its maximum recommended level. If the additive manufacturer believes that this maximum treatment rate will cause adverse effects to the test engine and/or that the engine's emissions may be subject to artifacts due to overuse of the additive, then the manufacturer may submit a request to EPA for modification of this requirement and related test procedures. Such request must include objective evidence that the modification(s) are needed, along with data demonstrating the maximum concentration of the additive which may actually reach the fuel tanks of vehicles in use.
 - (v) Additives produced exclusively for use in #1 diesel fuel shall be tested in the diesel base fuel specified in § 79.55(c), even though that base fuel is formulated with #2 diesel fuel. If a manufacturer is concerned that emissions generated from this combination of fuel and additive are subject to artifacts due to this blending, then that manufacturer may submit a request for a modification in test procedure requirements to the EPA. Any such request must include supporting test results and suggested test modifications.
 - (vi) Bulk additives which are used intermittently for the direct purpose of conditioning or treating a fuel during storage or transport, or for treating or maintaining the storage, pipeline, and/or other components of the fuel distribution system itself and not the vehicle/engine for which the fuel is ultimately intended, shall, for purposes of this program, be added to the base fuel at the maximum concentration recommended by the additive manufacturer for treatment of the fuel or distribution system component. However, if the additive manufacturer believes that this treatment rate will cause adverse effects to the test engine and/or that the engine's emissions may be subject to artifacts due to overuse of the additive, then the manufacturer may submit a request to EPA for modification of this requirement and related test procedures. Such request must include objective evidence that the modification(s) are needed, along with data demonstrating the maximum concentration of the additive which may actually reach the fuel tanks of vehicles in use.
- (2) EPA shall use emissions speciation and health effects data generated in the analysis of the applicable base fuel as control data for comparison with data generated for the additive/base fuel mixture.
- (i) The base fuel control data may be:
 - (A) Generated internally as an experimental control in conjunction with testing done in compliance with registration requirements for a specific additive; or
 - (B) Generated externally in the course of testing different additive(s) belonging to the same fuel family, or in the testing of a base fuel serving as representative of the baseline group for the respective fuel family pursuant to § 79.56(e)(4)(i).

- (ii) Control data generated using test equipment (including vehicle model and/or engine, or Evaporative Emissions Generator specifications, as appropriate) and protocols identical or nearly identical to those used in emissions and health effects testing of the subject additive/base fuel mixture would be most relevant for comparison purposes.
- (iii) If an additive manufacturer chooses the same vehicle/engine to independently test the base fuel as an experimental control prior to testing the additive/base fuel mixture, then the test vehicle/engine shall undergo two mileage accumulation periods, pursuant to § 79.57(c). The initial mileage accumulation period shall be performed using the base fuel alone. After base fuel testing, and prior to testing of the additive/base fuel mixture, a second mileage accumulation period shall be performed using the additive/base fuel mixture. The procedures outlined in this paragraph shall not preclude a manufacturer from testing a base fuel and the manufacturer's additive/base fuel mixture separately in identical, or nearly identical, vehicles/engines.

(i) **Multiple Test Potential for Non-Baseline Products.**

- (1) When the composition information reported in the registration application or basic registration data for a gasoline or diesel product meets criteria for classification as a non-baseline product (pursuant to § 79.56(e)(3)(i)(B) or § 79.56(e)(3)(ii)(B)), then the manufacturer is responsible for testing (or participating in group testing) of a separate formulation for each reported oxygenating compound, specified class of oxygenating compounds, or other substance which defines a separate non-baseline fuel/additive group pursuant to § 79.56(e)(4)(ii)(A) or (B). For each such substance, testing shall be performed on a mixture of the relevant substance in the appropriate base fuel, formulated according to the specifications for the corresponding group representatives in § 79.56(e)(4)(ii).
- (2) When the composition information reported in the registration application or basic registration data for a non-baseline gasoline product contains a range of total oxygenate concentration-in-use which encompasses gasoline formulations with less than 1.5 weight percent oxygen as well as gasoline formulations with 1.5 weight percent oxygen or more, then the manufacturer is required to test (or participate in applicable group testing of) a baseline gasoline formulation as well as one or more non-baseline gasoline formulations as described in paragraph (h)(1) of this section.
- (3) When the composition information reported in the registration application or basic registration data for a non-baseline diesel product contains a range of total oxygenate concentration-in-use which encompasses diesel formulations with less than 1.0 weight percent oxygen as well as diesel formulations with 1.0 weight percent oxygen or more, then the manufacturer is required to test (or participate in applicable group testing) of a baseline diesel formulation as well as one or more non-baseline diesel formulations as described in paragraph (h)(1) of this section.
- (4) The presence in a particular oxygenating additive of small amounts of other unintended oxygenate compounds as byproducts of the manufacturing process of the given oxygenating additive does not affect the grouping of that additive and does not create multiple testing responsibilities for manufacturers who blend that additive into fuel.

(j) **Multiple Test Potential for Atypical Fuel Formulations.** When the composition information reported in the registration application or basic registration data for a fuel product includes more than one atypical bulk additive product (pursuant to § 79.56(e)(2)(iii)), and when these additives belong to different fuel/additive groups (pursuant to § 79.56(e)(4)(iii)), then:

- (1) When such disparate additive products are for the same purpose-in-use and are not ordinarily used in the fuel simultaneously, the fuel manufacturer shall be responsible for testing (or participating in the group testing of) a separate formulation for each such additive product. Testing related to each additive product shall be performed on a mixture of the additive in the applicable base fuel, as described in paragraph (g)(1) of this section, or by participation in the costs of testing the designated representative of the fuel/additive group to which each separate atypical additive product belongs.
 - (2) When the disparate additive products are not for the same purpose-in-use, the fuel manufacturer shall nevertheless be responsible for testing a separate formulation for each such additive product, as described in paragraph (g)(1) of this section, if these additives are not ordinarily blended together in the same commercial formulation of the fuel.
 - (3) When the disparate additive products are ordinarily blended together in the same commercial formulation of the fuel, then the fuel manufacturer shall be responsible for the testing of a single test formulation containing all such simultaneously used atypical additive products. Alternatively, this responsibility can be satisfied by enrolling such fuel product in a group which includes other fuel or additive products with the same total combination of atypical elements as that occurring in the fuel product in question. If the basic registration data for the subject fuel includes any alternative additives which contain atypical elements not represented in the test formulation, then the fuel manufacturer is also responsible for testing a separate formulation for each such additional disparate additive product.
- (k) **Emission Control System Testing.** If any information submitted in accordance with this subpart or any other information available to EPA shows that a fuel or fuel additive may have a deleterious effect on the performance of any emission control system or device currently in use or which has been developed to a point where in a reasonable time it would be in general use were such effect avoided, EPA may, in its judgment, require testing to determine whether such effects in fact exist. Such testing will be required in accordance with such protocols and schedules as the Administrator shall reasonably require and shall be paid for by the fuel or fuel additive manufacturer.

[59 FR 33093, June 27, 1994, as amended at 61 FR 36511, July 11, 1996; 62 FR 12575, Mar. 17, 1997; 85 FR 7069, Feb. 6, 2020; 89 FR 88655, Nov. 8, 2024]

§ 79.52 Tier 1.

- (a) **General Specifications.** Tier 1 requires manufacturers of designated fuels or fuel additives (or groups of manufacturers pursuant to § 79.56) to supply to the Administrator the identity and concentration of certain emission products of such fuels or additives and any available information regarding the health and welfare effects of the whole and speciated emissions of such fuels or additives. In addition to any information required under § 79.59 and in conformance with the reporting requirements thereof, manufacturers shall provide, pursuant to the timing provisions of § 79.51(c), the following information.
- (b) **Emissions Characterization.** Manufacturers must provide a characterization of the emission products which are generated by evaporation (if required pursuant to § 79.58(b)) and by combustion of the fuel or additive/base fuel mixture in a motor vehicle. For this purpose, manufacturers may perform the characterization procedures described in this section or may rely on existing emission characterization data. To be considered adequate in lieu of performing new emission characterization procedures, the data must be the result of tests using the product in question or using a fuel or additive/base fuel mixture meeting the same grouping criteria as the product in question. In addition, the emissions must be

generated in a manner reasonably similar to those described in § 79.57, and the characterization procedures must be adequately performed and documented and must give results reasonably comparable to those which would be obtained by performing the procedures described herein. Reports of previous tests must be sufficiently detailed to allow EPA to judge the adequacy of protocols, techniques, and conclusions. After the manufacturer's submittal of such data, if EPA finds that the manufacturer has relied upon inadequate test data, then the manufacturer will not be considered to be in compliance until the corresponding tests have been conducted and the results submitted to EPA.

(1) **General Provisions.**

- (i) The emissions to be characterized shall be generated, collected, and stored according to the processes described in § 79.57. Characterization of combustion and evaporative emissions shall be performed separately on each emission sample collected during the applicable emission generation procedure.
 - (ii) As provided in § 79.57(d), if the emission generation vehicle/engine is ordinarily equipped with an emission aftertreatment device, then all requirements in this section for the characterization of combustion emissions must be completed both with and without the aftertreatment device in a functional state. The emissions shall be generated three times (on three different days) without a functional aftertreatment device and, if applicable, three times (on three different days) with a functional aftertreatment device, and each such time shall be analyzed according to the remaining provisions in this paragraph (b) of this section.
 - (iii) Measurement of background emissions: It is required that ambient/dilution air be analyzed for levels of background chemical species present at the time of emissions sampling (for both combustion and evaporative emissions) and that sample values be corrected by subtracting the concentrations contributed by the ambient/dilution air. Background chemical species measurement/analysis during the FTP is specified in §§ 86.109-94(c)(5) and 86.135-94 of this chapter.
 - (iv) Concentrations of emission products shall be reported either in units of grams per mile (g/mi) or grams per brake-horsepower/hour (g/bhp-hr) (for chassis dynamometer and engine dynamometer test configurations, respectively), as well as in units of weight percent of measured total hydrocarbons.
 - (v) Laboratory practice must be of high quality and must be consistent with state-of-the-art methods as presented in current environmental and analytical chemistry literature. Examples of analytical procedures which may be used in conducting the emission characterization/speciation requirements of this section can be found among the references in paragraph (b)(5) of this section.
- (2) Characterization of the combustion emissions shall include, for products in all fuel families (except when expressly noted in this section):
- (i) Determination of the concentration of the basic emissions as follows: total hydrocarbons, carbon monoxide, oxides of nitrogen, and particulates. Manufacturers are referred to the vehicle certification procedures in 40 CFR part 86, subparts B and D (§§ 86.101 through 86.145 and §§ 86.301 through 86.348) for guidance on the measurement of the basic emissions of interest to this subpart.
 - (ii) Characterization of the vapor phase of combustion emissions, as follows:

- (A) Determination of the identity and concentration of individual species of hydrocarbon compounds containing 12 or fewer carbon atoms. Such characterization shall begin within 30 minutes after emission collection is completed.
 - (B) Determination of the identity and concentration of individual species of aldehyde and ketone compounds containing eight or fewer carbon atoms. Characterization of these emissions captured in cartridges shall be performed within two weeks if the cartridge is stored at room temperature, and one month if the cartridge is stored at 0 °C or less. If the emissions are sampled using the impinger method, the sample must be stored in a capped sample vial at 0 °C or less and characterized within one week.
 - (C) Determination of the identity and concentration of individual species of alcohol and ether compounds containing six or fewer carbon atoms, for those fuels and additive/base fuel mixtures which contain alcohol and/or ether compounds containing from one to six carbon atoms in the uncombusted state. For fuel and additive formulations containing alcohols or ethers with more than six carbon atoms in the uncombusted state, alcohol and ether species with that higher number of carbon atoms or less must be identified and measured in the emissions. Such characterization shall begin within four hours after emission collection is completed.
- (iii) Characterization of the semi-volatile and particulate phases of combustion emissions to identify and measure polycyclic aromatic compounds, as follows:
- (A) Analysis for polycyclic aromatic compounds shall not be conducted at or soon after the start of a recommended engine lubricant change interval.
 - (B) Analysis for polycyclic aromatic hydrocarbons (PAHs) and nitrated polycyclic aromatic hydrocarbons (NPAHs), specified in paragraph (b)(2)(iii)(D) of this section, need not be done for any fuels and additives in the methane or propane fuel families, nor for fuels and additives in the atypical categories of any other fuel families, pursuant to the definitions of such families and categories in § 79.56.
 - (C) Analysis for poly-chlorinated dibenzodioxins and dibenzofurans (PCDD/PCDFs), specified in paragraph (b)(2)(iii)(E) of this section, is required only for fuels and additives which contain chlorine as an atypical element, pursuant to paragraph (b)(2)(iv) of this section, which requires all individual emission products containing atypical elements to be determined for atypical fuels and additives. However, manufacturers of baseline and nonbaseline fuels and fuel additives in all fuel families, except those in the methane and propane fuel families, are strongly encouraged to conduct these analyses on a voluntary basis.
 - (D) The analytical method used to measure species of PAHs and NPAHs should be capable of detecting at least 1 ppm (equivalent to 0.001 microgram (µg) of compound per milligram of organic extract) of these compounds in the extractable organic matter. The concentration of each individual PAH or NPAH compound identified shall be reported in units of microgram per mile or nanograms per brake-horsepower/hour (for chassis dynamometer and engine dynamometer test configurations, respectively). Each compound which is present at 0.001 µg per mile (0.5 nanograms per brake-horsepower/hour) or more must be identified, measured, and reported. The following individual species shall be measured:
 - (1) PAHs:

- (i) Benzo(a)anthracene;
 - (ii) Benzo[b]fluoranthene;
 - (iii) Benzo[k]fluoranthene;
 - (iv) Benzo(a)pyrene;
 - (v) Chrysene;
 - (vi) Dibenzo[a,h]anthracene; and
 - (vii) Indeno[1,2,3-c,d]pyrene.
- (2) NPAHs:
 - (i) 7-Nitrobenzo[a]anthracene;
 - (ii) 6-Nitrobenzo[a]pyrene;
 - (iii) 6-Nitrochrysene;
 - (iv) 2-Nitrofluorene; and
 - (v) 1-Nitropyrene.
- (E) The analytical method used to measure species and classes of PCDD/PCDFs should be capable of detecting at least 1 part per trillion (ppt) (equivalent to 0.001 picogram (pg) of compound per milligram of organic extract) of these compounds in the extractable organic matter. The concentration of each individual PCDD/PCDF compound identified shall be reported in units of picograms (pg) per mile or picograms per brake-horsepower/hour (for chassis dynamometer and engine dynamometer test configurations, respectively). Each compound which is present at 0.5 pg/mile (0.3 pg/bhp-hr) or more must be identified, measured, and reported.
 - (1) With respect to measurement of PCDD/PCDFs only, the liquid extracts from the particulate and semi-volatile emissions fractions may be combined into one sample for analysis.
 - (2) The manufacturer is referred to 40 CFR part 60, appendix A, Method 23 for a protocol which may be used to identify and measure any potential PCDD/PCDFs which might be present in exhaust emissions from a fuel or additive/base fuel mixture.
 - (3) The following individual compounds and classes of compounds of PCDD/PCDFs shall be identified and measured:
 - (i) Individual tetra-chloro-substituted dibenzodioxins (tetra-CDDs);
 - (ii) Individual tetra-chloro-substituted dibenzofurans (tetra-CDFs);
 - (iii) Penta-CDDs and penta-CDFs, as one class;
 - (iv) Hexa-CDDs and hexa-CDFs, as one class;
 - (v) Hepta-CDDs and hepta-CDFs as one class; and
 - (vi) Octo-CDDs and octo-CDFs as one class.

- (iv) With respect to all phases (vapor, semi-volatile, and particulate) of combustion emissions generated from those fuels and additive/base fuel mixtures classified in the atypical categories (pursuant to § 79.56), the identity and concentration of individual emission products containing such atypical elements shall also be determined.
- (3) For evaporative fuels and evaporative fuel additives, characterization of the evaporative emissions shall include:
- (i) Determination of the concentration of total hydrocarbons for the applicable vehicle type and class in 40 CFR part 86, subpart B (§§ 86.101 through 86.145).
 - (ii) Determination of the identity and concentration of individual species of hydrocarbon compounds containing 12 or fewer carbon atoms. Such characterization shall begin within 30 minutes after emission collection is completed.
 - (iii) In the case of those fuels and additive/base fuel mixtures which contain alcohol and/or ether compounds in the uncombusted state, determination of the identity and concentration of individual species of alcohol and ether compounds containing six or fewer carbon atoms. For fuel and additive formulations containing alcohols or ethers with more than six carbon atoms in the uncombusted state, alcohol and ether species with that higher number of carbon atoms or less must be identified and measured in the emissions. Such characterization shall begin within four hours after emission collection is completed.
 - (iv) In the case of those fuels and additive/base fuel mixtures which contain atypical elements, determination of the identity and concentration of individual emission products containing such atypical elements.
- (4) **Laboratory quality control.**
- (i) At a minimum, laboratories performing the procedures specified in this section shall conduct calibration testing of their emissions characterization equipment before each new fuel/additive product test start-up. Known samples representative of the compounds potentially to be found in emissions from the product to be characterized shall be used to calibrate such equipment.
 - (ii) Laboratories performing the procedures specified in this section shall agree to permit quality control inspections by EPA, and for this purpose shall admit any EPA Enforcement Officer, upon proper presentation of credentials, to any facility where vehicles are conditioned or where emissions are generated, collected, stored, sampled, or characterized in meeting the requirements of this section. Such laboratory audits may include EPA distribution of "blind" samples for analysis by participating laboratories.
- (5) **References.** For additional background information on the emission characterization procedures outlined in this paragraph, the following references may be consulted:
- (i) "Advanced Emission Speciation Methodologies for the Auto/Oil Air Quality Improvement Program—I. Hydrocarbons and Ethers," Auto Oil Air Quality Improvement Research Program, SP-920, 920320, SAE, February 1992.
 - (ii) "Advanced Speciation Methodologies for the Auto/Oil Air Quality Improvement Research Program—II. Aldehydes, Ketones, and Alcohols," Auto Oil Air Quality Improvement Research Program, SP-920, 920321, SAE, February 1992.

- (iii) ASTM D 5197-91, "Standard Test Method for Determination of Formaldehyde and Other Carbonyl Compounds in Air (Active Sampler Methodology)."
- (iv) Johnson J. H., Bagley, S. T., Gratz, L. D., and Leddy, D. G., "A Review of Diesel Particulate Control Technology and Emissions Effects—1992 Horning Memorial Award Lecture," SAE Technical Paper Series, SAE 940233, 1994.
- (v) Keith *et al.*, ACS Committee on Environmental Improvement, "Principles of Environmental Analysis," The Journal of Analytical Chemistry, Volume 55, pp. 2210-2218, 1983.
- (vi) Perez, J.M., Jabs, R.E., Leddy, D.G., eds. "Chemical Methods for the Measurement of Unregulated Diesel Emissions (CRC-APRAC Project No. CAPI-1-64), Coordinating Research Council, CRC Report No. 551, August, 1987.
- (vii) Schuetzle, D., "Analysis of Nitrated Polycyclic Aromatic Hydrocarbons in Diesel Particulates," Analytical Chemistry, Volume 54, pp. 265-271, 1982.
- (viii) Siegl, W.O., *et al.*, "Improved Emissions Speciation Methodology for Phase II of the Auto/Oil Air Quality Improvement Research Program—Hydrocarbons and Oxygenates", SAE Technical Paper Series, SAE 930142, 1993.
- (ix) Tejada, S. B. *et al.*, "Analysis of Nitroaromatics in Diesel and Gasoline Car Emissions," SAE Paper No. 820775, 1982.
- (x) Tejada, S. B. *et al.*, "Fluorescence Detection and Identification of Nitro Derivatives of Polynuclear Aromatic Hydrocarbons by On-Column Catalytic Reduction to Aromatic Amines," Analytical Chemistry, Volume 58, pp. 1827-1834, July 1986.
- (xi) "Test Method for Determination of C1-C4 Alcohols and MTBE in Gasoline by Gas Chromatography," 40 CFR part 80, appendix F.

(c) [Reserved]

(d) **Literature Search.**

- (1) Manufacturers of fuels and fuel additives shall conduct a literature search and compilation of information on the potential toxicologic, environmental, and other public welfare effects of the emissions of such fuels and additives. The literature search shall include all available relevant information from in-house, industry, government, and public sources pertaining to the emissions of the subject fuel or fuel additive or the emissions of similar fuels or additives, with such similarity determined according to the provisions of § 79.56.
- (2) The literature search shall address the potential adverse effects of whole combustion emissions, evaporative emissions, relevant emission fractions, and individual emission products of the subject fuel or fuel additive except as specified in the following paragraph. The individual emission products to be included are those identified pursuant to the emission characterization procedures specified in paragraph (b) of this section, other than carbon monoxide, carbon dioxide, nitrogen oxides, benzene, 1,3-butadiene, acetaldehyde, and formaldehyde.
- (3) In the case of the individual emission products of non-baseline or atypical fuels and additives (pursuant to § 79.56(e)(2)), the literature data need not be submitted for those emission products which are the same as the combustion emission products of the respective base fuel for the product's fuel family (pursuant to § 79.55). For this purpose, data on the base fuel emission products for the product's fuel family:

- (i) May be found in the literature of previously-conducted, adequate emission speciation studies for the base fuel, or for a fuel or additive/fuel mixture capable of grouping with the base fuel (see, for example, the references in paragraph (b)(5) of this section).
 - (ii) May be compiled while gathering internal control data during emissions characterization studies on the manufacturer's non-baseline or atypical product; or
 - (iii) May be obtained from various manufacturers in the course of their testing different additive(s) belonging to the same fuel family, or in the testing of a base fuel serving as representative of the baseline group for the respective fuel family.
- (e) **Data bases.** The literature search must include the results of searching appropriate commercially available chemical, toxicologic, and environmental databases. The databases shall be searched using, at a minimum, CAS numbers (when applicable), chemical names, and common synonyms.
- (f) **Search period.** The literature search shall cover a time period beginning at least thirty years prior to the date of submission of the reports specified in §§ 79.59(b) through (c) and ending no earlier than six months prior to the date on which testing is commenced or reports are submitted in compliance with this subpart.
- (g) **References.** Information on base fuel emission inventories may be found in references in paragraphs (b)(5)(i) through (xi) of this section, as well as in the following:
- (1) Auto/Oil Air Quality Improvement Research Program, Technical Bulletin #1, December 1990.
 - (2) Keith *et al.*, ACS Committee on Environmental Improvement, "Principles of Environmental Analysis," The Journal of Analytical Chemistry, Volume 55, pp. 2210-2218, 1983.
 - (3) "The Composition of Gasoline Engine Hydrocarbon Emissions—An Evaluation of Catalyst and Fuel Effects"—SAE 902074 and "Speciated Hydrocarbon Emissions from Aromatic, Olefin, and Paraffinic Model Fuels"—SAE 930373.

[59 FR 33093, June 27, 1994, as amended at 61 FR 36511, July 11, 1996; 62 FR 12571, Mar. 17, 1997]

§ 79.53 Tier 2.

- (a) **Generally.** Subject to the provisions of § 79.53(b) through (d), the combustion emissions of each fuel or fuel additive subject to testing under this subpart must be tested in accordance with each of the testing guidelines in §§ 79.60 through 79.68, except that fuels and additives in the methane and propane fuel families (pursuant to § 79.56(e)(1)(v) and (vi)) need not undergo the *Salmonella* mutagenicity assay in § 79.68). Similarly, subject to the provisions of § 79.53(b) through (d), the evaporative emissions of each designated evaporative fuel and each designated evaporative fuel additive subject to testing under this subpart must be tested according to each of the testing guidelines in §§ 79.60 through 79.67 (excluding § 79.68, *Salmonella typhimurium* Reverse Mutation Assay).
- (b) **Manufacturer Determination.** Manufacturers shall determine whether the information gathered pursuant to the literature search in § 79.52(d) contains the results of adequately performed and adequately documented previous testing which provides information reasonably comparable to that supplied by the health tests described in §§ 79.62 through 79.68 regarding the carcinogenicity, mutagenicity, neurotoxicity, teratogenicity, reproductive/fertility measures, and general toxicity effects of the emissions of the fuel or additive. When manufacturers make an affirmative determination, they need submit only the information gathered pursuant to § 79.52(d) for such tests. EPA maintains final authority in judging

whether the information is an adequate substitution in lieu of conducting the associated tests. EPA's determination of the adequacy of existing information shall be guided by the considerations described in paragraph (d) of this section. If EPA finds that the manufacturer has relied upon inadequate test data, then the manufacturer will not be considered to be in compliance until the corresponding tests have been conducted and the results submitted to EPA.

(c) **Testing.**

- (1) All testing required pursuant to this section must be done in accordance with the procedures, equipment, and facility requirements described in §§ 79.57, 79.60, and 79.61 regarding emissions generation, good laboratory practices, and inhalation exposure testing, respectively, as well as any other requirements described in this subpart. The laboratory conducting the animal studies shall be registered and in good standing with the United States Department of Agriculture and regularly inspected by United States Department of Agriculture veterinarians. In addition, the facility must be accredited by a generally recognized independent organization which sets laboratory animal care standards. Use of inadequate test protocols or substandard laboratory techniques in performing any testing required by this subpart may result in cancellation of all affected registrations.
- (2) Carcinogenic or mutagenic effects in animals from emissions exposures shall be determined pursuant to § 79.64 *In vivo* Micronucleus Assay, § 79.65 *In vivo* Sister Chromatid Exchange Assay, and § 79.68 *Salmonella typhimurium* Reverse Mutation Assay. Teratogenic effects and reproductive toxicity shall be examined pursuant to § 79.63 Fertility Assessment/Teratology. General toxicity and pulmonary effects shall be determined pursuant to § 79.62 Subchronic Toxicity Study with Specific Health Effect Assessments. Neurotoxic effects shall be determined pursuant to § 79.66 Neuropathology Assessment and § 79.67 Glial Fibrillary Acidic Protein Assay.

(d) **EPA Determination.**

- (1) After submission of all information and testing, EPA in its judgment shall determine whether previously conducted tests relied upon in the registration submission are adequately performed and documented and provide information reasonably comparable to that which would be provided by the tests described herein. Manufacturers' submissions shall be sufficiently detailed to allow EPA to judge the adequacy of protocols, techniques, experimental design, statistical analyses, and conclusions. Studies shall be performed using generally accepted scientific principles, good laboratory techniques, and the testing guidelines specified in these regulations.
- (2) EPA shall give appropriate weight when making this determination to the following factors:
 - (i) The age of the data;
 - (ii) The adequacy of documentation of procedures, findings, and conclusions;
 - (iii) The extent to which the testing conforms to generally accepted scientific principles and practices;
 - (iv) The type and number of test subjects;
 - (v) The number and adequacy of exposure concentrations, *i.e.*, emission dilutions;
 - (vi) The degree to which the tested emissions were generated by procedures and under conditions reasonably comparable to those set forth in § 79.57; and
 - (vii) The degree to which the test procedures conform to the testing guidelines set forth in §§ 79.60 through 79.68 and/or furnish information comparable to that provided by such testing.

- (3) The test animals shall be rodents, preferably a strain of rat, and testing shall include all of the endpoints covered in §§ 79.62 through 79.68. All studies shall be properly executed, with appropriate documentation, and in accord with the individual health testing guidelines (§§ 79.60 through 79.68) of this part, e.g., 90-day, 6-hour per day exposure, minimum.
- (4) In general, the data in a manufacturer's registration submittal shall be adequate if the duration of a test's exposure period is at least as long, in days and hours, as the inhalation exposure specified in the related health test guideline(s). Data from tests with shorter exposure durations than those specified in the guidelines may be acceptable if the test results are positive (i.e., exhibit adverse effects) and/or include a demonstrable concentration-response relationship.
- (5) Data in support of a manufacturer's registration submittal shall directly address the effects of inhalation exposure to the whole evaporative and exhaust emissions of the respective fuel or additive or to the whole evaporative and exhaust emissions of other fuels or additives which satisfy the criteria in § 79.56 for classification into the same group as the subject fuel or fuel additive. Data obtained in the testing of a raw liquid fuel or additive/base fuel mixture or a raw, aerosolized fuel or additive/base fuel mixture shall not be adequate to support a manufacturer's registration submittal. Data from testing of evaporative emissions cannot substitute for test data on combustion emissions. Data from testing of combustion emissions cannot substitute for test data on evaporative emissions.

§ 79.54 Tier 3.

(a) *General Criteria for Requiring Tier 3 Testing.*

- (1) Tier 3 testing shall be required of a manufacturer or group of manufacturers at EPA's discretion when remaining uncertainties as to the significance of observed health effects, welfare effects, and/or emissions exposures from a fuel or fuel/additive mixture interfere with EPA's ability to make reasonable estimates of the potential risks posed by emissions from the fuel or additive products. Tier 3 testing may be conducted either on an individual basis or a group basis. If performed on a group basis, EPA may require either the same representative to be used in Tier 3 testing as was used in Tier 2 testing or may select a different member or members of the group to represent the group in the Tier 3 tests.
- (2) In addition to the criteria specific to particular tests as summarized and detailed in the testing guidelines (§§ 79.62 through 79.68), EPA may consider a number of factors (including, but not limited to):
 - (i) The number of positive and negative outcomes related to each endpoint;
 - (ii) The identification of concentration-effect relationships;
 - (iii) The statistical sensitivity and significance of such studies;
 - (iv) The severity of the observed effects (e.g., whether the effects would be likely to lead to incapacitating or irreversible conditions);
 - (v) The type and number of species included in the reported tests;
 - (vi) The consistency and clarity of apparent mechanisms, target organs, and outcomes;
 - (vii) The presence or absence of effective health test control data for base-fuel-only versus additive/base fuel mixture comparisons;

- (viii) The nature and amount of known toxic agents in the emissions stream; and
- (ix) The observation of lesions which specifically implicate inhalation as an important exposure route.

(3) **Consideration of exposure.** EPA retains discretion to consider, in addition to available toxicity data, any Tier 1 data on potential exposures to emissions from a particular fuel or fuel additive (or group of fuels and/or fuel additives) in determining whether to require Tier 3 testing. EPA may consider, but is not limited to, the following factors:

- (i) Types and emission rates of speciated emission components;
- (ii) Types and emission rates of combinations of compounds or elements of concern;
- (iii) Historical and/or projected production volumes and market distributions; and
- (iv) Estimated population and/or environmental exposures obtained through extrapolation, modeling, or literature search findings on ambient, occupational, or epidemiological exposures.

(b) **Notice.**

- (1) EPA will determine whether Tier 3 testing is necessary upon receipt of a manufacturer's (or group's) submittal as prescribed under § 79.51(d). If EPA determines on the basis of the Tier 1 and 2 data submission and any other available information that further testing is necessary, EPA will require the responsible manufacturer(s) to conduct testing as described elsewhere in this section. EPA will notify the manufacturer (or group) by certified letter of the purpose and nature of any proposed testing and of the proposed deadline for completing the testing. A copy of the letter will be placed in the public record. EPA will provide the manufacturer a 60-day comment period after the manufacturer's receipt of such notice. EPA may extend the comment period if it appears from the nature of the issues raised that further discussion is warranted. In the event that no comment is received by EPA from the manufacturer (or group) within the comment period, the manufacturer (or group) shall be deemed to have consented to the adoption by EPA of the proposed Tier 3 requirements.
- (2) EPA will issue a notice in the FEDERAL REGISTER of its intent to require testing under Tier 3 for a particular fuel or additive manufacturer and that a copy of the letter to the manufacturer outlining the Tier 3 testing for that manufacturer is available in the public record for review and comment. The public shall have a minimum of thirty (30) days after the publication of this notice to comment on the proposed Tier 3 testing.
- (3) EPA will include in the public record a copy of any timely comments concerning the proposed Tier 3 testing requirements received from the affected manufacturer or group or from the public, and the responses of EPA to such comments. After reviewing all such comments received, EPA will adopt final Tier 3 requirements by sending a certified letter describing such final requirements to the manufacturer or group. EPA will also issue a notice in the FEDERAL REGISTER announcing that it has adopted such final Tier 3 requirements and that a copy of the letter adopting the requirements has been included in the public record.
- (4) Prior to beginning any required Tier 3 testing, the manufacturer shall submit detailed test protocols to EPA for approval. Once EPA has determined the Tier 3 testing requirements and approves the test protocols, any modification to the requirements shall be governed by § 79.51(f).

(c) **Carcinogenicity and Mutagenicity Testing.**

- (1) A potential need for Tier 3 carcinogenicity and/or mutagenicity testing may be indicated if the results of the *In vivo* Micronucleus Assay, required under § 79.64, the *In vivo* Sister Chromatid Exchange Assay, required under § 79.65, the Salmonella mutagenicity assay required under § 79.68, or relevant pathologic findings under § 79.62 demonstrate a statistically significant dose-related positive response as compared with appropriate controls. Alternatively, Tier 3 carcinogenicity testing and/or mutagenicity testing may be required if there are positive outcomes for at least one concentration in two or more of the tests required under §§ 79.64, 79.65, and 79.68.
- (2) The testing for carcinogenicity required under this paragraph may, at EPA's discretion, be conducted in accordance with 40 CFR 798.3300 or 798.3320, or their equivalents (see suggested references following each health effects testing guideline). The testing for mutagenicity required under this paragraph may likewise be conducted in accordance with 40 CFR 798.5195, 798.5500, 798.5955, 798.7100, and/or other suitable equivalent testing (see suggested references following each health effects testing guideline). EPA may supplement or modify guidelines as required to ensure that the prescribed testing addresses the identified areas of concern.

(d) ***Reproductive and Teratological Effects Testing.***

- (1) A potential need for Tier 3 testing may be indicated if the results of the Fertility Assessment/ Teratology study required under § 79.63 or relevant findings under § 79.62 demonstrate, in comparison with appropriate controls, a statistically significant dose-related positive response in one or more of the possible test outcomes. Similarly, Tier 3 testing may be indicated if statistically significant positive results are confined to either sex, or to the fetus as opposed to the pregnant adult.
- (2) The testing for reproductive and teratological effects required under this paragraph may, at EPA's discretion, be conducted in accordance with 40 CFR 798.4700 and/or by performance of a reproductive assay by continuous breeding. These guidelines may be modified or supplemented by EPA as required to ensure that the prescribed testing addresses the identified areas of concern.

(e) ***Neurotoxicity Testing.***

- (1) A potential need for Tier 3 neurotoxicity testing may be indicated if either the results of the Neuropathology Assessment required under § 79.67 shows concentration-related effects in exposed animals or the Glial Fibrillary Acidic Protein Assay required under § 79.66 demonstrates a statistically significant concentration-related positive response as compared with appropriate controls. Similarly, Tier 3 neurotoxicity testing may be indicated if relevant results under § 79.62 demonstrate a statistically significant positive response in comparison to appropriate controls.
- (2) The testing for neurotoxicity required under this paragraph may, at EPA's discretion, be conducted in accordance with 40 CFR 798.3260 and 40 CFR part 798 subpart G. These guidelines may be modified or supplemented by EPA as required to ensure that the prescribed testing addresses the identified areas of concern.

(f) ***General and Pulmonary Toxicity Testing.***

- (1) A potential need for Tier 3 general and/or pulmonary toxicity testing may be indicated if, in comparison with appropriate controls, the results of the Subchronic Toxicity Study, pursuant to § 79.62, demonstrate abnormal gross analysis or histopathological findings (especially as relates to lung pathology from whole-body preserved test animals) or persistence or delayed occurrence of toxic effects beyond the exposure period.

- (2) A potential need for Tier 3 testing with respect to other organ systems or endpoints not addressed by specific Tier 2 tests, e.g., hepatic, renal, or endocrine toxicity, may be demonstrated by findings in the Tier 2 Subchronic Toxicity Study (pursuant to § 79.62) or by findings in the Tier 1 literature search of adverse functional, physiologic, metabolic, or histopathologic effects of fuel or additive emissions to such other organ systems or any other information available to EPA. In addition, findings in the Tier 1 emission characterization of significant levels of a known toxicant to such other organ systems and endpoints may also indicate a need for relevant health effects testing. The testing required under this paragraph may include tests conducted in accordance with 40 CFR 798.3260 or 798.3320. These guidelines may be modified or supplemented by EPA as necessary to ensure that the prescribed testing addresses the identified areas of concern.
- (3) The testing for general/pulmonary toxicity required under this paragraph may, at EPA's discretion, be conducted in accordance with 40 CFR 798.2450 or 798.3260. These guidelines may be modified or supplemented by EPA as necessary to ensure that the prescribed testing addresses the identified areas of concern. Pulmonary function measurements, host defense assays, immunotoxicity tests, cell morphology/morphometry, and/or enzyme assays of lung lavage cells and fluids may be specifically required.

(g) Other Tier 3 Testing.

- (1) A manufacturer or group may be required to use up-to-date modeling, sampling, monitoring, and/or analytic approaches at the Tier 3 level to provide:
 - (i) Estimates of exposures to the emission products of a fuel or fuel additive or group of products;
 - (ii) The expected atmospheric transformation products of such emissions; and
 - (iii) The environmental partitioning of such emissions to the air, soil, water, and biota.
- (2) Additional emission characterization may be required if uncertainty over the identity of chemical species or rate of their emission interferes with reasonable judgments as to the presence and/or concentration of potentially toxic substances in the emissions of a fuel or fuel additive. The required tests may include characterization of additional classes of emissions, the characterization of emissions generated by additional vehicles/engines of various technology mixes (e.g., catalyzed versus non-catalyzed emissions), and/or other more precise analytic procedures for identification or quantification of emissions compounds. Additional emissions testing may also be required to evaluate concerns which may arise regarding the potential effects of a fuel or fuel additive on the performance of emission control equipment.
- (3) A manufacturer or group may be required to conduct biological and/or exposure studies at the Tier 3 level to evaluate directly the potential public welfare or environmental effects of the emissions of a fuel or additive, if significant concerns about such effects arise as a result of EPA's review of the literature search or emission characterization findings in Tier 1 or the results of the toxicological tests in Tier 2.
- (4) With regard to group submittals, Tier 3 studies on a fuel or additive product(s) other than the originally specified group representative may be required if specific differences in the product's composition indicate that its emissions may have different toxicologic properties from those of the original group representative.

- (5) Additional emission characterization and/or toxicologic tests may be required to evaluate the impact of different vehicle, engine, or emission control technologies on the observed composition or health or welfare effects of the emissions of a fuel or additive.
- (6) Toxicological tests on individual emission products may be required.
- (7) Upon review of information submitted for an aerosol product under § 79.58(e), emissions characterization, exposure, and/or toxicologic testing at a Tier 3 level may be required.
- (8) A manufacturer which qualifies for and has elected to use the special provisions for the products of small businesses (pursuant to § 79.58(d)) may be required to conduct emission characterization, exposure, and/or toxicologic studies at the Tier 3 level for such products, as specified in § 79.58(d)(4).
- (9) The examples of potential Tier 3 tests described in this section do not in any way limit EPA's broad discretion and authority under Tier 3.

§ 79.55 Base fuel specifications.

(a) *General Characteristics.*

- (1) The base fuel(s) in each fuel family shall serve as the group representative(s) for the baseline group(s) in each fuel family pursuant to § 79.56. Also, as specified in § 79.51(h)(1), for fuel additives undergoing testing, the designated base fuel for the respective fuel family shall serve as the substrate in which the additive shall be mixed prior to the generation of emissions.
- (2) Base fuels shall contain a limited complement of the additives which are essential for the fuel's production or distribution and/or for the successful operation of the test vehicle/engine throughout the mileage accumulation and emission generation periods. Such additives shall be used at the minimum effective concentration-in-use for the base fuel in question.
- (3) Unless otherwise restricted, the presence of trace contaminants does not preclude the use of a fuel or fuel additive as a component of a base fuel formulation.
- (4) When an additive is the test subject, any additive normally contained in the base fuel which serves the same function as the subject additive shall be removed from the base fuel formulation. For example, if a corrosion inhibitor were the subject of testing and if this additive were to be tested in a base fuel which normally contained a corrosion inhibitor, this test additive would replace the corrosion inhibitor normally included as a component of the base fuel.
- (5) Additive components of the methanol, ethanol, methane, and propane base fuels in addition to any such additives included below shall be limited to those recommended by the manufacturers of the vehicles and/or engines used in testing such fuels. For this purpose, EPA will review requests from manufacturers (or their agents) to modify the additive specifications for the alternative fuels and, if necessary, EPA shall change these specifications based on consistency of those changes with the associated vehicle manufacturer's recommendations for the operation of the vehicle. EPA shall publish notice of any such changes to a base fuel and/or its base additive package specifications in the FEDERAL REGISTER.

(b) *Gasoline Base Fuel.*

- (1)
- The gasoline base fuel is patterned after the reformulated gasoline summer baseline fuel as specified in CAA section 211(k)(10)(B)(i). The specifications and blending tolerances for the gasoline base fuel are listed in table F94-1. The additive types which shall be required and/or permissible in the gasoline base fuel are listed in table 1 as well.

TABLE F94-1—GASOLINE BASE FUEL PROPERTIES

API Gravity	57.4±0.3
Sulfur, ppm	339±25
Benzene, vol%	1.53±0.3
RVP, psi	8.7±0.3
Octane, (R + M)/2	87.3±0.5
Distillation Parameters:	
10%, °F	128±5
50%, °F	218±5
90%, °F	330±5
Aromatics, vol%	32.0±2.7
Olefins, vol%	9.2±2.5
Saturates, vol%	58.8±2.0
Additive Types:	
Required	Deposit Control Corrosion Inhibitor Demulsifier Anti-oxidant Metal Deactivator
Permissible	Anti-static

- (2)
- The additive components of the gasoline base fuel shall contain compounds comprised of no elements other than carbon, hydrogen, oxygen, nitrogen, and sulfur. Additives shall be used at the minimum concentration needed to perform effectively in the gasoline base fuel. In no case shall their concentration in the base fuel exceed the maximum concentration recommended by the additive manufacturer. The increment of sulfur contributed to the formulation by any additive shall not exceed 15 parts per million sulfur by weight and shall not cause the gasoline base fuel to exceed the sulfur specifications in table F94-1 of this section.

(c) Diesel Base Fuel.

- (1) The diesel base fuel shall be a #2 diesel fuel having the properties and blending tolerances shown in table F94-2 of this section. The additive types which shall be permissible in diesel base fuel are presented in table F94-2 as well.

TABLE F94-2—DIESEL BASE FUEL PROPERTIES

API Gravity	33±1
Sulfur, wt%	0.05±0.0025
Cetane Number	45.2±2
Cetane Index	45.7±2
Distillation Parameters:	
10%, °F	433±5
50%, °F	516±5
90%, °F	606±5
Aromatics, vol%	38.4±2.7
Olefins, vol%	1.5±0.4
Saturates, vol%	60.1±2.0
Additive Types:	
Required	Corrosion Inhibitor Demulsifier Anti-oxidant Metal Deactivator
Permitted	Anti-static Flow Improver
Not Permitted	Deposit Control

- (2) The additive components of the diesel base fuel shall contain compounds comprised of no elements other than carbon, hydrogen, oxygen, nitrogen, and sulfur. Additives shall be used at the minimum concentration needed to perform effectively in the diesel base fuel. In no case shall their concentration in the base fuel exceed the maximum concentration recommended by the additive manufacturer. The increment of sulfur contributed to the base fuel by additives shall not cause the diesel base fuel to exceed the sulfur specifications in table F94-2 of this section.

(d) **Methanol Base Fuels.**

- (1) The methanol base fuels shall contain no elements other than carbon, hydrogen, oxygen, nitrogen, sulfur, and chlorine.
- (2) The M100 base fuel shall consist of 100 percent by volume chemical grade methanol.
- (3) The M85 base fuel is to contain 85 percent by volume chemical grade methanol, blended with 15 percent by volume gasoline base fuel meeting the gasoline base fuel specifications outlined in paragraph (b)(1) of this section. Manufacturers shall ensure the methanol compatibility of lubricating oils as well as fuel additives used in the gasoline portion of the M85 base fuel.

- (4) The methanol base fuels shall meet the specifications listed in table F94-3.

TABLE F94-3—METHANOL BASE FUEL PROPERTIES

M100:	
Chemical Grade MeOH, vol%	100
Chlorine (as chlorides), wt%, max	0.0001
Water, wt%, max	0.5
Sulfur, wt%, max	0.002
M85	
Chemical Grade MeOH, vol%,	85
Gasoline Base Fuel, vol%	15
Chlorine (as chlorides), wt%, max	0.0001
Water, wt%, max	0.5
Sulfur, wt%, max	0.004

(e) **Ethanol Base Fuel.**

- (1) The ethanol base fuel, E85, shall contain no elements other than carbon, hydrogen, oxygen, nitrogen, sulfur, chlorine, and copper.
- (2) The ethanol base fuel shall contain 85 percent by volume chemical grade ethanol, blended with 15 percent by volume gasoline base fuel that meets the specifications listed in paragraph (b)(1) of this section. Additives used in the gasoline component of E85 shall be ethanol-compatible.
- (3) The ethanol base fuel shall meet the specifications listed in table F94-4.

TABLE F94-4—ETHANOL BASE FUEL PROPERTIES

E85:	
Chemical Grade EtOH, vol%, min	85
Gasoline Base Fuel, vol%	15
Chlorine (as chloride), wt%, max	0.0004
Copper, mg/L, max	0.07
Water, wt%, max	0.5
Sulfur, wt%, max	0.004

(f) **Methane Base Fuel.**

- (1) The methane base fuel is a gaseous motor vehicle fuel marketed commercially as compressed natural gas (CNG), whose primary constituent is methane.
- (2) The methane base fuel shall contain no elements other than carbon, hydrogen, oxygen, nitrogen, and sulfur. The fuel shall contain an odorant additive for leak detection purposes. The added odorant shall be used at a level such that, at ambient conditions, the fuel must have a distinctive odor potent enough for its presence to be detected down to a concentration in air of not over $\frac{1}{5}$ (one-fifth) of the lower limit of flammability. After addition of the odorant, the methane base fuel shall contain no more than 16 ppm sulfur by volume.
- (3) The methane base fuel shall meet the specifications listed in table F94-5.

TABLE F94-5—METHANE BASE FUEL SPECIFICATIONS

Methane, mole%, min	89.0
Ethane, mole%, max	4.5
Propane and higher HC, mole%, max	2.3
C6 and higher HC, mole%, max	0.2
Oxygen, mole%, max	0.6
Sulfur (including odorant additive) ppmv, max	16
Inert gases:	
Sum of CO ₂ and N ₂ , mole%, max	4.0

(g) *Propane Base Fuel.*

- (1) The propane base fuel is a gaseous motor vehicle fuel, marketed commercially as liquified petroleum gas (LPG), whose primary constituent is propane.
- (2) The propane base fuel may contain no elements other than carbon, hydrogen, oxygen, nitrogen, and sulfur. The fuel shall contain an odorant additive for leak detection purposes. The added odorant shall be used at a level such that at ambient conditions the fuel must have a distinctive odor potent enough for its presence to be detected down to a concentration in air of not over $\frac{1}{5}$ (one-fifth) of the lower limit of flammability. After addition of the odorant, the propane base fuel shall contain no more than 120 ppm sulfur by weight.
- (3) The propane base fuel shall meet the specifications listed in table F94-6.

TABLE F94-6—PROPANE BASE FUEL SPECIFICATIONS

Vapor pressure at 100-F, psig, max	208
Evaporative temperature, 95%, °F, max	-37

Propane, vol%, min	92.5
Propylene, vol%, max	5.0
Butane and heavier, vol%, max	2.5
Residue-evaporation of 100mL, max, mL	0.05
Sulfur (including odorant additive) ppmw, max	123

§ 79.56 Fuel and fuel additive grouping system.

- (a) Manufacturers of fuels and fuel additives are allowed to satisfy the testing requirements in §§ 79.52, 79.53, and 79.54 and the associated reporting requirements in § 79.59 on an individual or group basis, provided that such products meet the criteria in this section for enrollment in the same fuel/additive group. However, each manufacturer of a fuel or fuel additive must individually comply with the notification requirements of § 79.59(b). Further, if a manufacturer elects to comply by participation in a group, each manufacturer continues to be individually subject to the information requirements of this subpart.
- (1) The use of the grouping provision to comply with Tier 1 and Tier 2 testing requirements is voluntary. No manufacturer is prohibited from testing and submitting its own data for its own product registration, despite its qualification for membership in a particular group.
- (2) The only groups permitted are those established in this section.
- (b) Each manufacturer who chooses to enroll a fuel or fuel additive in a group of similar fuels and fuel additives as designated in this section may satisfy the registration requirements through a group submission of jointly-sponsored testing and analysis conducted on a product which is representative of all products in that group, provided that the group representative is chosen according to the specifications in this section.
- (1) The health effects information submitted by a group shall be considered applicable to all fuels and fuel additives in the group. A fuel or fuel additive manufacturer who has chosen to participate in a group may subsequently choose to perform testing of such fuel or fuel additive on an individual basis; however, until such independent registration information has been received and reviewed by EPA, the information initially submitted by the group on behalf of the manufacturer's fuel or fuel additive shall be considered applicable and valid for that fuel or fuel additive. It could therefore be used to support requirements for further testing under the provisions of Tier 3 or to support regulatory decisions affecting that fuel or fuel additive.
- (2) Manufacturers are responsible for determining the appropriate groups for their products according to the criteria in this section and for enrolling their products into those groups under industry-sponsored or other independent brokering arrangements.
- (3) Manufacturers who enroll a fuel or fuel additive into a group shall share the applicable costs according to appropriate arrangements established by the group. The organization and administration of group functions and the development of cost-sharing arrangements are the responsibility of the participating manufacturers. If manufacturers are unable to agree on fair and equitable cost sharing arrangements and if such dispute is referred by one or more manufacturers to EPA for resolution, then the provisions in § 79.56(c) (1) and (2) shall apply.

- (c) In complying with the registration requirements for a given fuel or fuel additive, notwithstanding the enrollment of such fuel or additive in a group, a manufacturer may make use of available information for any product which conforms to the same grouping criteria as the given product. If, for this purpose, a manufacturer wishes to rely upon the information previously submitted by another manufacturer (or group of manufacturers) for registration of a similar product (or group of products), then the previous submitter is entitled to reimbursement by the manufacturer for an appropriate portion of the applicable costs incurred to obtain and report such information. Such entitlement shall remain in effect for a period of fifteen years following the date on which the original information was submitted. Pursuant to § 79.59(b)(4)(ii), the manufacturer who relies on previously-submitted registration data shall certify to EPA that the original submitter has been notified and that appropriate reimbursement arrangements have been made.
- (1) When private efforts have failed to resolve a dispute about a fair amount or method of cost-sharing or reimbursement for testing costs incurred under this subpart, then any party involved in that dispute may initiate a hearing by filing two signed copies of a request for a hearing with a regional office of the American Arbitration Association and mailing a copy of the request to EPA. A copy must also be sent to each person from whom the filing party seeks reimbursement or who seeks reimbursement from that party. The information and fees to be included in the request for hearing are specified in 40 CFR 791.20(b) and (c).
- (2) Additional procedures and requirements governing the hearing process are those specified in 40 CFR 791.22 through 791.50, 791.60, 791.85, and 791.105, excluding 40 CFR 791.39(a)(3) and 791.48(d).
- (d) **Basis for classification.**
- (1) Rather than segregating fuels and fuel additives into separate groups, the grouping system applies the same grouping criteria and creates a single set of groups applicable both to fuels and fuel additives.
- (2) Fuels shall be classified pursuant to § 79.56(e) into categories and groups of similar fuels and fuel additives according to the components and characteristics of such fuels in their uncombusted state. The classification of a fuel product must take into account the components of all bulk fuel additives which are listed in the registration application or basic registration data submitted for the fuel product.
- (3) Fuel additives shall be classified pursuant to § 79.56(e) into categories and groups of similar fuels and fuel additives according to the components and characteristics of the respective uncombusted additive/base fuel mixture pursuant to § 79.51(h)(1).
- (4) In determining the category and group to which a fuel or fuel additive belongs, impurities present in trace amounts shall be ignored unless otherwise noted. Impurities are those substances which are present through contamination or which remain in the fuel or additive naturally after processing is completed.
- (5) **Reference standards.**
- (i) American Society for Testing and Materials (ASTM) standard D 4814-93a, "Standard Specification for Automotive Spark-Ignition Engine Fuel", used to define the general characteristics of gasoline fuels (paragraph (e)(3)(i)(A)(3) of this section) and ASTM standard D 975-93, "Standard Specification for Diesel Fuel Oils", used to define the general characteristics of diesel fuels (paragraph (e)(3)(ii)(A)(3) of this section) have been incorporated by reference.

(ii) 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 obtained from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103. 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.

(e) **Grouping criteria.** The grouping system is represented by a matrix of three fuel/additive categories within six specified fuel families (see table F94-7, Grouping System for Fuels and Fuel Additives). Each category may include one or more groups. Within each group, a representative may be designated based on the criteria in this section and joint registration information may be developed and submitted for member fuels and fuel additives.

TABLE F94-7—GROUPING SYSTEM FOR FUELS AND FUEL ADDITIVES

Category	Conventional Fuel Families		Alternative Fuel Families			
	Gasoline (A)	Diesel (B)	Methanol (C)	Ethanol (D)	Methane (CNG, LNG) (E)	Propane (LPG) (F)
Baseline	One group represented by gasoline base fuel	One group represented by diesel base fuel	Two groups: (1) M100 group (includes methanol-gasoline formulations with at least 96% methanol) represented by M100 base fuel (2) M85 (includes methanol-gasoline formulations with 50-95% methanol) represented by M85 base fuel	One group (includes ethanol-gasoline formulations with at least 50% ethanol) represented by E85 base fuel	One group (includes both CNG and LNG), represented by CNG base fuel	One group represented by LPG base fuel.
Non-baseline	One group for each gasoline-oxygenate	One group for each oxygen-contributing	One group for each individual non-	One group for each individual non-	One group to include methane	One group to include propane

Category	Conventional Fuel Families		Alternative Fuel Families			
	Gasoline (A)	Diesel (B)	Methanol (C)	Ethanol (D)	Methane (CNG, LNG) (E)	Propane (LPG) (F)
Atypical	blend or each gasoline-methanol/co-solvent blend; one group for each synthetic crude-derived fuel	compound or class of compounds; one group for each synthetic crude-derived fuel	methanol, non-gasoline component and one group for each unique combination of such components	ethanol, non-gasoline component and one group for each unique combination of such components	formulations exceeding the specified limit for non-methane hydrocarbons	formulations exceeding the specified limit for butane and higher hydrocarbons.
	One group for each atypical element/characteristic, or unique combination of atypical elements/characteristics	One group for each atypical element/characteristic, or unique combination of atypical elements/characteristics	One group for each atypical element/characteristic, or unique combination of atypical elements/characteristics	One group for each atypical element/characteristic, or unique combination of atypical elements/characteristics	One group for each atypical element/characteristic, or unique combination of atypical elements/characteristics	One group for each atypical element/characteristic, or unique combination of atypical elements/characteristics.

- (1) **Fuel families.** Each of the following six fuel families (Table F94-7, columns A-F) includes fuels of the type referenced in the name of the family as well as bulk and aftermarket additives which are intended for use in those fuels. When applied to fuel additives, the criteria in these descriptions refer to the associated additive/base fuel mixture, pursuant to § 79.51(h)(1). One or more base fuel formulations are specified for each fuel family pursuant to § 79.55.
- (i) The Gasoline Family includes fuels composed of more than 50 percent gasoline by volume and their associated fuel additives. The base fuel for this family is specified in § 79.55(b).
 - (ii) The Diesel Family includes fuels composed of more than 50 percent diesel fuel by volume and their associated fuel additives. The Diesel fuel family includes both Diesel #1 and Diesel #2 formulations. The base fuel for this family is specified in § 79.55(c).
 - (iii) The Methanol Family includes fuels composed of at least 50 percent methanol by volume and their associated fuel additives. The M100 and M85 base fuels are specified in § 79.55(d).
 - (iv) The Ethanol Family includes fuels composed of at least 50 percent ethanol by volume and their associated fuel additives. The base fuel for this family is E85 as specified in § 79.55(e).
 - (v) The Methane Family includes compressed natural gas (CNG) and liquefied natural gas (LNG) fuels containing at least 50 mole percent methane and their associated fuel additives. The base fuel for the family is a CNG formulation specified in § 79.55(f).

- (vi) The Propane Family includes propane fuels containing at least 50 percent propane by volume and their associated fuel additives. The base fuel for this family is a liquefied petroleum gas (LPG) as specified in § 79.55(g).
- (vii) A manufacturer seeking registration for formulation(s) which do not fit the criteria for inclusion in any of the fuel families described in this section shall contact EPA at the address in § 79.59(a)(1) for further guidance in classifying and testing such formulation(s).
- (2) **Fuel/additive categories.** Fuel/additive categories (Table F94-7, rows 1-3) are subdivisions of fuel families which represent the degree to which fuels and fuel additives in the family resemble the base fuel(s) designated for the family. Three general category types are defined in this section. When applied to fuel additives, the criteria in these descriptions refer to the associated additive/base fuel mixture, pursuant to § 79.51(h)(1).
 - (i) Baseline categories consist of fuels and fuel additives which contain no elements other than those permitted in the base fuel for the respective fuel family and conform to specified limitations on the amounts of certain components or characteristics applicable to that fuel family.
 - (ii) Non-Baseline Categories consist of fuels and fuel additives which contain no elements other than those permitted in the base fuel for the respective fuel family, but which exceed one or more of the limitations for certain specified components or characteristics applicable to baseline formulations in that fuel family.
 - (iii) Atypical Categories consist of fuels and fuel additives which contain elements or classes of compounds other than those permitted in the base fuel for the respective fuel family or which otherwise do not meet the criteria for either baseline or non-baseline formulations in that fuel family. A fuel or fuel additive product having both non-baseline and atypical characteristics pursuant to § 79.56(e)(3), shall be considered to be an atypical product.
- (3) This section defines the specific categories applicable to each fuel family. When applied to fuel additives, the criteria in these descriptions refer to the associated additive/base fuel mixture, pursuant to § 79.51(h)(1).
 - (i) **Gasoline Categories.**
 - (A) The Baseline Gasoline category contains gasoline fuels and associated additives which satisfy all of the following criteria:
 - (1) Contain no elements other than carbon, hydrogen, oxygen, nitrogen, and/or sulfur.
 - (2) Contain less than 1.5 percent oxygen by weight.
 - (3) Sulfur concentration is limited to 1000 ppm per the specifications cited in the following paragraph.
 - (4) Possess the physical and chemical characteristics of unleaded gasoline as specified by ASTM standard D 4814-93a (incorporated by reference, pursuant to paragraph (d)(5) of this section), in at least one Seasonal and Geographical Volatility Class.
 - (5) Derived only from conventional petroleum, heavy oil deposits, coal, tar sands, and/or oil sands.

- (B) The Non-Baseline Gasoline category is comprised of gasoline fuels and associated additives which conform to the specifications in paragraph (e)(3)(i)(A) of this section for the Baseline Gasoline category except that they contain 1.5 percent or more oxygen by weight and/or may be derived from sources other than those listed in paragraph (e)(3)(i)(A)(5) of this section.
- (C) The Atypical Gasoline category is comprised of gasoline fuels and associated additives which contain one or more elements other than carbon, hydrogen, oxygen, nitrogen, and sulfur.

(ii) ***Diesel Categories.***

- (A) The Baseline Diesel category is comprised of diesel fuels and associated additives which satisfy all of the following criteria:
 - (1) Contain no elements other than carbon, hydrogen, oxygen, nitrogen, and/or sulfur. Pursuant to 40 CFR 80.29, highway diesel sold after October 1, 1993 shall contain 0.05 percent or less sulfur by weight;
 - (2) Contain less than 1.0 percent oxygen by weight;
 - (3) Diesel formulations containing more than 0.05 percent sulfur by weight are precluded by 40 CFR 80.29;
 - (4) Possess the characteristics of diesel fuel as specified by ASTM standard D 975-93 (incorporated by reference, pursuant to paragraph (d)(5) of this section); and
 - (5) Derived only from conventional petroleum, heavy oil deposits, coal, tar sands, and/or oil sands.
- (B) The Non-Baseline Diesel category is comprised of diesel fuels and associated additives which conform to the specifications in paragraph (e)(3)(ii)(A) of this section for the Baseline Diesel category except that they contain 1.0 percent or more oxygen by weight and/or may be derived from sources other than those listed in paragraph (e)(3)(ii)(A)(5) of this section.
- (C) The Atypical Diesel category is comprised of diesel fuels and associated additives which contain one or more elements other than carbon, hydrogen, oxygen, nitrogen, and sulfur.

(iii) ***Methanol categories.***

- (A) The Baseline Methanol category is comprised of methanol fuels and associated additives which contain at least 50 percent methanol by volume, no more than 4.0 percent by volume of substances other than methanol and gasoline, and no elements other than carbon, hydrogen, oxygen, nitrogen, sulfur, and/or chlorine. Baseline methanol shall contain no more than 0.004 percent by weight of sulfur or 0.0001 percent by weight of chlorine.
- (B) The Non-Baseline Methanol category is comprised of fuel blends which contain at least 50 percent methanol by volume, more than 4.0 percent by volume of a substance(s) other than methanol and gasoline, and meet the baseline limitations on elemental composition in paragraph (e)(3)(iii)(A) of this section.

- (C) The Atypical Methanol category consists of methanol fuels and associated additives which do not meet the criteria for either the Baseline or the Non-Baseline Methanol category.

(iv) *Ethanol categories.*

- (A) The Baseline Ethanol category is comprised of ethanol fuels and associated additives which contain at least 50 percent ethanol by volume, no more than five (5) percent by volume of substances other than ethanol and gasoline, and no elements other than carbon, hydrogen, oxygen, nitrogen, sulfur, chlorine, and copper. Baseline ethanol formulations shall contain no more than 0.004 percent by weight of sulfur, 0.0004 percent by weight of chlorine, and/or 0.07 mg/L of copper.
- (B) The Non-Baseline Ethanol category is comprised of fuel blends which contain at least 50 percent ethanol by volume, more than five (5) percent by volume of a substance(s) other than ethanol and gasoline, and meet the baseline limitations on elemental composition in paragraph (e)(3)(iv)(A) of this section.
- (C) The Atypical Ethanol category consists of ethanol fuels and associated additives which do not meet the criteria for either the Baseline or the Non-Baseline Ethanol categories.

(v) *Methane categories.*

- (A) The Baseline Methane category is comprised of methane fuels and associated additives (including at least an odorant additive) which contain no elements other than carbon, hydrogen, oxygen, nitrogen, and/or sulfur, and contain no more than 20 mole percent non-methane hydrocarbons. Baseline methane formulations shall not contain more than 16 ppm by volume of sulfur, including any sulfur which may be contributed by the odorant additive.
- (B) The Non-Baseline Methane category consists of methane fuels and associated additives which conform to the specifications in paragraph (e)(3)(v)(A) of this section for the Baseline Methane category except that they exceed 20 mole percent non-methane hydrocarbons.
- (C) The Atypical Methane category consists of methane fuels and associated additives which contain one or more elements other than carbon, hydrogen, oxygen, nitrogen, and/or sulfur, or exceed 16 ppm by volume of sulfur.

(vi) *Propane categories.*

- (A) The Baseline Propane category is comprised of propane fuels and associated additives (including at least an odorant additive) which contain no elements other than carbon, hydrogen, oxygen, nitrogen, and/or sulfur, and contain no more than 20 percent by volume non-propane hydrocarbons. Baseline Propane formulations shall not contain more than 123 ppm by weight of sulfur, including any sulfur which may be contributed by the odorant additive.
- (B) The Non-Baseline Propane category consists of propane fuels and associated additives which conform to the specifications in paragraph (e)(3)(vi)(A) of this section for the Baseline Propane category, except that they exceed the 20 percent by volume limit for butane and higher hydrocarbons.

- (C) The Atypical Propane category consists of propane fuels and associated additives which contain elements other than carbon, hydrogen, oxygen, nitrogen, and/or sulfur, or exceed 123 ppm by weight of sulfur.

(4) **Fuel/additive groups.** Fuel/additive groups are subdivisions of the fuel/additive categories. One or more group(s) are defined within each category in each fuel family according to the presence of differing characteristics in the fuel or additive/base fuel mixture. For each group, one formulation (either a base fuel or a member fuel or additive product) is chosen to represent all the member products in the group in any tests required under this subpart. The section which follows describes the fuel/additive groups.

(i) **Baseline groups.**

- (A) The Baseline Gasoline category comprises a single group. The gasoline base fuel specified in § 79.55(b) shall serve as the representative of this group.
- (B) The Baseline Diesel category comprises a single group. The diesel base fuel specified in § 79.55(c) shall serve as the representative of this group.
- (C) The Baseline Methanol category includes two groups: M100 and M85. The M100 group consists of methanol-gasoline formulations containing at least 96 percent methanol by volume. These formulations must contain odorants and bitterants (limited in elemental composition to carbon, hydrogen, oxygen, nitrogen, sulfur, and chlorine) for prevention of purposeful or inadvertent consumption. The M100 base fuel specified in § 79.55(d) shall serve as the representative for this group. The M85 group consists of methanol-gasoline formulations containing at least 50 percent by volume but less than 96 percent by volume methanol. The M85 base fuel specified in § 79.55(d) shall serve as the representative of this group.
- (D) The Baseline Ethanol category comprises a single group. The E85 base fuel specified in § 79.55(e) shall serve as the representative of this group.
- (E) The Baseline Methane category comprises a single group. The CNG base fuel specified in § 79.55(f) shall serve as the representative of this group.
- (F) The Baseline Propane category comprises a single group. The LPG base fuel specified in § 79.55(g) shall serve as the representative of this group.

(ii) **Non-baseline groups —**

- (A) **Non-Baseline Gasoline.** The Non-Baseline gasoline fuels and associated additives shall sort into groups according to the following criteria:
 - (1) For gasoline fuel and additive products which contain 1.5 percent oxygen by weight or more, a separate non-baseline gasoline group shall be defined by each oxygenate compound or methanol/co-solvent blend listed as a component in the registration application or basic registration data of any such fuel or additive.
 - (i) Examples of oxygenates occurring in non-baseline gasoline formulations include ethanol, methyl tertiary butyl ether (MTBE), ethyl tertiary butyl ether (ETBE), tertiary amyl methyl ether (TAME), diisopropyl ether (DIPE), dimethyl

ether (DME), tertiary amyl ethyl ether (TAE), and any other compound(s) which increase the oxygen content of the gasoline formulation. A separate non-baseline gasoline group is defined for each such oxygenating compound.

(ii) Each unique methanol and co-solvent combination (whether one, two, or more additional oxygenate compounds) used in a non-baseline fuel shall also define a separate group. An oxygenate compound used as a co-solvent for methanol in a non-baseline gasoline formulation must be identified as such in its registration. If the oxygenate is not identified as a methanol co-solvent, then the compound shall be regarded by EPA as defining a separate non-baseline gasoline group. Examples of methanol/co-solvent combinations occurring in non-baseline gasoline formulations include methanol/isopropyl alcohol, methanol/butanol, and methanol with alcohols up to C8/octanol (Octamix).

(iii) For each such group, the representative to be used in testing shall be a formulation consisting of the gasoline base fuel blended with the relevant oxygenate compound (or methanol/co-solvent combination) in an amount equivalent to the highest actual or recommended concentration-in-use of the oxygenate (or methanol/co-solvent combination) recorded in the basic registration data of any member fuel or additive product. In the event that two or more products in the same group contain the same and highest amount of the oxygenate or methanol/co-solvent blend, then the representative shall be chosen at random for such candidate products.

(2) An oxygenate compound or methanol/co-solvent combination to be blended with the gasoline base fuel for testing purposes shall be chemical-grade quality, at a minimum, and shall not contain a significant amount of other contaminating oxygenate compounds.

(3) Separate non-baseline gasoline groups shall also be defined for gasoline formulations derived from each particular petroleum source not listed in paragraph (e)(3)(i)(A)(5) of this section.

(i) Such groups may include, but are not limited to, those derived from shale, used oil, waste plastics, and other recycled chemical/petrochemical products.

(4) Pursuant to § 79.51(i), non-baseline gasoline products may belong to more than one fuel/additive group.

(B) **Non-Baseline Diesel.** The Non-Baseline diesel fuels and associated additives shall sort into groups according to the following criteria:

(1) For diesel fuel and additive products which contain 1.0 percent or more oxygen by weight in the form of alcohol(s) and/or ether(s):

(i) A separate non-baseline diesel group shall be defined by each individual alcohol or ether listed as a component in the registration application or basic registration data of any such fuel or additive.

- (ii) For each such group, the representative to be used in testing shall be a formulation consisting of the diesel base fuel blended with the relevant alcohol or ether in an amount equivalent to the highest actual or recommended concentration-in-use of the alcohol or ether recorded in the basic registration data of any member fuel or additive product.
- (2) A separate non-baseline diesel group is also defined for each of the following classes of oxygenating compounds: mixed nitroso-compounds; mixed nitro-compounds; mixed alkyl nitrates; mixed alkyl nitrites; peroxides; furans; mixed alkyl esters of plant and/or animal origin (biodiesel). For each such group, the representative to be used in testing shall be formulated as follows:
 - (i) From the class of compounds which defines the group, a particular oxygenate compound shall be chosen from among all such compounds recorded in the registration application or basic registration data of any fuel or additive in the group.
 - (ii) The selected compound shall be the one recorded in any member product's registration application with the highest actual or recommended maximum concentration-in-use.
 - (iii) In the event that two or more oxygenate compounds in the relevant class have the highest recorded concentration-in-use, then the oxygenate compound to be used in the group representative shall be chosen at random from the qualifying candidate compounds.
 - (iv) The compound thus selected shall be the group representative, and shall be used in testing at the following concentration:
 - (A) For biodiesel groups, the representative shall be 100 percent biodiesel fuel.
 - (B) Otherwise, the group representative shall be the selected compound mixed into diesel base fuel at the maximum recommended concentration-in-use.
- (3) Separate non-baseline diesel groups shall also be defined for diesel formulations derived from each particular petroleum source not listed in paragraph (e)(3)(i)(A)(5) of this section.
 - (i) Such groups may include, but are not limited to, those derived from shale, used oil, waste plastics, and other recycled chemical/petrochemical products.
 - (ii) In any such group, the first product to be registered or to apply for EPA registration shall be the representative of that group. If two or more products are registered or apply for first registration simultaneously, then the representative shall be chosen by a random method from among such candidate products.
- (4) Pursuant to § 79.51(i), non-baseline diesel products may belong to more than one fuel/additive group.

- (C) **Non-baseline methanol.** The Non-Baseline methanol formulations are sorted into groups based on the non-methanol, non-gasoline component(s) of the blended fuel. Each such component occurring separately and each unique combination of such components shall define a separate group.
 - (1) The representative of each such non-baseline methanol group shall be the group member with the highest percent by volume of non-methanol, non-gasoline component(s).
 - (2) In case two or more such members have the same and highest concentration of non-methanol, non-gasoline component(s), the representative of the group shall be chosen at random from among such equivalent member products.
- (D) **Non-Baseline Ethanol.** The Non-Baseline ethanol formulations are sorted into groups based on the non-ethanol, non-gasoline component(s) of the blended fuel. Each such component occurring separately and each unique combination of such components shall define a separate group.
 - (1) The representative of each such non-baseline ethanol group shall be the group member with the highest percent by volume of non-ethanol, non-gasoline component(s).
 - (2) In case two or more such members have the same and highest concentration of non-ethanol, non-gasoline component(s), the representative of the group shall be chosen at random from among such equivalent member products.
- (E) **Non-Baseline Methane.** The Non-Baseline methane category consists of one group. The group representative shall be the member fuel or fuel/additive formulation containing the highest concentration-in-use of non-methane hydrocarbons. If two or more member products have the same and the highest concentration-in-use, then the representative shall be chosen at random from such products.
- (F) **Non-baseline propane.** The Non-Baseline propane category consists of one group. The group representative shall be the member fuel or fuel/additive formulation containing the highest concentration-in-use of butane and higher hydrocarbons. If two or more products have the same and the highest concentration-in-use, then the representative shall be chosen at random from such products.

(iii) **Atypical groups.**

- (A) As defined for each individual fuel family in § 79.56(e)(3), fuels and additives meeting any one of the following criteria are considered atypical.
 - (1) Gasoline Atypical fuels and additives contain one or more elements in addition to carbon, hydrogen, oxygen, nitrogen, and sulfur.
 - (2) Diesel Atypical fuels and additives contain one or more element in addition to carbon, hydrogen, oxygen, nitrogen, and sulfur.
 - (3) Methanol Atypical fuels and additives contain:
 - (i) one or more element in addition to carbon, hydrogen, oxygen, nitrogen, sulfur, and chlorine, and/or

- (ii) sulfur in excess of 0.004 percent by weight, and/or
 - (iii) chlorine in excess of 0.0001 percent by weight.
 - (4) Ethanol Atypical fuels and additives contain:
 - (i) one or more element in addition to carbon, hydrogen, oxygen, nitrogen, sulfur, chlorine, and copper, and/or
 - (ii) sulfur in excess of 0.004 percent by weight, and/or
 - (iii) contain chlorine (as chloride) in excess of 0.0004 percent by weight, and/or
 - (iv) contain copper in excess of 0.07 mg/L.
 - (5) Methane Atypical fuels and additives contain:
 - (i) one or more element in addition to carbon, hydrogen, oxygen, nitrogen, and sulfur, and/or
 - (ii) sulfur in excess of 16 ppm by volume.
 - (6) Propane Atypical fuels and additives contain:
 - (i) one or more element in addition to carbon, hydrogen, oxygen, nitrogen, and sulfur, and/or
 - (ii) sulfur in excess of 123 ppm by weight.
- (B) General rules for sorting these atypical fuels and additives into separate groups are as follows:
 - (1) Pursuant to § 79.51(j), a given atypical product may belong to more than one atypical group.
 - (2) Fuels and additives in different fuel families may not be grouped together, even if they contain the same atypical element(s) or other atypical characteristic(s).
 - (3) A fuel or additive containing one or more atypical elements attached to a polymer compound must be sorted into a separate group from atypical fuels or fuel additives containing the same atypical element(s) in non-polymer form. However, the occurrence of a polymer compound which does not contain an atypical element does not affect the grouping of a fuel or additive.
- (C) Specific rules for sorting each family's atypical fuels and additives into separate groups, and for choosing each such group's representative for testing, are as follows:
 - (1) A separate group is created for each atypical element (or other atypical characteristic) occurring separately, i.e., in the absence of any other atypical element or characteristic, in one or more fuels and/or additives within a given fuel family.
 - (i) Consistent with the basic grouping guidelines provided in § 79.56(d), a fuel product which is classified as atypical because its basic registration data or application lists a bulk additive containing an atypical characteristic, may be grouped with that additive and/or with other fuels and additives containing the same atypical characteristic.

- (ii) Within a group of products containing only one atypical element or characteristic, the fuel or additive/base fuel mixture with the highest concentration-in-use or recommended concentration-in-use of the atypical element or characteristic shall be the designated representative of that group. In the event that two or more fuels or additive/base fuel mixtures within the group contain the same and highest concentration of the single atypical element or characteristic, then the group representative shall be selected by a random method from among such candidate products.
- (2) A separate group is also created for each unique combination of atypical elements (and/or other specified atypical characteristics) occurring together in one or more fuels and/or additives within a given fuel family.
 - (i) Consistent with the basic grouping guidelines provided in § 79.56(d), a fuel which is classified as atypical because its basic registration data lists one bulk additive containing two or more atypical characteristics, may be grouped with that additive and/or with other fuels and/or additives containing the same combination of atypical characteristics. Grouping of fuels containing more than one atypical additive shall be guided by provisions of § 79.51(j).
 - (ii) Within a group of such products containing a unique combination of two or more atypical elements or characteristics, the designated representative shall be the product within the group which contains the highest total concentration of the atypical elements or characteristics.
 - (iii) In the event that two or more products within a given atypical group contain the same and highest concentration of the same atypical elements or characteristics then, among such candidate products, the designated representative shall be the product which, first, has the highest total concentration of metals, followed in order by highest total concentration of halogens, highest total concentration of other atypical elements (including sulfur concentration, as applicable), highest total concentration of polymers containing atypical elements, and, lastly, highest total concentration of oxygen.
 - (iv) If two or more products have the same and highest concentration of the variable identified in the preceding paragraph, then, among such products, the one with the greatest concentration of the next highest variable on the list shall be the group representative.
 - (v) This decision-making process shall continue until a single product is determined to be the representative. If two or more products remain tied at the end of this process, then the representative shall be chosen by a random method from among such remaining products.

[59 FR 33093, June 27, 1994, as amended at 62 FR 12571, Mar. 17, 1997]

§ 79.57 Emission generation.

This section specifies the equipment and procedures that must be used in generating the emissions which are to be subjected to the characterization procedures and/or the biological tests specified in §§ 79.52(b) and 79.53 of these regulations. When applicable, they may also be required in conjunction with testing under §§ 79.54 and 79.58(c). Additional requirements concerning emission generation, delivery, dilution, quality control, and safety practices are outlined in § 79.61.

(a) *Vehicle and engine selection criteria.*

- (1) All vehicles and engines used to generate emissions for testing a fuel or additive/fuel mixture must be new (i.e., never before titled) and placed into the program with less than 500 miles on the odometer or 12 hours on the engine chronometer. The vehicles and engines shall be unaltered from the specifications of the original equipment manufacturer.
- (2) The vehicle/engine type, vehicle/engine class, and vehicle/engine subclass designated to generate emissions for a given fuel or additive shall be the same type, class, and subclass which, over the previous three years, has consumed the most gallons of fuel in the fuel family applicable to the given fuel or additive. No distinction shall be made between light-duty vehicles and light-duty trucks for purposes of this classification.
- (3) Within this vehicle/engine type, class, and subclass, the specific vehicles and engines acceptable for emission generation are those that represent the most common fuel metering system and the most common of the most important emission control system devices or characteristics with respect to emission reduction performance for the model year in which testing begins. These vehicles will be determined through a survey of the previous model year's vehicle/engine sales within the given subclass. These characteristics shall include, but need not be limited to, aftertreatment device(s), fuel aspiration, air injection, exhaust gas recirculation, and feedback type.
- (4) Within the applicable subclass, the five highest selling vehicle/engine models that contain the most common such equipment and characteristics shall be determined. Any of these five models of the current model year (at the time testing begins) may be selected for emission generation.
 - (i) If one or more of the five models is not available for the current model year, the choice of model for emission generation shall be limited to those remaining among the five.
 - (ii) If fewer than five models of the given vehicle/engine type are available for the current model year, all such models shall be eligible.
- (5) When the fuel or fuel additive undergoing testing is not commonly used or intended to be used in the vehicle/engine types prescribed by this selection procedure, or when rebuilding or alteration is required to obtain a suitable vehicle/engine for emission generation, the manufacturer may submit a request to EPA for a modification in test procedure requirements. Any such request must include objective test results which support the claim that a more appropriate vehicle/engine type is needed as well as a suggested substitute vehicle/engine type. The vehicle/engine selection in this case shall be approved by EPA prior to the start of testing.
- (6) Once a particular model has been chosen on which to test a fuel or additive product, all mileage accumulation and generation of emissions for characterization and biological testing of such product shall be conducted on that same model.

- (i) If the initial test vehicle/engine fails or must be replaced for any reason, emission generation shall continue with a second vehicle/engine which is identical to, or resembles to the greatest extent possible, the initial test vehicle/engine. If more than one replacement vehicle/engine is necessary, all such vehicles/engines shall be identical, or resemble to the greatest extent possible, the initial test vehicle/engine.
- (ii) Manufacturers are encouraged to obtain, at the start of a test program, more than one emission generation vehicle/engine of the identical model, to ensure the availability of back-up emission generator(s). All backup vehicles/engines must be conditioned and must have their emissions fully characterized, as done for the initial test vehicle/engine, prior to their use as emission generators for biological testing. Alternating between such vehicles/engines regularly during the course of testing is permissible and advisable, particularly to allow regular maintenance on such vehicles/engines during prolonged health effects testing.

(b) ***Vehicle/engine operation and maintenance.***

- (1) For the purpose of generating combustion emissions from a fuel or additive/base fuel mixture for which the relevant class is light duty, either a light-duty vehicle shall be operated on a chassis dynamometer or a light-duty engine shall be operated on an engine dynamometer. When the relevant class is heavy duty, the emissions shall be generated on a heavy-duty engine operated on an engine dynamometer. In both cases, the vehicle or engine model shall be selected as described in paragraph (a) of this section and shall have all applicable fuel and emission control systems intact.
- (2) Except as provided in § 79.51(h)(2)(iii), the fuel or additive/base fuel mixture being tested shall be used at all times during operation of the test vehicle or engine. No other fuels or additives shall be used in the test vehicle or engine once mileage accumulation has begun until emission generation for emission characterization and biological testing purposes is completed.
 - (i) A vehicle or engine may be used to generate emissions for the testing of more than one fuel or additive, provided that all such fuels and additives belong to the same fuel family pursuant to § 79.56(e)(i), and that, once a vehicle or engine has been used to generate emissions for an atypical fuel or additive (pursuant to § 79.56(e)(2)(iii)), it shall not be used in the testing of any other fuel or additive. Paragraphs (a) (2) and (3) of this section shall apply only to the first fuel or additive tested.
 - (ii) Prior to being used to generate emissions for testing an additional fuel or additive, a vehicle or engine which has previously been used for testing a different fuel or additive shall undergo an effective intermediate preconditioning cycle to remove the previously used fuel and its emissions from the vehicle's fuel and exhaust systems and from the combustion emission and evaporative emission control systems, if any.
 - (iii) Such preconditioning shall include, at a minimum, the following steps:
 - (A) The canister (if any) shall be removed from the vehicle and purged with 300 °F nitrogen at 20 liters per minute until the incremental weight loss of the canister is less than 1 gram in 30 minutes. This typically takes 3-4 hours and removes 100 to 120 grams of adsorbed gasoline vapors.
 - (B) The fuel tank shall be drained and filled to capacity with the new test fuel or additive/fuel mixture.

- (C) The vehicle or engine shall be operated until at least 95% of the fuel tank capacity is consumed.
 - (D) The purged canister shall be returned to the vehicle.
 - (E) The fuel tank shall be drained and filled to 40% capacity with test fuel.
 - (F) Two-hour fuel tank heat builds from 72-120 °F shall be performed repeatedly as necessary to achieve canister breakthrough. The fuel tank must be drained and filled prior to each heat build.
- (3) Scheduled and unscheduled vehicle/engine maintenance.
- (i) During emission generation, vehicles and engines must be maintained in good condition by following the recommendations of the original equipment manufacturer (OEM) for scheduled service and parts replacement, with repairs performed only as necessary. Modifications, adjustments, and maintenance procedures contrary to procedures found in 40 CFR part 86 for the maintenance of test vehicles/engines or performed solely for the purpose of emissions improvement are not allowed.
 - (ii) If unscheduled maintenance becomes necessary, the vehicle or engine must be repaired to OEM specifications, using OEM or OEM-approved parts. In addition, the tester is required to measure the basic emissions pursuant to § 79.52(b)(2)(i) after the unscheduled maintenance and before resuming testing to ensure that the post-maintenance emissions shall be within 20 percent of pre-maintenance emissions levels. If the basic emissions cannot be brought within 20 percent of their previous levels, then the manufacturer shall restart the emissions characterization and health testing of its products combustion emissions using a new vehicle/engine.

(c) **Mileage accumulation.**

- (1) A vehicle/engine break-in period is required prior to generating emissions for characterization and/or biological testing under this subpart. The required mileage accumulation may be accomplished on a test track, on the street, on a dynamometer, or using any other conventionally accepted method.
- (2) Vehicles to be used in the evaluation of baseline and non-baseline fuels and fuel additives shall accumulate 4,000 miles prior to emission testing. Engines to be used in the evaluation of baseline and non-baseline fuels and fuel additives shall accumulate 125 hours of operation on an engine dynamometer prior to emission testing.
- (3) When the test formulation is classified as an atypical fuel or fuel additive formulation (pursuant to definitions in § 79.56(e)(4)(iii)), the following additional mileage accumulation requirements apply:
 - (i) The test vehicle/engine must be operated for a minimum of 4,000 vehicle miles or 125 hours of engine operation.
 - (ii) Thereafter, at intervals determined by the tester, all emission fractions (*i.e.*, vapor, semi-volatile, and particulate) shall be sampled and analyzed for the presence and amount of the atypical element(s) and/or other atypical constituents. Pursuant to paragraph (d) of this section, the sampled emissions must be generated in the absence of an intact aftertreatment device. Immediately before the samples are taken, a brief warmup period (at least ten miles or the engine equivalent) is required.

- (iii) Mileage accumulation shall continue until either 50 percent or more of the mass of each atypical element (or other atypical constituent) entering the engine can be measured in the exhaust emissions (all fractions combined), or the vehicle/engine has accumulated mileage (or hours) equivalent to 40 percent of the average useful life of the applicable vehicle/engine class (pursuant to regulations in 40 CFR part 86). For example, the maximum mileage required for light-duty vehicles is 40 percent of 100,000 miles (*i.e.*, 40,000 miles), while the maximum time of operation for heavy-duty engines is the equivalent of 40 percent of 290,000 miles (*i.e.*, the equivalent in engine hours of 116,000 miles).
- (iv) When either condition in paragraph (c)(3)(iii) of this section has been reached, additional emission characterization and biological testing of the emissions may begin.

(d) *Use of exhaust aftertreatment devices.*

- (1) If the selected test vehicle/engine, as certified by EPA, does not come equipped with an emissions aftertreatment device (such as a catalyst or particulate trap), such device shall not be used in the context of this program.
- (2) Except as provided in paragraph (d)(3) of this section for certain specialized additives, the following provisions apply when the test vehicle/engine, as certified by EPA, comes equipped with an emissions aftertreatment device.
 - (i) For mileage accumulation:
 - (A) When the test formulation does not contain any atypical elements (pursuant to definitions in § 79.56(e)(4)(iii)), an intact aftertreatment device must be used during mileage accumulation.
 - (B) When the test formulation does contain atypical elements, then the manufacturer may choose to accumulate the required mileage using a vehicle/engine equipped with either an intact aftertreatment device or with a non-functional aftertreatment device (*e.g.*, a blank catalyst without its catalytic wash coat). In either case, sampling and analysis of emissions for measurement of the mass of the atypical element(s) (as described in § 79.57(c)(3)) must be done on emissions generated with a non-functional (blank) aftertreatment device.
 - (1) If the manufacturer chooses to accumulate mileage without a functional aftertreatment device, and if the manufacturer wishes to do this outside of a laboratory/test track setting, then a memorandum of exemption for product testing must be obtained by applying to the Director of the Field Operations and Support Division (see § 79.59(a)(1)).
 - (2) [Reserved]
 - (ii) For Tier 1 (§ 79.52), the total set of requirements for the characterization of combustion emissions (§ 79.52(b)) must be completed two times, once using emissions generated with the aftertreatment device intact and a second time with the aftertreatment device rendered nonfunctional or replaced with a non-functional aftertreatment device as described in paragraph (d)(2)(i)(B) of this section.
 - (iii) For Tier 2 (§ 79.53), the standard requirements for biological testing of combustion emissions shall be conducted using emissions generated with a non-functioning aftertreatment device as described in paragraph (d)(2)(i)(B) of this section.

- (iv) For alternative Tier 2 requirements (§ 79.58(c)) or Tier 3 requirements (§ 79.54) which may be prescribed by EPA, the use of functional or nonfunctional aftertreatment devices shall be specified by EPA as part of the test guidelines.
- (v) In the case where an intact aftertreatment device is not in place, all other manufacturer-specified combustion characteristics (e.g., back pressure, residence time, and mixing characteristics) of the altered vehicle/engine shall be retained to the greatest extent possible.
- (3) Notwithstanding paragraphs (d)(1) and (d)(2) of this section, when the subject of testing is a fuel additive specifically intended to enhance the effectiveness of exhaust aftertreatment devices, the related aftertreatment device may be used on the emission generation vehicle/engine during all mileage accumulation and testing.

(e) *Generation of combustion emissions* —

(1) *Generating combustion emissions for emission characterization.*

- (i) Combustion emissions shall be generated according to the exhaust emission portion of the Federal Test Procedure (FTP) for the certification of new motor vehicles, found in 40 CFR part 86, subpart B for light-duty vehicles/engines, and subparts D, M and N for heavy-duty vehicles/engines. The Urban Dynamometer Driving Schedule (UDDS), pursuant to 40 CFR part 86, appendix I(a), shall apply to light-duty vehicles/engines and the Engine Dynamometer Driving Schedule (EDS), pursuant to 40 CFR part 86, appendix I(f)(2), shall apply to heavy-duty vehicles/engines. The motoring portion of the heavy-duty test cycle may be eliminated, at the manufacturer's option, for the generation of emissions.
 - (A) For light-duty engines operated on an engine dynamometer, the tester shall determine the speed-torque equivalencies ("trace") for its test engine from valid FTP testing performed on a chassis dynamometer, using a test vehicle with an engine identical to that being tested. The test engine must then be operated under these speed and torque specifications to simulate the FTP cycle.
 - (B) Special procedures not included in the FTP may be necessary in order to characterize emissions from fuels and fuel additives containing atypical elements or to collect some types of emissions (e.g., particulate emissions from light-duty vehicles/engines, semi-volatile emissions from both light-duty and heavy-duty vehicles/engines). Such alterations to the FTP are acceptable.
 - (C) For Tier 2 testing, the engines shall operate on repeated bags 2 and 3 of the UDDS or back to back repeats of the heavy-duty transient cycle of the EDS.
- (ii) Pursuant to § 79.52(b)(1)(i) and § 79.57(d)(2)(ii), emission generation and characterization must be repeated three times when the selected vehicle/engine is normally operated without an emissions aftertreatment device and six times when the selected vehicle/engine is normally operated with an emissions aftertreatment device. In the latter case, the emission generation and characterization process shall be repeated three times with the intact aftertreatment device in place and three times with a non-functioning (blank) aftertreatment device in place.

- (iii) From both light-duty and heavy-duty vehicles/engines, samples of vapor phase, semi-volatile phase, and particulate phase emissions shall be collected, except that semi-volatile phase, and particulate emissions need not be sampled for fuels and additives in the methane and propane families (pursuant to § 79.56(e)(1)(v) and (vi)). The number and type of samples to be collected and separately analyzed during one emission generation/characterization process are as follows:
 - (A) In the case of combustion emissions generated from light-duty vehicles/engines, the samples consist of three bags of vapor emissions (one from each segment of the light-duty exhaust emission cycle) plus one sample of particulate-phase emissions and one sample of semi-volatile-phase emissions (collected over all segments of the exhaust emission cycle). If the mass of particulate emissions or semi-volatile emissions obtained during one driving cycle is not sufficient for characterization, up to three driving cycles may be performed and the extracted fractions combined prior to chemical analysis. Particulate-phase emissions shall not be combined with semi-volatile-phase emissions. The test laboratory should focus on the characterization of the limit of detection for particulates and semi-volatile emissions.
 - (B) In the case of combustion emissions generated from heavy-duty engines, the samples consist of one sample of each emission phase (vapor, particulate, and semi-volatile) collected over the entire cold-start cycle and a second sample of each such phase collected over the entire hot-start cycle (see 40 CFR 86.334 through 86.342).
- (iv) *Emission collection and storage.*
 - (A) Vapor phase emissions shall be collected and stored in Tedlar bags for subsequent chemical analysis. Storage conditions are specified in § 79.52(b)(2).
 - (B) Particulate phase emissions shall be collected on a particulate filter (or more than one, if required) using methods described in 40 CFR 86.1301 through 86.1344. These methods, ordinarily applied only to heavy-duty emissions, are to be adapted and used for collection of particulates from light-duty vehicles/engines, as well. The particulate matter may be stored on the filter in a sealed container, or the soluble organic fraction may be extracted and stored in a separate sealed container. Both the particulate and the extract shall be shielded from ultraviolet light and stored at -20 °C or less. Particulate emissions shall be tested no later than six months from the date they were generated.
 - (C) Semi-volatile emissions shall be collected immediately downstream from the particulate collection filters using porous polymer resin beds, or their equivalent, designed for their capture. The soluble organic fraction of semi-volatile emissions shall be extracted immediately and tested within six months of being generated. The extract shall be stored in a sealed container which is shielded from ultraviolet light and stored at -20 °C or less.
 - (D) Particulate and semi-volatile phase emission collection, handling and extraction methods shall not alter the composition of the collected material, to the extent possible.
 - (v) Additional requirements for combustion emission sampling, storage, and characterization are specified in § 79.52(b).

(2) *Generating whole combustion emissions for biological testing.*

- (i) Biological tests requiring whole combustion emissions shall be conducted using emissions generated from the test vehicle or engine operated in accordance with general FTP requirements.
- (ii) Light-duty test vehicles/engines shall be repeatedly operated over the Urban Dynamometer Driving Schedule (UDDS) (or equivalent engine dynamometer trace, per paragraph (e)(1)(i)(A) of this section) and heavy-duty test engines shall be repeatedly operated over the Engine Dynamometer Schedule (EDS) (see 40 CFR part 86, appendix I).
 - (A) The tolerances of the driving cycle shall be two times those of the Federal Test Procedure and must be met 95 percent of the time.
 - (B) The UDDS or EDS shall be repeated as many times as required for the biological test session.
 - (C) Light-duty dynamometers shall be calibrated prior to the start of a biological test (40 CFR 86.118-78), verified weekly (40 CFR 86.118-78), and recalibrated as required. Heavy-duty dynamometers shall be calibrated and checked prior to the start of a biological test (40 CFR 86.1318-84), recalibrated every two weeks (40 CFR 86.1318-84(a)) and checked as stated in 40 CFR 86.1318-84(b) and (c).
 - (D) The fuel reservoir for the test vehicle/engine shall be large enough to operate the test vehicle/engine throughout the daily biological exposure period, avoiding the need for refueling during testing.
- (iii) An apparatus to integrate the large concentration swings typical of transient-cycle exhaust is to be used between the source of emissions and the exposure chamber containing the animal test cages(s). The purpose of such apparatus is to decrease the variability of the biological exposure atmosphere and achieve the necessary concentration of CO or NO_x, whichever is limiting.
 - (A) A large mixing chamber is suggested for this purpose. The mixing chamber would be charged from the CVS at a constant rate determined by the exposure chamber purge rate. Flow to the exposure chamber would begin at the conclusion of the initial transient cycle with the associated mixing chamber charge.
 - (B) A potential alternative apparatus is a mini-diluter (see, for example, AIGER/CRADA, February, 1994 in § 79.57(g)).
 - (C) [Reserved]
- (iv) **Emission dilution.**
 - (A) Dilution air can be pre-dried to lower the relative humidity, thus permitting a lower dilution rate and a higher concentration of hydrocarbons to be achieved without condensation of water vapor.
 - (B) These procedures include requirements that the mean exposure concentration in the inhalation test chamber on 90 percent or more of the exposure days shall be controlled as follows:
 - (1) If the species being controlled is hydrocarbon or particulate, the mean exposure concentration must be within 15 percent of the target concentration for the single species being controlled.

- (2) For other species, the mean exposure concentration must be within 10 percent of the target concentration for the single species being controlled.
 - (3) For all species, daily monitoring of CO, CO₂, NO_x, SO_x, and total hydrocarbons in the exposure chamber shall be required. Analysis of the particle size distribution shall also be performed to establish the stability and consistency of particle size distribution in the test exposure.
 - (C) After the initial exhaust dilution to preserve the character of the exhaust, the exhaust stream can be further diluted in the mixing chamber (and/or after leaving the chamber) to achieve the desired biological exposure concentrations.
- (v) **Verification procedures.**
- (A) The entire system used to dilute and transport whole combustion emissions (*i.e.*, from exhaust pipe to outlet in the biological testing chamber) shall be verified before any animal exposures begin, and verified at least weekly during testing. (See procedures at 40 CFR 86.119-90 for light-duty vehicles and § 86.1319-90 for heavy-duty engines.) Verification testing shall be accomplished by introducing a known sample at the end of the vehicle/engine exhaust pipe into the dilution system and measuring the amount exiting the system. For example, an injected hydrocarbon sample could be detected with a gas chromatograph (GC) and flame ionization detector (FID) to determine the recovery factor.
 - (B) [Reserved]
- (vi) **Emission exposure quality control.**
- (A) The tester shall incorporate the additional quality assurance and safety procedures outlined in § 79.61(d) to control variability of emissions during the generation of exposure emissions during health effect testing.
 - (B) These procedures include requirements that the mean exposure concentration in the inhalation test chamber on 90 percent or more of the exposure days shall be controlled as follows:
 - (1) If the species being controlled is hydrocarbon or particulate, the mean exposure concentration must be within 15 percent of the target concentration for the single species being controlled.
 - (2) For other species, the mean exposure concentration must be within 10 percent of the target concentration for the single species being controlled.
 - (3) For all species, daily monitoring of CO, CO₂, NO_x, SO_x, and total hydrocarbons in the exposure chamber shall be required. Analysis of the particle size distribution shall also be performed to establish the stability and consistency of particle size distribution in the test exposure.
 - (C) The testing facility shall allow an audit of its premises, the qualifications, *e.g.*, curriculum vitae, of its staff assigned to testing, and the specimens and records of the testing for registration purposes (as specified in § 79.60).

- (vii) To allow for customary laboratory scheduling and unforeseen problems affecting the combustion emission generation or dilution equipment, biological exposures may be interrupted on limited occasions, as specified in § 79.61(d)(5). Interruptions exceeding these limitations shall cause the affected test(s) to be void. Testers shall be aware of concerns for backup vehicles/engines cited in paragraph (a)(7)(ii) of this section.

(3) ***Generating particulate and semi-volatile emissions for biological testing.***

- (i) Salmonella mutagenicity testing, pursuant to § 79.68, shall be conducted on extracts of the particulate and semi-volatile emission phases separately. These emissions shall be generated by operating the test vehicle/engine over the appropriate FTP driving schedule (see paragraph (e)(2)(ii) of this section) and collected and analyzed according to methods described in 40 CFR 86.1301 through 1344 (further information on this subject may be found in Perez, *et al.* CRC Report No. 551, 1987 listed in § 79.57(g)).
 - (A) Particulate emissions shall be collected on particulate filters and extracted from the collection equipment for use in biological tests. The number of repetitions of the applicable driving schedule required to collect sufficient quantities of the particulate emissions will vary, depending on the characteristics of the engine, the test fuel, and the requirements of the biological test protocol. The particulate sample may be collected on one or more filters, as necessary.
 - (B) Semi-volatile emissions shall be collected immediately downstream from the particulate collection filters using porous polymer resin beds, or their equivalent, designed for their capture. Semi-volatile phase emissions shall be collected on one apparatus. The time spent collecting sufficient quantities of the test substances in emissions samples will vary, depending on the emission characteristics of the engine and fuel or additive/base fuel mixture and on the requirements of the biological test protocol.
- (ii) The extraction method shall be determined by the specifications of the biological test for which the emissions are used.
- (iii) Particulate and semi-volatile emission storage requirements are as specified in § 79.57(e)(1)(iv).
- (iv) Particulate and semi-volatile phase emission collection, handling and extraction methods shall not alter the composition of the collected material, to the extent possible.
- (v) Particulate emissions shall not be combined with semi-volatile phase emissions.

(f) ***Generation of evaporative emissions for characterization and biological testing.***

- (1) Except as provided in paragraph (f)(5) of this section, an evaporative emissions generator shall be used to volatilize samples of a fuel or additive/base fuel mixture for evaporative emissions characterization and biological testing. Emissions shall be collected and sampled using equipment and methods appropriate for use with the compounds being characterized and the requirements of the emission characterization analysis. In the case of potentially explosive test substance concentrations, care must be taken to avoid generating explosive atmospheres. The tester is referred to § 79.61(d)(8) for considerations involving explosivity.

- (2) **Evaporative Emissions Generator (EEG) Description.** An EEG is a fuel tank or vessel to which heat is applied causing a portion of the fuel to evaporate at a desired rate. The manufacturer has flexibility in designing an EEG for testing a particular fuel or fuel additive. The sample used to generate emissions in the EEG shall be renewed at least daily.
- (i) The evaporation chamber shall be made from materials compatible with the fuels and additives being tested and shall be equipped with a drain.
 - (ii) The chamber shall be filled to 40 ± 5 percent of its interior volume with the fuel or additive/base fuel mixture being tested, with the remainder of the volume containing air.
 - (iii) The concentration of the evaporated fuel or additive/base fuel mixture in the vapor space of the evaporation chamber during the time emissions are being withdrawn for testing shall not vary by more than 10 percent from the equilibrium concentration in the vapor space of emissions generated from the fresh fuel or additive/base fuel mixture in the chamber.
 - (A) During the course of a day's emission generation period, the level of fuel in the EEG shall be maintained to within 7 percent of its height at the start of the daily exposure period.
 - (B) The fuel used in the EEG shall be drained at the end of each daily exposure. The EEG shall be refilled with a fresh supply of the test formulation before the start of each daily exposure.
 - (C) The vapor space of the evaporation chamber shall be well mixed throughout the time emissions are being withdrawn for testing.
 - (iv) The size of the evaporation chamber shall be determined by the rate at which evaporative emissions shall be needed in the test animal exposure chambers and the rate at which the fuel or the additive/base fuel mixture evaporates. The rate of evaporative emissions may be adjusted by altering the size of the EEG or by using one or more additional EEG(s). Emission rate modifications shall not be adjusted by temperature control or pressure control.
 - (v) The temperature of the fuel or additive/base fuel mixture in the evaporation chamber shall be $130 \text{ }^{\circ}\text{F} \pm 5 \text{ }^{\circ}\text{F}$. The vapors shall maintain this temperature up to the point in the system where the vapors are diluted.
 - (vi) The pressure in the vapor space of the evaporation chamber and the dilution and sampling apparatus shall stay within 10 percent of ambient atmospheric pressure.
 - (vii) There shall be no controls or equipment on the evaporation chamber system that change the concentration or composition of the vapors generated for testing.
 - (viii) Manufacturers shall perform verification testing of evaporative emissions in a manner analogous to the verification testing performed for combustion emissions.
- (3) For biological testing, vapor shall be withdrawn from the EEG at a constant rate, diluted with air as required for the particular study, and conducted immediately to the biological testing chamber(s) in a manner similar to the method used in § 79.57(e), excluding the mixing chamber therein. The rate of emission generation shall be high enough to supply the biological exposure chamber with sufficient emissions to allow for a minimum of fifteen air changes per exposure chamber per hour. To allow for customary laboratory scheduling and for unforeseen problems with the evaporative emission

generation or dilution equipment, biological exposures may be interrupted on limited occasions, as specified in § 79.61(d)(5). Interruptions exceeding these limitations shall cause the affected test(s) to be void.

- (4) For characterization of evaporative emissions, samples of equilibrated emissions to the vapor space of the EEG shall be withdrawn into Tedlar bags, then stored and analyzed as specified in § 79.52(b).
- (5) A manufacturer (or group of manufacturers) may submit to EPA a request for approval of an alternative method of generating evaporative emissions for use in emission characterization and biological tests required under this subpart.

- (i) To be approved by EPA, the request must fully explain the rationale for the proposed method as well as the technical procedures, quality control, and safety precautions to be used, and must demonstrate that the proposed method will meet the following criteria:

- (A) The emission mixture generated by the proposed procedures must be reasonably similar to the equilibrium composition of the vapor which occurs in the vehicle fuel tank head space when the subject fuel or additive/base fuel mixture is in use and near-maximum in-use temperatures are encountered.

- (B) The emissions mixture generated by the proposed method must be sufficiently concentrated to provide adequate exposure levels in the context of the required toxicologic tests.

- (C) The proposed method must include procedures to ensure that the emissions delivered to the biologic exposure chambers will provide a reasonably constant exposure atmosphere over time.

- (ii) If EPA approves the request, EPA will place in the public record a copy of the request, together with all supporting procedural descriptions and justifications, and will notify the public of its availability by publishing a notice in the FEDERAL REGISTER.

(g) **References.** For additional background information on the emission generation procedures outlined in this paragraph (g), the following references may be consulted. Additional references can be found in § 79.61(f).

- (1) AIGER/CRADA (American Industry/Government Emissions Research Cooperative Research and Development Agreement, "Specifications for Advanced Emissions Test Instrumentation" AIGER PD-94-1, Revision 5.0, February, 1994
- (2) Black, F. and R. Snow, "Constant Volume Sampling System Water Condensation" SAE #940970 in "Testing and Instrumentation" SP-1039, Society of Automotive Engineers, Feb. 28-Mar. 3, 1994.
- (3) Perez, J.M., Jass, R.E., Leddy, D.G., eds. "Chemical Methods for the Measurement of Unregulated Diesel Emissions (CRC-APRAC Project No. CAPI-1-64), Coordinating Research Council, CRC Report No. 551, August, 1987.
- (4) Phalen, R.F., "Inhalation Studies: Foundations and Techniques", CRC Press, Inc., Boca Raton, Florida, 1984.

[59 FR 33093, June 27, 1994, as amended at 61 FR 36511, July 11, 1996; 63 FR 63792, Nov. 17, 1998]

§ 79.58 Special provisions.

- (a) **Relabeled Additives.** Sellers of relabeled additives (pursuant to § 79.50) are not required to comply with the provisions of § 79.52, 79.53 or 79.59, except that such sellers are required to comply with § 79.59(b).
- (b) **Low Vapor Pressure Fuels and Additives.** Fuels which are not designated as “evaporative fuels” and fuel additives which are not designated as “evaporative fuel additives” pursuant to the definitions in § 79.50 need not undergo the emission characterization or health effects testing specified in §§ 79.52 and 79.53 for evaporative emissions. At EPA's discretion, the evaporative emissions of such fuels and additives may be required to undergo Tier 3 testing, pursuant to § 79.54.
- (c) **Alternative Tier 2 Provisions.** At EPA's discretion, EPA may modify the standard Tier 2 health effects testing requirements for a fuel or fuel additive (or group). Such modification may encompass substitution, addition, or deletion of Tier 2 studies or study specifications, and/or changes in underlying engine or equipment requirements, except that a Tier 2 endpoint will not be deleted in the absence of existing information deemed adequate by EPA or alternative testing requirements for such endpoint. If warranted by the particular requirements, EPA will allow additional time for completion of the alternative Tier 2 testing program.
 - (1) When EPA intends to require testing in lieu of or in addition to standard Tier 2 health testing, EPA will notify the responsible manufacturer (or group) by certified letter of the specific tests which EPA is proposing to require in lieu of or in addition to Tier 2, and the proposed schedule for completion and submission of such tests. A copy of the letter will be placed in the public record. EPA intends to send the notification prior to November 27, 1995, or in the case of new fuels and additives (as defined in § 79.51(c)(3)), within 18 months of EPA's receipt of an intent to register such product. However, EPA's notification to the manufacturer (or group) may occur at any time up to EPA's receipt of Tier 2 data for the product(s) in question. EPA will provide the manufacturer with 60 days from the date of receipt of the notice to comment on the tests which EPA is proposing to require and on the proposed schedule. If the manufacturer believes that undue costs or hardships will occur as a result of EPA's delay in providing notification of alternative Tier 2 requirements, then the manufacturer's comments should describe and include evidence of such hardship. In particular, if the standard Tier 2 toxicology testing for the fuel or additive in question has already begun at the time the manufacturer receives EPA's notification of proposed alternative Tier 2 requirements, then EPA shall refrain from requiring alternative Tier 2 tests provided that EPA receives the standard Tier 2 data and report (pursuant to § 79.59(c)) within one year of the date on which the toxicology testing began.
 - (2) EPA will issue a notice in the FEDERAL REGISTER announcing its intent to require special testing in lieu of or in addition to the standard Tier 2 testing for a particular fuel or additive manufacturer or group, and that a copy of the letter to the manufacturer or group describing the proposed alternative Tier 2 testing for that manufacturer or group is available in the public record for review and comment. The public shall have a minimum of 30 days after the publication of this notice to comment on the proposed alternative Tier 2 testing.
 - (3) EPA will include in the public record a copy of any timely comments concerning the proposed alternative Tier 2 testing requirements received from the affected manufacturer or group or from the public, and the responses of EPA to such comments. After reviewing all such comments received, EPA may adopt final alternative Tier 2 requirements by sending a certified letter describing such final requirements to the manufacturer or group. In that event, EPA will also issue a notice in the FEDERAL REGISTER announcing that it has adopted final alternative Tier 2 requirements and that a copy of the letter adopting the requirements has been included in the public record.

- (4) After EPA's receipt of a manufacturer's (or group's) submittals, EPA will notify the responsible manufacturer (or group) regarding the adequacy of the submittal and potential Tier 3 testing requirements according to the same relative time intervals and by the same procedures as specified in § 79.51 (c) and (d) for routine Tier 1 and Tier 2 submittals.

(d) ***Small Business Provisions.***

- (1) For purposes of these provisions, when subsidiary, divisional, or other complex business arrangements exist, *manufacturer* is defined as the business entity with ultimate ownership of all related parents, subsidiaries, divisions, branches, or other operating units. *Total annual sales* means the average of the manufacturer's total sales revenue, excluding any revenue which represents the collection of Federal, State, or local excise taxes or sales taxes, in each of the three years prior to such manufacturer's submittal to EPA of the basic registration information pursuant to § 79.59(b)(2) through (b)(5).
- (2) ***Provisions Applicable to Baseline and Non-baseline Products.*** A manufacturer with total annual sales less than \$50 million is not required to meet the requirements of Tier 1 and Tier 2 (specified in §§ 79.52 and 79.53) with regard to such manufacturer's fuel and/or additive products which meet the criteria for inclusion in a Baseline or Non-baseline group pursuant to § 79.56. Upon such manufacturer's satisfactory completion and submittal to EPA of basic registration data specified in § 79.59(b), the manufacturer may request and EPA shall issue a registration for such product, subject to § 79.51(c) and paragraphs (d)(4) and (d)(5) of this section.
- (3) ***Provisions Applicable to Atypical Products.*** A manufacturer with total annual sales less than \$10 million is not required to meet the requirements of Tier 2 (specified in § 79.53) in regard to such manufacturer's fuel and/or additive products which meet the criteria for inclusion in an Atypical group pursuant to § 79.56. Upon such manufacturer's satisfactory completion and submittal to EPA of basic registration data specified in § 79.59(b) and Tier 1 information specified in § 79.52 for an Atypical fuel or additive, the manufacturer may request and EPA shall issue a registration for such product, subject to § 79.51(c) and paragraphs (d)(4) and (d)(5) of this section. Compliance with Tier 1 requirements under this paragraph may be accomplished by the individual manufacturer or as a part of a group pursuant to § 79.56.
- (4) Any registration granted by EPA under the provisions of this section are conditional upon satisfactory completion of any Tier 3 requirements which EPA may subsequently impose pursuant to § 79.54. In such circumstances, the Tier 3 requirements might include (but would not necessarily be limited to) information which would otherwise have been required under the provisions of Tier 1 and/or Tier 2.
- (5) The provisions in paragraphs (d)(2) and (d)(3) of this section are voluntary on the part of qualifying small manufacturers. Such manufacturers may choose to fulfill the standard requirements for their fuels and additives, individually or as a part of a group, rather than satisfying only the requirements specified in paragraphs (d)(2) and/or (d)(3) of this section. If a qualifying small manufacturer elects these special provisions rather than the standard requirements for a product, then EPA will generally assume that any additional information submitted by other manufacturers, for fuels and additives meeting the same grouping criteria (under § 79.56) as that of the small manufacturer's product, is pertinent to further testing and/or regulatory decisions that may affect the small manufacturer's product.
- (6) In the case of an additive for which the manufacturer is not required to meet the requirements of Tier 2 pursuant to paragraph (d)(3) of this section:

- (i) A fuel manufacturer which blends such an additive into fuel shall not be required to meet the requirements of Tier 2 with respect to such additive/fuel mixture.
- (ii) An additive manufacturer which blends such an additive with one or more other registered additive products and/or with substances containing only carbon and/or hydrogen shall not be required to meet the requirements of Tier 2 with respect to such additive or additive blend.

(e) **Aftermarket Aerosol Additives.**

- (1) To obtain registration for an aftermarket aerosol fuel additive, the manufacturer shall provide existing information in the form of a literature search, a discussion of the potential exposure(s) to such product, and the basic registration data specified in § 79.59(b).
- (2) The literature search shall include existing data on potential health and welfare effects due to exposure to the aerosol product itself and its raw (uncombusted) components. The analysis for potential exposures shall be based on the actual or anticipated production volume and market distribution of the particular aerosol product, and its estimated frequency of use. Other Tier 1 and Tier 2 requirements are not routinely required for aerosol products. EPA will review the submitted information and, at EPA's discretion, may require from the manufacturer further information and/or testing under Tier 3 on a case-by-case basis.

[59 FR 33093, June 27, 1994, as amended at 62 FR 12571, Mar. 17, 1997]

§ 79.59 Reporting requirements.

(a) **Timing.**

- (1) The manufacturer of each designated fuel or fuel additive shall submit to EPA the basic registration data detailed in paragraph (b) of this section. Forms for submitting this data may be obtained from EPA at the following address: Attn: Fuel/Additives Registration, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW, Mail Code 6405A, Washington, DC 20460.
 - (i) For existing products (pursuant to § 79.51(c)(1)), manufacturers shall submit the basic registration data as specified in § 79.59(b) to EPA by November 28, 1994.
 - (ii) For registrable products (pursuant to § 79.51(c)(2)), manufacturers shall submit the basic registration data as specified in § 79.59(b) to apply for registration for such product.
 - (iii) For new products (pursuant to § 79.51(c)(3)), manufacturers are strongly encouraged to notify EPA of an intent to obtain product registration by submitting the basic registration data as specified in § 79.59(b) prior to starting Tiers 1 and 2.
- (2) The information specified in paragraph (c) of this section shall be submitted to the address in paragraph (a)(1) of this section at the conclusion of activities performed in compliance with Tiers 1 and 2 under the provisions of §§ 79.52 and 79.53, according to the time constraints specified in § 79.51 (c) through (d).
- (3) The information specified in paragraph (d) of this section shall be submitted to EPA at the address in paragraph (a)(1) of this section at the conclusion of activities performed in compliance with Tier 3 under the provisions of § 79.54.

- (b) **Basic Registration Data.** Each manufacturer of a designated fuel or fuel additive shall submit the following data in regard to such fuel or fuel additive:

- (1) The information specified in § 79.11 or § 79.21. If such information has already been submitted to EPA in compliance with subpart B or C of this part, and if such previous information is accurate and up-to-date, the manufacturer need not resubmit this information.
- (2) Annual production volume of the fuel or fuel additive product, in units of gallons per year if most commonly sold in liquid form or kilograms per year if most commonly sold in solid form. For fuels and fuel additives already in production, the most recent annual production volume and the volume projected to be produced in the third subsequent year shall be provided. For products not yet in production, the best estimate of expected annual volume during the third year of production shall be provided.
- (3) **Market distribution of the product.** For fuels and bulk additives, this information shall be presented as the percent of total annual sales volume marketed in each Petroleum Administration for Defense District (PADD). The States comprising each PADD are listed in the following section. For aftermarket additives, the distribution data shall be presented as the percent of total annual sales volume marketed in each State. For a product not yet in production, the manufacturer shall present the distribution (by PADD or State, as applicable) projected to occur during the third year of production.

(i) The following States and jurisdictions are included in PADD I:

Connecticut
Delaware
District of Columbia
Florida
Georgia
Maine
Maryland
Massachusetts
New Hampshire
New Jersey
New York
North Carolina
Pennsylvania
Rhode Island
South Carolina
Vermont
Virginia
West Virginia

(ii) The following States are included in PADD II:

Illinois
Indiana
Iowa
Kansas
Kentucky
Michigan
Minnesota

Missouri
Nebraska
North Dakota
Ohio
Oklahoma
South Dakota
Tennessee
Wisconsin

(iii) The following States are included in PADD III:

Alabama
Arkansas
Louisiana
Mississippi
New Mexico
Texas

(iv) The following States are included in PADD IV:

Colorado
Idaho
Montana
Utah
Wyoming

(v) The following States are included in PADD V:

Alaska
Arizona
California
Hawaii
Nevada
Oregon
Washington

(4) Any applicable information pursuant to the grouping provisions in § 79.56, as follows:

- (i) If the manufacturer has enrolled or intends to enroll the product in a fuel/additive group, the relevant group and the person(s) or entity expected to submit information on behalf of the group must be identified.
- (ii) If the manufacturer intends to rely on registration information previously submitted by another manufacturer (or group) for registration of other product(s) in the same fuel/additive group, then the original submitter and its product (or product group) shall be identified. In such cases, the manufacturer shall provide evidence that the original submitter has been notified of the use of its registration data and that the manufacturer has complied or intends to comply with the proportional reimbursement required under § 79.56(c) of this rule.

(5) Any applicable information pursuant to the special provisions in § 79.58, as follows:

- (i) If the manufacturer claims applicability of the special provisions for relabeled additives, pursuant to § 79.58(a), then the manufacturer and brand name of the original product shall be given.
 - (ii) If the manufacturer claims applicability of any small business provisions pursuant to § 79.58(d), the average of the manufacturer's total annual sales revenue for the previous three years shall be given.
 - (iii) If the manufacturer claims applicability of the special provisions for aerosol products, pursuant to § 79.58(e), then the purpose and recommended frequency of use shall be given.
- (c) **Tier 1 and Tier 2 Reports.** If the results of Tiers 1 and 2 are reported to EPA at the same time, then the report shall include the following documents in paragraphs (c)(1) through (7) of this section. If Tier 1 and Tier 2 results are submitted to EPA separately, then the separate Tier 1 report shall include only documents in paragraphs (c) (1) through (4), (c)(6), and associated appendices in paragraphs (c)(7) of this section, and the separate Tier 2 report shall include only documents in paragraphs (c)(1) through (3), (c)(5), (c)(6), and associated appendices in paragraph (c)(7) of this section. In addition, manufacturers complying with Tier 2 requirements according to one of the time schedules specified in § 79.51(c)(1)(ii)(B), § 79.51(c)(1)(vi)(B)(2), or § 79.51(c)(1)(vii)(B)(2) must submit evidence of a suitable arrangement for completion of Tier 2 (e.g., a copy of a signed contract with a qualified laboratory for applicable Tier 2 services) by the date specified in the applicable time schedule.

(1) **Cover page.**

- (i) Identification of test substance,
- (ii) Name and address of the manufacturer of the test substance,
- (iii) Name and phone number of a designated contact person,
- (iv) Group information, if applicable, including:
 - (A) Group name or grouping criteria,
 - (B) Name and address of responsible organization or entity reporting for the group,
 - (C) Product trade name and manufacturer of each member fuel and additive to which the report pertains.

(2) **Executive Summary.** Text overview of the significant results and conclusions obtained as a result of completing the requirements of Tier 1 and/or Tier 2, including references if used to support such results and conclusions.

(3) **Test Substance Information.** Test substance description, including, as applicable,

- (i) Base fuel parameter values (including types and concentrations of base fuel additives) or test fuel composition (if a fuel other than the base fuel is used in testing). These values must be provided for each of the fuel parameters specified in § 79.55 for the applicable fuel family.
- (ii) Test additive composition and concentration

(4) **Summary of Tier 1.**

- (i) **Literature Search.** Pursuant to § 79.52(d), the literature search shall include a text summary of the methods and results of the literature search, including the following:

- (A) Identification of person(s) performing the literature search,
 - (B) Description of data sources accessed, search strategy used, search period, and terms included in literature search,
 - (C) Documentation of all unpublished in-house and other privately-conducted studies,
 - (D) Tables summarizing the protocols and results of all cited studies,
 - (E) Summary of significant results and conclusions with respect to the effects of the emissions of the subject fuel or fuel additive on the public health and welfare, including references if used to support such results and conclusions.
 - (F) Statement of the extent to which the literature search has produced adequate information comparable to that which would otherwise be obtained through the performance of applicable emission characterization requirements under § 79.52(b) and/or health effects testing requirements under § 79.53, including justifications and specific references.
- (ii) **Emission Characterization.** Pursuant to § 79.52(b), the emission characterization shall include:
- (A) Name, address, and telephone number of the laboratory performing the characterization,
 - (B) Name and description of analytic methods used for characterization.
- (5) **Summary of Tier 2.** For each health effects test performed pursuant to the provisions of § 79.53, the Tier 2 summary shall contain the following information:
- (i) Name, address, and telephone number of the testing facility,
 - (ii) Summary of procedures (including quality assurance, quality control and compliance with Good Laboratory Practice Standards as specified in § 79.60), findings, and conclusions, including references if used to support such results and conclusions,
 - (iii) Description of any problems and their resolution.
- (6) **Conclusions.** The conclusions shall identify the need for further testing, if that need exists, or justify that current testing and/or available information is adequate for the tier(s) included in the report.
- (7) **Appendices.** The appendices shall contain detailed documentation related to the summary information described in this section, including, at a minimum, the following five appendices:
- (i) Literature search appendices shall contain:
 - (A) Copies of literature source outputs, including reference lists and associated abstracts from database searches, printed or on 3¹/₂ inch IBM-compatible computer diskettes;
 - (B) Summary tables organized by health or welfare endpoint and type of emission (e.g., combustion, evaporation, individual emission product), presenting in tabular form the following information at a minimum: number and species of test subjects, exposure concentrations/duration, positive (i.e., abnormal) findings including numbers of test subjects involved, and bibliographic references;
 - (C) Complete documentation and/or reprints of articles for any previous study relied upon for satisfying emission characterization and/or Tier 2 test requirements; and
 - (D) Full reports for unpublished/in-house studies.

(ii) Emissions characterization appendices shall contain:

- (A) Complete laboratory reports, including documentation of calibration and verification procedures;
- (B) Documentation of the emissions generation procedures used; and
- (C) Lists of speciated emission products and their emission rates reported in units of grams/mile.

(iii) [Reserved]

(iv) Tier 2 appendices shall contain, for each test performed:

- (A) Complete protocol used;
- (B) Documentation of emission generation procedures; and
- (C) Complete laboratory report in compliance with the reporting standards in § 79.60, including detailed test results and conclusions, and descriptions of any problems encountered and their resolution.

(v) Laboratory certification/accreditation information, personnel credentials, and statements of compliance with the Good Laboratory Practices Standards specified in § 79.60 and the requirements in § 79.53(c)(1).

(d) **Tier 3 Report.** Subject to applicability as specified in § 79.54, each manufacturer of a designated fuel or fuel additive, or each group of such manufacturers pursuant to the provisions of § 79.56, shall submit the following information with respect to each Tier 3 test conducted for such fuels or fuel additives:

- (1) The test objectives, including a summary of the reason(s) why such additional testing, beyond Tiers 1 and 2, was required;
- (2) Name, address, and telephone number of each testing facility;
- (3) Summary of test procedures, results and conclusions;
- (4) Complete documentation of test protocols and emission generation procedures, complete laboratory reports in compliance with the reporting standards of § 79.60, detailed test results and conclusions, including references if used to support such results and conclusions, and descriptions of any problems encountered and their resolution; and
- (5) Laboratory certification information, personnel credentials, and statements of compliance with the Good Laboratory Practices Standards specified in § 79.60.

(e) **Availability of Information.**

- (1) All health and safety test data and other information concerning health and welfare effects which is submitted by any manufacturer or group pursuant to §§ 79.52(c), 79.53, or 79.54, shall be considered to be public information and shall be made available to the public by EPA upon request. A reasonable fee may be charged by EPA for copying such materials. Any manufacturer or group who claims that any information concerning the composition of a fuel or fuel additive product, or any other information, submitted under this subpart is confidential business information must state this claim in writing at the time of the submittal.

- (2) To assert a business confidentiality claim concerning any information submitted under this subpart, the submitter must:
 - (i) Clearly mark the information as confidential at each location it appears in the submission; and
 - (ii) Submit with the information claimed as confidential a separate document setting forth the claim and listing each location at which the information appears in the submission.
- (3) If any person subsequently requests access to information submitted under this subpart (other than health and safety test data and other information concerning health and welfare effects), and such information is subject to a claim of business confidentiality, the request and any subsequent disclosure shall be governed by the provisions of 40 CFR part 2.

[59 FR 33093, June 27, 1994, as amended at 62 FR 12572, 12576, Mar. 17, 1997; 85 FR 7069, Feb. 6, 2020]

§ 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 or 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.

§ 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:

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

(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 $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and for rabbits, it is $20^{\circ}\text{C} \pm 3^{\circ}\text{C}$. 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, NO_x, 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 turbinometers (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.

- (1) Barr, E.B. (1988) Operational Limits for Temperature and Percent Oxygen During HM Nose-Only Exposures—Emergency Procedures [interoffice memorandum]. Albuquerque, NM: Lovelace Inhalation Toxicology Research Institute; May 13.
- (2) Barr, E.B.; Cheng, Y.S.; Mauderly, J.L. (1990) Determination of Oxygen Depletion in a Nose-Only Exposure Chamber. Presented at: 1990 American Association for Aerosol Research; June; Philadelphia, PA: American Association for Aerosol Research; abstract no. P2e1.
- (3) Barrow, C.S. (1989) Generation and Characterization of Gases and Vapors. In: McClellan, R.O., Henderson, R.F. ed. Concepts in Inhalation Toxicology. New York, NY: Hemisphere Publishing Corp., 63-84.
- (4) Benedict, R.P. (1984) Fundamentals of Temperature, Pressure, and Flow Measurements. 3rd ed. New York, NY: John Wiley and Sons.
- (5) Cannon, W.C.; Blanton, E.F.; McDonald, K.E. The Flow-Past Chamber. (1983) An Improved Nose-Only Exposure System for Rodents. Am. Ind. Hyg. Assoc. J. 44: 923-928.
- (6) Chaddock, J.B. ed. (1985) Moisture and humidity. Measurement and Control in Science and Industry: Proceedings of the 1985 International Symposium on Moisture and Humidity; April 1985; Washington, D.C. Research Triangle Park, NC: Instrument Society of America.
- (7) Cheng, Y.S.; Barr, E.B.; Carpenter, R.L.; Benson, J.M.; Hobbs, C.H. (1989) Improvement of Aerosol Distribution in Whole-Body Inhalation Exposure Chambers. Inhal. Toxicol. 1: 153-166.
- (8) Cheng, Y.S.; Moss, O.R. (1989) Inhalation Exposure Systems. In: McClellan, R.O.; Henderson, R.F. ed. Concepts in Inhalation Toxicology. New York, NY: Hemisphere Publishing Corp., 19-62.
- (9) Cheng, Y.S.; Yeh, H.C.; Mauderly, J.L.; Mokler, B.V. (1984) Characterization of Diesel Exhaust in a Chronic Inhalation Study. Am. Ind. Hyg. Assoc. J. 45: 547-555.
- (10) Gillum, D.R. (1982) Industrial Pressure Measurement. Research Triangle Park, NC: Instrument Society of America.
- (11) Hinnners, R.G.; Burkart, J.K.; Malanchuk, M. (1979) Animal Exposure Facility for Diesel Exhaust Studies.
- (12) Kittelson, D.B.; Dolan, D.F. (1979) Diesel exhaust aerosols. In Willeke, K. ed. Generation of Aerosols and Facilities for Exposure Experiments. Ann Arbor, MI: Ann Arbor Science Publishers Inc., 337-360.
- (13) Mokler, B.V.; Archibeque, F.A.; Beethe, R.L.; Kelly, C.P.J.; Lopez, J.A.; Mauderly, J.L.; Stafford, D.L. (1984) Diesel Exhaust Exposure System for Animal Studies. Fundamental and Applied Toxicology 4: 270-277.

- (14) Moore, W.; et al. (1978) Preliminary finding on the Deposition and Retention of Automotive Diesel Particulate in Rat Lungs. Proc. of Annual Meeting of the Air Pollution Control Assn, 3, paper 78-33.7.
- (15) Raabe, O.G., Bennick, J.E., Light, M.E., Hobbs, C.H., Thomas, R.L., Tillery, M.I. (1973) An Improved Apparatus for Acute Inhalation Exposure of Rodents to Radioactive Aerosols. Toxicol & Applied Pharmacol.; 1973; 26: 264-273.
- (16) Rao, G.N. (1986) Significance of Environmental Factors on the Test System. In: Hoover, B.K.; Baldwin, J.K.; Uelner, A.F.; Whitmire, C.E.; Davies, C.L.; Bristol, D.W. ed. Managing conduct and data quality of toxicology studies. Raleigh, NC: Princeton Scientific Publishing Co., Inc.: 173-185.
- (17) Spitzer, D.W. (1984) Industrial Flow Measurement. Research Triangle Park, NC: Instrument Society of America.
- (18) 40 CFR part 798, Health effects testing guidelines.
- (19) 29 CFR part 1910, Occupational safety and health standards for general industry.
- (20) FEDERAL REGISTER, 42 FR 26748, May 25, 1977.

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

§ 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 determination 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 (zygomatic and extraocular lacrimal 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]

§ 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 lethalties 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]

§ 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.

§ 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 bromodeoxyuridine (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: Assessment 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).

§ 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 glutaraldehyde 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).

[59 FR 33093, June 27, 1994, as amended at 63 FR 63793, Nov. 17, 1999]

§ 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 [¹²⁵I] 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 [I^{125}] Protein A (2-10 μ Ci/ μ g), Anti-sera to GFAP, nitrocellulose paper (0.1 or 0.2 μ m 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 $MgCl_2$), 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 μ g/20 μ l).
 - (v) **Preparation of Standard Curve.** Dilute a single control sample in sample buffer to give at least five standards, between 1 and 10 μ g total protein per 20 μ l. The suggested values of total protein per 20 μ l sample buffer are 1.25, 2.50, 3.25, 5.0, 6.25, 7.5, 8.75, and 10.0 μ g.
 - (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 μ l portions to the center of each square for a total sample volume of 20 μ l. 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 μ l 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.

- (1) Brock, T.O and O'Callaghan, J.P. 1987. Quantitative changes in the synaptic vesicle proteins, synapsin I and p38 and the astrocyte specific protein, glial fibrillary acidic protein, are associated with chemical-induced injury to the rat central nervous system, *J. Neurosci.* 7:931-942.
- (2) Jahn, R., Schiebler, W. Greengard, P. 1984. A quantitative dot-immunobinding assay for protein using nitrocellulose membrane filters. *Proc. Natl. Acad. Sci. U.S.A.* 81:1684-1687.
- (3) O'Callaghan, J.P. 1988. Neurotypic and gliotypic protein as biochemical markers of neurotoxicity. *Neurotoxicol. Teratol.* 10:445-452.
- (4) O'Callaghan, J.P. 1991. Quantification of glial fibrillary acidic protein: comparison of slot-immunobinding assays with a novel sandwich ELISA. *Neurotoxicol. Teratol.* 13:275-281.
- (5) O'Callaghan, J.P. and Miller, D.B. 1985. Cerebellar hypoplasia in the Gunn rat is associated with quantitative changes in neurotypic and gliotypic proteins. *J. Pharmacol. Exp. Ther.* 234:522-532.
- (6) Sette, W.F. "Pesticide Assessment Guidelines, Subdivision 'F', Hazard Evaluation: Human and Domestic Animals, Addendum 10, Neurotoxicity, Series 81, 82, and 83" US-EPA, Office of Pesticide Programs, EPA-540/09-91-123, March 1991.
- (7) Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Annal. Biochem.* 150:76-85.

§ 79.68 *Salmonella typhimurium* reverse mutation assay.

- (a) **Purpose.** The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay which measures $\text{his}^- \rightarrow \text{his}^+$ 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.

- (1) 40 CFR 798.5265, The *Salmonella typhimurium* reverse mutation assay.
- (2) Ames, B.N., McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test," Mutation Research 31:347-364 (1975).
- (3) Huisingh, J.L., et al., "Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions: Study Design, Sample Generation, Collection, and Preparation". In: Health Effects of Diesel Engine Emissions, Vol. II, W.E. Peplko, R., M., Danner and N. A. Clarke (Eds.), US EPA, Cincinnati, EPA-600/9-80-057b, pp. 788-800 (1980).
- (4) [Reserved]
- (5) Claxton, L.D., Allen, J., Auletta, A., Mortelmans, K., Nestmann, E., Zeiger, E. "Guide for the *Salmonella typhimurium*/mammalian microsome tests for bacterial mutagenicity" Mutation Research 189(2):83-91 (1987).

- (6) Claxton, L., Houk, V.S., Allison, J.C., Creason, J., "Evaluating the relationship of metabolic activation system concentrations and chemical dose concentrations for the Salmonella Spiral and Plate Assays" Mutation Research 253:127-136 (1991).
- (7) Claxton, L., Houk, V.S., Monteith, L.G., Myers, L.E., Hughes, T.J., "Assessing the use of known mutagens to calibrate the *Salmonella typhimurium* mutagenicity assay: I. Without exogenous activation." Mutation Research 253:137-147 (1991).
- (8) Claxton, L., Houk, V.S., Warner, J.R., Myers, L.E., Hughes, T.J., "Assessing the use of known mutagens to calibrate the *Salmonella typhimurium* mutagenicity assay: II. With exogenous activation." Mutation Research 253:149-159 (1991).
- (9) Claxton, L., Creason, J., Lares, B., Augurell, E., Bagley, S., Bryant, D.W., Courtois, Y.A., Douglas, G., Clare, C.B., Goto, S., Quillardet, P., Jagannath, D.R., Mohn, G., Neilsen, P.A., Ohnishi, Y., Ong, T., Pederson, T.C., Shimizu, H., Nylund, L., Tokiwa, H., Vink, I.G.R., Wang, Y., Warshawsky, D., "Results of the IPCS Collaborative Study on Complex Mixtures" Mutation Research 276:23-32 (1992).
- (10) Claxton, L., Douglas, G., Krewski, D., Lewtas, J., Matsushita, H., Rosenkranz, H., "Overview, conclusions, and recommendations of the IPCS Collaborative Study on Complex Mixtures" Mutation Research 276:61-80 (1992).
- (11) Houk, V.S., Schalkowsky, S., and Claxton, L.D., "Development and Validation of the Spiral Salmonella Assay: An Automated Approach to Bacterial Mutagenicity Testing" Mutation Research 223:49-64 (1989).
- (12) Jones, E., Richold, M., May, J.H., and Saje, A. "The Assessment of the Mutagenic Potential of Vehicle Engine Exhaust in the Ames Salmonella Assay Using a Direct Exposure Method" Mutation Research 97:35-40 (1985).
- (13) Maron, D., and Ames, B. N., Revised methods for the Salmonella mutagenicity test, Mutation Research, 113:173-212 (1983).
- (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).
- (15) Rosenkranz, H.S., et.al. "Nitropyrenes: Isolation, identification, and reduction of mutagenic impurities in carbon black and toners" Science 209:1039-43 (1980).
- (16) Stump, F., Snow, R., et.al., "Trapping gaseous hydrocarbons for mutagenic testing" SAE Technical Paper Series, No. 820776 (1982).
- (17) Vogel, H.J., Bonner, D.M. "Acetylornithinase of *E. coli*: partial purification and some properties," Journal of Biological Chemistry. 218:97-106 (1956).

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