Designation: E 1687 – 98

An American National Standard

Standard Test Method for Determining Carcinogenic Potential of Virgin Base Oils in Metalworking Fluids[1]

This standard is issued under the fixed designation E 1687; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers a microbiological test procedure based upon the *Salmonella* mutagenesis assay of Ames et al **(1)**[2] (see also Maron et al **(2)**). It can be used as a screening technique to detect the presence of potential dermal carcinogens in virgin base oils used in the formulation of metalworking oils. Persons who perform this test should be well-versed in the conduct of the Ames test and conversant with the physical and chemical properties of petroleum products.

1.2 The test method is not recommended as the sole testing procedure for oils which have viscosities less than 18 cSt (90 SUS) at 40°C, or for formulated metalworking fluids.

1.3 The values stated in SI units are to be regarded as the standard. The values given in parentheses are provided for information only.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Section 7 provides general guidelines for safe conduct of this test method.

2. Referenced Documents

2.1 *ASTM Standards:*

29 CFR 1910.1450 Occupational Exposure to Hazardous Chemical in Laboratories[3]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *base stock, n*—the refined oil component of metalworking fluid formulations.

3.1.2 *PCA (Polycyclic Aromatics), n*—For the purposes of this test method, PCA refers to fused-ring polycyclic aromatic compounds with three or more rings. For example, the hydrocarbon series is represented by phenanthrene (3), pyrene (4), benzopyrene (5), dibenzopyrene (6), coronene (7). Heterocyclic polynuclear compounds are also included in the definition.

3.1.3 *promutagenic compounds, promutagens, n*—compounds that are not directly mutagenic but require metabolism for expression of mutagenic activity.

3.1.4 *Reference Oil 1, n*—straight-run naphthenic vacuum distillate (heavy vacuum gas oil) of known MI and PNA content recommended for use as a reference standard for the modified Ames test.

3.2 *Abbreviations:Abbreviations:*

3.2.1 *DMSO (Dimethyl Sulfoxide), n*—extraction agent used in the preparation of aromatic-enriched oil fractions for mutagenicity testing.

3.2.2 *G-6-P (Glucose-6-Phosphate), n*—substrate required for the operation of the NADPH generating system involved in the biological oxidations described above.

3.2.3 *MI (Mutagenicity Index), n*—the slope of the dose-response curve for mutagenicity in the modified Ames test.

3.2.3.1 *Discussion*—MI is an index of relative mutagenic potency.

3.2.4 *NADP (Nicotinamide Adenine Dinucleotide Phosphate)*—required cofactor for the biological oxidations involved in activation of PNA to their mutagenic forms.

3.2.5 *PNA (Polynuclear Aromatics; also termed CA), n*—polynuclear aromatic compounds.

3.2.6 *S-9, n*—fraction prepared from hamster liver which contains the enzymes required for metabolic activation of PNAs to their mutagenic forms.

4. Summary of Test Method

4.1 The Ames *Salmonella* mutagenicity assay is the most widely used short-term *in vitro* genotoxicity test. The assay employs specific strains of the bacterium *Salmonella typhimurium* that have been mutated at a genetic locus precluding the biosynthesis of the amino acid histidine which is required for growth and reproduction. Additional genetic alterations, some of which are important markers of strain identity, are also present.

4.2 The mutagenicity assay relies upon treating the bacteria with test material over a range of doses immediately below the concentration showing significant toxicity to the bacteria. Treated bacteria are then grown on agar plates deficient in

[1] This test method is under the jurisdiction of ASTM Committee E34 on Occupational Health and Safety and is the direct responsibility of Subcommittee E34.50 on Health and Safety Standards for Metal Working Fluids.

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[2] The boldface numbers refer to the list of references at the end of this standard.

[3] Available from Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

histidine. Bacteria possessing the original mutation in the histidine locus cannot form colonies under these growth conditions, but a certain fraction of treated bacteria which have undergone a second mutation in the histidine locus revert to histidine-independence and are able to grow and form visible colonies. The number of such revertant colonies per agar plate is an indicator of the mutagenic potency of the test material.

4.3 Typically, the test is conducted using a number of bacterial strains selectively sensitive to various chemical classes of mutagens. Treatment with test compound is carried out in the presence and absence of a rodent liver extract capable of mimicking *in vivo* metabolic activation of promutagenic compounds (see 3.2 for a listing of terms and abbreviations used.) With this combination of test conditions, the Ames test becomes a very effective screening tool for chemical mutagens. Moreover, because many mutagens are also carcinogens, the test is often used as a screen for carcinogenic potential.

4.4 Although the ability of the Ames test to assess carcinogenic potential is good for many classes of compounds, it has been shown to be generally unsuited to the testing of water-insoluble complex mixtures such as mineral oils. To circumvent poor solubility and other difficulties, this test method employs an extraction of the test oil with DMSO to produce aqueous-compatible solutions which readily interact with the metabolic activation system (S-9) and with the tester bacteria. The concentration of S-9 and of NADP cofactor are increased relative to the unmodified assay, and hamster rather than rat liver S-9 is used. The slope of the dose response curve relating mutagenicity (TA98 revertants per plate) to the dose of extract added is used as an index of mutagenic potency (MI).

4.5 In this test method, the MI (the slope of the dose response curve, and a measure of mutagenic potency) of a DMSO extract of an oil is compared to the mutagenicity indices of other oil extracts whose dermal carcinogenicities are known. By correlation, the potential dermal carcinogenicity of the test oil can be assessed.

5. Significance and Use

5.1 The test method is based on a modification of the Ames *Salmonella* mutagenesis assay. As modified, there is good correlation with mouse skin-painting bioassay results for samples of raw and refined lubricating oil process streams.

5.2 Mutagenic potency in this modified assay and carcinogenicity in the skin-painting bioassay also correlate with the content of 3 to 7 ring PNAs, which include polynuclear aromatic hydrocarbons and their heterocyclic analogs. The strength of these correlations implies that PNAs are the principal mutagenic and carcinogenic species in these oils. Some of the methods that have provided evidence supporting this view are referenced in Appendix X1.

6. Interferences

6.1 The test method is designed to detect mutagenicity mediated by PNAs derived from petroleum. The assay is disproportionately sensitive to nitroaromatic combustion products and as yet unidentified components of catalytically or thermally cracked stocks such as light or heavy cycle oils. The latter materials are not known to occur in virgin base oils.

6.2 For petroleum refinery streams distilling in the range associated with the production of naptha or kerosine or the light end of atmospheric gas oil (that is, median boiling point <250°C; viscosity< 18 cStat 40°C), the assay is sensitive to detecting carcinogenicity related to the presence of polycyclic aromatic compounds. However, streams in the range, even those with MI less than 1.0, can produce tumors in a standard mouse dermal carcinogenicity assay through alternative non-genotoxic mechanisms.

7. Hazards

7.1 The test materials and positive control compounds used in this assay may present a carcinogenic hazard by ingestion or skin contact. Avoid all contact with test oils and Reference Oil No. 1.

7.2 The tester bacteria are attenuated and unlikely to cause illness. However, gloves should be worn during handling of bacteria, and care should be taken to avoid injuries with syringes and hypodermic needles contaminated with bacterial cultures. Waste material generated during testing should be regarded as a potential biohazard and disposed of accordingly. **Reference 3** provides general guidelines for safe use of this test method.

7.3 Provisions for the safe use of this test method should be incorporated into the employer's compliance with 29 CFR 1910.1450.

8. Materials and Methods

8.1 *Test Organism*—Methods for storage, culture, and characterization of the test organism are exactly as described by Ames et al **(1)**. The test organism used in this assay is *Salmonella typhimurium* strain TA98 derived from an original stock produced and supplied by B. N. Ames, University of California, Berkeley. Strain TA98 was selected for the test because it is the most sensitive to the class of mutagens present in petroleum materials (PNAs) (Hermann et al **(4)**).

8.1.1 Strain TA98 was derived from strain TA1538, and has the same genetic markers as that strain, including histidine/biotin requirement, crystal violet sensitivity, and ultraviolet sensitivity. In addition, TA98 contains plasmid pKM101, which confers ampicillin resistance. Full characterization of strain TA98 has been published by Ames et al **(1)**.

8.1.2 Strain TA98 can be inoculated, either from frozen stocks maintained at − 80 ± 5°C or from master plates maintained at approximately 4°C, into 25 mL of Oxoid No. 2 nutrient broth in a 125 mL erlenmeyer flask equipped with a screw cap. The flask is placed into a shaker-incubator set at approximately 37°C and 100 to 120 rpm. Sixteen hours later, 2 mL of the culture is diluted into 8 mL of fresh Oxoid No. 2, and allowed to regrow for 3 h, or until the turbidity of the regrown culture, measured spectrophotometrically at 650 nm, is in the range from 1.0 to 2.0 absorbance units. A second check on cell density may be obtained by serially diluting the culture by a factor of 10^7 into phosphate-buffered saline (PBS), and plating 1 mL of the resultant dilution onto nutrient agar plates containing 0.5 % NaCl. After 44 to 48 h incubation at approximately 37°C, the number of colonies can be determined immediately, or the plates may be refrigerated at 5 ± 3°C for

up to five days, and the cell density of the culture calculated from the net dilution factor. Acceptable values range from 1 to 3×10^9 cells/mL.

8.2 *Sampling and Handling of Oils*—Sampling of oils should be performed with consideration of viscosity and other physical properties to ensure that test specimens are representative. Whenever possible, oils should be stored at room temperature in amber bottles under nitrogen to avoid photoreactivity.

8.3 *Preparation of DMSO Extract*—The mutagenic components of oils are extracted into DMSO prior to testing. For oils with viscosities low enough to permit accurate volumetric dispensing (< approximately 200 cSt at 40°C), 1 mL of the oil is measured into a 15 mL tube, and 5 mL of reagent grade DMSO added. Volumes of oil rather than 1 mL may be used so long as the 1:5 volume ratio of oil to DMSO is preserved. The tube is vortexed vigorously either continuously or intermittently for a 30-min period to ensure thorough contact between the oil and DMSO layers. The sample is then centrifuged for 10 min in a table-top centrifuge to effect phase separation ($200 \times g$). A portion of the lower, DMSO layer, is withdrawn with a pipet and reserved for testing.

8.4 *Preparation of Metabolic Activation Mixture (S-9)*:

8.4.1 Aroclor 1254-induced liver S-9 from Syrian golden hamsters is prepared according to the following procedure: Adult male hamsters, weighing between 90 and 100 g, are induced by a single intraperitoneal injection of Aroclor 1254 at a dose of 500 mg/kg body weight. Five days after induction, the hamsters are sacrificed, the livers are aseptically removed and rinsed in cold, sterile suspending buffer (isotonic KCl) and homogenized in a Polytron Tissuemizer at a concentration of 1:3 (wet liver wt:volume of suspending buffer).

8.4.2 The supernatant fraction (S-9) is collected following centrifugation at $9000 \times g$ for 10 min in a centrifuge maintained at approximately 4°C. The supernatant is then portioned into aliquots of 5 mL each and stored frozen at -80 ± 5°C until used.

8.4.3 S-9 is thawed at approximately 4°C on the day of the test, and metabolic activation mixture sufficient for one test article prepared is as follows:

8.4.4 To a sterile container at approximately 4°C are added in sequence 1.5 mL of 1 M sodium phosphate buffer, pH 7.4; 0.3 mL 0.25 M glucose-6-phosphate; 0.6 mL 0.2 M NADP; 0.6 mL of a salt solution of 0.2 M $MgCl_2$/0.825 M KCl. To the resulting solution, 12 mL of S-9 are added with gentle swirling.

8.4.5 All steps in the preparation and dispensing of S-9 and S-9 mixture must be performed at approximately 4°C, and all reagents used in preparing the S-9 mixture should be maintained at approximately 4°C. S-9 mixture should not be stored for longer than 2 h prior to use; excess mixture should be discarded when the test is completed.

8.5 *Calibration and Standardization*:

8.5.1 *Reference Standards and Blanks*—The reference standard for this test method is a vacuum distillate designated Reference Oil No. 1.[4] This oil is tested as part of each assay according to the procedures outlined in 8.6.

8.5.2 Assay acceptability is determined using the data generated for Reference Oil No. 1. An assay is deemed acceptable only if both of the following criteria are met:

8.5.2.1 Revertant colony counts for the DMSO extract of Reference Oil No. 1, diluted 1:3 (one volume of oil plus three volumes of DMSO), must reach, in a dose-responsive manner, at least twice the representative mean solvent control value for the method, that is, must exceed $2 \times 46 = 92$ revertant colonies/plate.

8.5.2.2 No more than three doses may produce mean revertant counts more than 15 % below the representative mean at that dose. The representative data to be used in this analysis are provided in Table A2.1.

8.5.3 For assays done with a single extract and an independent repeat, three solvent control plates per assay serve as a blank (see 8.5.2.1). If a single assay is done on three extracts of the test material, two solvent control plates per extract should be used. The mean revertant count for these plates should not fall below 30 colonies/plate or exceed 60 colonies/plate. If either of these conditions occur, the effect on the dose response curves of Reference Oil No. 1 and the test materials should be assessed. If there is a significant change in the slopes of those curves, which is directly attributable to the effects of the out-of-range solvent controls, then the assay should be repeated.

9. Procedure

9.1 Perform the following steps in order:

9.1.1 Prepare dosing solutions for the test article and Reference Oil No. 1 by diluting the DMSO extracts with DMSO to give individual doses deliverable in 60 µL. A typical dosing schedule is shown in Table 1 but other dosing protocols are acceptable if they provide at least four doses on the linear portion of the dose-response curve. For materials which produce a curvilinear dose response, the original DMSO extract should be diluted with DMSO to yield a linear dose-response over the 0 to 60 µL range. In general, oils with MIs greater than 1.0 will require dilution. A preliminary one plate/dose range-finding assay may be done to determine the point at which the dose response begins to curve. Based on the results of this assay, the extract is diluted sufficiently to

[4] Available upon request from Stonybrook Laboratories Inc., P.O. Box 1029, Princeton, NJ 08543.

TABLE 1 Dosing Solutions[A]

	Dose, µL/Plate					
	0	12	24	36	48	60
µL Extract	0	36	72	108	144	180
µL DMSO	180	144	108	72	36	0

[A] Other dosing regimens over the range 0 to 60 µL may be used.

produce approximately 100 to 120 revertants/plate at the 60 µL dose in the full assay.

9.1.2 Either of the following procedures may be used. For single-extract assays with independent repeat, dose three 13 by 100 mm sterile glass test tubes with 60 µL of each dosing solution. Measure doses with a positive displacement micropipet, and place in a dry block at approximately 37°C. The outside of the pipet tip is wiped free of adherent liquid using a disposable paper wipe in order to ensure accurate dosing. All tubes for a day's test may be dosed together, but the following steps should be performed one test article (30 tubes) at a time.

9.1.3 Add 0.5 mL of S-9 mix to the bottom of each tube.

9.1.4 Add 0.1 mL of a well-mixed suspension of strain TA98 bacteria prepared as described in 8.1.2 to the bottom of each tube. Bacteria should be maintained at ice temperature until used.

9.1.5 Incubate tubes at approximately 37°C on a gyratory shaker-incubator at 150 rpm for 20 to 30 min; then return tubes to a dry block at approximately 37°C for plating.

9.1.6 Add 2.0 mL of top agar to each tube (see Note 1). During dispensing, the top agar is placed on a dry block maintained at approximately 37°C. Vortex the mix gently, and pour the resulting agar mixture onto a 100 mm petri plate containing 30 mL of bottom agar consisting of 1.5 % bacteriological grade agar in Vogel-Bonner Minimal E medium supplemented with 2 % dextrose.

NOTE 1—Each 100 mL of top agar contains 0.6 g bacteriological grade agar and 0.5 g NaCl. Top agar is melted, equilibrated to approximately 45°C, and supplemented by addition of a volume of 0.5 millimolar histidine -0.5 millimolar biotin equal to 10 % of the original agar volume. After mixing (and approximately 20 min prior to dispensing), the top agar is reequilibrated to approximately 40°C.

9.1.7 Swirl the plate to obtain a layer of top agar of even thickness across the plate.

9.1.8 Allow to cool and harden on a level surface, and incubate inverted in an incubator at approximately 37°C for 44 to 48 h.

9.1.9 Remove plates from incubator; count colonies immediately or store at 5 ± 3°C for up to five days before evaluation. Colonies are enumerated using an automatic marking pen or similar manual counting device. An automatic colony counter may be used if the results are demonstrably equivalent to those obtained by manual counting.

10. Calculation and Interpretation of Results

10.1 *Calculation*:

10.1.1 The raw data from this test method are in the form of mean bacterial colony counts for each of the doses of the test material and the solvent control. It is recommended that analysis of this data should follow the following sequence:

10.1.1.1 Determine the acceptability of the assay using the criteria in 8.5.1.

10.1.1.2 If the assay meets the criteria in 8.5.1, a plot of colony counts or their means against dose is used to generate a dose response curve for mutagenesis. Linear regression analysis of this curve (see 10.1.2) produces a slope (coefficient of the x-term of the regression equation) with units of revertants/µL DMSO extract. This slope is the fundamental measurement obtained through the use of this test method.

10.1.2 DMSO extracts of all oils should be diluted sufficiently that the dose-response for mutagenicity is linear over at least four doses.

10.1.3 If data on diluted extracts are not available, nonlinear dose-responses may be truncated and the initial linear region fit by linear regression analysis. Methods such as those of Bernstein et al **(6)** and Skisak et al **(7)** are good examples of this approach. The latter procedure was designed specifically for the treatment of data from this test method.

10.2 *Interpretation of Data*:

10.2.1 Based upon previous studies using this test method, categories of response in the assay can be used to determine the likelihood of a carcinogenic response in a mouse skin-painting bioassay. (Categories are based on MI values rather than other measures of mutagenic potency since the original correlations with mouse skin-painting data are based on these values (Blackburn et al **(8, 9)**, Roy et al **(10)**)) Other measures of potency can be normalized against MI or can be directly related to carcinogenicity if skin-painting data are available for sufficient similar oils to establish an independent correlation.

10.2.2 The following guidelines for interpretation of data are based on the historical database for use of this test method, and should be used with the understanding that any changes in practice since the database was developed, either in the mutagenicity or carcinogenicity assays, may affect the MI ranges of the categories. It should also be understood that oils producing MIs close to the values separating categories may be indiscernibly different in a carcinogenicity assay from oils having MIs on the other side of that boundary.

10.2.2.1 Oils with MI < 1.0 have a high probability of being noncarcinogenic in a mouse skin-painting bioassay.

10.2.2.2 Oils with MI ≥ 1.0 but ≤ 2.0 may or may not be carcinogenic in a mouse skin-painting bioassay. Whenever possible, corroborative data from PNA analyses or additional biological testing should be used in categorizing such oils for carcinogenic potential.

10.2.2.3 Oils with MI > 2.0 have a high probability of being carcinogenic in a mouse skin-painting bioassay.

11. Report

11.1 Report the following information:

11.1.1 Counts of revertant colonies per plate for each dose of the test article and for the solvent control (DMSO) plates.

11.1.2 Counts of revertant colonies per plate for each dose of Reference Oil No. 1. One test of the positive control oil will serve for all test articles concurrently assayed.

11.1.3 A mutagenicity index (MI), mutagenic potency index (MPI) or other quantitative estimate of mutagenicity calculated by suitable regression analysis of the dose-response curve for mutagenicity (10.1).

11.1.4 Categorization of the probable dermal carcinogenic potential of the test article, using the criteria cited in 10.2.

12. Precision and Bias

12.1 *Precision*:

12.1.1 The fundamental data produced from the use of this test method is an estimate of the mutagenic potency of test oils (MI). This value, which is calculated by the procedure detailed in 10.1.1, is used to categorize oils according to their potential