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An American National Standard

Standard Practice for Determination of Endotoxin Concentrations in Water-Miscible Metalworking Fluids[1]

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1. Scope

1.1 This practice covers quantitative methods for the sampling and determination of bacterial endotoxin concentrations in water miscible metalworking fluids (MWF).

1.2 Users of this practice need to be familiar with the handling of MWF.

1.3 This practice gives an estimate of the endotoxin concentration in the sampled MWF.

1.4 This practice replaces Method E2250.

1.5 This practice seeks to minimize inter-laboratory variation of endotoxin data but does not ensure uniformity of results.

1.6 *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.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D4840 Guide for Sample Chain-of-Custody Procedures

E1488 Guide for Statistical Procedures to Use in Developing and Applying Test Methods

E1497 Practice for Selection and Safe Use of Water-Miscible and Straight Oil Metal Removal Fluids

E1542 Terminology Relating to Occupational Health and Safety

E2250 Method for Determination of Endotoxin Concentration in Water Miscible Metal Working Fluids (Withdrawn 2008)[3]

2.2 *Government Standard:*[4]

29 CFR 1910.1450 Occupational Exposure to Hazardous Chemicals in Laboratories

2.3 *Other Documents:*[5]

Criteria Document for a Recommended Standard: Occupational Exposure to Metalworking Fluids, 1998 NIOSH

Manual of Analytical Methods (NMAM), 4th ed., Eller and Cassinelli, Eds., 1994

3. Terminology

3.1 For definitions of terms relating to this practice, refer to Terminology E1542.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *control standard endotoxin (CSE), n*—a purified preparation of endotoxin based on the USP Reference Standard Endotoxin (RSE); used in laboratories to prepare standard solutions.

3.2.2 *endotoxin, n*—pyrogenic high molar mass lipopolysaccharide (LPS) complex associated with the cell wall of gram-negative bacteria.

3.2.2.1 *Discussion*—Though endotoxins are pyrogens, not all pyrogens are endotoxins. Endotoxins are specifically detected through a Limulus Amoebocyte Lysate (LAL) test.

3.2.3 *endotoxin unit (EU), n*—a biological potency unit equivalent to the FDA Reference Standard Endotoxin (RSE).

3.2.3.1 *Discussion*—The current RSE (EC-6) is equivalent to 1ng = 10 EU.

3.2.4 *geometric mean (GM), n*—the central tendency of a set of numbers expressed as the nth root of their product.

3.2.5 *geometric standard deviation (GSD), n*—the spread of data in a set of numbers expressed as a geometric mean.

3.2.6 *Gram-negative bacteria, n*—prokaryotic cells that have a complex cell wall structure that stains characteristically when subjected to the differential Gram staining procedure.

[1] This practice 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] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

[4] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401, http://www.access.gpo.gov.

[5] Available from CDC/NIOSH, 4676 Columbia Pkwy, Cincinnati, OH 45226-1998.

3.2.7 *inhibition/enhancement phenomenon, n*—conditions or artifacts in sample solutions that cause endotoxin concentration data from LAL assays to be less than or more than the concentration of endotoxin actually present in a given aqueous sample.

3.2.8 *Limulus amebocyte lysate (LAL) assay, n*—a biological assay dependent on a series of cascading enzyme reactions that occur when Limulus blood cell (amebocyte) lysate combines with endotoxin.

3.2.9 *metalworking fluid (MWF), n*—any fluid used for the purpose of cooling or treating metal surfaces during metal removal, metal forming or surface protection or preservation.

3.2.10 *metal removal fluid (MRF), n*—any fluid in the subclass of metalworking fluids used to cut, or otherwise take away material or piece of stock.

3.2.10.1 *Discussion*—Metal removal fluids include straight or neat oils (D2881), not intended for further dilution with water, and water miscible soluble oils, semisynthetics and synthetics, which are intended to be diluted with water before use. Metal removal fluids become contaminated during use in the workplace with a variety of workplace substances including, but not limited to, abrasive particles, tramp oils, cleaners, dirt, metal fines and shavings, dissolved metal and hard water salts, bacteria, fungi, microbiological decay products, and waste. These contaminants can cause changes in the lubricity and cooling ability of the metal removal fluid as well as have the potential to adversely affect the health and welfare of employees in contact with the contaminated metal removal fluid.

3.2.11 *Operator-dependent assay, n*—an assay performed by a technician in such a manner to cause significant influence(s) on the resultant data.

3.2.12 *pyrogen-free (PF), adj*—material(s) devoid of measurable endotoxin activity.

3.2.13 *pyrogen-free water (PFW), n*—processed water that is devoid of measurable endotoxin activity.

3.2.14 *sensitivity range, n*—a span of endotoxin measurements expressed as EU/mL or λ.

4. Summary of Practice

4.1 Serial dilutions of CSE in PFW in borosilicate glass test tubes are prepared to construct a calibration curve.

4.2 The metalworking fluid sample is sonicated, centrifuged, and the supernatant retained.

4.3 Triplicates of the sample supernate, standard serial dilutions, blanks, and positive control solutions are subjected to the kinetic chromogenic LAL assay.

4.4 If data indicate interferences are present, MWF supernate is diluted and assay is performed with diluted supernate.

4.5 The reaction of Limulus amebocyte lysate with sample endotoxin imparts a proportional yellow color to the analyte solution that is measured photometrically at 405 nm.

4.6 The measured endotoxin concentration is reported as EU/mL.

5. Significance and Use

5.1 The determination of endotoxin concentrations in MWF is a parameter that can be used in decision-making for prudent fluid management practices (fluid draining, cleaning, recharging or biocide dosages).

5.2 This standard provides a practice for analysts who perform quantitative endotoxin analyses of water-miscible MWF.

6. Interferences

6.1 Data from samples analyzed by LAL methodologies are prone to variations due to batch differences in lysate composition/processing, non-optimal pH and temperatures of assay solutions.

6.2 In the event that the phenomenon of inhibition/enhancement influences this practice, endotoxin concentration data will be less than or more than actual concentrations present in a given MWF sample.

6.3 LAL assays are highly influenced by the skill/experience level of the analyst.

7. Apparatus

7.1 *Sampling:*

7.1.1 *Sample Collection Container,* pyrogen-free, wide-mouth, stainless steel sealable container, at least 100 mL capacity.

7.1.2 *Glass Pipet,* pyrogen-free, 50 mL.

7.1.3 *Battery-Powered Aspirator Unit (or suction bulb),* compatible with 100 mL glass pipet.

7.2 *Extraction:*

7.2.1 *Centrifuge,* minimum rotational speed of 5000 rpm.

7.2.2 *Ultrasonic Water Bath,* ultrasonic/water bath apparatus with a minimum peak frequency of 40 kHz with cavitation adjustment and thermostat control; use pyrogen-free glass containers only.

7.3 *Analysis:*

7.3.1 *Incubating/Shaking Microplate Reader,* spectrophotometric at 405 nm.

7.3.2 *Statistical Analysis Software Package for Microplate Reader.*

7.3.3 *Vortexer,* variable speed.

7.3.4 *Microtiter Plates,* flat-bottomed, pyrogen-free, 96-well.

7.3.5 *Dilution Tubes,* pyrogen-free, 13 by 100 mm.

7.3.6 *Borosilicate Glass Test Tubes,* pyrogen-free, screw caps, 10 by 75 mm.

7.3.7 *Single-Channel Micropipetor(s),* 0.5-10 µL.

7.3.8 *Eight-Channel Micropipetor,* 100 µL.

7.3.9 *Pipet Tips,* pyrogen-free, 300 µL.

7.3.10 *Glass Rod,* pyrogen-free.

7.3.11 *Reagent Reservoir,* pyrogen-free, 8-channel multipipettor compatible.

7.3.12 *Parafilm M.*

8. Reagents and Materials

8.1 *Control Standard Endotoxin (CSE),* referenced to most current Federal Drug Administration (FDA) Reference Standard Endotoxin (RSE).

8.2 *Limulus Amebocyte Lysate (LAL),* unexpired with stated potency.

8.3 *Dilution Water,* pyrogen-free (*PFW*).

8.4 *MWF Concentrate,* concentrated, unused MWF as supplied.

9. Hazards

9.1 Aerosols of endotoxin preparations pose a potential respiratory hazard to susceptible laboratory personnel who are directly involved with an endotoxin assay.

9.2 Inhalation or dermal exposure to metalworking fluids pose potential health problems for personnel involved in MWF sampling. Provision of personal protective equipment (PPE) in the form of respirators or protective clothing, or both, is potentially indicated (see Practice E1497 and Criteria Document for a Recommended Standard: Occupational Exposure to Metalworking Fluids).

9.3 Follow good laboratory procedures for worker protection and waste disposal, including 29 CFR 1910.1450.

9.4 Review material safety data sheets (MSDS) for materials in use at a facility to identify potential hazards to determine appropriate PPE (see 29 CFR 1910.1000).

10. Sampling Procedure

10.1 *Sampling Site:*

10.1.1 Select sampling site that will yield a representative MWF sample.

10.1.2 Select individual sump(s) or central system(s) that has actively circulating fluids. If possible, draw sample from the mid-point of the fluid reservoir. Otherwise, draw sample below the surface of the metalworking fluid volume of interest and avoid the aspiration of extraneous floating biomass.

10.1.3 Use aseptic techniques with pyrogen-free apparatus to aspirate a 100-mL grab sample with a glass pipet into a suitable pyrogen-free 250-mL container and then seal securely with a pyrogen-free lid or Parafilm M. Avoid touching inner lid and interior container areas with hands/gloves or nonpyrogenic labware.

11. Sample Storage/Shipment

11.1 For best results, LAL analysis of the sample within 24 hours is advisable. However, if this is not feasible, store the sealed sample container in a plastic bag and then refrigerate or pack in crushed ice at 4 ± 2°C. Avoid freezing sample, since this will adversely affect resultant data.

11.2 If the sample is shipped to an analytical laboratory, pack its container securely in cold packs (or portable refrigeration) and expedite shipment time so that the sample arrives at the laboratory no later than 24 hours after its acquisition.

11.3 Maintain procedures for sample custody in accordance with accepted chain of custody procedures (see Guide D4840).

12. Preparation of Labware

12.1 A critical consideration of quantitative LAL analyses is that the sample must be protected against the indiscriminate introduction of exogenous sources of endotoxin:

12.1.1 Commercially packaged labware used in LAL analyses shall be clearly marked as “pyrogen-free,” “endotoxin-free,” “depyrogenated,” or clearly identified as suitable for use in LAL analyses. A certificate of authentication shall accompany labware that attests to its pyrogen-free condition. Manufacturer ID, lot numbers, expiration dates, and authentication/certification information shall be recorded in laboratory notebooks.

12.1.2 Commercially packaged labware that is nominally described or labeled as “sterile,” “sterilized,” “disinfected,” or otherwise identified as suitable for routine microbiological usage only shall not be used in this standard practice, due to the possibility of the presence of residual endotoxin on critical labware surfaces.

12.1.3 Prior to use in this standard practice, non-pyrogen-free glass or metal labware that will be used in LAL analyses shall be subjected to the depyrogenation procedure described in Section 13 of this standard. The analyst shall not use plastic labware, due to the possibility of introducing non-specific assay interferences, or causing container-related adsorption of endotoxin onto surfaces, or both.

13. Depyrogenation Procedure

13.1 Thoroughly clean labware and then rinse twice in pyrogen-free water.

13.2 Bake glassware at 250°C for 1 h in a laboratory convection-type oven. As part of quality assurance procedures, check oven heating performance with a NTIS-calibrated thermometer before each depyrogenation batch run.

13.3 The analyst shall avoid indiscriminate contamination of depyrogenated labware.

14. Extraction Procedure

14.1 This critical procedure shall be performed by a single, experienced analyst only.

14.2 Open the container with collected sample in a negative-pressure biosafety cabinet (or under a chemical fume evacuation hood), and stir the sample vigorously with a pyrogen-free glass rod for 1 min.

14.3 Aspirate 20 mL of MWF (center, midway depth) and transfer to a pyrogen-free test tube.

14.4 Bath sonicate sample in test tube at a minimum peak frequency of 40 kHz for 1 h at 25 ± 2°C (or place on a mechanical shaker/vortexer for 1 h).

14.5 Centrifuge solution in a pyrogen-free tube at 1000 g for at least 15 min.

14.6 Remove centrifuge tube and note zoning layers: tramp oil (upper layer); MWF (middle layer); suspended solids (bottom layer).

14.7 Pipet and discard tramp oil layer with a pyrogen-free pipet tip.

14.8 Pipet MWF layer with a pyrogen-free pipet tip.

15. Microtiter Plate Template Set-up

15.1 Record microtiter well assignments for the 96-well microtiter plate for each set of analytical solutions (in triplicate) in laboratory notebook.

15.1.1 Samples (in triplicate).

15.1.2 Standard serial solutions (in triplicate for each concentration in the dilution series).

15.1.3 PFW blanks (in triplicate).

15.1.4 Positive control (in triplicate – record endotoxin spike concentration).

NOTE 1—If Parafilm M is utilized to cover vessels containing sample material, the extraction procedure needs to be conducted on a 1-cm^2 piece, and triplicates of the extract shall be subjected to LAL analysis.

15.2 Program microtiter plate well locations into plate reader software in accordance with predetermined template assignments.

16. Preparation of Assay Solutions

16.1 Use 1.0 N HCl or 1.0 N NaOH for pH adjustment of the MWF sample to pH 7.5.

16.2 Use fresh (non-expired) PFW for use as a diluent for lyophilized Control Standard Endotoxin (CSE), Limulus amebocyte lysate (LAL), blanks, and positive controls. PFW (8.3) from the same lot shall be used for all analyte solutions per assay.

16.3 Reconstitute CSE as per manufacturer's directions and adjust to pH 7.5.

16.4 *Standard Solutions:*

16.4.1 Use CSE solution to prepare serial dilutions with concentrations of 5.0, 0.5, 0.05, and 0.005 EU/mL.

16.4.2 Dispense 100 µL of each CSE dilution (in triplicate) in designated LAL reagent grade microtiter plate wells.

16.5 *Blanks:*

16.5.1 Dispense 100 µL of 7.5 pH-adjusted PFW (in triplicate) in designated microtiter plate wells.

16.6 *Positive Controls:*

16.6.1 Dispense 100 µL of extract solution (in triplicate) into assigned microtiter plate wells, then add 10 µL of a 5-EU/mL CSE spike into each well.

16.7 *Sample Extract:*

16.7.1 To identify potential interferences in the MWF sample that may adversely affect resultant data, a validation procedure shall be performed:

16.7.1.1 Mix 20 mL of PFW with 1 mL of fresh MWF concentrate sample (8.4) to prepare a 5 % solution.

16.7.1.2 Subject the 5 % solution to the extraction procedure (Section 14).

16.7.1.3 Dispense 100 µL directly of the extracted 5 % solution (in triplicate) in assigned wells, then add 10 µL of a 5-EU/mL CSE spike into each well.

16.7.1.4 Test in addition different dilutions (1:10; 1:100; 1:1000) of 5 % solution (in triplicate) in assigned wells, then add 10 µL of a 5-EU/mL CSE spike into each well.

NOTE 2—Due to indeterminate interference factors present in the sample matrix that have the potential to adversely affect data from LAL assays, it is possible that it will be necessary to test successively increased dilutions of the sample in order to identify the MWF concentration yield unbiased data – dilutions at or below which 16.7.1 results are not affected by MWF dilution factor.

16.7.2 Dispense 100 µL of metalworking fluid extract (in triplicate) in designated microtiter plate wells.

17. Analysis of Solutions

17.1 *Program Microplate Reader:*

17.1.1 Follow manufacturer's directions for set-up for microplate reader shaking, heating, and analysis modes.

17.1.1.1 Pre-set plate reader shaking platform mode for 15 min duration.

17.1.1.2 Pre-heat plate reader analysis chamber to 37°C.

17.2 Use an 8-channel multipipettor with pyrogen-free tips to dispense 100 µL of reconstituted LAL (warmed to 37°C) into each analyte microtiter plate well.

17.3 Insert microtiter plate (with cover) into microplate reader analysis chamber and commence shaking mode.

17.4 Activate automated kinetic chromogenic analysis according to plate reader instructions.

18. Quality Assurance

18.1 Ensure that validation and maintenance procedures have been conducted in accordance with microtiter plate reader manufacturer's directions.

18.2 The correctness of software calculations shall be validated at least once each calendar quarter by checking selected generated data with other software or calculators.

18.3 Individuals who perform endotoxin assays shall be appropriately trained, and it is advisable to assign a dedicated analyst to perform the assay in order to reduce variation in data sets due to operator dependency factors.

18.4 Avoid exposing samples and extracts to extreme temperatures (freezing or above ambient).

18.5 Ensure that linearity of standard curve, spike recovery and other quality control measures meet LAL kit manufacturer's specifications.

19. Calculation or Interpretation of Results

19.1 Endotoxin concentration of standards and samples shall be determined in accordance with kit manufacturer's directions.

19.2 The acceptable correlation coefficient of the calibration curve (log-log) linearity shall be ≥0.980 (r).

19.3 The acceptable coefficient of variation for all triplicate samples and positive controls shall be ≤10 %.

19.4 Resultant endotoxin concentration data shall be expressed in endotoxin units per millilitre (EU/mL).

19.5 Currently, there is no consensus occupational exposure limit for exposure to endotoxin in metalworking fluids in machine sumps or other sectors of fluid distribution systems.

20. Precision and Bias

20.1 *Precision*—Repeatability and Reproducibility depend on the MWF being tested. Consequently, there is no reference MWF from which to base generally applicable precision assessment for this practice.

20.1.1 Users are advised to refer to Guide E1488.

20.2 *Bias*—In accordance with Note 2, MWF are likely to introduce formulation-specific bias to the test results.

20.2.1 Instructions for identifying and eliminating bias are provided in 16.7.1.

21. Keywords

21.1 bioaerosols; endotoxin; endotoxin assay; endotoxin units (EU); gram-negative bacteria; Limulus amebocyte lysate (LAL); metalworking fluids; pyrogen