Designation: E2694 – 11

An American National Standard

Standard Test Method for Measurement of Adenosine Triphosphate in Water-Miscible Metalworking Fluids[1]

This standard is issued under the fixed designation E2694; 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 The method provides a protocol for capturing, extracting and quantifying the adenosine triphosphate (ATP) content associated with microorganisms found in water-miscible metalworking fluids (MWF).

1.2 The ATP is measured using a bioluminescence enzyme assay, whereby light is generated in amounts proportional to the concentration of ATP in the samples. The light is produced and measured quantitatively as relative light units (RLU) which are converted by comparison with an ATP standard and computation to pg ATP/mL.

1.3 This method is equally suitable for use in the laboratory or field.

1.4 The method detects ATP concentrations in the range of 4.0 pg ATP/mL to 400,000 pg ATP/mL.

1.5 Providing interferences can be overcome, bioluminescence is a reliable and proven method for qualifying and quantifying ATP. The method does not differentiate between ATP from different sources, for example, from different types of microorganisms, such as bacteria and fungi.

1.6 The values stated in SI are to be regarded as standard.

1.7 *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]

D1129 Terminology Relating to Water

D4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water

D4840 Guide for Sample Chain-of-Custody Procedures

D6161 Terminology Used for Microfiltration, Ultrafiltration, Nanofiltration and Reverse Osmosis Membrane Processes

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E1326 Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria

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

E2523 Terminology for Metalworking Fluids and Operations

2.2 *Government Standards:*[3]

29 CFR 1910.1000 Occupational Safety and Health Standards; Air contaminants

29 CFR 1910.1450 Occupational Exposure to Hazardous Chemicals in Laboratories

3. Terminology

3.1 *Definitions*:

For definition of terms used in this method, refer to Terminology standards D1129, D6161, and E2523.

3.2 *adenosine triphosphate (ATP)*, *n*—a molecule comprised of a purine and three phosphate groups that serves as the primary energy transport molecule in all biological cells.

3.3 *adenosine monophosphate (AMP)*, *n*—the molecule formed by the removal of two molecules of phosphate (one pyrophosphate molecule) from ATP.

3.4 *aseptic*, *adj*—sterile, free from viable microbial contamination.

3.5 *bioluminescence*, *n*—the production and emission of light by a living organism as the result of a chemical reaction during which chemical energy is converted to light energy.

3.6 *biomass*, *n*—any matter which is or was a living organism or excreted from a microorganism (D6161).

[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.

Current edition approved Aug. 1, 2011. Published August 2011. Originally approved in 2009. Last previous edition approved in 2009 as E2694 - 09. DOI:10.1520/E2694-11.

[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] 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.

***A Summary of Changes section appears at the end of this standard.**

3.7 *culturable*, *adj*—microorganisms that proliferate as indicated by the formation of colonies on solid growth media or the development of turbidity in liquid growth media under specific growth conditions.

3.8 *Luciferase*, *n*—a general term for a class of enzymes that catalyze bioluminescent reactions.

3.9 *Luciferin*, *n*—a general term for a class of light-emitting biological pigments found in organisms capable of bioluminescence.

3.10 *luminometer*, *n*—an instrument capable of measuring light emitted as a result of non-thermal excitation.

3.11 *relative light unit (RLU)*, *n*—an instrument-specific unit of measurement reflecting the number of photons emitted by the Luciferin-Luciferase driven hydrolysis of ATP to AMP plus pyrophosphate.

3.11.1 *Discussion*—RLU is not an SI unit, however, RLU are proportional to ATP concentration.

3.12 *viable microbial biomass*, *n*—metabolically active (living) microorganisms

3.13 *Acronyms:*

3.13.1 *AMP*—adenosine monophosphate

3.13.2 *ATP*—adenosine triphosphate

3.13.3 *HDPE*—high density polyethylene

3.13.4 *MWF*—metalworking fluid

3.13.5 *PP*—polypropylene

3.13.6 *RLU*—relative light unit

4. Summary of Test Method

4.1 A control assay is performed using 100 µL of 1.0 ng ATP/mL standard.

4.2 A 5.0 mL sample of MWF is placed into a syringe and then pressure- filtered through a 0.7 µm, glass-fiber, in-line depth filter.

4.3 The retentate is then washed with a reagent to remove extra-cellular ATP and other contaminants that might otherwise interfere with the ATP assay.

4.4 The filter is air-dried.

4.5 A lysing reagent is used to release ATP from microbial cells that have been captured on the glass-fiber filter, and the filtrate is dispensed into an unused culture tube.

4.6 The filtrate is diluted 1+9 with a buffer solution.

4.7 A100-µL volume of diluted filtrate is transferred to an unused culture tube into which 100 µL of Luciferin-Luciferase reagent has previously been dispensed.

4.8 The culture tube is placed into a luminometer and the light intensity is read in RLU.

4.9 RLU are converted to Log_{10}[pg ATP/mL] of sample by computation.

5. Significance and Use

5.1 This method measures the concentration of ATP present in the sample. ATP is a constituent of all living cells, including bacteria and fungi. Consequently, the presence of ATP is an indicator of total microbial contamination in metalworking fluids. ATP is not associated with matter of non-biological origin.

5.2 Method D4012 validated ATP as a surrogate for culturable bacterial data (Guide E1326).

5.3 This method differs from Method D4012 in that it eliminates interferences that have historically rendered ATP testing unusable with complex organic fluids such as MWF.

5.4 The ATP test provides rapid test results that reflect the total bioburden in the sample. It thereby reduces the delay between test initiation and data capture, from the 36 h to 48 h (or longer) required for culturable colonies to become visible, to approximately five minutes.

5.5 Although ATP data generally covary with culture data in MWF[4], different factors affect ATP concentration than those that affect culturability.

5.5.1 Culturability is affected primarily by the ability of captured microbes to proliferate on the growth medium provided, under specific growth conditions. It have been estimated that less than 1 % of the species present in an environmental sample will form colonies under any given set of growth conditions.[5]

5.5.2 ATP concentration is affected by: the microbial species present, the physiological states of those species, and the total bioburden (See Appendix X1).

5.5.2.1 One example of the species effect is that the amount of ATP per cell is substantially greater for fungi than bacteria.

5.5.2.2 Within a species, cells that are more metabolically active will have more ATP per cell than dormant cells.

5.5.2.3 The greater the total bioburden, the greater the ATP concentration in a sample.

5.5.3 The possibility exists that the rinse step (11.15) may not eliminate all chemical substances that can interfere with the bioluminescence reaction (11.39).

5.5.3.1 The presence of any such interferences can be evaluated by performing a standard addition test series as described in Appendix X3.

5.5.3.2 Any impact of interfering chemicals will be reflected as bias relative to data obtained from fluid that does not contain interfering chemicals.

6. Apparatus

6.1 *Culture tube*, PP, 12 by 55 mm.

6.2 *Culture tube*, PP, 17 by 100 mm with caps.

6.3 *Filter*, 25 mm, sterile, disposable, in- line, 0.7 µm pore-size, glass-fiber, depth-type with Luer-Lok inlet.

6.4 *Luminometer*, using photomultiplier tube, capable of detecting light emission at 420 nm and with a cuvette chamber that can hold a 12 by 55-mm culture tube.

6.5 *Macropipeter*, adjustable, 1.0 to 5.0 mL.

6.6 *Micropipeter*, adjustable, 100 to 1000 µL.

6.7 *Pipet tips*, sterile, disposable, PP, 100 to 1000 µL.

6.8 *Pipet tips*, sterile, disposable, PP, 1.0 to 5.0 mL.

6.9 *Sample collection container*, sterile, wide-mouth bottle, 100 mL.

NOTE 1—ATP can adsorb onto glass surfaces. Consequently, PP or HDPE containers are strongly preferred.

[4] Passman et al. "Real-time Testing of Bioburdens in Metalworking Fluids using Adenosine Triphosphate as a Biomass Indicator," 2009 STLE Annual Meeting, Orlando, FL.

[5] Sloan, W. T., C. Quince and Curtis, T. P., "The Uncountables," *Accessing Uncultivated Microorganisms*, ASM Press, Washington, DC, 2008, p. 35.

6.10 *Syringe*, Luer-Lok , 20 mL, PP, sterile, disposable.
6.11 *Syringe*, Luer-Lok, 60 mL, PP, sterile disposable.
6.12 *Test tube rack*, 12 mm.
6.13 *Test tube rack*, 17 mm.
6.14 *Waste receptacle*, any container suitable for receiving and retaining filtrate fluid for ultimate disposal.

7. Reagents and Materials

7.1 *ATP standard*, 1 ng ATP/mL
7.1.1 Commercially available[6]; or
7.1.2 Dilute 1 mg ATP into 1000 mL ATP dilution buffer to get a 1000-ng ATP/mL stock solution. Then, dilute 1.0 mL of 1000 ng ATP/mL stock solution into 999.0 mL ATP dilution buffer to get a 1 ng ATP/mL ATP standard.
7.2 *ATP extract dilution buffer*[6] (proprietary)
7.3 *ATP extraction reagent*[6] (proprietary)
7.4 *Filter wash reagent*[6] (proprietary)
7.5 *Luciferin-Luciferase reagent*[6] (proprietary); store between -20°C and 4°C; allow to equilibrate to ambient temperature before using.

8. Hazards

8.1 The analyst must know and observe good laboratory safety practice in accordance with 29 CFR 1910.1450.
8.2 Inhalation or dermal exposure to MWF can pose health problems for personnel involved with MWF sampling. Provision of personal protective equipment (PPE) in the form of respirators, protective clothing or both may be indicated (see Practice E1497).
8.3 Review material safety data sheets for materials in use at the facility to identify potential hazards in order to determine appropriate PPE (see 29 CFR 1910.1000).

9. Sampling and Test Specs and Units

9.1 *Sampling Site*:
9.1.1 Select sampling site that will yield a representative MWF sample.
9.1.2 For routine condition monitoring, select individual sump(s) or central systems that have actively circulating fluid.
9.1.3 For diagnostic testing, select zones of pooled or stagnant MWF.
9.2 *Sampling*:
9.2.1 If practical, draw sample from the mid-point of the fluid reservoir, otherwise draw sample from below surface of the MWF at an accessible location.
9.2.1.1 Microbial contamination will vary considerably within the fluid system and it is important to be consistent in selecting the sampling location; this should be appropriate for the analysis objectives.
9.2.2 Collect sample by removing lid from sample container, immersing the open container (6.9), opening-down, below the fluid surface and inverting the container to allow it to fill with the sampled fluid.
9.2.3 If the fluid depth is insufficient to permit 9.2.1, use a sterile pipet to draw sample from the fluid and dispense it into the sample container; collecting at least 25 mL of sample.
9.3 *Sample Storage/Shipment*:
9.3.1 Label the sample container and follow accepted chain-of-custody procedures (Guide D4840).
9.3.2 Optimally samples should be tested on-site as soon as possible (<4 h) after testing.
9.3.3 If testing is to be delayed for longer than 4 h, or to be performed by an outside testing facility, samples may be stored on ice or in a refrigerator for up to 24 h. Samples older than 24 h are unlikely to microbiologically representative of the MWF at the time it was collected.

10. Calibration and Standardization

10.1 Turn on power to luminometer (6.4) and allow instrument to warm-up, in accordance with manufacturer's recommendations.
10.2 Ensure that all reagents have equilibrated to ambient temperature before running any tests.
10.3 Use a micropipeter (6.6) with a new 100 to 1000-µL tip (6.7) to dispense 100 µL Luciferin- Luciferase reagent (7.5) to an unused 12 by 55-mm culture tube (6.1).
10.4 Replace the micropipeter tip with a fresh tip.
10.5 Dispense 100 µL of 1 ng ATP/mL standard solution (7.1) into the culture tube.
10.6 Swirl gently for five times.
10.7 Place the culture tube into the luminometer.
10.8 Read and record RLU (RLU_{ctrl}).

11. Procedure

11.1 Use aseptic procedure while performing this test method; ATP from analyst's hands, sputum, etc. can contaminate the sample with ATP from sources other than the sample itself.
11.2 Remove plunger from a new 20-mL syringe (6.10) and place onto a 17-mm test tube rack so that plunger tip does not contact any surfaces.
11.3 Affix filter (6.3) onto the 20-mL syringe.
11.4 Place a fresh 1.0 to 5.0-mL tip (6.8) onto the macropipeter (6.5).
11.5 Shake sample for 15 seconds to ensure homogeneity.
11.6 With minimal delay, remove lid from sample container and, using the macropipeter, transfer 5.0 mL of sample to the 20-mL syringe barrel.
11.7 While holding the barrel over the waste receptacle (6.14), replace the plunger into the 20-mL syringe.
11.8 Apply even pressure to the 20-mL syringe plunger to pressure filter MWF sample, having filtrate discharge into the waste receptacle.
11.9 Remove filter from the 20-mL syringe and place onto a 17-mm test tube rack so that filter outlet does not contact any surfaces.
11.10 Remove plunger from the 20-mL syringe (6.10) and place onto a 17-mm test tube rack so that the plunger tip does not contact any surfaces.
11.11 Replace filter onto the end of the syringe barrel.

[6] The sole source of supply of the proprietary ATP dilution buffer, ATP extraction reagent, filter wash reagent, and Luciferin-Luciferase reagent, is LuminUltra Technologies Ltd., Fredericton, New Brunswick, Canada, www.luminultra.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend.