Designation: D7463 – 21

Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Fuel, Fuel/Water Mixtures, and Fuel Associated Water[1]

This standard is issued under the fixed designation D7463; 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 provides a protocol for capturing, concentrating, and testing the adenosine triphosphate (ATP) present in a fuel system sub-sample (that is, test specimen) associated with:

1.1.1 Microorganisms and hydrophilic particles found in liquid fuels as described in Table X6.1, or

1.1.2 Microorganisms and hydrophilic particles found in mixture of fuel and associated bottom water or just associated bottom water.

1.1.3 ATP detected by this bioluminescence test can be derived from cellular ATP, extra-cellular ATP, or some combination of both.

1.1.4 Cellular and extra-cellular ATP utilized to perform ATP bioluminescence are captured and concentrated from a fuel system sample into an aqueous test specimen (that is, sub-sample) for testing. For example, for a fuel system sample that does not contain any visible fuel associated bottom water, the aqueous test specimen is the capture solution itself described in 8.2.1.1. For fuel system samples that are a mixture of fuel and associated bottom water (that is, free water), the test specimen is an aliquant of the capture solution and associated bottom water.

1.2 The ATP is measured using a patented bioluminescence enzyme assay, whereby light is generated in amounts proportional to the concentration of ATP in the sample. The light is produced and measured quantitatively using dedicated ATP test pens[2] and a dedicated luminometer[2] and reported in (instrument specific) Relative Light Units.

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

1.4 Although bioluminescence is a reliable and proven technology, this method does not differentiate ATP from bacteria or fungi.

1.5 For water or capture solution samples, the concentration range of ATP detectable by this test method is 1×10^{-11} M to 3×10^{-8} M which is equivalent to 1×10^{-14} moles/mL to 3×10^{-11} moles/mL for water samples or capture solution. Assuming testing on fuel phase is performed on a 500 mL volume of fuel the equivalent concentrations is fuel would be: 6×10^{-11} M to 2×10^{-14} M.

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6.1 There is one exception—Relative Light Unit (RLU) as defined in 3.1.19.

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D396 Specification for Fuel Oils
D975 Specification for Diesel Fuel
D1655 Specification for Aviation Turbine Fuels
D2880 Specification for Gas Turbine Fuel Oils
D4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water

[1] This test method is under the jurisdiction of ASTM Committee D02 on Petroleum Products, Liquid Fuels, and Lubricants and is the direct responsibility of Subcommittee D02.14 on Stability, Cleanliness and Compatibility of Liquid Fuels.

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[2] The sole source of supply, repair, recertification, and technical support of the apparatus or test pen known to the committee at this time is Merck KGaA, 64271 Darmstadt, Germany (Worldwide) or Fuel Quality Services, Inc., 4584 Cantrell Rd., Flowery Branch, GA 30542 (USA). 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,[1] which you may attend.

[3] 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.

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

D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants
D6300 Practice for Determination of Precision and Bias Data for Use in Test Methods for Petroleum Products, Liquid Fuels, and Lubricants
D7464 Practice for Manual Sampling of Liquid Fuels, Associated Materials and Fuel System Components for Microbiological Testing
D7467 Specification for Diesel Fuel Oil, Biodiesel Blend (B6 to B20)

3. Terminology

3.1 *Definitions:*

3.1.1 For definition of terms used in this test method, refer to Terminology D4175.

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

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

3.1.4 *aseptic, adj*—sterile, free from viable microbiological contamination.

3.1.5 *bioluminescence, n*—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.

3.1.6 *biomass, n*—biological material including any material other than fossil fuels which is or was a living organism or component or product of a living organism.

3.1.7 *capture solution, n*—aqueous solution of proprietary composition used to capture and concentrate hydrophilic compounds and particles from liquid fuels.

3.1.8 *cellular adenosine triphosphate (cellular-ATP), n*—ATP present in whole cells, whether they are living or dead.

3.1.8.1 *Discussion*—Cellular-ATP is released upon intentional lysis (rupturing) of microbial cells during the sample preparation process. Microbially infected fluids contain both cellular (cell-associated/cell-bound) and extra-cellular ATP.

3.1.9 *culturable, adj*—(microorganisms that are) able to proliferate as indicated by the formation of colonies on or in solid, semi-solid, or liquid growth media under specific growth conditions, as indicated by the formation of colonies, the development of turbidity, or other indicators.

3.1.10 *extracellular ATP, n*—ATP that is not contained inside a cell.

3.1.10.1 *Discussion*—ATP is released into the environment when cells die and break open (lyse), for example, as when they are killed by exposure to some microbicides. ATP released into the environment can persist for several days after a cell has been lysed. Consequently extracellular ATP must be subtracted from total ATP to determine the concentration of viable cell-associated (biomass associated) ATP. However, extracellular ATP can also be an indicator of "distant" biomass, for example, biofilm in the system.

3.1.11 *free water, n*—water that exists as a separate phase.

3.1.11.1 *Discussion*—Water present in fuel such as hydrocarbon diesel fuel that can be present as suspended haze, nonvisible suspended water droplets, as droplets on the walls of the vessel, or as a separate layer on the bottom of the vessel or sample container.

3.1.12 *fungus, (pl. fungi), n*—single cell (yeasts) or filamentous (molds) microorganisms that share the property of having the true intracellular membranes (organelles) that characterize all higher life forms (*Eukaryotes*).

3.1.13 *hydrophilic particles, n*—compounds such as ATP, NAD^+, $NADP^+$, NADH, NADPH, enzymes, free fatty acids, preservatives, biocides, salts, as well as microorganisms or other articles are often dispersed or distributed in hydrophobic liquid matrices such as crude oil, vegetable oil, petrol, and kerosine.

3.1.14 *invert emulsion layer, n*—interface between the water phase and fuel phase of a fuel water sample which consists of water micelles dispersed in the fuel.

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

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

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

3.1.18 *pyrogen free, n*—free of substances which can induce fever.

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

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

3.1.20 *test specimen, n*—a representative piece of a sample.

3.1.20.1 *Discussion*—For this test method, the test specimen is an aqueous sub-sample drawn from the fuel system sample that is tested for the presence of cellular and/or extra-cellular ATP. In the case of a fuel system sample that is fuel only in the absence of associated bottom water, the test specimen is the capture solution (3.1.7). For fuel system samples that contain associated bottom water, the test specimen is an aliquant of the capture solution and associated bottom water (3.1.11).

3.1.21 *viable microbial biomass, n*—metabolically active (living) micro-organisms

3.2 *Abbreviations:*

3.2.1 *AMP*—adenosine monophosphate

3.2.2 *ATP*—adenosine triphosphate

3.2.3 *HDPE*—high density polyethylene

3.2.4 *NAD^+*—nicotinamide adenine dinucleotide, oxidized form

3.2.5 *NADH*—nicotinamide adenine dinucleotide, reduced form

3.2.6 *$NADP^+$*—nicotinamide adenine dinucleotide phosphate, oxidized form

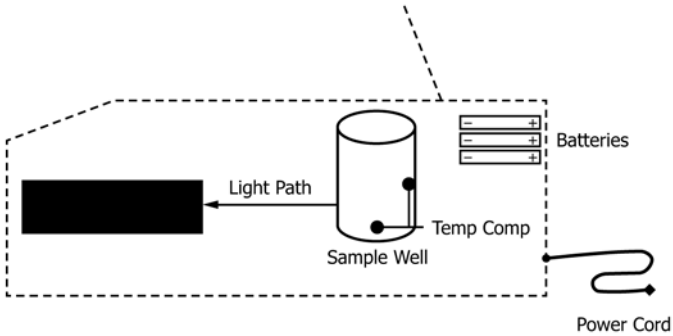

FIG. 1 Luminometer

3.2.7 *NADPH*—nicotinamide adenine dinucleotide phosphate, reduced form

3.2.8 *PP*—polypropylene

3.2.9 *RLU*—relative light units

4. Summary of Test Method

4.1 A fuel system sample is obtained either for condition monitoring or for diagnostic testing, for example, fuel from a fuel system that is exhibiting problems such as sediment formation or filter plugging where the presence of microorganisms is suspected.

4.2 Microbial ATP is captured from the fuel system sample, concentrated into a test specimen, and tested using a bioluminescence reaction. The light generated by the luminescence reaction is proportional to the amount of ATP present in the test specimen as measured in a luminometer.[2]

4.3 Test results should be documented for evaluation and trending.

4.4 Specialized test methods for fuel samples, water samples, extracellular determination, or resolving potential matrix interference in bottom water samples are described in Appendix X4 and Appendix X5.

5. Significance and Use

5.1 This test 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 a reliable indicator of microbial contamination in fuel systems. ATP is not associated with matter of non-biological origin.

5.2 This test method differs from Test Method D4012 as follows:

5.2.1 By providing for the rapid determination of ATP present in a fuel (petroleum) sample, a fuel and water mixture sample, fuel-associated bottom water sample, and extracellular ATP freely available in the fuel or aqueous sample matrix;

5.2.2 By providing for a method to capture, extract, and quantify ATP using self-contained test device and luminometer;

5.2.3 By providing a method of quantifying ATP present in fuel or water matrices in generally less than 10 min; and

5.2.4 By providing for the rapid separation of the ATP from chemical interferences that have previously prevented the use of ATP determinations in complex fluids containing hydrocarbons and other organic molecules.

5.3 This test method does not require the use of hazardous materials and does not generate biohazard waste.

5.4 This test method can be used to estimate viable microbial biomass, to evaluate the efficacy of antimicrobial pesticides, and to monitor microbial contamination in fuel storage and distribution systems.

6. Interferences

6.1 Sample containers and sampling devices shall be clean and free of both ATP and microbial contamination.

6.2 Ensure that the sampling stick on the ATP Test Pen does not come into contact with any contaminating surfaces. Contact with a surface or substance can cause contamination with high levels of ATP, giving erroneous results.

6.3 Luciferase is an enzyme, which can be inhibited or denatured by high temperatures, the presence of heavy metals, and high salt concentrations in the sample. These conditions are unlikely to occur except in samples containing large volumes of bottom-water samples from storage tanks and similar systems.

6.3.1 For samples in which inhibition is suspected or likely to occur, testing of a dilution of the sample is described in Appendix X4.

7. Apparatus

7.1 An example of the luminometer[2] is shown as a diagram in Fig. 1.

7.2 **Warning—**The apparatus is not explosion-proof. The instrument should not be operated in explosive atmospheres or in locations where there may be explosive fumes, as it cannot be grounded.

7.3 Sample bottle, round wide-mouth, nominal capacity 500 mL or 1000 mL, HDPE (High Density Poly Ethylene) or equivalent. There shall be sufficient excess volume in the sample bottle so that there is at least 10 % head space in addition to the 500 mL or 1000 mL sample volume to facilitate the shearing and mixing of the capture solution.