Designation: D7463 – 08

StandardTest 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, extracting and quantifying the adenosine triphosphate (ATP) content associated with:

1.1.1 Microorganisms found in conventional liquid fuels with kinematic viscosities (at 40°C) of ≤ 8 $mm^2 \cdot s^{-1}$ as described in Table X6.1,

1.1.2 Microorganisms found in fuel-associated bottom water, and

1.1.3 Extracellular (non-cellular) ATP present in the sample matrix.

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 method for qualifying and quantifying ATP, this method does not differentiate between ATP from different sources, for example, from different types of microorganism such as 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.

1.7 *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:*[3]

D396 Specification for Fuel Oils
D975 Specification for Diesel Fuel Oils
D1129 Terminology Relating to Water
D1655 Specification for Aviation Turbine Fuels
D2069 Specification for Marine Fuels (Withdrawn 2003)[4]
D2880 Specification for Gas Turbine Fuel Oils
D3699 Specification for Kerosine
D4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water
D4057 Practice for Manual Sampling of Petroleum and Petroleum Products
D4175 Terminology Relating to Petroleum, Petroleum Products, and Lubricants
D6161 Terminology Used for Microfiltration, Ultrafiltration, Nanofiltration and Reverse Osmosis Membrane Processes
D6469 Guide for Microbial Contamination in Fuels and Fuel Systems
D6751 Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels

3. Terminology

3.1 *Definitions:*

3.1.1 For definition of terms used in this test method, refer to Terminologies D1129 and D4175, and Guide D6469.

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

Current edition approved Dec. 1, 2008. Published February 2009. DOI: 10.1520/D7463-08.

[2] The sole source of supply of the apparatus known to the committee at this time is Merck KGaA, 64271 Darmstadt, Germany. 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.

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

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

3.1.3 *adenosine monophosphate, n*—molecule formed by the removal of two (2) molecules of phosphate (one pyrophosphate molecule) from ATP.

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*—any matter which is or was a living organism or excreted from a microorganism. D6161

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

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

3.1.9.1 *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.

3.1.10 *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*).

3.1.11 *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.

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

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

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

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

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

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

3.1.17 *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 *PP*—polypropylene

3.2.5 *RLU*—relative light units

4. Summary of Test Method

4.1 A fuel 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 micro-organisms is suspected.

4.2 Microbial ATP is captured, extracted, and quantified using a bioluminescence reaction. The light generated by the luminescence reaction is measured in a luminometer.[2]

4.3 Specialized test methods for fuel samples, water samples, extracellular determination or resolving potential matrix interference in bottom water samples are described in the appendixes.

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.

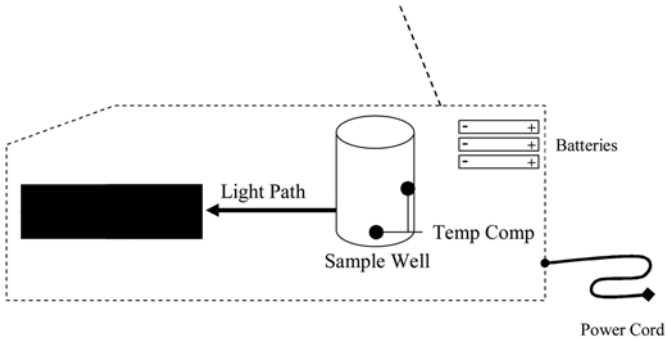

FIG. 1 Luminometer

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, 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 sample volume to facilitate the shearing and mixing of the capture solution.

7.3.1 Sample bottles may be reused provided they are cleaned and dried correctly. Refer to test supplier's information regarding recommended cleaning procedure.

7.4 Pipettors, fixed volume or adjustable capable of providing discreet volumes of bottom water to determine the presence of matrix interference as described in Appendix X4. Example pipettor volumes include 10 µL, 50 µL, and 100 µL.

8. Reagents and Materials

8.1 *Reagents:*

8.1.1 *ATP di-sodium salt.*

8.1.2 *Water, Pyrogen free.*

8.2 *Materials:*

8.2.1 ATP test pens:[2]

8.2.1.1 HY-LiTE[5] Fuel Test Pen, as shown in Fig. 2.

8.2.1.2 HY-LiTE[5] Free ATP Pen, as shown in Fig. 2.

8.2.2 Pasteur pipettes, sterile, disposable, polyethylene, 1.0 mL.

8.2.3 Pasteur pipettes sterile, disposable, polyethylene, 10.0 mL.

9. Sampling, Test Specimens, and Test Units

9.1 Samples shall be drawn in accordance with Practice D4057 as amplified by Hill.[6]

9.2 To reduce the risk of accidental contamination, samples intended for microbiological testing shall not be used for other tests until after they are no longer needed for microbiological testing.

9.3 It may be possible to accidentally cross contaminate the sample under field conditions. To reduce risk of potential cross-contamination, rinse the sample device(s) and sample container(s) with a 70 % alcohol (ethanol, methanol, or isopropanol) and water solution and let air dry. All devices (except factory new, clean bottles) should be disinfected is this manner to minimize the likelihood of cross-contamination. Use care to not touch the interior of the freshly decontaminated sample devices or sample bottles. Remove the container lid immediately before dispensing the sample into the container and replace the lid on the container as soon as possible.

9.4 Microbial contaminant populations are dynamic. Microbes within the sample can proliferate or die during the interval between collection and testing. Consequently, samples shall be processed within 24 h after collection.

9.5 If samples are to be tested later than 4 h after collection, store the samples either on ice, or refrigerated at >0 to 5°C until tested. Avoid freezing samples. Allow samples to equilibrate to room temperature before testing.

10. Calibration and Standardization

10.1 The luminometer[2] which is specific to this test is factory calibrated and temperature compensated to give a linear

[5] Registered trademark of Merck KGaA, 64271 Darmstadt, Germany.

[6] Hill, G., "Sampling Methods for Detecting Microbial Contamination in Fuels and Fuel Systems," *Fuels and Fuel Systems Microbiology: Fundamentals, Diagnosis, and Contamination Control, ASTM MNL 47,* ASTM International, West Conshohocken, PA, 2002, p. 14.