Designation: ~~D7463 – 14~~ D7463 – 14a

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, ~~extracting~~concentrating, and ~~quantifying~~testing the adenosine triphosphate (ATP) ~~content~~ present in a fuel system sub-sample (that is, test specimen) associated with:

1.1.1 Microorganisms and hydrophilic particles found in ~~conventional liquid fuels with kinematic viscosities (at 40°C) of ≤ 8 mm~~liquid fuels ~~^{2} · s^{-1}~~as described in Table X6.1, or

1.1.2 Microorganisms ~~found in fuel-associated bottom water, and~~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 ~~Extracellular (non-cellular) ATP present~~ 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~~the sample matrix.~~. 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 ~~method for qualifying and quantifying ATP,~~ technology, this method does not differentiate ~~between ATP from different sources, for example, from different types of microorganism such as~~ 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 and health practices and determine the applicability of regulatory limitations prior to use.*

[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 and Cleanliness of Liquid Fuels.

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[2] The sole source of ~~supply~~ 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.~~ 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.

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

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D396 Specification for Fuel Oils
D910 Specification for Aviation Gasolines
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~~D4814 ~~Terminology Used for Microfiltration, Ultrafiltration, Nanofiltration and Reverse Osmosis Membrane Processes~~Specification for Automotive Spark-Ignition Engine Fuel
~~D6469~~D6227 ~~Guide for Microbial Contamination in Fuels and Fuel Systems~~Specification for Unleaded Aviation Gasoline Containing a Non-hydrocarbon Component
D6615 Specification for Jet B Wide-Cut Aviation Turbine Fuel
D6751 Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels
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 ~~Terminologies~~Terminology ~~D1129 and~~ D4175~~, and Guide D6469~~.

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~~ biological material including any material other than fossil fuels which is or was a living organism or ~~excreted from a microorganism.~~component or product of a living organism. ~~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 *cellular adenosine triphosphate (cellular-ATP), n*—ATP present in whole cells, whether they are living or dead.

[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.

3.1.8.1 *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 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.

3.1.10 *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*—undissolved water present in a hydrophobic material.

3.1.11.1 *Discussion*—

Free water in fuel such as hydrocarbon diesel fuel can be present as a suspended haze, as droplets on the walls of the vessel, or as a separate layer on the bottom of the vessel.

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-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

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 micro-organisms is suspected.

4.2 Microbial ATP is ~~captured, extracted, and quantified~~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.

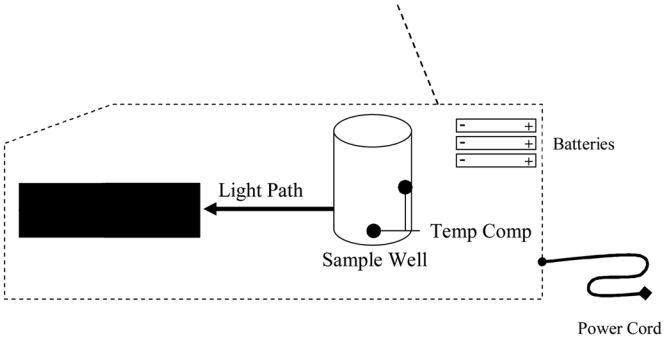

FIG. 1 Luminometer

4.4 Specialized test methods for fuel samples, water samples, extracellular determination, or resolving potential matrix interference in bottom water samples are described in ~~the~~Appendix X4 ~~appendixes.~~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.~~ 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~~discrete 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.*

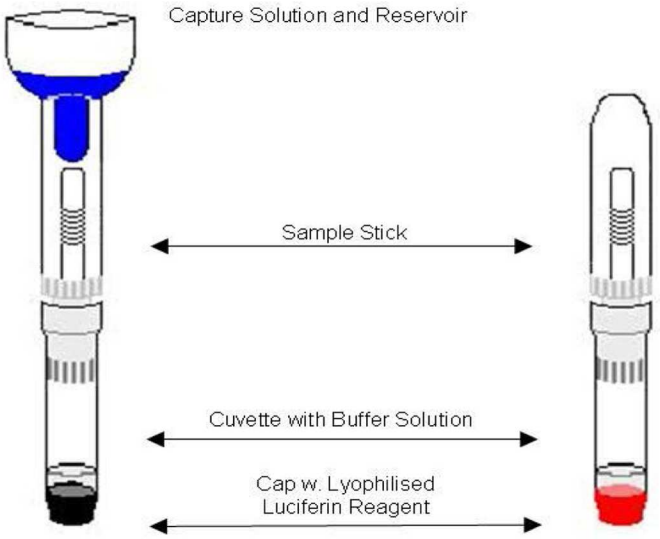

FIG. 2 Fuel Test Pen and Free ATP Test Pen

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 (isopropyl alcohol or ethanol) and water solution and let air dry. All devices (except factory new, clean bottles) should be disinfected ~~is~~in 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,~~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 response from ~~0-99,000~~ 0 to 99 000 RLU at temperatures between 41 and 95°F (5 and 35°C). No calibration is necessary because calibrations checks are performed automatically during start-up.

10.2 RLU data may be converted to ATP concentration by interpolating from a standard curve as described in Appendix X5.

10.3 1 RLU is equivalent to approximately 5×10^{-15} grams ATP.

[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.

11. Procedure

11.1 *Analysis of Fuel and Combined Fuel and Water Samples:*

11.1.1 Collect sample according to 9.1.

11.1.1.1 If sample is ≤500 mL, collect dispense directly into clean sample bottle (7.3).

11.1.1.2 If total sample is >500 mL, transfer 500 mL to a clean sample bottle (7.3).

11.1.2 Obtain the small pipette from the fuel test kit.

11.1.3 Using a clean implement (for example, scissors or knife), cut the protective plastic sleeve open at the bulb-end and remove the pipette. Do not touch the tip and lower stem of the pipette by hand or against any surfaces.

11.1.4 Using the small sterile pipette, transfer the capture solution from the fuel test pen reservoir into the bottle that contains the sample.

11.1.5 Rinse the interior of the pipette with sample to ensure maximum transfer of the capture solution to the sample.

11.1.6 Dispose of the pipette as solid (fuel-contaminated) waste according to local regulations.

11.1.7 Close the lid securely on the sample vessel.

11.1.8 Shake the sample vigorously for 30 s.

11.1.9 Place the sample vessel on level surface and let stand for 5 min.

NOTE 1—The capture solution will readily dissolve into the free water associated with the fuel sample or those samples that contain only water. The presence of water in the sample will cause the diluted capture solution to look paler than the undiluted capture solution.

NOTE 2—Highly colored or hazy samples may color the capture solution. This will not affect the RLU reading.

11.1.10 Ensure that the luminometer is powered on and has successfully completed the self-check and is ready for analysis.

11.1.11 After the 5 min standing as prescribed in 11.1.9,

11.1.11.1 Obtain the large pipette from the fuel test kit,

11.1.11.2 Using a clean implement (for example, scissors or knife), cut the plastic protective sleeve open at the bulb-end and remove the pipette. Do not touch the tip and lower stem of the pipette by hand or against any surfaces.

11.1.12 Coalesce the capture solution and any water phase present into a single drop or phase and use the large pipette to retrieve and transfer a sample aliquant to the fuel test pen reservoir for testing.

11.1.13 The level of capture solution sample must at least reach up to the bottom of the bowl shape on the sampling tube. If excess fuel phase (more than 1 mm visible above blue phase) enters the sampling tube, use the same pipette to remove free fuel prior to testing.

11.1.14 Close the lid on the sampling tube reservoir and separate the sampling pen from the sampling tube to expose the sterile white sampling stick.

11.1.14.1 Do not touch the sampling stick by hand or against any surface.

11.1.15 Open the lid of the fuel test pen to access the reservoir and dip the sampling stick into the capture solution until it touches the bottom of the tube.

11.1.16 Holding the tube and reservoir vertically, carefully remove the sampling stick from the reservoir without touching the stick against the sides of the sampling tube.

11.1.17 Keep the pen vertical and sampling stick pointing downward. Do not shake the pen. The capture solution should be thoroughly deposited between all the ridges on the lower half of the sampling stick.

NOTE 3—If the capture solution sample contained excess fuel, this will show up as white or pale “patches” in the rings of blue liquid.

11.1.17.1 If fuel is present, dip the stick again and move up and down several times in the capture solution before removing the sampling stick again. This will usually rinse off most of the fuel phase and ensure that the capture solution is evident on all of the ridges of the sample stick.

11.1.18 With the white sample stick pointing down, hold the pen cuvette firmly in a fist and firmly press the tip of the sampling stick vertically against a hard, flat, level surface, until the sampling stick retracts completely into the pen cuvette (chamber).

11.1.19 Activate the pen by pressing and turning the white pen collar clock-wise until finger-tight.

11.1.20 Hold the sample cuvette between the thumb and forefinger and shake it in an end-to-end motion for 10 to 20 s.

NOTE 4—Good shaking of the pen is crucial to obtain complete reconstitution and mixing of the freeze-dried reagents deposited in the pen cap. Insufficient mixing can typically be diagnosed by the light signal increasing with time for up to several minutes after the initial measurement.

11.1.20.1 Remove gloves that may have been worn during steps that involve potential direct contact with sample. This will prevent the risk of static discharge while inserting test-pen into luminometer.

11.1.21 Place the pen in the cuvette holder of the luminometer and close the lid to initiate the reading.

11.1.21.1 Measure the light signal immediately after activation of the test pens. Results are displayed and the instrument lid opens after approximately 15 s.

11.1.22 Record RLU.

11.1.23 Remove the pen and discard according to local regulations.

11.1.24 Calculate and report the results as described in Section 12.