Designation: D7687 – 17

Standard Test Method for Measurement of Cellular Adenosine Triphosphate in Fuel and Fuel-associated Water With Sample Concentration by Filtration[1]

This standard is issued under the fixed designation D7687; 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 covers a protocol for capturing, extracting and quantifying the cellular adenosine triphosphate (cellular-ATP) content associated with microorganisms found in fuels and fuel-associated water.

1.2 The ATP is measured using a bioluminescence enzyme assay, whereby light is generated in amounts proportional to the concentration of cellular-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, computation to pg ATP/mL and optional further transformation to Log_{10}[pg ATP/mL].

1.3 This test method is equally suitable for use as a laboratory or portable method.

1.4 This test method is limited to fuels with a nominal viscosity ≤75 cSt at test temperature.

1.5 This test method detects ATP concentrations in the range of 5.0 pg ATP/mL (≈0.699 $\log_{10}$[pg ATP/mL]) to 100 000 pg ATP/mL (≈5.000 $\log_{10}$[pg ATP/mL]) for 20 mL samples of fuel and 20 pg ATP/mL (≈1.301 $\log_{10}$[pg ATP/mL]) to 400 000 pg ATP/mL (≈5.602 $\log_{10}$[pg ATP/mL]) for 5 mL samples of fuel-associated water.

NOTE 1—These ranges were calculated with the formula for calculating sample ATP in pg/mL provided in 12.1 based on the minimum recommended RLU for a 1 ng/mL ATP standard when using the reagents specified in Section 7 and the luminometer specified in 6.4 and corrected with a reagent-method blank as determined in Appendix X5.

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

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

1.8 *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.*

1.9 *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:*[2]

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)[3]
D2880 Specification for Gas Turbine Fuel Oils
D3699 Specification for Kerosine
D4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water
D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants
D6161 Terminology Used for Microfiltration, Ultrafiltration, Nanofiltration and Reverse Osmosis Membrane Processes
D6300 Practice for Determination of Precision and Bias Data for Use in Test Methods for Petroleum Products and Lubricants
D6751 Specification for Biodiesel Fuel Blend Stock (B100)

[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] 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.

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

for Middle Distillate Fuels
D7463 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Fuel, Fuel/Water Mixtures, and Fuel Associated Water
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)
D7847 Guide for Interlaboratory Studies for Microbiological Test Methods
D7978 Test Method for Determination of the Viable Aerobic Microbial Content of Fuels and Associated Water—Thixotropic Gel Culture Method
E2523 Terminology for Metalworking Fluids and Operations
E2694 Test Method for Measurement of Adenosine Triphosphate in Water-Miscible Metalworking Fluids
F1671 Test Method for Resistance of Materials Used in Protective Clothing to Penetration by Blood-Borne Pathogens Using Phi-X174 Bacteriophage Penetration as a Test System

3. Terminology

3.1 *Definitions:*

3.1.1 For definition of terms used in this test method, refer to Terminology D1129, D4175, D6161, and E2523.

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 microbial contamination.

3.1.5 *background RLU, n*—quantity of relative light units resulting from running the test method without incorporation of the sample.

3.1.6 *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.

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

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 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 on solid growth media or the development of turbidity in liquid growth media under specific growth conditions.

3.1.10 *extra-cellular, adj*—molecules or substances that are either excreted by living cells or released from microbial cells that have lysed (see 3.1.14) in the sample.

3.1.10.1 *Discussion*—Extra-cellular ATP is ATP that has been released from microbial cells that have either fully or partially lysed in the sample, the upstream fluid (fuel or water phase), or both.

3.1.10.2 *Discussion*—Lysis can occur due to natural life cycle process, antimicrobial treatment or a combination of these factors. Extra-cellular ATP can under certain circumstances persist for periods greater than 24 h after cell lysis depending on physical/chemical conditions.

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

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

3.1.13 *luminometer, n*—instrument capable of measuring light emitted as a result of nonthermal excitation.

3.1.14 *lysis, n*—disintegration or destruction of whole bacterial cells. **F1671**

3.1.15 *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.

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

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

3.2 *Acronyms:*

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 unit.

4. Summary of Test Method

4.1 A control assay is performed using 100 µL of 1.0 ng ± 0.05 ng ATP/mL standard to produce RLU_{ctrl}.

4.2 A 20 mL sample of fuel or 5.0 mL bottom-water 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 non-ATP contaminants that might otherwise interfere with the cellular-ATP assay.

4.4 The filter is air-dried.

4.5 A lysing reagent is used to release cellular-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 to 10 with a buffer solution.

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

4.8 The culture tube is placed into a luminometer and the light intensity is read as RLU_{obs}.

4.9 RLU_{obs} is normalized to an actual pg ATP/mL concentration through an equation that accounts for the result of the control assay (RLU_{ctrl}), the volume of the sample processed, and the method dilution factor.

NOTE 2—Optionally, for condition monitoring purposes, pg ATP/mL are converted to Log_{10}[pg ATP/mL] of sample by computation.

5. Significance and Use

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

5.2 This test method is similar to Test Method E2694 except for the volumes sampled.

5.3 This test method differs from Test Method D4012 in that it utilizes filtration and wash steps designed to eliminate interferences that have historically rendered ATP testing unusable with complex organic fluids such as fuel and fuel-associated water.

5.4 This test method differs from Test Method D7463 in several regards:

5.4.1 Test Method D7463 reports relative light units (RLU). Consistent with Test Methods D4012 and E2694, this test method reports ATP concentration.

5.4.2 This test method detects only cellular-ATP and it can be used to detect cellular-ATP in fuels and fuel stocks from which small quantities of water do not separate readily (for example, ethanol blended gasoline containing ≥5 % v/v ethanol). Test Method D7463 cannot be used to recover ATP from fuels from which small quantities of water do not separate readily (for example, ethanol blended gasoline containing ≥5 % v/v ethanol).

5.4.3 This test method measures cellular-ATP in a single measurement (as pg ATP/mL). Test Method D7463 detects total ATP (as RLU) and extra-cellular ATP (as RLU) using two separate analyses and permits computation of cellular-ATP (as RLU) as the difference between total and extracellular ATP.

5.4.4 Test Method D7463 suggests a nominal 500 mL fuel sample volume. This test method suggests a nominal 20 mL fuel sample.

5.5 This test method can be used with all fuels specified in Specifications D396, D975, D1655, D2069, D2880, D3699, D6751, and D7467 and other fuels with nominal viscosities ≤75 cSt at 20° ± 2°.

5.6 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 5 min.

5.7 Although ATP data generally covary with culture data in fuel and fuel-associated water, different factors affect ATP concentration than those that affect culturability.

5.7.1 Culturability is affected primarily by the ability of captured microbes to proliferate on the growth medium provided, under specific growth conditions. Consequently, a proportion of the active or inactive microbial population present in a sample may be viable but not detected by any one culture test.[4]

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

5.7.2.1 One example of the species effect is that the amount of ATP per cell is substantially greater for active fungal cells than bacteria.

5.7.2.2 Within a species, cells that are more metabolically active will have more ATP per cell than dormant cells, such as fungal spores. Because fungal spores are more hydrophobic than active fungal material (mycelium), spores may be the only indicator of fungal proliferation when fuel samples are taken from some fuel systems, but they will not be detected by a test for ATP.

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

5.7.3 The possibility exists that the rinse step (11.15) may not eliminate all chemical substances that can interfere with the bioluminescence reaction (11.37).

5.7.3.1 The presence of any such interferences can be evaluated by performing a standard addition test series or dilution series as described in Appendix X4. The precision statement in Section 13 will not apply.

5.8 As explained in Test Method D7978, there are inherent difficulties in assessing precision of microbiological procedures for fuels on account of the inherent variability of the determinant and various determinable and indeterminable sources of inaccuracy (see Guide D7847).

5.8.1 The precision of any microbiological analytical method will generally be considerably less than that of methods widely used in the petroleum industry for analysis of physical and chemical properties of fuels.

6. Apparatus

6.1 *Culture Tube,* sterile, disposable, PP, 12 mm by 55 mm.

6.2 *Culture Tube,* sterile, disposable, PP, 17 mm by 100 mm with caps.

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

6.4 *Luminometer,* using photomultiplier tube, having a spectral range between 300 nm and 600 nm, and with a cuvette chamber that can hold and provide an unobstructed line of sight to the reactants in a 12 mm by 55 mm test culture tube (6.1), providing a ratio of $RLU_{background}$ /RLU_{ctrl} (refer to Section 10 and Appendix X5) ≤0.01 and optimally having five decades of linearity (refer to Appendix X2).

NOTE 3—Although this test method is optimized to function on this luminometer, users may examine the use of other luminometers according to key performance criteria, including linear measurement range (Appendix X2) and $RLU_{background}$ level (Appendix X5). The precision statement in Section 13 will not apply to the test result(s).

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

6.4.1 ILS-1259 was run using PhotonMaster[5] (trademark) luminometers, which met 6.4 specifications.

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

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

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

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

6.9 *Sample Collection Container,* sterile, wide-mouth bottle, PP or HDPE, 100 mL.

NOTE 4—ATP can adsorb onto glass surfaces. Consequently, PP or HDPE containers are strongly preferred.

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,* 1.0 ng ± 0.05 ng ATP/mL.

7.2 *ATP Extract Dilution Buffer,* (proprietary),[6] predispensed as 9.0 mL dilution 4 7.3 ATP Extraction Reagent, (proprietary). 4 blanks in 17 mm x 100 mm culture tubes (6.2).

7.3 *ATP Extraction Reagent,* (proprietary).[6]

7.4 *Filter Wash Reagent,* (proprietary).[6]

7.5 *Luciferin-Luciferase Reagent,* (proprietary).[6] Store between –20 °C and 4 °C; allow to equilibrate to ambient temperature before using.

8. Hazards

8.1 Inhalation or dermal exposure to fuels can pose health problems for personnel involved with fuel sampling. Provision of personal protective equipment (PPE) in the form of respirators, protective clothing or both may be indicated.

8.2 **Warning—**PhotonMaster[5] is not explosion-proof and shall not be operated in explosive atmospheres or in locations where there may be explosive fumes, as it cannot be grounded. Consult the manufacturer's guidelines for further information.

9. Sampling, Test Specimens, and Test Units

9.1 Samples shall be drawn in accordance with Practice D7464.

NOTE 5—If the sample contains two or more phases (for example: fuel and water), each phase can be transferred to a separate, appropriate container to facilitate testing single-phase specimens per Section 11 and 11.5.

[5] *PhotonMaster*, is a trademark of LuminUltra Technologies Ltd., Fredericton, New Brunswick, Canada, www.luminultra.com, LuminUltra Technologies Ltd.

[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,[1] which you may attend.

10. Calibration and Standardization

10.1 It is necessary to perform only one calibration during each set of tests performed on the same day, using the same reagent batches, under the same temperature, using the same materials and the same luminometer. In general, one calibration per day of testing is sufficient so long as the aforementioned variables are kept the same.

NOTE 6—Although multi-point calibration is not required, guidance on performing a multi-point calibration is provided in Appendix X2.

10.2 Turn on power to luminometer (6.4) and allow instrument to equilibrate, in accordance with manufacturer's recommendations.

10.2.1 It is possible, that during prolonged use, over the course of several hours, some luminometer models will heat up sufficiently to affect the temperature of the reactants (11.36) to affect the test result (11.37). Excessive on-time can also stimulate the photomultiplier tube to produce increased instrument background noise. Consequently, it is advisable to repeat 10.7 through 10.12 after every 3 h of continuous operation.

10.3 Ensure that all reagents have equilibrated to ambient temperature (22 °C ± 3 °C) before running any tests.

NOTE 7—The kinetics of the enzymatic reaction measured by this procedure are affected by temperature.

10.4 It is important to ensure that the assay is performed at constant temperature (T ± 3 °C), that is, the instrument and all reagents must remain at same temperature during the measurement series.

10.5 Likewise, if the measurement results are to be compared to a standard curve or control samples these must be measured at the same conditions and temperature as the test sample.

10.6 Changes in the instrument, diluted sample, ATP standard (7.1) or Luciferin-Luciferase reagent (7.5) temperature while testing multiple samples will affect the RLU and consequently introduce a bias to the test results.

10.7 Use a micropipeter (6.6) with a new 100 µL to 1000 µL tip (6.7) to dispense 100 µL Luciferin-Luciferase reagent (7.5) to an unused 12 mm by 55 mm culture tube (6.1).

10.8 Replace the micropipeter tip with a fresh 100 µL to 1000 µL tip.

10.9 Dispense 100 µL of 1 ng ATP/mL standard solution (7.1) into the culture tube.

10.10 Swirl gently for five times.

10.11 Place the culture tube into the luminometer.

10.12 Read and record RLU (RLU_{ctrl}).

11. Procedure

11.1 Use aseptic procedure while performing this test method; cellular-ATP from analyst's hands, sputum, etc. can contaminate the sample with cellular-ATP from sources other than the sample itself.

11.2 Remove plunger from a new 20 mL syringe (6.10) and place onto 17 mm test tube rack so that plunger tip does not contact any surfaces so as to avoid contamination with ATP.

11.3 Affix filter (6.3) onto the 20 mL syringe.

11.4 Place a fresh 1.0 mL to 5.0 mL tip (6.8) onto the macropipeter (6.5).

11.5 In order to test fuel specimens, confirm that the sample contains no visible free-water.

11.5.1 If visible free-water is present, separate the fuel-phase from the aqueous-phase.

11.5.2 Shake fuel sample for 15 s to ensure homogeneity.

11.5.3 With minimal delay, remove lid from specimen container and, using the macropipeter, transfer four volumes of 5.0 mL (20 mL total) of fuel to the 20 mL syringe barrel (6.10).

11.5.4 If desired, consult Appendix X3 for guidelines on adjusting method detection range by modifying the standard sample volume. The precision statement in Section 13 will not apply to the test result(s).

11.6 In order to test fuel-associated water specimens, either:

11.6.1 Collect aqueous-phase sample per 11.5.1, or

11.6.2 Use a pipet to draw ≥20 mL (or maximum volume available, whichever is less) of aqueous-phase fluid and transfer it into a separate specimen container.

11.6.3 Shake aqueous-phase sample for 15 s to ensure homogeneity.

11.6.4 With minimal delay, remove lid from sample container and, using the macropipeter, transfer 5.0 mL of aqueous-phase fluid to the 20 mL syringe barrel (6.10).

11.6.5 If desired, consult Appendix X3 for guidelines on adjusting method detection range by modifying the standard sample volume. The precision statement in Section 13 will not apply to the test result(s).

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 the sample, having filtrate discharge into the waste receptacle.

NOTE 8—For samples having high particulate loads, it may not be possible to filter the full subsample. When this occurs, record the actual volume filtered and substitute this value for "V" in Eq 1 (12.1). The precision statement in Section 13 will not apply to the test result(s).

11.9 Remove filter from the 20 mL syringe and place onto 17 mm test tube rack so that filter outlet does not contact any surfaces.

11.10 Remove plunger from the 20 mL syringe and place onto 17 mm test tube rack so that plunger tip does not contact any surfaces so as to avoid contamination with ATP.

11.11 Replace filter onto the end of the 20 mL syringe barrel.

11.12 Place a 1.0 mL to 5.0 mL fresh tip onto the macropipeter.

11.13 Transfer 5 mL of filter wash reagent (7.4) into the syringe barrel.

11.14 While holding the barrel over the waste receptacle (6.14), replace the 20 mL syringe plunger.

11.15 Apply even pressure to syringe plunger to pressure filter sample, having filtrate discharge into the waste receptacle.

11.16 Remove filter from the 20 mL syringe. Place the 20 mL syringe to the side for later use (11.24).

11.17 Remove plunger from a 60 mL syringe (6.11) and place onto 17 mm test tube rack so that plunger tip does not contact any surfaces.

11.18 Attach the filter onto the 60 mL syringe.

11.19 While holding the barrel over the waste receptacle (6.14), replace the 60 mL syringe plunger.

11.20 Apply even pressure to the 60 mL syringe plunger to air dry the filter.

11.21 Repeat steps 11.17 through 11.20 one more time, first separating the filter before removing the plunger from the 60 mL syringe.

11.22 Remove filter from the 60 mL syringe and place onto 17 mm test tube rack so that filter outlet does not contact any surfaces. Place the 60 mL syringe to the side for later use (11.17).

11.23 Place an unused 17 mm by 100 mm culture tube containing 9.0 mL ATP extract dilution buffer (7.2) into 17 mm test tube rack.

11.24 Remove the plunger from the 20 mL syringe (11.16) and place onto 17 mm test tube rack so that barrel tip does not contact any surfaces.

11.25 Attach filter from step 11.22 onto end of the 20 mL syringe.

11.26 Place a fresh 100 µL to 1000 µL pipet tip onto the micropipeter.

11.27 Use micropipeter to dispense 1.0 mL of ATP extraction reagent (7.3) into the 20 mL syringe barrel.

11.28 While holding the barrel over the 17 mm by 100 mm culture tube (11.23), replace the 20 mL syringe plunger.

11.29 Apply even pressure to the 20 mL syringe plunger, to dispense ATP extraction reagent and extracted ATP into the 17 mm by 100 mm culture tube containing 9.0 mL ATP extract dilution buffer (7.2).

NOTE 9—Alternatively, dispense ATP extraction reagent and extracted ATP into an empty 17 mm by 100 mm culture tube (6.2). At this point in the protocol, this ATP extract may be stored for up to seven days at 2 °C to 8 °C prior to completing the test. The precision statement in Section 13 will not apply to the test result(s).

11.30 If not already performed (10.2), turn power on luminometer (6.4) and allow instrument to equilibrate, in accordance with manufacturer's recommendations.

11.31 Place cap on culture tube and invert three times to mix well.

NOTE 10—Diluted ATP extract is stable for at least 4 h at room temperature (20 °C ± 2 °C).

11.32 Place one 12 mm by 55 mm culture tube into the 12 mm test tube rack (6.12).

11.33 As in 10.7, use a micropipeter with a fresh 100 µL to 1000 µL tip to dispense 100 µL of Luciferin-Luciferase reagent into the 12 mm by 55 mm culture tube.

11.34 Using a fresh pipet tip, use micropipeter to transfer 100 µL of diluted sample to a 12 mm by 55 mm culture tube containing 100 µL of Luciferin-Luciferase reagent (11.33).

11.35 Remove the culture tube from the test tube rack and swirl gently five times.

11.36 Place culture tube into luminometer chamber.

11.37 Read and record RLU_{obs}.

11.37.1 If RLU are outside of the luminometer's range (that is, below the background level or greater than the maximum read-out), see Appendix X3 for guidance on steps to prepare sample so that RLU reading is within the luminometer's measurement range. The precision statement in Section 13 does not apply to the test result(s).

11.38 When testing multiple samples, perform steps 11.1 through 11.29 in sequence for each sample after turning on the luminometer (11.30), perform steps 11.26 through 11.37 for each prepared ATP extract.

12. Calculation of Results

12.1 Compute cellular-ATP_{Sample} in pg ATP/mL:

$$cATP_{Sample}\,(pg\ ATP/mL) = \frac{RLU_{obs}}{RLU_{ctrl}} \times \frac{10\,000\,(pg\ ATP)}{V_{Sample}\,(mL)} \quad (1)$$

where:

RLU_{obs} = the sample RLU reading (11.37),
RLU_{ctrl} = the RLU for the 1 ng ATP/mL control (10.12), and
V_{Sample} = sample volume in mL (20 mL or 5.0 mL in accordance with 11.6).

10 000 pg ATP is derived from:

$$10\,000\,pg\ ATP = (1000\,pg\ ATP/ng\ ATP) \times 1.00\,ng\ ATP \times dilution\ factor \quad (2)$$

where:

1000 pg ATP/ng ATP = unit conversion factor,
1.00 ng ATP = concentration of the ATP standard used to acquire RLU_{ctrl} (10.9), and
dilution factor = 10 (1.0 mL ATP extract (11.29) in 9.0 mL ATP extract dilution buffer).

12.1.1 Report result as ATP_{Sample} in pg ATP/mL.

12.1.1.1 When RLU_{obs} is ≤10 times $RLU_{background}$ for the luminometer specified in 6.4, subtract $RLU_{background}$ (obtained through the procedure outlined in Appendix X5) from RLU_{obs} prior to computing pg ATP/mL.

NOTE 11—Because [cATP] can range across multiple orders of magnitude, as a matter of convenience and to enhance control chart ease of reading, linear data can be transformed to Log_{10} values, report only the first three digits as significant. All other whole number digits to the right of the third digit should be entered as "0". If data are transformed to Log_{10} digits beyond the first two places to the right of the decimal point are meaningless.

NOTE 12—Reporting results on a Log_{10} basis facilitates comparison of total microbial population data via cellular-ATP on the same scale as traditional culturability methods.

13. Precision and Bias[7]

13.1 *Precision*—The following precision was determined in accordance with Practice D6300.

NOTE 13—The repeatability and intermediate precision statements were based on data obtained during an ILS[7] performed at CBAI Biosciences, Egham, UK. Four representative fuel grades, plus one representative bottoms-water were used. A retail fuel system, bottoms-water, uncharacterized population was used as the inoculum. Each sample type was tested at three biobuden levels: negligible, moderate, and high. Eight ILS participants ran Test Method D7687 on triplicate samples of each sample type/bioburden combination, generating a total of 360 test results.

13.1.1 *Repeatability*—The difference between repetitive results obtained by the same operator in a given laboratory applying the same test method with the same apparatus under constant operating conditions on identical test material within short intervals of time would in the long run, in the normal and correct operation of the test method, exceed the values determined using the equations in 13.1.3 only in 1 case in 20.

13.1.2 *Intermediate Precision (Between Operator/Apparatus Repeatability)*—The difference between two single and independent results obtained by different operators applying the same test method in same laboratory using different apparatus on identical test material within short intervals of time would, in the long run, in the normal and correct operation of the test method, exceed the values determined using the equations in 13.1.3 only in 1 case in 20.

13.1.3 *Precision Equations:*

13.1.3.1 Fuel and Fuel-Associated Water:

$$\text{Repeatability (r) in fuel or fuel-associated water} = 6.1387 \cdot X^{0.8559}\,\text{pg ATP/mL} \quad (3)$$

$$\text{Between Operator/Apparatus Repeatability} = 6.7528 \cdot X^{0.8559}\,\text{pg/mL}$$

where:

X = average of two test results.

13.2 *Reproducibility*—Due to the instability of the measure and of this test method over time, it is not possible to determine reproducibility of this test method using traditional interlaboratory studies in strict adherence with Practice D6300.

13.3 *Bias*—Since there is no accepted reference material suitable for determining the bias of this test method, bias cannot be determined.

14. Keywords

14.1 adenosine triphosphate; ATP; bacteria; bioburden; biodeterioration; biodiesel; biofuels; biomass; cellular; cellular-ATP; diesel; fuel; fuel-oil; fungi; gasoline; microbial contamination; microbiology; microorganisms

[7] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D02-1854. Contact ASTM Customer Service at service@astm.org.