Designation: D7687 – 11

Standard Test Method for Measurement of Cellular Adenosine Triphosphate in Fuel, Fuel/Water Mixtures, 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, fuel/water mixtures 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 ≤ 75cSt 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 or fuel/water mixtures, 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 ATP/mL 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.*

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, Petroleum Products, 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) 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)

[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.

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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.

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 ± 0.05 ng ATP/mL standard to produce RLU_{ctrl}.

4.2 A 20 mL sample of fuel or fuel/water mixture 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, fuel/water mixtures 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 Method 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 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, fuel/water mixtures, 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 (11.39).

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.

6. Apparatus

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

6.2 *Culture Tube,* sterile, disposable, PP, 17 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 and 600 nm, and with a cuvette chamber that can hold and provide an unobstructed line of sight to the reactants in a 12 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 5 decades of linearity (refer to Appendix X2).

NOTE 3—The preliminary interlaboratory study and data presented in Appendix X6 and Table X4.2, respectively, were developed using a Kikkoman Lumitester C-110, which provides nominally a 5000 RLU_{ctrl} and 50 $RLU_{background}$. 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).

NOTE 4—It is the responsibility of the user to ensure that the luminometer selected for use meets the criteria listed in 6.4 and to consult with the luminometer manufacturer to ensure that use of the luminometer with the apparatus, reagents and materials described in Sections 6 and 7 does not result in the inability of the instrument manufacturer to provide technical support or loss of instrument warranty.

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, PP or HDPE, 100 mL.

NOTE 5—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.

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

7. Reagents and Materials

7.1 *ATP Standard,* 1.0 ± 0.05 ng ATP/mL.

7.2 *ATP Extract Dilution Buffer,* (proprietary).[5]

7.3 *ATP Extraction Reagent,* (proprietary).[5]

7.4 *Filter Wash Reagent,* (proprietary).[5]

7.5 *Luciferin-Luciferase Reagent,* (proprietary).[5] Store between -20 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—**Not all luminometers are explosion proof. Luminometers that are not explosion-proof should 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.

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 (11.38) to affect the test result (11.39). 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 ± 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 (11.32), 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 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).

10.8 Replace the micropipeter tip with a fresh 100 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 to 5.0 mL tip (6.8) onto the macropipeter (6.5).

11.5 Shake sample for 15 s to ensure homogeneity.

11.6 With minimal delay, remove lid from sample container and, using the macropipeter, transfer four volumes of 5.0 mL (20 mL total) of fuel or fuel/water mixture sample, or one volume of 5.0 mL of fuel-associated water sample to the 20 mL syringe barrel.

11.6.1 If desired, consult Appendix X3 for guidelines on adjusting method detection range by modifying the standard sample volume.

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.

11.8.1 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).

[5] 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.

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 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.

11.17.1 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 (11.17.1).

11.23 Place an unused 17 by 100 mm culture tube (6.2) 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 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 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 by 100 mm culture tube.

NOTE 8—At this point in the protocol, this ATP extract may be stored for up to seven days at 2 to 8°C prior to completing the test.

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 a fresh 1.0 to 5.0 mL pipet tip onto the macropipeter.

11.32 Use macropipeter to dispense two 4.5 mL portions (9.0 mL total) of ATP extract dilution buffer (7.2) into the culture tube to prepare diluted ATP extract.

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

NOTE 9—Diluted ATP extract is stable for at least 4 h at room temperature (20 ± 2°C).

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

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

11.36 Using a fresh pipet tip, use micropipeter to transfer 100 µL of diluted sample (11.32) to a 12 by 55 mm culture tube containing 100 µL of Luciferin-Luciferase reagent (11.35).

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

11.38 Place culture tube into luminometer chamber.

11.39 Read and record RLU_{obs}.

11.39.1 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.

11.40 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 11.31 through 11.39 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 (11.39),
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 10—Optionally, report result as Log_{10} [pg ATP/mL]. If data are not transformed into 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} report the first three places to the right of the decimal point.

NOTE 11—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

13.1 *Precision—Interim Repeatability*—The difference between repetitive results, from nondiluted samples, 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 following values only in one case in 20.

$$Repeatability\ (r)\ in\ fuel\ and\ fuel-associated\ water = 0.313*X\ pg\ ATP/mL \quad (3)$$

where:

X = average of three test results.

13.1.1 Computation of interim repeatability was based on triplicate analysis of 24 samples by a single analyst in accordance with Practice D6300. A total of 18 fuel and 6 bottom-water samples were tested (Table X6.2).

13.2 Reproducibility (R) of the procedure in this test method for measuring cellular-ATP in fuels and bottom-water will be determined as part of an interlaboratory study and will be reported within five years of the publication date of this test method.

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

APPENDIXES

(Nonmandatory Information)

X1. RELATIONSHIP BETWEEN ATP CONCENTRATION AND POPULATION DENSITY

X1.1 Bacterial cells typically contain 0.5 to 5 fg ATP/cell (1 fg = 10^{-15} g). Fungal cells can have 10 to 100 times as much ATP/cell as bacteria. Conversely, bacterial or fungal spores can have 100 to 1000 times less ATP/cell as bacteria. Consequently, although ATP concentration tends to covary with culturability (CFU/mL) data, it is inappropriate to attempt to convert ATP data into CFU/mL data mathematically.

X1.2 Based on the information provided above in X1.1:

X1.2.1 For 20 mL fuel and fuel water mixture volumes the 5.0 pg ATP/mL (0.70 log_{10}[pg ATP/mL]) lower detection limit for this test method ranges from 1000 to 10 000 bacteria/mL and 10 to 1000 fungal cells/mL.

X1.2.2 For 5 mL fuel-associated water volumes the 20.0 pg ATP/mL (1.3 log_{10}[pg ATP/mL]) lower detection limit for this test method ranges from 4000 to 40 000 bacteria/mL and 40 to 4000 fungal cells/mL.

X1.3 Without first determining the actual cell count (cells/mL), it is inadvisable to correlate ATP concentration to cell counts of CFU/mL.

X1.4 As for all condition monitoring parameters, ATP data are best used based on data trends. Upper control limits (UCL) should be established after determining normal fluctuation in a well controlled fuel system. Baseline values (average and standard deviation) are most reliable when they are based on ≥50 samples. UCL should be at least two standard deviations greater than the average baseline value.

X2. ATP STANDARD CURVE METHOD

X2.1 Acquire standard solutions of 100 000 pg ATP/mL, 10 000 pg ATP/mL, 1000 pg ATP/mL, 100 pg ATP/mL, and 10 pg ATP/mL.

X2.1.1 Available commercially,[5] or

X2.1.2 Dilute 1.2 mg ATP disodium salt (having approximately 12% free moisture) into 1000 mL ATP dilution buffer to get a 1 000 000 pg ATP/mL stock solution. Create standard solutions series via serial dilution of 1.0 mL parent solution in 9.0 mL ATP dilution buffer down to 10 pg ATP/mL.

X2.2 Follow 10.2 through 10.12 to determine RLU_{ctrl} for each ATP standard solution. Perform each in triplicate and report RLU_{ctrl} for each as the average of triplicate measurements.

X2.3 To evaluate linearity, plot $\log_{10}[RLU_{ctrl}]$ versus $\log_{10}$[pg ATP/mL] and obtain the R2 value for a linear best-fit line. Ideally, this value should be 1.0 for the dynamic range of the luminometer.

X2.3.1 If RLU_{ctrl} values are not available for all of the standards (for example, if the 100 000 pg ATP/mL standard is over-scale on the luminometer), or is the R^2 value is not 1.0 for the entire range of standards, determine the appropriate operating range or obtain a new luminometer.

X2.3.2 For maximum accuracy, the RLU_{ctrl} should be corrected by subtracting RLU background at the lowest end of the standard scale (for example, this subtraction likely will improve the accuracy in the measurement of ATP in solutions at concentrations near 10 pg/mL). The reagent blank can be obtained by measuring the RLU of ATP extract dilution buffer (7.2) and using the average of triplicate measurements to calculate the reagent blank.

X3. ADJUSTING METHOD DETECTION RANGE

X3.1 Increasing Test Sensitivity (Increasing RLU_{obs} by Filtration)

X3.1.1 Although the detection limit of this test method is 5.0 or 20 pg ATP/mL, RLU_{obs} values that are $\leq$ 0.01 RLU_{ctrl} are generally considered to be background noise.

X3.1.2 To increase accuracy at the low end of detection and therefore the test sensitivity, increase the volume of fuel, fuel/water mixture or fuel-associated water that is filtered in steps 11.6 through 11.8. Using a 60 mL syringe (6.10), up to 50 mL of sample can be filtered. Moreover, additional aliquants of sample can be processed successively through the filter so that the total volume filtered can be >50 mL.

X3.1.3 Report actual volume filtered, and use this volume in Eq 1 (12.1).

X3.2 Decreasing Test Sensitivity (Decreasing RLU_{obs} by Dilution)

X3.2.1 Many luminometers have an upper detection limit of <100 000 RLU. If the luminescence is greater than the upper detection limit, the RLU display defaults to an overload signal.

X3.2.2 If the original test result yields a value greater than the luminometer's upper detection limit, run a 1/10 dilution of the diluted ATP extract (11.32).

X3.2.2.1 To an unused 17 by 100 mm culture tube, add 9.0 mL of ATP extract dilution buffer (7.2).

X3.2.2.2 Use the micropipeter to transfer 1.0 mL of the diluted ATP extract (11.32) to the 9.0 mL of ATP extract dilution buffer prepared in X4.2.2.

X3.2.2.3 Follow steps 11.33 through 11.39.

X3.2.3 If the RLU_{obs} is still greater than the luminometer's upper detection limits, run a 1/100 dilution of the diluted ATP extract (11.32).

X3.2.3.1 To an unused 17 by 100 mm culture tube, add 9.9 mL of ATP extract dilution buffer (7.2).

X3.2.3.2 Use the micropipeter to transfer 0.1 mL of the diluted sample (11.32) to the 9.9 mL of ATP Extract Dilution Buffer prepared in X3.2.3.1.

X3.2.3.3 Follow steps 11.33 through 11.39.

X3.2.4 Additional dilutions of the X4.2.2 dilution can be made if necessary, until RLU_{obs} is below the luminometer's upper detection limit.

X3.2.5 Observe the RLU value from the luminometer and multiply it by the appropriate dilution factor (that is, 10 for a 1/10 dilution; 100 for a 1/100 dilution). Record the result as RLU_{obs} and use this value in Eq 1.

X4. EVALUATION OF ASSAY INTERFERENCES

X4.1 Two methods of evaluation are provided. The first addresses samples that are uncontaminated, while the second one addresses samples suspected to be contaminated.

X4.2 Uncontaminated Sample Evaluation by Standard Addition Method

X4.2.1 Obtain neat stocks of the target sample(s) and use sample(s) to prepare ATP extracts via steps 11.1 through 11.29.

X4.2.2 Prepared ATP extracts are diluted (11.31 and 11.32) and doped with ATP/mL stock solutions to give 10 000, 1000, 100 and 10 pg ATP/mL concentrations. It is recommended that the ATP stock solutions be diluted by at least 100 fold when doping the extracts.

X4.2.3 These doped, diluted ATP extracts are analyzed for ATP concentration via steps 11.33 through 11.39.

X4.2.4 Results from each sample are compared with the Standard Curve Method (Appendix X2) to evaluate interferences in the analysis. The recommended comparison includes a one-way ANOVA analysis of the data for each ATP concentration tested.

X4.2.5 In the example provided below, data from ATP standard additions to 87 Octane Gasoline are compared with data obtained in accordance with Appendix X2.

X4.2.5.1 Table X4.1 and Table X4.2 present the ANOVA test results. In this example, there were no significant matrix effects at any of the tested ATP concentrations. All computed F-values were less than Fcritical (the criterion value of the F-statistic when variable 1 has one degree of freedom, variable 2 has 4 degrees of freedom and the confidence level (α) is 0.95.

TABLE X4.1 RLU Comparison between ATP Standard Curve and ATP Standard Additions to Gasoline Sample Extract

[ATP] (pg/mL)	Rep	Prepared Standards	Gasoline
10	1	135	142
	2	128	147
	3	133	118
100	1	1263	1229
	2	1271	1255
	3	1368	1361
1000	1	15 215	14 071
	2	14 133	14 679
	3	14 114	14 870
10000	1	139 699	152 332
	2	145 559	128 631
	3	144 991	147 077

TABLE X4.2 ANOVA Summary – Evaluation of Matrix Effect of Gasoline

pg ATP/mL	10	100	1000	10 000
F-matrix Effect	0.222	0.130	0.015	0.010
P-matrix effect	0.662	0.736	0.910	0.926
F-critical	7.708647			

X4.3 Contaminated Sample Evaluation by Dilution Series Method

X4.3.1 For samples suspected to be contaminated, prepare diluted sample ATP extracts via steps 11.1 through 11.29 and 11.33 through 11.33.

X4.3.2 Prepare a ½ dilution of the extract from X4.3.1 by adding 1 mL of diluted sample ATP extract to 1 mL ATP extract dilution buffer (7.2).

X4.3.3 Analyze both the parent and the diluted extract dilutions via steps 11.34 through 11.40 and calculate the ATP concentration of the samples via step 12. If the results agree within 10%, use the average of the two results. If the ½ dilution calculation gives an ATP result that is more than 10% higher, continue with ½ serial dilutions until the dilution required to remove inhibition is determined. Use this dilution on all future analyses of this sample, providing the results are above 50 RLU. In situations where the results of diluted samples are less than 50 RLU, the standard addition method described in X4.2 is recommended.

X5. DETERMINATION OF BACKGROUND RLU

X5.1 The purposes of determining background RLU ($RLU_{background}$) are to provide a correction factor when measuring sample ATP near the detection limit of this test method and to serve as quality control procedure to reveal potential contamination of reagents or testing apparatus, or both. An alternative procedure to eliminate the requirement of determining the background RLU for routine sample analyses of relatively uncontaminated samples is to increase RLU_{obs} by filtering more sample (see Appendix X3).

X5.2 A background RLU refers to the relative light units produced by this test method without incorporation of the sample.

X5.3 To measure $RLU_{background}$, follow steps 11.1 through 11.4 followed by steps 11.13 through 11.39. Record RLU_{obs}. Perform in triplicate and report $RLU_{background}$ as the average of triplicate measurements. When measuring sample ATP concentrations near the reagent-method blank (50 RLU for the Kikkoman Lumitester C-110), subtract $RLU_{background}$ from the RLU_{obs} of the sample prior to calculation of cellular ATP (12).

X6. PRELIMINARY INTERLABORATORY STUDY TO ESTABLISH REPEATABILITY FOR MEASUREMENT OF CELLULAR ATP IN FUELS AND FUEL-ASSOCIATED WATER WITH SAMPLE CONCENTRATION BY FILTRATION

X6.1 This Interlaboratory Study (ILS) was conducted to establish a preliminary repeatability statement for Test Method D7687.[2]

TABLE X6.1 List of Sample Types in ILS

Sample Type
E-0 gasoline
E-10 gasoline
ULSD
Jet A
B-5
B-20
E-10 Bottom water
ULSD Bottom water
Jet A Bottom-water

X6.2 *The following laboratory participated in this ILS*—Analyst–Vikram Datta, LuminUltra Technologies Ltd., 440 King St., King Tower, Suite 630, Fredericton, NB E3B 5H8, Canada.

X6.3 Description of Samples

X6.3.1 Table X6.1 provides a list of fuels and fuel-associated waters included in this ILS.

X6.3.2 For each sample type (fuel + bioburden) and bioburden concentration (low, medium, high), three replicate, 25 mL portions were dispensed into sterile, 30 mL vials. Each replicate was doped with a preconditioned inoculum (see X6.4 and Table X6.1). Each replicate subsample was tested once for cellular ATP.

X6.3.3 Challenge populations were prepared by inoculating 400 mL of either E-0 gasoline (for gasoline and gasoline-associated water-samples) or ULSD (for diesel, biodiesel, Jet A and their respective bottom-water samples) over 100 mL of bottled spring water. Rid-X,[6] 1.46 mg was used as the inoculum for the challenge microcosms. In previous studies (Passman, et al, 2007[7]) Rid-X has be found to be a reliable and consistent source of an uncharacterized, mixed microbial population of organisms capable of degrading fats, oils and greases.

X6.3.4 Challenge population microcosms were incubated at room temperature (28 ± 2°C) on a gyrorotary shaker (140 rpm) for three weeks, until the bottom-water ATP concentration was > 100 ng ATP/mL (105 pg ATP/mL; 468 000 pg ATP/mL E-0 bottom-water and 480 000 pg ATP/mL ULSD bottom-water).

[6] The sole source of supply of the material known to the committee at this time is Reckitt Benckiser, Inc., Parsippany, NJ, 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.

[7] Passman, F. J., English, E. Lindhardt, C. "Using Adenosine Triphosphate Concentration as a Measure of Fuel Treatment Microbicide Performance," in Morris, R. E., Ed., Proceedings of the 10th International Conference on the Stability and Handling of Liquid Fuels, Oct. 7-11, 2007, Tucson, AZ.

TABLE X6.2 Preliminary Repeatability Data – Cellular ATP in Fuels and Fuel-Associated Water (ATP Data are in pg ATP/mL)

Sample	Replicate			avg	range
	1	2	3		
E-0 gasoline – A	33	31	27	30	6
E-0 gasoline –B	1100	1100	1300	1200	200
E-0 gasoline – C	20 000	21 000	15 000	18 000	6000
E-10 gasoline – A	39	27	28	31	13
E-10 gasoline – B	1000	1100	920	1000	180
E-10 gasoline – C	20 000	17 000	18 000	18 000	3000
ULSD – A	37	42	33	37	9
ULSD – B	890	1100	1100	1020	210
ULSD – C	24 000	22 000	25 000	24 000	3000
Jet A – A	31	30	24	28	7
Jet A – B	1000	1300	1300	1200	300
Jet A – C	21 000	20 000	19 000	20 000	2000
B-5 – A	42	34	41	39	8
B-5 – B	950	1200	1000	1100	250
B-5 – C	21 000	23 000	21 000	22 000	2000
B-20 – A	41	34	44	40	10
B-20 – B	1100	1300	1300	1200	200
B-20 – Cs	17 000	22 000	23 000	21 000	6000
E-10 Bottom water – A	37	36	28	34	8
E-10 Bottom water – B	22 000	21 000	20 000	21 000	2000
ULSD Bottom water – A	35	43	42	40	9
ULSD Bottom-water – B	18 000	17 000	15 000	17 000	3000
Jet A Bottom-water – A	37	37	49	41	13
Jet A Bottom-water – B	16 000	20 000	19 000	18 000	4000

X6.4 ILS Instructions

X6.4.1 The analyst was instructed to follow the sample preparation steps defined in Section 11.

X6.5 Description of Equipment/Apparatus

X6.5.1 All tests were performed using a LuminUltra Technologies Ltd. QGO-M test kit. A Kikkoman C-110 Lumitester (Tokyo, Japan), in accordance with Note 3, was used to measure luminescence at 420 nm.

X6.6 Test data were reported in an Excel workbook spreadsheet. The data are presented in Table X6.2.

X6.7 Statistical Data Summary

X6.7.1 Table X6.2 data set. as well as raw RLU data, was sent to Dr. Alex Lau (D02.94) for statistical analysis. Dr. Lau reported that the coefficient of variation for repeatability (CVr), where CVr = r AVG regression of CVr v. measured pg ATP/mL is not significantly different from zero.

X6.8 Preliminary Repeatability Statement:

X6.8.1 The repeatability coefficient (r) is the average of the CVr for all sets of triplicate analysis:

$$Repeatability(r)\ in\ fuel\ and\ fuel-associated\ water = 0.313*X\ pg\ ATP/mL \quad (X6.1)$$

where:

X = the average of three test results.