Designation: D4012 – 15

Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water[1]

This standard is issued under the fixed designation D4012; 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 (cATP) content associated with microorganisms normally found in laboratory cultures, waters, wastewaters, and in plankton and periphyton samples from waters.

1.2 The ATP is measured using a bioluminescence enzyme assay, whereby light is generated in amounts proportional to the concentration of 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 and computation to pg ATP/mL.

1.3 This method does not remove all known chemical interferences, known to either luminesce in the 530 ± 20 nm range, or to quench light emitted in that range. It should not be used to determine ATP concentrations in samples with dissolved organic compounds, heavy metals or >10 000 ppm total dissolved solids. Alternative methods have been developed for determining ATP concentrations in fluids samples likely to contain such interferences (Test Methods D7687 and E2694).

1.4 Knowledge of the concentration of ATP can be related to viable biomass or metabolic activity of microorganisms (Appendix X1).

1.5 This test method offers a high degree of sensitivity, rapidity, accuracy, and reproducibility.

1.6 The analyst should be aware that the precision statement pertains only to determinations in reagent water and not necessarily in the matrix being tested.

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

1.8 The method normally detects cATP concentrations in the range of 0.1 pg cATP/mL (-1.0Log_{10} [pg cATP/mL]) to 4 000 000 pg cATP/mL (6.6 Log_{10} [pg cATP/mL]) in 50 mL water samples.

1.9 Providing interferences can be overcome, bioluminescence is a reliable and proven method for qualifying and quantifying ATP, although the method does not differentiate between ATP from different sources, for example, from different types of microorganisms, such as bacteria, fungi, algae and protozoa.

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

1.11 *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]

D1129 Terminology Relating to Water
D1193 Specification for Reagent Water
D1601 Test Method for Dilute Solution Viscosity of Ethylene Polymers
D4175 Terminology Relating to Petroleum, Petroleum Products, and Lubricants
D5847 Practice for Writing Quality Control Specifications for Standard Test Methods for Water Analysis
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
D7687 Test Method for Measurement of Cellular Adenosine Triphosphate in Fuel, Fuel/Water Mixtures, and Fuel-Associated Water with Sample Concentration by Filtration
E2694 Test Method for Measurement of Adenosine Triphosphate in Water-Miscible Metalworking Fluids
F1671 Test Method for Resistance of Materials Used in

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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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.

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 definitions of terms used in this standard, refer to Terminologies D1129 and D4175.

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

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

3.1.4 *aseptic, adj*—sterile, free from viable 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; c-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 are) able to 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, n*—adenosine monophosphate.

3.2.2 *ATP, n*—adenosine triphosphate.

3.2.3 *HDPE, n*—high density polyethylene.

3.2.4 *PP, n*—polypropylene.

3.2.5 *pg, n*—picogram (1×10^{-12} g).

3.2.6 *RLU, n*—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 50 mL sample of water is placed into a syringe and then pressure-filtered through a 0.7 µm, glass-fiber, in-line, depth filter.

4.3 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.

4.4 The filtrate is diluted 1 to 10 with a buffer solution.

4.5 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.

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

4.7 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 1—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 A rapid and routine procedure for determining biomass of the living microorganisms in cultures, waters, wastewaters, and in plankton and periphyton samples taken from surface waters is frequently of vital importance. However, classical techniques such as direct microscope counts, turbidity, organic chemical analyses, cell tagging, and plate counts are expensive, time-consuming, or tend to underestimate total numbers. In addition, some of these methods do not distinguish between living and nonliving cells.

5.2 This test method measures the concentration of cellular-ATP present in the sample. ATP is a constituent of all living cells, including bacteria, algae, protozoa, and fungi. Consequently, the presence of cellular-ATP is an indicator of

total metabolically active microbial contamination in water. ATP is not associated with matter of non-biological origin.

5.3 The ATP (luciferin-luciferase) method is a rapid, sensitive determination of viable microbial biomass. ATP is the primary energy donor for life processes, does not exist in association with nonliving detrital material, and the amount of ATP per unit of biomass (expressed in weight) is relatively constant. (ATP per cell varies with species and physiological state of the organism.)

5.4 This test method can be used to:

5.4.1 Estimate viable microbial biomass in cultures, waters, and wastewaters.

5.4.2 Estimate the amount of total viable biomass in plankton and periphyton samples.

5.4.3 Estimate the number of viable cells in a unispecies culture if the cATP content (or if the average amount of cATP) per cell is known.

5.4.4 Estimate and differentiate between zooplanktonic, phytoplanktonic, bacterial, and fungal cATP through size fractionation of water, and wastewater samples.

5.4.5 Measure the mortality rate of microorganisms in toxicity tests in entrainment studies, and in other situations where populations or assemblages of microorganisms are placed under stress.

5.5 This test method is similar to Test Methods D7687 and E2694 except for the volumes sampled, and omission of wash and drying steps used in Test Methods D7687 and E2694 to remove interferences (1.3).

5.6 Although ATP data generally covary with culture data in water samples, different factors affect cATP concentration than those that affect culturability.

5.6.1 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.[3]

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

5.6.2.1 One example of the species effect is that the amount of ATP per cell is substantially greater for active fungal cells than bacteria (Appendix X1).

5.6.2.2 Within a species, cells that are more metabolically active will have more ATP per cell than dormant cells, such as fungal spores.

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

6. Interferences

6.1 Reagents must be of high purity so that background light emission is held to a minimum for the measurement of ATP.

6.2 ATP-free glassware, prepared by the procedure in 7.5, is required for the determination of ATP.

6.3 Luciferase is a protein and as such can be inhibited or denatured through the presence of heavy metals, high salt (for example, NaCl) concentrations, and organic solvents in the sample. Additionally, high amounts of color or turbidity in the sample can impede light transmission, causing a negative bias. Although the method described herein is designed to mitigate such interferences in most types of water, for samples with high amounts of such interferences consider the use of Test Methods D7687 and E2694.

6.4 Other energy-mediating compounds, such as adenosine diphosphate, cytidine-5-triphosphate, and inosine-5-triphosphate also react with luciferase to produce light, but as compared to ATP they are usually present only in small amounts and do not constitute a significant source of error.

7. Apparatus

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

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

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

7.4 *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 (7.1), 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 2—It is the responsibility of the user to ensure that the luminometer selected for use meets the criteria listed in 7.4 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.

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,[4] 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).

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

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

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

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

7.9 *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.

7.10 *Syringe, Luer-Lok*, 60 mL, PP, sterile disposable.

7.11 *Test Tube Rack*, 12 mm.

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

[4] The sole source of supply of the Kikkoman Lumitester C-110 apparatus known to the committee at this time is Hach Company, Colorado. 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.

7.12 *Test Tube Rack*, 17 mm.

7.13 *Waste Receptacle*, any container suitable for receiving and retaining filtrate fluid for ultimate disposal.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall conform to Specification D1193, Type II.

8.3 *ATP Standard Solution, 1.0 ± 0.05 ng ATP/mL*—Weigh 119.3 mg of crystalline adenosine 5'-triphosphate-disodium salt using ATP-free glassware. Dissolve the ATP in 100 mL of fresh 0.02 *M* tris buffer containing 29.2 mg of EDTA ($Na_2H_2EDTA{\cdot}2H_2O$) and 120 mg of $MgSO_4$ (the resulting concentration is 1 mg of ATP/mL). The material may be dispensed in 1.0-mL aliquots and stored at –20°C until required.

8.4 *ATP Extract Dilution Buffer,* (proprietary).[6]

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

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

NOTE 5—Follow manufacturers' instructions regarding product storage and shelf life.

9. Hazards

9.1 **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.

10. Precaution

10.1 This standard may involve the use of hazardous materials, operations, and equipment. It is the responsibility of whoever uses this standard to establish appropriate safety practices and to determine the applicability of regulatory limitations prior to use.

[5] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[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, http://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. Sample Collection

11.1 The sample sites should correspond as closely as possible to those selected for chemical, biological, and microbiological sampling, so that there is maximum correlation of results. The sample collection method will be determined by study objectives. To collect a sample, use a nonmetallic water sampling bottle. Extraction procedures should be performed immediately after collection. The sample may be stored 2 to 3 h if necessary if the temperature and lighting conditions are maintained; for example, do not place a warm sample from a well-lighted area into a cool, dark ice chest.

12. Calibration and Standardization

12.1 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.

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

12.2.1 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 (13.25) to affect the test result (13.25). Excessive on-time can also stimulate the photomultiplier tube to produce increased instrument background noise. Consequently, it is advisable to repeat 12.6 – 12.9 after every 3 h of continuous operation.

12.3 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.

12.3.1 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.

12.3.2 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.

12.3.3 Changes in the instrument, diluted sample (13.19) ATP standard (8.3) or Luciferin-Luciferase reagent (8.6) temperature while testing multiple samples will affect the RLU and consequently introduce a bias to the test results.

12.4 Use a micropipeter (7.6) with a new 100 to 1000 µL tip (7.7) to dispense 100 µL Luciferin-Luciferase reagent (8.6) to an unused 12 by 55 mm culture tube (7.1).

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

12.6 Dispense 100 µL of 1 ng ATP/mL standard solution (8.3) into the culture tube.

12.7 Swirl gently for five times.

12.8 Place the culture tube into the luminometer.

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

13. ATP Measurement Procedure

13.1 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.

13.2 Remove plunger from a new 60-mL syringe (7.10) and place onto 17-mm test tube rack so that plunger tip does not contact any surfaces so as to avoid contamination with ATP.

13.3 Affix filter (7.3) onto the 60-mL syringe.

13.4 Place a fresh 1.0 to 5.0-mL tip (7.8) onto the macropipeter (7.5).

13.5 Shake sample for 15 s to ensure homogeneity.

13.6 With minimal delay, remove lid from sample container and, using the macropipeter, transfer ten volumes of 5.0-mL (50 mL total) water sample, or one to the 60-mL syringe barrel.

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

13.7 While holding the barrel over the waste receptacle (7.13), replace the plunger into the 60-mL syringe.

13.8 Apply even pressure to the 60-mL syringe plunger to pressure filter the sample, having filtrate discharge into the waste receptacle.

13.8.1 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 (14.1).

13.9 Remove filter from the 60-mL syringe and place onto 17-mm test tube rack (7.12) so that filter outlet does not contact any surfaces.

13.10 Remove plunger from the 60-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.

13.11 Replace filter onto the end of the 60-mL syringe barrel.

13.12 Place an unused 17 by 100-mm culture tube (7.2) into 17-mm test tube rack.

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

13.14 Use micropipeter to dispense 1.0 mL of ATP extraction reagent (8.5) into the 60-mL syringe barrel.

13.15 While holding the barrel over the 17 by 100-mm culture tube (13.12), replace the 60-mL syringe plunger.

13.16 Apply even pressure to the 60-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.

13.17 If not already performed (12.2), turn power on luminometer (7.4) and allow instrument to equilibrate, in accordance with manufacturer's recommendations.

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

13.19 Use macropipeter to dispense two 4.5-mL portions (9.0 mL total) of ATP extract dilution buffer (8.4) into the culture tube to prepare diluted ATP extract.

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

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

13.21 Place one 12 by 55-mm culture tube into the 12-mm test tube rack (7.11).

13.22 As in 12.7, 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.

13.23 Using a fresh pipet tip, use micropipeter to transfer 100 µL of diluted sample (13.20) to a 12 by 55-mm culture tube containing 100 µL of Luciferin-Luciferase reagent (13.22).

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

13.25 Place culture tube into luminometer chamber.

13.26 Read and record RLU_{obs}.

13.26.1 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.

13.27 When testing multiple samples, perform steps 13.1 – 13.27 in sequence for each sample. After turning on the luminometer (7.4), perform steps 13.21 – 13.24 for each diluted ATP extract.

14. Calculation of Results

14.1 Compute cellular-ATP Sample in pg ATP/mL:

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

where:

RLU_{obs} = the sample RLU reading (13.24),
RLU_{ctrl} = the RLU for the 1 ng ATP/mL control (7.4), and
V_{Sample} = sample volume in mL (50 mL in accordance with 13.6).

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

where:

1000 pg ATP/ng ATP = unit conversion factor,
1.00 ng ATP = concentration of the ATP standard used to acquire RLU_{ctrl} (7.4), and
dilution factor = 10 (1.0 mL ATP extract (13.16) in 9.0-mL ATP extract dilution buffer).

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

14.1.1.1 When RLU_{obs} is ≤10 times $RLU_{background}$ for the luminometer specified in 8.4, 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 by means of cellular-ATP on the same scale as traditional culturability methods.

15. Precision and Bias

15.1 *Precision—Interim Repeatability*—The difference between repetitive results, from undiluted 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.

15.1.1 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 ultrapure water samples were tested (Table X6.2).

15.1.2 The repeatability coefficient (r) is 2.80 s_r for duplicate sets of triplicate analysis:

$$\text{Repeatability } (r) \text{ in water samples} = 1.23 - 1.10\text{Log}_{10} X \text{ pg ATP/mL} \quad (3)$$

where:

X = average and s_r is the standard deviation of six test results.

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

16. Quality Control

16.1 In accordance with Practice D5847, the following quality control guidance is provided.

16.2 *Initial Demonstration of Laboratory Capability*—Laboratories performing this method for the first time shall prepare a dilution curve in accordance with instructions provided in Appendix X2. Each individual analyst who will be performing the test shall perform triplicate tests at each ATP concentration. The repeatability standard deviation (s_r) and reproducibility standard deviation (s_R) shall not exceed the values obtained during the interlaboratory study.[7] Additionally the correlation coefficient between pg ATP/mL and average observed RLU shall be ≤0.98.

16.3 *Batch QC*—The coefficient of variation for replicate tests of a single sample shall not exceed 10 %. For the purposes of precision evaluation, no fewer than triplicate replicates shall be tested.

16.4 *Method Blank*—Background RLU shall be determined by placing 100 µL luciferin-luciferase reagent (8.6) into a 12 by 55-mm culture tube (7.1) and determining the RLU (13.25 and 13.26). The background RLU shall not exceed 20 RLU. If the background RLU does exceed 20 RLU, replace the luciferin-luciferase reagent with freshly prepared reagent.

16.5 *Laboratory Control Sample (LCS)*—Calibration is achieved in accordanc with Section 12. When RLU of 1 ng/mL reference standard is <5000, replace luciferin-luciferase reagent (8.6) with freshly prepared reagent.

16.6 *Matrix Spike (MS)*—Follow guidance provided in Appendix X4 to test for interferences or other matrix effects.

16.7 *Independent Reference Sample (IRS)*—There is no known independent reference sample other than the 1 ± 0.05 ng ATP/mL standard (8.3) listed in this test method. Users who are aware of alternative reference standards are advised to provide this information to ASTM International Headquarters. All comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

17. Keywords

17.1 adenosine triphosphate; algae; ATP; bacteria; bioburden; biomass; biomass; cellular; cellular-ATP; microbial contamination; microbiology; microorganisms; protozoa

[7] Supporting data are being filed at ASTM International.

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 in Appendix X1:

X1.2.1 For 50 mL water mixture volumes the 0.1 pg ATP/mL (0.22 Log_{10} [pg ATP/mL]) lower detection limit for this test method ranges from 50 to 500 bacteria/mL and 5 to 50 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 10 000 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 0000 pg ATP/mL stock solution. Create standard solutions series by means of serial dilution of 1.0-mL parent solution in 9.0-mL ATP dilution buffer down to 10 pg ATP/mL.

X2.2 Follow 12.2 – 12.9 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 R^2 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 (8.4) 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 0.1 pg ATP/mL, RLUobs values that are ≤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 water that is filtered in steps 13.6 – 13.8. Using a 60-mL syringe (7.10), up to 50 mL of sample can be filtered, per syringe load. 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 (14.1).

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 (13.19).

X3.2.2.1 To an unused 17 by 100-mm culture tube, add 9.0 mL of ATP extract dilution buffer (8.4).

X3.2.2.2 Use the micropipeter to transfer 1.0 mL of the diluted ATP extract (13.19) to the 9.0 mL of ATP extract dilution buffer prepared in X4.2.2.

X3.2.2.3 Follow steps 13.21 – 13.26.

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 (13.19).

X3.2.3.1 To an unused 17 by 100-mm culture tube, add 9.9 mL of ATP extract dilution buffer (8.4).

X3.2.3.2 Use the micropipeter to transfer 0.1 mL of the diluted sample (13.19) to the 9.9 mL of ATP Extract Dilution Buffer prepared in X3.2.3.1.

X3.2.3.3 Follow steps 13.21 – 13.27.

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 by means of steps 13.1 – 13.19.

X4.2.2 Prepared ATP extracts are diluted (13.19 and 13.20) 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 by means of steps 13.21 – 13.27.

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 Tables X4.1 and 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 F-critical (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	Purified Water
10	1	140	134
	2	133	136
	3	138	140
100	1	1263	1315
	2	1271	1400
	3	1368	1298
10 00	1	15215	12998
	2	14133	13872
	3	14114	14157
10 000	1	139699	143861
	2	145559	150062
	3	144991	139907

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

pg ATP/mL	10	100	1 000	10 000
F-statistic	0.015	0.640	2.590	0.120
P-value	0.910	0.470	0.180	0.750
F-critical	7.709			

X4.3 Contaminated Sample Evaluation by Dilution Series Method

X4.3.1 For samples suspected to be contaminated, prepare diluted sample ATP extracts by means of steps 13.1 – 13.27.

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 (8.4).

X4.3.3 Analyze both the parent and the diluted extract dilutions by means of steps 13.21 – 13.27 and calculate the ATP concentration of the samples by means of step Section 14. 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. Tables X4.1 and X4.2 illustrate the evaluation of gasoline as an interference. In this example, the matrix effect was not significant at the 95 % confidence level.

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 12.2 – 12.6 followed by steps 12.4, 12.5, 12.8, and 12.9. 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[4]), subtract $RLU_{background}$ from the RLU_{obs} of the sample prior to calculation of cellular ATP (Section 12).

X6. PRELIMINARY INTERLABORATORY STUDY

X6.1 This Interlaboratory Study (ILS) was conducted to establish a preliminary repeatability statement for Test Method D4012.

X6.2 The following laboratory participated in this ILS—Analyst – Jennifer Paul, Centre for Alternative Wastewater Treatment, Fleming College, 200 Albert Street South, PO Box 8000, Lindsay, Ontario, K9V 5E6, Canada.

X6.3 Description of Samples

X6.3.1 Table X6.1 provides a list of waters samples included in this ILS.

X6.3.2 For each sample three replicate, 50 mL portions were tested by each of three analysts. Each replicate subsample was tested once for cellular ATP.

X6.4 ILS Instructions

X6.4.1 The analysts were instructed to follow the sample preparation steps defined in Section 13.

X6.5 Description of Equipment/Apparatus

X6.5.1 All tests were performed using a LuminUltra Technologies Ltd. QGA test kit. A Kikkoman C-110 Lumitester[4] (Tokyo, Japan), in accordance with Note 3, was used to measure luminescence at 520 nm.

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

TABLE X6.1 List of Sample Types in ILS

Sample
Ultrapure water
Reverse osmosis (RO) water
Raw hatchery water
Ultrafiltered (UF) water

TABLE X6.2 Preliminary Repeatability Data – Cellular ATP in Water (ATP Data are in pg ATP/mL)

Analyst	Water Sample (pg ATP/mL)			
	Ultrapure	RO	Raw Hatchery	UF
1	0.08	1.02	169	133
	0.21	1.14	263	105
	0.09	2.27	214	152
2	0.07	1.36	189	102
	0.08	1.17	227	82
	0.07	1.15	213	124
3	0.32	0.97	286	164
	0.14	1.18	257	128
	0.29	1.55	181	89

X6.7 Statistical Data Summary

X6.7.1 Table X6.2 data set was analyzed in accordance with Test Method D1601.

X6.8 Preliminary Repeatability Statement

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

$$\text{Repeatability } (r) \text{ in water samples} = 0.27\ X \text{ pg ATP/mL} \quad \text{(X6.1)}$$

where:

X = average of three test results.

X6.9 Preliminary Reproducibility Statement

X6.9.1 The reproducibility coefficient (R) is the average of the CV_R for all sets of analysis.

$$\text{Repeatability } (R) \text{ in water samples} = 0.35\ X \quad \text{(X6.2)}$$

where:

X = average of nine test results.