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.