



Designation: D4012 – 81(Reapproved 2009)

Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water¹

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 the measurement of adenosine triphosphate (ATP) in microorganisms in concentrations normally found in laboratory cultures, waters, wastewaters, and in plankton and periphyton samples from waters.

1.2 Knowledge of the concentration of ATP can be related to *viable biomass* or metabolic activity, or by utilizing an average concentration (or amount) of ATP per cell, an estimated count of microorganisms can be obtained in the case of unispecies cultures.

1.3 This test method offers a high degree of sensitivity, rapidity, accuracy, and reproducibility. However, extreme care must be taken at each step in the analysis to ensure meaningful and reliable results.

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

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

1.6 *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

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.

Current edition approved May 1, 2009. Published June 2009. Originally approved in 1981. Last previous edition approved in 2002 as D4012 – 81 (2002). DOI: 10.1520/D4012-81R09.

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. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D1129.

4. Summary of Test Method

4.1 The biomass in the sample can be determined by direct ATP extraction when cell counts are greater than 20 000 microorganisms per millilitre. When the cell counts are less than 20 000 microorganisms per millilitre, the sample may be concentrated using either centrifugation or filtration prior to ATP extraction.

4.2 The ATP is extracted from the sample with boiling 0.02 M tris buffer.

4.3 A carefully measured aliquot of the ATP extract is mixed with a standard quantity of buffered luciferin-luciferase reaction mixture and the light produced in the resulting reaction is measured with an appropriate photometric analyzer.

4.4 The data obtained from the test can be expressed in terms of ATP content or biomass.

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 The ATP firefly (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.)

5.3 This test method can be used to:

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

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

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

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

5.3.5 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.

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 by the presence of heavy metals, high salt (NaCl) concentrations, and organic solvents, in the sample. The ATP luciferase reaction is also affected by certain phosphate buffers, inorganic salts, and by high magnesium concentrations.

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.

6.5 High-viscosity samples may not mix adequately with the reagents upon injection. If this occurs, reaction rate may be reduced (reaction will go to completion, but the reaction rate will be decreased with improper mixing) or the results may not be reproducible.

7. Apparatus

7.1 *ATP Photometers or Liquid Scintillation Spectrometers*—may be used. The stability of the instrument should be checked before each use with a standard light source available from the manufacturer. It is advisable to maintain a record of the instrument response to permit detection of any unstability or changes in response levels.

7.2 *Vacuum Filtration System* (0.45- μ m membrane filters).

7.3 *Precision Syringe*, 50- μ L. A constant-rate injection attachment is recommended.

7.4 *Automatic Pipets and Disposable Tips*.

7.5 *ATP-Free Glassware*—Rinse chemically clean glassware three times with 0.2 *N* HCl, rinse three times with tris buffer (8.8), and rinse three times with low-response water (8.6).

7.6 *Reaction Vial*, 6 by 49-mm.

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 Commit-

tee on Analytical Reagents of the American Chemical Society, where such specifications are available.³ 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*—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 ($\text{Na}_2\text{H}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$) 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 *Extraction Reagent*—ATP can be extracted from samples by various reagents and procedures. The most commonly used extracting reagent is boiling tris buffer (see 8.8).

8.5 *Hydrochloric Acid* (17 mL/L)—Add 17.0 mL of HCl (sp gr 1.19) to a 1-L volumetric flask and bring to volume with water.

8.6 (*LR*) *Water; Low-Response*—(Sterile ATP-free water may be prepared by treatment in a suitable system involving carbon treatment with deionization, filtration glass distillation, or sterilization by autoclaving and stored under refrigeration in stoppered flasks.

8.7 *Luciferase/Luciferin Reaction Mixture*—This material is commercially available and should be prepared in accordance with the supplier's instructions. Note the following when preparing this material:

8.7.1 Clean glassware must be used.

8.7.2 The luciferase/luciferin reaction mixture must be mixed gently without shaking.

8.8 *Tris Buffer* (0.02 *M*) (*Tris(Hydroxymethyl) Aminomethane*)—Dissolve 2.5 g of the buffer crystals in 1 L of deionized water. Bring to pH 7.75 using HCl (pH meter). Sterilize by autoclaving for 30 min at 121°C , 15 psi (103 kPa) pressure, and store refrigerated in stoppered flasks.

NOTE 1—Bacteria may live and multiply in the LR water and tris buffer; this can introduce an ATP interference. The quality of the LR water and tris buffer should be periodically tested.

9. Precaution

9.1 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.

³ *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.