Designation: D 4012 – 81(Reapproved 2002)

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

This standard is issued under the fixed designation D 4012; 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 *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:*

D 1129 Terminology Relating to Water[2]

D 1193 Specification for Reagent Water[2]

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D 1129.

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

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

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[2] *Annual Book of ASTM Standards*, Vol 11.01.

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-μm membrane filters).

7.3 *Precision Syringe*, 50-μL. 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 Committee 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 D 1193, 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 (Na_2H_2 EDTA·$2H_2O$) 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 *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.

10. Collection

10.1 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.

11. ATP Extraction Procedures

11.1 Accurate determinations of ATP require quantitative extraction of ATP from the sample. Separate the cells from any possible free (extracellular) ATP and other interfering materials by filtration, centrifugation, sample washing, etc. Omit the separation step if the sample is known to be free of soluble ATP or interfering material. After separation, lyse the cell wall to free ATP for subsequent analysis. Perform three replicate

[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 "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

(triplicate) analyses on each sample to ensure the efficiency, reliability, and reproducibility of the method employed.

11.2 *Procedure A—Boiling 0.02 M Tris Filtration Method*:

11.2.1 Filter 100 mL of sample through a 0.45-μm membrane filter.

11.2.2 Remove the filter as soon as the filtration is complete. Do not allow the filter to dry. Break the vacuum just as the last of the water passes through the filter and quickly transfer the filter and place in 5.0 mL of boiling 0.02 *M* tris buffer.

11.2.3 Heat for 5 to 10 min at 100°C in a water bath.

11.2.4 If the analysis is not to be performed immediately, the extracted sample may be stored at – 20°C for a period up to 6 months.

11.3 *Procedure B—Boiling 0.02 M Tris Buffer Without Filtration Method*:

11.3.1 Add 1.0 mL of sample to approximately 35 mL of 0.02 *M* tris buffer pH 7.75 in a 50-mL Erlenmeyer flask that has reached a temperature of at least 98°C in a boiling water bath.

11.3.2 Maintain boiling temperature for 2 to 4 min.

11.3.3 Cool to room temperature.

11.3.4 Analytically transfer to a 50-mL volumetric flask and bring up to volume with 0.02 *M* tris buffer. If the analysis is not to be performed immediately, the extracted sample may be stored at – 20°C for a period up to 6 months.

11.3.5 If marine water samples are extracted directly without filtrations, it is especially important to dilute the sample with 0.02 *M* tris buffer to avoid inhibition of the luminescence reaction by NaCl.

12. Standardization Curve

12.1 Pipet 1.0 mL of the standard ATP solution containing 1 mg of ATP/mL into a 1-L volumetric flask and bring up to volume with 0.02 *M* tris buffer. Call this Solution A. Solution A will contain 1.00 μg ATP/mL. Then make the following serial dilutions:

12.1.1 1.0 mL of Solution A + 9 mL of 0.02 *M* tris = Solution B, Solution B = 1.00×10^{-1} μg ATP/mL.

12.1.2 1.0 mL of Solution A + 99 mL 0.02 *M* tris = Solution C, Solution C = 1.00×10^{-2} μg ATP/mL.

12.1.3 1.0 mL of Solution C + 9 mL 0.02 *M* tris = Solution D, Solution D = 1.00×10^{-3} μg ATP/mL.

12.2 The above concentrations should be used when preparing a curve for normal laboratory samples. For oligotrophic waters additional dilutions are required. Standards can be prepared and frozen, then thawed as needed.

12.3 A minimum of three replicate determinations of each of the standard solutions (Solutions A, B, C, and D) should be used to prepare a calibration curve. These solutions should be chosen so that they contain concentrations of ATP at the lower end, upper end, and midway in the range of ATP concentrations that the analyst suspects (or knows) to be present in the samples to be analyzed.

12.4 Determine (triplicate measurements) the instrument response to the reagent blank, consisting of sterile extractant. Subtract the instrument response to the blank from the response to dilutions of the standard and plot the results versus ATP concentration on log-log paper. If the microorganisms are not concentrated by filtration before the ATP is extracted, test the water carrying the microorganisms for the presence of agents that might interfere with the ATP-luciferase reaction. This is done by spiking a suitable volume of filtered water, that does not contain soluble extracellular ATP (see 10.1) from the samples and the unfiltered sample with a known amount of ATP to determine the percent recovery.

13. ATP Measurement

13.1 Rinse the microlitre syringe three times with 0.2 *N* HCl (8.5) by drawing acid into the entire 50 μL; rinse three times with 0.02 *M* tris buffer solution to neutralize any remaining acid.

13.2 Add sufficient volume of extract from 11.2 or 11.3 to the luciferin-luciferase mixture and measure the response with a suitable ATP analyzer.

NOTE 2—Some systems required the luciferin-luciferase mixture to be injected into the sample.

13.3 Repeat rinse (13.1) between each sample.

13.4 Convert the instrument reading to ATP units per millilitre using the standard curve. Account for the total sample volume filtered or the volume actually analyzed, or both, as appropriate.

14. Precision and Bias

14.1 The precision data were obtained by using standard ATP solutions as it was not possible to prepare a standard reference water sample for ATP content, which would represent a true environmental sample. Furthermore, because of the unstable nature of ATP, it was not possible to prepare and ship "unknown" standard solutions to all participating laboratories. For these reasons, the following procedure was used:

14.2 Each participating laboratory prepared and calibrated a luminescence meter covering the concentration range from 0.5 to 100μ g/L ATP. Participating laboratories were instructed to prepare" Pseudo Unknown" ATP samples with concentration levels of 0.4, 4.0, 16.0, 64.0, and 96 μg/L in 0.02 *M* tris buffer. These solutions were prepared by an independent person in the laboratory and the "Pseudo Unknown" ATP samples were then analyzed for 3 days. Each sample was run in triplicate and ATP concentration was determined using the calibration curve prepared during the same day. The round robin included seven people and six laboratories. The results are tabulated in Table 1.

TABLE 1 Precision and Bias

Known Concentration, µg/L	Single-Operator Precision, S_o, µg/L	Overall Precision, S_T	± % Bias, µg/L
0.4	0.166	0.162	6.9
4.0	0.540	0.521	0.3
16.0	1.171	1.479	0.7
64.0	3.180	4.251	0.8
96.0	5.516	5.343	0.9

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