Designation: E2694 – 21

An American National Standard

Standard Test Method for Measurement of Adenosine Triphosphate in Water-Miscible Metalworking Fluids[1]

This standard is issued under the fixed designation E2694; 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 provides a protocol for capturing, extracting, and quantifying the adenosine triphosphate (ATP) content associated with microorganisms found in water-miscible metalworking fluids (MWFs).

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 (RLUs) which are converted by comparison with an ATP standard and computation to pg ATP/mL.

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

1.4 The test method detects ATP concentrations in the range of 4.0 pg ATP/mL to 400 000 pg ATP/mL.

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

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

1.7 *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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water

D4840 Guide for Sample Chain-of-Custody Procedures

D6161 Terminology Used for Microfiltration, Ultrafiltration, Nanofiltration, and Reverse Osmosis Membrane Processes

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E1326 Guide for Evaluating Non-culture Microbiological Tests

E1497 Practice for Selection and Safe Use of Water-Miscible and Straight Oil Metal Removal Fluids

E2523 Terminology for Metalworking Fluids and Operations

2.2 *Government Standards:*[3]

29 CFR 1910.1000 Air Contaminants

29 CFR 1910.1450 Occupational Exposure to Hazardous Chemicals in Laboratories

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this test method, refer to Terminologies D1129, D6161, and E2523.

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

3.1.3 *adenosine triphosphate (ATP), n*—a 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.

[1] This test method is under the jurisdiction of ASTM Committee E34 on Occupational Health and Safety and is the direct responsibility of Subcommittee E34.50 on Health and Safety Standards for Metal Working Fluids.

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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] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401, http://www.access.gpo.gov.

***A Summary of Changes section appears at the end of this standard**

3.1.5 *bioluminescence, n*—the 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.

3.1.6 *biomass, n*—any matter which is or was a living organism or excreted from a microorganism (D6161).

3.1.7 *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.

3.1.8 *Luciferase, n*—a general term for a class of enzymes that catalyze bioluminescent reactions.

3.1.9 *Luciferin, n*—a general term for a class of light-emitting biological pigments found in organisms capable of bioluminescence.

3.1.10 *luminometer, n*—an instrument capable of measuring light emitted as a result of non-thermal excitation.

3.1.11 *relative light unit (RLU), n*—an instrument-specific unit of measurement reflecting the number of photons emitted by the Luciferin-Luciferase driven hydrolysis of ATP to AMP plus pyrophosphate.

3.1.11.1 *Discussion*—RLU is not an SI unit, however, RLU is proportional to ATP concentration.

3.1.12 *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 *MWF*—metalworking fluid

3.2.5 *PP*—polypropylene

3.2.6 *RLU*—relative light unit

4. Summary of Test Method

4.1 A control assay is performed using 100 µL of 1.0 ng ATP/mL standard.

4.2 A 5.0 mL sample of MWF 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 extracellular ATP and other contaminants that might otherwise interfere with the ATP assay.

4.4 The filter is air dried.

4.5 A lysing reagent is used to release 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+9 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 previously been dispensed.

4.8 The culture tube is placed into a luminometer and the light intensity is read in RLU.

4.9 RLU is converted to Log_{10} [pg ATP/mL] of sample by computation.

4.10 A procedure for differentiating between bacterial and fungal cATP biomass is provided in Appendix X4.

4.11 A procedure for determining the total ATP (tATP) biomass on MWF system surfaces is provided in Appendix X5.

5. Significance and Use

5.1 This method measures the concentration of ATP present in the sample. ATP is a constituent of all living cells, including bacteria and fungi. Consequently, the presence of ATP is an indicator of total microbial contamination in metalworking fluids. ATP is not associated with matter of non-biological origin.

5.2 Test Method D4012 validated ATP as a surrogate for culturable bacterial data (Guide E1326).

5.3 This method differs from Test Method D4012 in that it eliminates interferences that have historically rendered ATP testing unusable with complex organic fluids such as MWFs.

5.4 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 h to 48 h (or longer) required for culturable colonies to become visible, to approximately 5 min.

5.5 Although ATP data generally covary with culture data in MWF,[4] different factors affect ATP concentration than those that affect culturability.

5.5.1 Culturability is affected primarily by the ability of captured microbes to proliferate on the growth medium provided, under specific growth conditions. It has been estimated that less than 1 % of the species present in an environmental sample will form colonies under any given set of growth conditions.[5]

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

5.5.2.1 One example of the species effect is that the amount of ATP per cell is substantially greater for fungi than bacteria.

5.5.2.2 Within a species, cells that are more metabolically active will have more ATP per cell than dormant cells.

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

5.5.3 The possibility exists that the rinse step (11.15) may not eliminate all chemical substances that can interfere with the bioluminescence reaction (11.39).

5.5.3.1 The presence of any such interferences can be evaluated by performing a standard addition test series as described in Appendix X3.

5.5.3.2 Any impact of interfering chemicals will be reflected as bias relative to data obtained from fluid that does not contain interfering chemicals.

[4] Passman, et al., "Real-Time Testing of Bioburdens in Metalworking Fluids using Adenosine Triphosphate as a Biomass Indicator," 2009 STLE Annual Meeting, Orlando, FL.

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

6. Apparatus

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

6.2 *Culture Tube,* PP, 17 by 100 mm with caps.

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

6.4 *Luminometer,* using photomultiplier tube, capable of detecting light emission at 420 nm and with a cuvette chamber that can hold a 12 by 55 mm culture tube.

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, 100 mL.

NOTE 1—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.

7. Reagents and Materials

7.1 *ATP Standard,* 1 ng ATP/mL.

7.1.1 Commercially available;[6] or

7.1.2 Dilute 1 mg ATP into 1000 mL ATP dilution buffer to get a 1000 ng ATP/mL stock solution. Then, dilute 1.0 mL of 1000 ng ATP/mL stock solution into 999.0 mL ATP dilution buffer to get a 1 ng ATP/mL ATP standard.

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

7.3 *ATP Extraction Reagent*[6] (proprietary).

7.4 *Filter Wash Reagent*[6] (proprietary).

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

8. Hazards

8.1 The analyst must know and observe good laboratory safety practice in accordance with 29 CFR1910.1450.

8.2 Inhalation or dermal exposure to MWF can pose health problems for personnel involved with MWF sampling. Provision of personal protective equipment (PPE) in the form of respirators, protective clothing, or both may be indicated (see Practice E1497).

8.3 Review material safety data sheets for materials in use at the facility to identify potential hazards in order to determine appropriate PPE (see 29 CFR 1910.1000).

9. Sampling and Test Specs and Units

9.1 *Sampling Site:*

9.1.1 Select sampling site that will yield a representative MWF sample.

9.1.2 For routine condition monitoring, select individual sump(s) or central systems that have actively circulating fluid.

9.1.3 For diagnostic testing, select zones of pooled or stagnant MWF.

9.2 *Sampling:*

9.2.1 If practical, draw sample from the midpoint of the fluid reservoir; otherwise draw sample from below surface of the MWF at an accessible location.

9.2.1.1 Microbial contamination will vary considerably within the fluid system and it is important to be consistent in selecting the sampling location; this should be appropriate for the analysis objectives.

9.2.2 Collect sample by removing lid from sample container, immersing the open container (6.9), opening-down, below the fluid surface and inverting the container to allow it to fill with the sampled fluid.

9.2.3 If the fluid depth is insufficient to permit 9.2.1, use a sterile pipet to draw sample from the fluid and dispense it into the sample container, collecting at least 25 mL of sample.

9.3 *Sample Storage/Shipment:*

9.3.1 Label the sample container and follow accepted chain-of-custody procedures (Guide D4840).

9.3.2 Optimally samples should be tested onsite as soon as possible (<4 h) after testing.

9.3.3 If testing is to be delayed for longer than 4 h, or to be performed by an outside testing facility, samples may be stored on ice or in a refrigerator for up to 24 h. Samples older than 24 h are unlikely to microbiologically representative of the MWF at the time it was collected.

10. Calibration and Standardization

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

10.2 Ensure that all reagents have equilibrated to ambient temperature before running any tests.

10.3 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).

10.4 Replace the micropipeter tip with a fresh tip.

10.5 Dispense 100 µL of 1 ng ATP/mL standard solution (7.1) into the culture tube.

10.6 Swirl gently five times.

10.7 Place the culture tube into the luminometer.

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

[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, 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. Procedure

11.1 Use aseptic procedure while performing this test method; ATP from analyst's hands, sputum, etc. can contaminate the sample with ATP from sources other than the sample itself.

11.2 Remove plunger from a new 20 mL syringe (6.10) and place onto a 17 mm test tube rack so that plunger tip does not contact any surfaces.

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 5.0 mL of sample to the 20 mL syringe barrel.

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 MWF sample, having filtrate discharge into the waste receptacle.

11.9 Remove filter from the 20 mL syringe and place onto a 17 mm test tube rack so that filter outlet does not contact any surfaces.

11.10 Remove plunger from the 20 mL syringe (6.10) and place onto a 17 mm test tube rack so that the plunger tip does not contact any surfaces.

11.11 Replace filter onto the end of the syringe barrel.

11.12 Place a fresh 1.0 to 5.0 mL 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 MWF 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 (11.25).

11.17 Remove plunger from a 60 mL syringe (6.11) and place onto a 17 mm test tube rack so that barrel tip does not contact any surfaces.

NOTE 2—The 60 mL syringe used for the air-drying step may be used for multiple samples. However, used syringes should not be stored overnight for reuse.

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 – 11.20 one more time, first separating the filter before removing the plunger from the 60 mL syringe.

11.22 Remove the filter from the 60 mL syringe and place onto a 17 mm test tube rack so that the filter outlet does not contact any surfaces. Place the 60 mL syringe to the side for later use (see Note 2).

11.23 Place an unused 17 by 100 mm culture tube (6.2) into the 17 mm test tube rack.

11.24 Remove the barrel from the 20 mL syringe (11.16) and place onto the 17 mm test tube rack so that the 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 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 3—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 (10.1), turn on power to luminometer (6.4) and allow instrument to warm up, in accordance with the 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 mL total) of ATP extract dilution buffer (7.2) into the culture tube to prepare the diluted ATP extract.

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

NOTE 4—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 10.3, 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 100 to 1000 µL pipet tip, use micropipeter to transfer 100 µL of diluted sample (11.33) to the 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}.

NOTE 5—If RLU is outside of the luminometer's range (that is, below the background level or greater than the maximum readout), see Appendix X2 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 – 11.29 in sequence for each sample. After turning on the luminometer (11.30), perform steps 11.31 – 11.39 for each prepared ATP extract.

12. Calculation or Interpretation of Results

12.1 Compute ATP_{Sample} in pg ATP/mL:

$$ATP_{Sample}\,(pgATP/mL) = \frac{RLU_{obs}}{RLU_{ctrl}} \times \frac{10{,}000\,(pgATP)}{V_{Sample}(mL)} \quad (1)$$

where:

RLU_{obs} = the sample RLU reading (11.39),
RLU_{ctrl} = the RLU for the 1 ng ATP/mL control (10.8),
V_{Sample} = the sample volume in mL (5 mL per 11.6), and 10 000 pg ATP is derived from:

$$10{,}000\,pg\,ATP = \left(\frac{1000\,pg\,ATP}{ng\,ATP}\right) \times 1\,ng\,ATP \times dilution\,factor \quad (2)$$

where:

1000 pg ATP/ng ATP = a unit conversion factor,
1 ng ATP = the concentration of the ATP standard used to acquire RLU_{ctrl} (10.8), and
dilution factor = 10 (1.0 mL ATP extract (11.29) in 9.0 mL ATP extract dilution buffer.

12.2 Transform and report results as Log_{10} (pg ATP/mL).

13. Precision and Bias[7]

13.1 The precision of this test method is based on an interlaboratory study of ASTM E2694 conducted in 2011. Ten laboratories tested 22 different metalworking fluids for ATP content. Every "test result" represents an individual determination. All labs were asked to submit triplicate test results for each material tested (see Table 1). Practice E691 was followed for the overall design and analysis of the data; the details are given in ASTM Research Report No. E34-1002.

13.2 *Repeatability Limit (r)*—Two test results obtained within one laboratory shall be judged not equivalent if they differ by more than the "r" value for that material; "r" is the interval representing the critical difference between two test results for the same material, obtained by the same operator using the same equipment on the same day in the same laboratory.

13.2.1 Repeatability limits are listed in Table 1 below.

13.3 *Reproducibility Limit (R)*—Two test results shall be judged not equivalent if they differ by more than the "R" value for that material; "R" is the interval representing the critical difference between two test results for the same material, obtained by different operators using different equipment in different laboratories.

13.3.1 Reproducibility limits are listed in Table 1 below.

13.4 The above terms (repeatability limit and reproducibility limit) are used as specified in Practice E177.

13.5 Any judgment in accordance with statements 13.2 and 13.3 would have an approximate 95 % probability of being correct.

14. Keywords

14.1 adenosine triphosphate; ATP; bacteria; bioburden; biodeterioration; biomass; fungi; metalworking; microbial contamination; microbiology; microorganisms

[7] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:E34-1002. Contact ASTM Customer Service at service@astm.org.

TABLE 1 Precision Data for Log_{10} pg ATP/mL

Material	Average $\bar{x}$	Repeatability Standard Deviation s_r	Reproducibility Standard Deviation s_R	Repeatability Limit r	Reproducibility Limit R
EO0101	4.38	0.07	0.14	0.18	0.40
EO0102	3.35	0.07	0.12	0.18	0.35
E00103	1.85	0.30	0.32	0.83	0.91
E00104	0.93	0.13	0.33	0.37	0.92
SS0101	4.85	0.06	0.14	0.17	0.39
SS0102	4.26	0.67	0.95	1.89	2.66
SS0103	3.16	0.40	0.42	1.11	1.18
SS0104	1.03	0.26	0.46	0.74	1.28
SO0101	4.59	0.07	0.10	0.21	0.28
SO0102	3.90	0.06	0.21	0.17	0.58
SO0103	1.66	0.09	0.29	0.25	0.82
SO0104	0.89	0.25	0.53	0.70	1.48
EO0201	4.21	0.06	0.67	0.16	1.87
EO0202	2.46	0.15	0.56	0.42	1.58
EO0203	1.06	0.25	0.39	0.70	1.09
EO0204	0.80	0.26	0.55	0.74	1.53
SS0201	1.74	0.44	0.49	1.22	1.36
SS0204	0.74	0.29	0.59	0.82	1.66
SO0201	3.31	0.27	0.27	0.75	0.75
SO0202	2.12	0.16	0.25	0.45	0.69
SO0203	1.26	0.14	0.39	0.39	1.09

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. 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 X1.1, the 4.0 pg ATP/mL lower detection limit for this method ranges from 800 to 8000 bacteria/mL and 8 to 800 fungal cells/mL. Without first determining the actual cell count (cells/mL), it is impossible to correlate ATP concentration to cell counts of CFU/mL. However, Passman et al. have demonstrated strong correlations between ATP data and other commonly used MWF condition monitoring parameters, including: CFU bacteria/mL, biocide concentration, and pH.[4]

X1.3 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 MWF 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. ADJUSTING METHOD DETECTION RANGE

X2.1 Increasing Test Sensitivity (Increasing RLU_{obs} by Filtration)

X2.1.1 Although the detection limit of this method is 4.0 pg ATP/mL (Log_{10} pg ATP/mL = 0.60), RLU_{obs} values that are ≤0.01 RLU_{ctrl} are generally considered to be background noise.

X2.1.2 To increase accuracy at the low end of detection and therefore the test sensitivity, increase the volume of MWF that is filtered in steps 11.6 – 11.8. Using a 60 mL syringe (6.11), up to 50 mL of MWF can be filtered. Moreover, additional aliquants of the sample can be processed successively through the filter so that the total volume filtered can be >50 mL.

X2.1.3 Report actual volume filtered, and use this volume in Eq 1 (12.1).

X2.2 Decreasing Test Sensitivity (Decreasing RLU_{obs} by Dilution)

X2.2.1 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.

X2.2.2 If the original test result yields a value greater than the luminometer's upper detection limit, run a 1+9 dilution of the diluted ATP extract (11.33).

X2.2.2.1 To an unused 17 by 100 mm culture tube, add 9.0 mL of ATP extract dilution buffer (7.2).

X2.2.2.2 Use the micropipeter to transfer 1.0 mL of the diluted ATP extract from step 11.33 to the 9.0 mL of ATP extract dilution buffer prepared in X2.2.2.1.

X2.2.2.3 Follow steps 11.33 – 11.39.

X2.2.3 If the RLU_{obs} is still greater than the luminometer's upper detection limits, run a 1+99 dilution of the diluted ATP extract (11.33).

X2.2.3.1 To an unused 17 by 100 mm culture tube, add 9.9 mL of ATP extract dilution buffer (7.2).

X2.2.3.2 Use the micropipeter to transfer 0.1 mL of the diluted ATP extract from step 11.33 to the 9.9 mL of ATP extract dilution buffer prepared in X2.2.3.1.

X2.2.3.3 Follow steps 11.33 – 11.39.

X2.2.4 Additional dilutions of the X2.2.3.2 dilution can be made if necessary, until the RLU_{obs} is below the luminometer's upper detection limit.

X2.2.5 Observe the RLU value from the luminometer and multiply it by the appropriate dilution factor (that is, 10 for a 1+9 dilution; 100 for a 1+99 dilution). Record the result as RLU_{obs} and use this value in Eq 1.

X3. EVALUATING THE RELATIONSHIP BETWEEN LOG_{10} RLU AND LOG_{10} PG ATP/ML IN EMULSIFIED OIL, SEMI-SYNTHETIC, AND SYNTHETIC MWF

X3.1 Neat stocks of emulsifiable oil (EO), semi-synthetic MWF (SS), and synthetic MWF (S) were used to prepare ATP extracts by way of steps 11.1 – 11.29.

X3.2 Prepared ATP extracts were diluted (11.31 – 11.33) and doped with a 100 000 pg ATP/mL stock solution to give 10 000, 1000, 100, and 10 pg ATP/mL.

X3.3 These doped diluted ATP extracts were analyzed for ATP concentration by way of steps 11.34 – 11.39. Test results are shown in Table X3.1 and Fig. X3.1.

X3.4 Two-way analysis of variance (ANOVA) was computed to determine whether fluid type affected the relationship between RLU and ATP concentration. The ANOVA summary (Table X3.2) demonstrates that the only significant source of variation was the ATP concentration. There was no significant effect due to fluid type.

TABLE X3.1 ATP Standard Addition Test Results in EO, SS, and S

Log pg ATP/mL	Log_{10} RLU		
	EO	SS	S
4.000	5.190	5.185	5.099
4.000	5.122	5.083	5.099
3.000	4.124	4.156	4.259
3.000	4.158	4.146	4.068
2.000	3.164	3.150	3.136
2.000	3.128	3.142	3.009
1.000	2.179	2.294	2.201
1.000	2.117	2.236	2.158
0.000	2.137	1.792	1.462