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LABORATORY PROCEDURES MANUAL FOR THE FIREFLY LUCIFERASE ASSAY FOR ADENOSINE TRIPHOSPHATE (ATP)

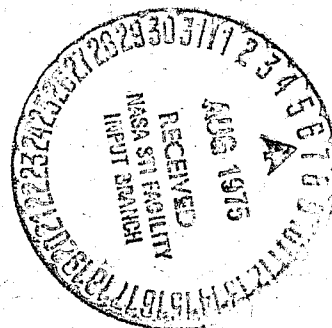
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GODDARD SPACE FLIGHT CENTER
GREENBELT, MARYLAND

LABORATORY PROCEDURES MANUAL
FOR THE
FIREFLY LUCIFERASE ASSAY FOR ADENOSINE TRIPHOSPHATE (ATP)

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January 1975

PREFACE

This manual is the outgrowth of many years experience of working with bioluminescent systems for various applications. Goddard Space Flight Center has been directly involved in the development of procedures and instruments for the adenosine triphosphate (ATP) luciferase assay. As a consequence of this work, a considerable amount of information has been compiled from internal and external sources, as well as from practical experience in the laboratory.

This manual provides detailed descriptions of those laboratory procedures developed and in use at the Goddard Space Flight Center laboratory. The manual has been prepared to provide that type of information that is necessary for a new worker in the field of bioluminescent systems; it is the "sine quo non" for a successful experiment. We hope this manual will be useful and will be a handy reference for laboratory workers. Some information may have been inadvertently omitted or incompletely covered which, if available, would enhance the procedures and descriptions presented. In order to improve future editions of this manual, comments and queries will be most appreciated. Future developments in the ATP luciferase assay will also be reflected in future editions.

The perception and judgement brought to bear by so many of our technical staff and collaborators over the years is greatly appreciated. We also thank V. N. Bush, M. Rindler, and S. A. Tuttle for their help in compiling this manual. Support for this work was furnished by the NASA Technology Utilization Office and Technology Applications Program and by the HEW Regional Medical Program Service.

Emmett W. Chappelle and Grace Lee Picciolo
Goddard Space Flight Center

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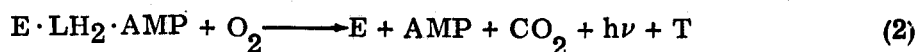
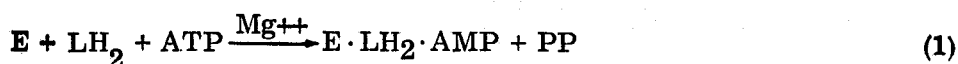
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SECTION 1

INTRODUCTION

The ubiquity and functional significance of adenosine triphosphate (ATP) in metabolism allows its assay to be an excellent monitor of the amount of biological material in a specimen. Because it was established that the firefly luciferase reaction, where light is produced by an enzymatic reaction, is specific for ATP, many investigators use this reaction to determine the amount of bacteria or biological mass present in a specimen (Beutler and Mathai, 1967; Brewer and Knutsen, 1966; Cole et al., 1967; Ebadi et al., 1971; Freese et al., 1969; Holm-Hansen and Booth, 1966; Lee et al., 1971; Patterson et al., 1970).

The reaction mechanism and kinetics have been determined by several investigators (McElroy et al., 1969; Strehler and McElroy, 1957). The reaction can be summarized in two steps (Plant et al., 1968):



where:

- E = firefly luciferase
- LH₂ = reduced luciferin
- AMP = adenosine monophosphate
- PP = pyrophosphate
- T = thiazolinone (dehydroluciferin)
- hν = light (550 nm)

Conditions are here prescribed where this enzymatic reaction can be used as a rapid, sensitive, and easy-to-use assay for the quantity of bacteria (Chappelle and Levin, 1964). When all reagents are present in excess, the light production is proportional to the ATP concentration, which is proportional to the bacterial cell concentration in the specimen. The plot of bacterial cell concentration versus light units shows a linear response over a functional range (Chappelle and Levin, 1968).

The ATP content of an average bacterium is about 2.5×10^{-10} μg per organism. The ATP content varies somewhat through a growth cycle (Freese et al., 1969;

Klofat et al., 1969) and varies with species from 0.28 to 8.9×10^{-10} μg for the 19 species tested (Chappelle and Levin, 1968).

One of the major problems in determining the relationship between ATP levels and bacterial cell number is the presence in the sample of large quantities of soluble ATP. Additionally, there may be numbers of nonbacterial cells containing ATP such as blood and other tissue cells. These amounts of ATP would far exceed the bacterial ATP levels in specimens such as blood and urine.

The luciferase procedures in this manual describe the methods to chemically remove the ATP associated with nonbacterial cells and any soluble ATP (Picciolo et al., 1971; Chappelle and Picciolo, 1973; Chappelle, Picciolo, and Kelbaugh, 1970). Treating the specimen with a nonionic detergent selectively ruptures mammalian cells and does not disturb the cell membranes of bacteria. The addition of an ATPase then hydrolyzes all the free ATP. This action is then inhibited, the bacterial cells are ruptured, and their ATP assayed by the luciferase light reaction.

SECTION 2

INSTRUMENTATION

Many types of photometric instruments exist for quantitating light. Those suitable for accurately measuring low levels of light as are emitted by the small amounts of ATP present in bacteria, i. e., picomoles (10^{-12} moles) of ATP, employ a photomultiplier tube as a detector and a dc amplifier in conjunction with some readout device, for example, recorder, digital readout, etc. Several companies manufacture light measuring instruments; however, we know of only American Instrument Co. (Aminco), E. I. DuPont de Nemours & Co., Inc., and JRB Inc. which manufacture instruments specifically designed for the ATP assay, providing a means to inject a sample into luciferase while in a light-tight configuration in place before a photocathode surface. Three instruments have been used and evaluated in the GSFC laboratory: the Luminescence 760 Biometer manufactured by E. I. DuPont de Nemours & Co., Inc.; the Chem-Glow Photometer manufactured by Aminco division of Travenol Labs, Inc.; and the ATP Photometer, manufactured by JRB, Inc. Information bulletins for these instruments are reproduced in Appendix A.

The Biometer is a photometer specifically designed for measurement of luminescent reactions. An analog signal from the photomultiplier tube is amplified and utilized to charge a "memory" capacitor. This design allows the peak to be accumulated for 3 seconds and the convenience of a digital display. The instrument also provides automatic range change over five decades to accommodate a wide range of light intensities.

The Chem-Glow Photometer consists of a reaction chamber-photomultiplier assembly which is used with the Aminco 10-222 microphotometer. Either peak height or area under the curve of bioluminescence can be measured, the readout being by meter, strip chart recorder, or oscilloscope. Four decades of range change are provided; however, range selection is manual, and if the range selected is inappropriate for the sample being measured, the entire reaction must be repeated after changing the range. Aminco has developed an integrator that will sum the area under the curve as well as measuring peak height and provides four decades of range.

JRB's ATP Photometer uses a photomultiplier and a voltage-to-frequency converter to measure the light produced by the luciferase ATP reaction. This measurement is in the form of a digital display that is proportional to the amount of ATP in the sample. This ATP photometer is capable of measuring either the peak height of light response or the total area under the light reaction curve and provides six decades of range.

LUMINESCENCE BIOMETER, DUPONT, INC.

USE OF THE DUPONT 760 BIOMETER

The DuPont Biometer provides five decades of range with range selection being automatic. The light units are presented on a digital display that is proportional to the amount of ATP in the sample. The calibration, upkeep, and operation of the Biometer will be discussed below, supplementing the instruction manual supplied by DuPont with the Biometer.

Before beginning to assay, read the instruction manual carefully or have an experienced individual explain the operation of the Biometer. The following procedures have been modified for the applications at GSFC and are not necessarily endorsed by the instrument manufacturers.

1. It is advisable to leave the Biometer turned on continuously. If it should be turned off for any length of time, allow at least a one-hour warm-up period. When replacing the diaphragm, the power is turned off momentarily; no warm-up period is necessary.
2. Check the dark current (as shown in Example 1) to ensure that it is within acceptable limits. The limits for each machine are established by recording daily fluctuations.
3. Check diaphragm for light leakage by measuring dark current with drum in EXPOSE position. If a reading higher than the normal dark current is displayed, the diaphragm must be changed. To replace it:
 - Turn off power.
 - Unscrew syringe housing. A metal tube has been devised to accommodate a 1.0-cc disposable syringe with a 22-g, 1 1/2 in. needle. It is fitted above the cuvette and diaphragm and holds the syringe perpendicular to guide the needle through the diaphragm and into the cuvette. It is referred to hereafter as the syringe housing, and its dimensions are 11 × 105 mm (outside diameter).
 - Remove used diaphragm with forceps and insert new diaphragm.
 - Replace syringe housing and turn on power.
4. Check radioactive light standard No. 2. The light standards used at GSFC contain C^{14} labeled glucose dispersed in a liquid scintillation fluid. This is sealed in a glass cuvette fitted to the instruments. If the instrument is calibrated and warmed up, the light standard should read $2.00 \times 10^7 \pm 0.1$.

5. The luciferase is dispensed with a 1.0-ml pipette into cuvettes for assay. Do not use the last tenth milliliter from a blow-out pipette because this is not an accurate volume. A disposable 1-ml syringe is considered too inaccurate for use in dispensing luciferase.
6. Insert cuvette containing 0.1-ml luciferase into Biometer. Insertion of a cuvette forces the previous cuvette to drop into the discharge tray. If a cuvette does not load freely, do not force it. If a cuvette should break, the glass chips must be removed immediately to prevent severe damage to the drum.
7. Check the inherent light of the luciferase and determine if the machine has zeroed it. If there is carry-over light, hold the luciferase at room temperature until the machine is able to clear it to within the range of the dark current. (See section on Controls).
8. Zero the cuvette in the EXPOSE position and fill a 1.0-ml disposable tuberculin syringe. Fill syringe by drawing sample past the 0.1-ml mark. Remove air bubbles by flicking the syringe expelling the excess sample until the syringe contains exactly 0.1 ml. There should be no air in the needle or syringe because this will drastically affect the amount of sample displaced during injection.
9. Insert syringe and needle into the syringe housing on the Biometer. Press down on the syringe to force the needle through the diaphragm and inject. Injection speed is critical.

-NOTE-

If the sample is injected too slowly, it will not form a uniform mixture with the luciferase and replication will be poor. Too rapid injection will cause undesirable foaming in the cuvette. An automatic injector is being developed to minimize individual variation in filling the syringe and in injection speed.

10. Press COUNT button. Press again. Repeat until digital readout remains constant. Record on data form shown in Figure 2-1. To interpret the results see "Calculations" in Section 7.
11. Remove syringe and discard. Turn drum to LOAD position and continue with next sample.

EXAMPLES

The following are some examples of data obtained in the GSFC laboratory using GSFC standard procedures for the Biometer. These are not the best data possible, but are results with typical coefficient of variation (CV) percentages that could be expected. Figure 2-1 is the form of recording Biometer readings which is used in the laboratory.

LIGHT READINGS

DATE: _____ TECHNICIAN: _____
INSTRUMENT: _____ CALIBRATION: _____
SETTINGS: _____ EXPT: _____

READINGS

SAMPLE: _____	SAMPLE: _____
1. _____	1. _____
2. _____	2. _____
3. _____	3. _____
4. _____	4. _____
5. _____	5. _____
\bar{x} _____	\bar{x} _____
σ _____	σ _____
CV% _____	CV% _____
1. _____	1. _____
2. _____	2. _____
3. _____	3. _____
4. _____	4. _____
5. _____	5. _____
\bar{x} _____	\bar{x} _____
σ _____	σ _____
CV% _____	CV% _____

Figure 2-1. Form for Recording Biometer Readings

Example 1: Measurement of Biometer Dark Current

The purpose of the measurement of Biometer dark current is to measure instrument electrical noise, which shows the lowest limits of the machine; that is, only a reading of greater than the dark current is a valid reading of light, and the dark current must be subtracted from this reading. See Figure 2-2 for sample recorded readings.

Procedure for Measurement of Biometer Dark Current

1. Rotate drum to LOAD position.
2. Zero Biometer and clear the digital readout by pressing the DARK CURRENT switch to zero position for 3 seconds and by releasing again to hold position. Three seconds are necessary to ensure zeroing, especially after a high reading.
3. Press the COUNT button located on the top of the Biometer. The digital readout that appears on the screen is the dark current.

LIGHT READINGS	
DATE: <u>7/1/74</u>	TECHNICIAN: <u>Rebecca</u>
INSTRUMENT: <u>Du Pont Biometer</u>	CALIBRATION: <u>Cal to read</u>
SETTINGS: <u>Coarse</u>	EXPT: <u>Dark Current</u>
<small>Sens 5.0</small>	<small>2x10⁷ using ALS #2</small>
READINGS	
SAMPLE: <u>Dark Current</u>	SAMPLE: <u>Dark Current</u>
1. <u>0.03 X 10⁵</u>	1. <u>0.02 X 10⁵</u>
2. <u>0.01 X 10⁵</u>	2. <u>0.03 X 10⁵</u>
3. <u>0.03 X 10⁵</u>	3. <u>0.04 X 10⁵</u>
4. <u>0.02 X 10⁵</u>	4. <u>0.01 X 10⁵</u>
5. <u>0.01 X 10⁵</u>	5. <u>0.01 X 10⁵</u>
\bar{x} <u>2 X 10³</u>	\bar{x} <u>2.2 X 10³</u>
σ <u>1 X 10³</u>	σ <u>1.3 X 10³</u>
CV% <u>50%</u>	CV% <u>59%</u>
1. _____	1. _____
2. _____	2. _____
3. _____	3. _____
4. _____	4. _____
5. _____	5. _____
\bar{x} _____	\bar{x} _____
σ _____	σ _____
CV% _____	CV% _____

Figure 2-2. Recorded Biometer Readings from Example 1

Example 2. Measurement of Biometer Lamp Cal

The purpose of measurement of Lamp Cal is to check calibration of the lamp, which should be standardized to the appropriate light standard (i. e., if RLS No. 2 is to read 2.00×10^7 , then lamp cal should read 2.00×10^7). A sample record is shown in Figure 2-3.

Procedure for the Measurement of Biometer Lamp Cal

1. Rotate drum to LOAD position.
2. Zero Biometer and clear the digital readout by pressing DARK CURRENT switch to zero position for 3 seconds and releasing again to hold position.
3. Press RESET button.
4. Press Lamp Cal READ button and hold down until a digital readout appears on the screen.

LIGHT READINGS

DATE: 2/10/74 TECHNICIAN: DANA

INSTRUMENT: DUPONT Biometer CALIBRATION: CALIBRATED TO READ 2×10^7 USING RLS #2

SETTINGS: Course Sensitivity EXPT: LAMP CAL REPRODUCIBILITY
5.0

READINGS

<p>SAMPLE: <u>Lamp CAL</u></p> <p>1. <u>1.97×10^7</u></p> <p>2. <u>1.98×10^7</u></p> <p>3. <u>1.96×10^7</u></p> <p>4. <u>1.97×10^7</u></p> <p>5. <u>1.97×10^7</u></p> <p>\bar{x} <u>1.97×10^7</u></p> <p>σ <u>7.07×10^4</u></p> <p>CV% <u>0.4%</u></p>	<p>SAMPLE: <u>Lamp CAL</u></p> <p>1. <u>1.97×10^7</u></p> <p>2. <u>1.95×10^7</u></p> <p>3. <u>1.98×10^7</u></p> <p>4. <u>1.99×10^7</u></p> <p>5. <u>1.97×10^7</u></p> <p>\bar{x} <u>1.97×10^7</u></p> <p>σ <u>1.48×10^5</u></p> <p>CV% <u>0.7%</u></p>
<p>1. _____</p> <p>2. _____</p> <p>3. _____</p> <p>4. _____</p> <p>5. _____</p> <p>\bar{x} _____</p> <p>σ _____</p> <p>CV% _____</p>	<p>1. _____</p> <p>2. _____</p> <p>3. _____</p> <p>4. _____</p> <p>5. _____</p> <p>\bar{x} _____</p> <p>σ _____</p> <p>CV% _____</p>

Figure 2-3. Recorded Biometer Readings from Example 2

Example 3: Measurement of Radioactive Light Standard

The purpose of the measurement of radioactive light standard (RLS) is to check the range and sensitivity of the instrument. This is also a double check on the Lamp Cal. The Lamp Cal is calibrated to read 2×10^7 light units using radioactive light standard No. 2. Sample record is shown in Figure 2-4. Radioactive light standard values from the Biometer are compared to those from the JRB ATP photometer and the Chem-Glow in Figure 2-5.

Procedure

1. Rotate drum to LOAD position.
2. Load radioactive light standard in drum. Clear the digital readout, by pressing the DARK CURRENT switch to zero position for 3 seconds and releasing again to hold position.
3. Rotate drum to EXPOSE position and press COUNT button, located on top of the Biometer. The digital readout that will appear on the screen

LIGHT READINGS

DATE: 4/14/74 TECHNICIAN: Corinne

INSTRUMENT: DuPont Biometer CALIBRATION: to read 2×10^7 with

SETTINGS: Range - ON EXPT: Replicate light RLS # 2

Course sens. - 5.0 Power - ON READINGS standards

<p>SAMPLE: <u>lt. std. #2</u></p> <p>1. <u>2.13×10^7</u></p> <p>2. <u>2.10×10^7</u></p> <p>3. <u>2.11×10^7</u></p> <p>4. <u>2.11×10^7</u></p> <p>5. <u>2.10×10^7</u></p> <p>\bar{x} <u>2.11×10^7</u></p> <p>σ <u>1.22×10^5</u></p> <p>CV% <u>0.6%</u></p> <p>1. _____</p> <p>2. _____</p> <p>3. _____</p> <p>4. _____</p> <p>5. _____</p> <p>\bar{x} _____</p> <p>σ _____</p> <p>CV% _____</p>	<p>SAMPLE: <u>lt. std. #2</u></p> <p>1. <u>2.09×10^7</u></p> <p>2. <u>2.11×10^7</u></p> <p>3. <u>2.10×10^7</u></p> <p>4. <u>2.09×10^7</u></p> <p>5. <u>2.09×10^7</u></p> <p>\bar{x} <u>2.10×10^7</u></p> <p>σ <u>8.94×10^4</u></p> <p>CV% <u>0.4%</u></p> <p>1. _____</p> <p>2. _____</p> <p>3. _____</p> <p>4. _____</p> <p>5. _____</p> <p>\bar{x} _____</p> <p>σ _____</p> <p>CV% _____</p>
--	--

Figure 2-4. Recorded Biometer Readings from Example 3

is the radioactive light standard value; taken only one time. If count button is repeatedly pushed, the RLS reading will continue to rise slightly.

-NOTE-

Care should be taken to avoid breaking the radioactive light standard when removing it from the Biometer. When removing the RLS, should it become trapped in the reflectorized chamber, it can be removed by opening a trap located behind the sliding panel on the front of the Biometer.

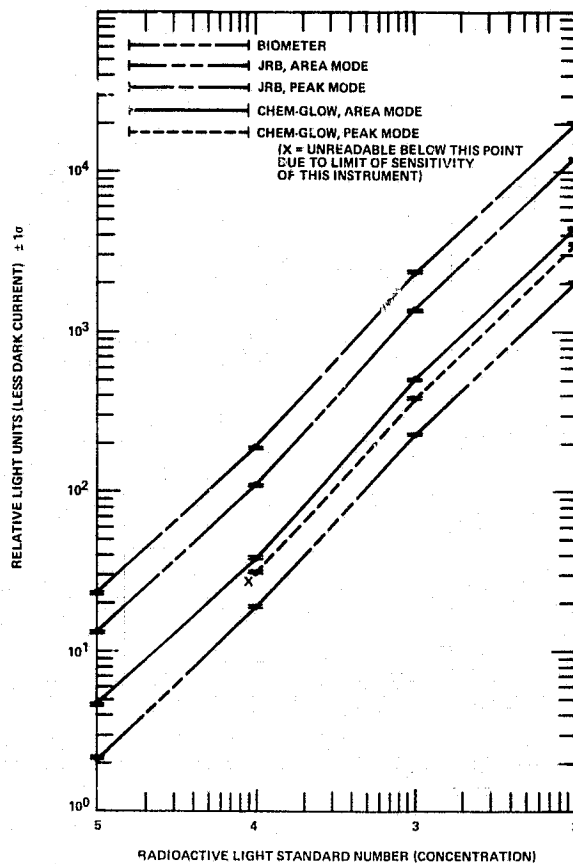


Figure 2-5. Comparison of Radioactive Light Standard Values (Because the graphs overlap they have been separated to show linearity. The numerical relationship between the graphs is arbitrary.)

Example 4: Standard ATP in H₂O

The purpose of measuring standard ATP in H₂O is to check reproducibility of injection. Ten replicate injections were made—five by each of two technicians. A sample record is shown in Figure 2-6.

Procedure

1. Dilute ATP in H₂O to 10⁻² μg/ml.
2. Inject 0.1 ml of 10⁻² μg ATP/ml into luciferase rehydrated in 0.05 M TRIS, 0.01 M MgSO₄ pH 7.75.

LIGHT READINGS

DATE: 2-18-74 TECHNICIAN: Carol & Ann
 INSTRUMENT: Biometer CALIBRATION: BLS #2 = 2 x 10⁷
 SETTINGS: Course Set 5.0 EXPT: Standard ATP in H₂O

READINGS

<p>SAMPLE: <u>Carol</u></p> <p>1. <u>1.09 x 10⁸</u> 2. <u>9.96 x 10⁷</u> 3. <u>1.11 x 10⁸</u> 4. <u>1.06 x 10⁸</u> 5. <u>1.14 x 10⁸</u> \bar{x} <u>1.08 x 10⁸</u> σ <u>5.49 x 10⁶</u> CV% <u>5.1%</u></p> <p>1. _____ 2. _____ 3. _____ 4. _____ 5. _____ \bar{x} _____ σ _____ CV% _____</p>	<p>SAMPLE: <u>Ann</u></p> <p>1. <u>9.97 x 10⁷</u> 2. <u>1.00 x 10⁸</u> 3. <u>1.12 x 10⁸</u> 4. <u>1.05 x 10⁸</u> 5. <u>9.8 x 10⁷</u> \bar{x} <u>1.03 x 10⁸</u> σ <u>5.66 x 10⁶</u> CV% <u>5.5%</u></p> <p>1. _____ 2. _____ 3. _____ 4. _____ 5. _____ \bar{x} _____ σ _____ CV% _____</p>
--	---

Figure 2-6. Recorded Biometer Readings from Example 4

CALIBRATION OF THE BIOMETER

Introduction

The sensitivity and range of the Biometer is dependent upon the calibration of the instrument. It is possible to calibrate the instrument with either an ATP standard solution containing all reagents to be used or a radioactive light standard of a known quantity. At GSFC, it has been found that the radioactive light standard is more dependable because the light reading of the ATP standard solution is dependent on the activity of the luciferase and the inhibitors in the solution. Different lot numbers of DuPont luciferase vary somewhat in activity and with time after rehydration.

When using the ATP standard solution for calibration the following procedure is used.

Procedure for ATP Standard Solution

1. Prepare an ATP standard solution by diluting ATP to 2×10^{-1} $\mu\text{g}/\text{ml}$. (1.0 ml of 1.0 μg ATP/ml + 4.0 ml H_2O).
2. Place in a 12-ml centrifuge tube 2.0 ml 0.15 M Na_2SO_4 .
3. Add 2.0 ml 0.1 N HNO_3 .
4. Add 0.5 ml 2×10^{-1} μg ATP/ml.
Vortex.
5. Assay by injecting 0.1 ml into luciferase, rehydrated in 0.25 M TRIS, 0.01 M MgSO_4 pH 8.20.

Calibration

When calibrating the Biometer with the ATP standard solution, it is calibrated to give a final readout of 2.00×10^8 fg/ml ± 0.05 . When using radioactive light standard No. 2, the instrument is calibrated to give a final readout of 2.00×10^7 fg/ml ± 0.05 . The sensitivity and range of the instrument when calibrated using light standard No. 2 is comparable to the calibration by the ATP standard solution. This range has been found adequate for the Biometer to zero out the inherent light of the luciferase reconstituted in 0.25 M TRIS, 0.01 M MgSO_4 at pH 8.20 and allowed to sit 45 minutes.

The importance of zeroing out this inherent light is apparent in lower light readings. The carryover light would have the effect of increasing the lower light readings. This could give false ATP values and thus a false indication of the number of microorganisms present in the sample.

Calibration Procedure with the ATP Standard Solution for DuPont 760 Biometer

1. Place DARK CURRENT switch in manual zero position.
2. Adjust INTENSITY dial to midpoint by turning dial to the STOP position in either direction and then reversing five turns.
3. Place RANGE switch in ON position.
4. Release lock on COARSE SENSITIVITY. Adjust COARSE SENSITIVITY to 4.00.
5. Adjust FINE SENSITIVITY to midpoint.
6. Rotate EXPONENT WHEEL to read 5 when instrument is zeroed (Quadrant V).
7. With DRUM in LOAD position, insert a cuvette containing the reaction mixture until edge of cuvette is flush with top of collating drum. Manually zero machine with DARK CURRENT switch so digits read 0.00×10^5 . Rotate DRUM to EXPOSE position.
8. Take 0.1 ml of ATP standard solution and inject into the cuvette. Leave syringe in place.
9. Depress COUNT button. Repeat until reading is stabilized. Record digital readout
10. Remove syringe and rotate DRUM to LOAD position and insert a new cuvette of luciferase.
11. Rotate the DRUM again to EXPOSE position and repeat steps 8, 9, and 10. When three consecutive readouts show a count variation of no more than 0.15 proceed to step 12.

12. Calculate the mean of the three ATP injections to obtain the mean light response.
13. Rotate DRUM into LOAD position and clear memory by depressing RESET button.
14. Check the lamp's intensity by depressing the READ button and holding it down until a new readout appears.
15. Adjust INTENSITY dial until the light reading of the lamp corresponds to the mean light response.
16. Between adjustments of the INTENSITY dial, clear memory circuits by depressing RESET button. Repeat step 14 until desired readout appears and is repeatable. Remember to hold down READ button until readout appears.
17. When the READ button gives reproducible readouts of the mean light response, the calibration lamp matches the intensity of the ATP standard solution flash. During the remainder of the calibration procedure, the internal lamp is used instead of an injection of ATP standard solution.
18. Leave DRUM in LOAD position and clear the instrument with RESET button.
19. Using the READ button and the sensitivity dials, adjust the COARSE SENSITIVITY to obtain a readout of ± 0.02 of the desired readout. As before, between adjustments of the COARSE and FINE SENSITIVITY dials, first clear the memory by depressing the RESET button. Then check the result of the adjustment by means of the READ button.
20. Lock the COARSE SENSITIVITY dial by means of the lever on the side of the dial.
21. Make final adjustment with the FINE SENSITIVITY dial.

Calibration Check

To check the calibration procedure, inject ATP standard solution as at the beginning of the procedure. If a readout of ± 0.05 of the desired conversion factor is obtained, the instrument is calibrated properly.

Should excessive variation occur, repeat calibration procedure. In this event, substitute the means of the readouts of these last ATP injections for the mean light response in step 12.

Calibration Procedure with Radioactive Light Standard for DuPont 760 Biometer

1. Place DARK CURRENT switch in manual ZERO position.
2. Adjust INTENSITY dial to midpoint by turning dial to the STOP position in either direction and then reversing five turns.
3. Place RANGE switch in ON position.
4. Release lock on COARSE SENSITIVITY. Adjust COARSE SENSITIVITY to 4.00.
5. Adjust FINE SENSITIVITY to midpoint.
6. Rotate EXPONENT wheel to read 5 when instrument is zeroed (Quadrant V).
7. With DRUM in LOAD position, insert a cuvette containing the radioactive light standard until edge of cuvette is flush with top of collating drum. Manually zero machine so digits read 0.00×10^5 . Rotate DRUM to EXPOSE position.
8. Depress COUNT button only once. (Because the light standard is a known constant light output and the Biometer will keep summing the light and read peak levels, to keep depressing the COUNT button is only showing machine fluctuation in the digital readout.)
9. After recording the digital readout, rotate DRUM to LOAD position and manually zero digital readout.
10. Rotate DRUM once more to EXPOSE position and depress COUNT button. Record results.
11. Calculate the mean of the three readouts to obtain mean light response.
12. Rotate DRUM into LOAD position and clear memory by depressing RESET button.

13. Check the lamp's intensity by depressing the READ button and by holding it down until a new readout appears.
14. Adjust INTENSITY dial until the light reading of the lamp corresponds to the mean light response.
15. Between adjustments of the INTENSITY dial, clear memory circuits by depressing RESET button. Repeat step 13 until desired readout appears and is repeatable. Remember to hold down READ button until readout appears.
16. When the READ button gives reproducible readouts of the mean light response, the calibration lamp matches the intensity of the radioactivity light standard. During the remainder of the calibration procedure, the internal lamp is used instead of the radioactive light standard.
17. Leave DRUM in LOAD position and clear the instrument with RESET button.
18. Using the READ button and the sensitivity dials, adjust the COARSE SENSITIVITY to obtain a readout of ± 0.02 of the desired readout.
19. As before, between adjustments of the COARSE and FINE SENSITIVITY dials, first clear the memory by depressing the RESET button. Then check the result of the adjustment by means of the READ button.
20. Make final adjustment with the FINE SENSITIVITY dial.

Calibration Check

To check the calibration procedure, repeat digital readouts of the radioactive light standard as at the beginning of the procedure. If a readout of ± 0.05 of the desired conversion factor is obtained, the instrument is calibrated properly.

Should excessive variation occur, repeat calibration procedure. In this event, substitute the means of the readouts of these last digital readouts for the mean light response in step 11.

CHEM-GLOW PHOTOMETER, AMINCO, INC.

USE OF THE CHEM-GLOW PHOTOMETER

The Aminco Chem-Glow photometer is capable of measuring peak height of light response on the area under the light reaction curve. Four decades of range selection are provided, with range selection being manual. The read-out is by meter, strip chart recorder, or oscilloscope. The newly developed integrator (September 1974) provides digital display and three decades of automatic ranging. Operation and calibration of the Chem-Glow is discussed below and supplements the manual supplied by Aminco with the Chem-Glow instrument. The following procedures have been modified for the applications at GSFC and are not necessarily endorsed by the instrument manufacturers.

Before Assaying

This instrument requires that the housing be grounded before use. This can be accomplished by inserting the three-pronged power plug into a properly grounded receptical or, if an adapter is used, the external lead of the adapter must be grounded properly.

The technician should turn the power on at least 30 minutes prior to assaying to allow for instrument stabilization. Before assaying, the power switch must be turned to HV (high voltage), sending high voltage to the photomultiplier, thereby activating it.

When the damping switch is on, a 2-second delay constant is employed in the readings. For peak light readings, which are of short lived duration, the damping switch must be off.

Sensitivity and Relative Intensity

The multiplier dial controls the sensitivity of this instrument and it has seven calibrated sensitivity settings. There are two relative intensity scales, one 0 to 30 and the other 0 to 100, of which only one should be read depending on which sensitivity setting is used. The sensitivities are all relative to a meter reading of 100 on a multiplier setting of 100. The following chart represents the proper meter scale to be used in relation to the multiplier setting:

<u>Multiplier Dial Setting</u>	<u>Read Scale</u>
100	0-100
30	0-30
10	0-100
3	0-30
1	0-100
0.3	0-30
0.1	0-100

The Diaphragm

After repeatedly injecting samples into the Chem-Glow, it may become necessary to change the rubber diaphragm which is located at the bottom of the syringe housing situated on top of the instrument. Symptoms of an excessively worn diaphragm include encountering very little resistance when pushing the syringe through the diaphragm or experiencing light leaks. Light leaks are verified by a meter reading (taken when no sample is placed in the reaction chamber) which disappears when the hole at the top of the syringe housing is tightly covered. The technician may simply use his thumb to cover the tube when checking for a light leak due to diaphragm wear. Care must be exercised not to expose the photomultiplier tube to strong light while the high voltage to the photomultiplier is turned on.

When changing the diaphragm, the power to the instrument must be turned off. The syringe housing is unscrewed, and the rubber diaphragm is replaced. Replace syringe housing and turn on power.

Blank Adjustments

The Chem-Glow photometer has blank adjustment settings which are adjustable to three intensities: low, medium, and high. These settings are used in conjunction with the fine adjustment dial to zero either the dark current or the inherent light of the enzyme or both. The intensity used depends on the intensity of the signal. A zero adjustment made on low intensity will not necessarily provide a zero reading when switched to high intensity; however, a zero adjustment when made on high intensity will always give a zero reading on medium or low intensity.

ASSAYING WITH THE CHEM-GLOW PHOTOMETER

After the instrument has been warmed for approximately 30 minutes and the dark current (any reading which may appear when there is no sample in the instrument) is zeroed, the instrument is ready to use. Comparison of radioactive light standard values are shown in Figure 2-5.

Procedure

1. Place a cuvette containing enzyme into the cuvette receptical and turn the bar handle until the reaction chamber comes to a stop under the syringe housing.
2. Zero the inherent light of the enzyme by turning either the FINE or COARSE BLANK ADJUSTMENT dials. The FINE ADJUST dial will usually be sufficient. The meter reading should be adjusted just to the right side of 0 on either meter scale.
3. Place syringe containing sample into syringe housing (11 x 89 mm, outside diameter) located on top of the instrument and inject the sample into the cuvette containing the enzyme.
4. Visually observe needle deflection and record peak height.
5. Turn the bar handle fully to the right exposing the used cuvette.
6. Replace with a fresh cuvette and continue with next sample.

This instrument may be used in conjunction with a strip chart recorder which eliminates the need to manually record the results of each injection. A sample data record for the Aminco Chem-Glow photometer is shown in Figure 2-7.

If it is necessary to have a larger volume of reaction mixture, Aminco will supply a reaction chamber which accommodates a larger cuvette. The larger cuvettes hold approximately 2.5 ml.

THE ATP PHOTOMETER, JRB INC.

USE OF THE ATP PHOTOMETER

The JRB ATP photometer has been used in the laboratories at GSFC; calibration and operation of this instrument are discussed below. The following procedures have been modified for the applications at GSFC and are not necessarily endorsed

LIGHT READINGS

DATE: 1-8-74 TECHNICIAN: Grace
 INSTRUMENT: Chem-Glow CALIBRATION: FLSA = 3 sec 5mV
 SETTINGS: 5.0V, low adj, w and multiplier at 0.1
 EXPT: light standard
 replication

READINGS

SAMPLE: <u>light standard # A</u>	SAMPLE: # A
1. <u>356</u>	1. <u>356</u>
2. <u>354</u>	2. <u>351</u>
3. <u>355</u>	3. <u>350</u>
4. <u>349</u>	4. <u>350</u>
5. <u>352</u>	5. <u>352</u>
\bar{x} <u>354</u>	\bar{x} <u>352</u>
σ <u>3.05</u>	σ <u>2.49</u>
CV% <u>0.9%</u>	CV% <u>0.7%</u>
1. _____	1. _____
2. _____	2. _____
3. _____	3. _____
4. _____	4. _____
5. _____	5. _____
\bar{x} _____	\bar{x} _____
σ _____	σ _____
CV% _____	CV% _____

Figure 2-7. Sample Data Record from the Chem-Glow Photometer

by the manufacturers. The measurement of the light units, as with the Biometer, is presented on a digital display which is proportional to the amount of ATP in the sample.

The ATP photometer is equipped with two modes of operation: the integral mode and the peak mode. The integral mode measures the total area under the light reaction curve and must be used in conjunction with the 60-second interval. The peak mode measures the peak of the light reaction and must be used in conjunction with the 6-second interval.

The 60-second mode has a 15-second delay during which the sample may be injected with the cap off the reaction chamber. When assaying a sample, the cover must be locked in place. This will open the shutter. This instrument has a safety lock on the cover that prevents damage to the photomultiplier tube from excessive light. This device prevents the cover from being removed when the shutter is open. To open the shutter and thus expose the photomultiplier tube to the sample, the safety latch must be in the locked position.

After setting the instrument at the preferred sensitivity, it is necessary to zero the dark current. The dark current may be eliminated by turning the zero dial. The dark current is zeroed by turning the zero adjustment until counts appear and then backing off until the empty chamber gives a reading of less than 10.

This instrument has the capability of displaying a six-digit number. It is not necessary to provide the JRB ATP photometer with power when not in use, although a warm-up period of about 30 minutes is required prior to use. An electrical transformer, one which will provide a constant voltage source, may be needed to protect the instrument from electrical power surges, although there is one built in. Data records for the following procedures are shown in Figure 2-8.

LIGHT READINGS		LIGHT READINGS	
DATE: <u>6/28/74</u>	TECHNICIAN: <u>David Nisley</u>	DATE: <u>6/28/74</u>	TECHNICIAN: <u>David Nisley</u>
INSTRUMENT: <u>JRB Photometer</u>	CALIBRATION: <u>RLS #2 using 6 sec. mode = 2000.0</u>	INSTRUMENT: <u>JRB Photometer</u>	CALIBRATION: <u>6 sec. mode = 2000.0</u>
SETTINGS: <u>60 Sec Area Mode</u>	EXPT: <u>Light Standard Replication</u>	SETTINGS: <u>6 Sec Peak Mode</u>	EXPT: <u>Light Standard Replication</u>
READINGS		READINGS	
SAMPLE: <u>Light STAND #2</u>	SAMPLE: <u>Light STAND #2</u>	SAMPLE: <u>Light STANDARD #2</u>	SAMPLE: <u>Light STANDARD #2</u>
1. <u>10, 204</u>	1. <u>10, 185</u>	1. <u>2062</u>	1. <u>2064</u>
2. <u>10, 202</u>	2. <u>10, 184</u>	2. <u>2055</u>	2. <u>2060</u>
3. <u>10, 195</u>	3. <u>10, 170</u>	3. <u>2061</u>	3. <u>2075</u>
4. <u>10, 190</u>	4. <u>10, 166</u>	4. <u>2060</u>	4. <u>2070</u>
5. <u>10, 189</u>	5. <u>10, 169</u>	5. <u>1966</u>	5. <u>2073</u>
\bar{x} <u>10, 196</u>	\bar{x} <u>10, 175</u>	\bar{x} <u>2041</u>	\bar{x} <u>2063</u>
σ <u>6.8</u>	σ <u>8.9</u>	σ <u>41</u>	σ <u>6</u>
CV% <u>.07%</u>	CV% <u>.09%</u>	CV% <u>.2%</u>	CV% <u>.3%</u>
1. _____	1. _____	1. _____	1. _____
2. _____	2. _____	2. _____	2. _____
3. _____	3. _____	3. _____	3. _____
4. _____	4. _____	4. _____	4. _____
5. _____	5. _____	5. _____	5. _____
\bar{x} _____	\bar{x} _____	\bar{x} _____	\bar{x} _____
σ _____	σ _____	σ _____	σ _____
CV% _____	CV% _____	CV% _____	CV% _____

Figure 2-8. Comparison of RLS Readings on 6-second Peak Mode with 60-second Area Mode

INHERENT LIGHT PRODUCED BY THE ENZYME

For linear and accurate results, the inherent light of the enzyme must be zeroed before each injection. The procedure is identical to zeroing dark current. The technician must decide when inherent light counts warrant zeroing. An inherent light reading of 2 or 3 in conjunction with an ATP count of 600 would be negligible. An inherent light count of 4 in conjunction with an ATP count of 7 would warrant zeroing the inherent light to obtain a real value of ATP induced light. Before beginning the assay, the radioactive light standard must be checked. Figure 2-5 compares the light standard values from the JRB ATP photometer with those from the Biometer and the Chem-Glow photometer.

PROCEDURE FOR THE 6-SECOND PEAK MODE

1. Remove the cover of the reaction chamber.
2. Insert cuvette containing luciferase.
3. Inject the sample.
4. Replace the reaction chamber cover and lock in place. This will open the shutter.
5. Press ASSAY button one time.
6. Record the digital display and press RESET button.
7. Remove used cuvette and continue with next sample.

PROCEDURE FOR THE 60-SECOND AREA MODE

The 60-second mode has a 15-second delay during which the sample is injected.

1. Remove the cover of reaction chamber.
2. Insert cuvette containing luciferase.
3. Press ASSAY button.

-NOTE-

The user now has 15 seconds to inject the sample and lock the cap in place. The assay starts automatically after the end of the delay period.

4. Record the digital display and press RESET button.
5. Remove used cuvette and repeat with next sample.

SUMMARY

The following graph (Figure 2-9) represents an ATP concentration curve using the three described photometers.

Serial dilutions of ATP and water were made and equal volumes of the varying ATP concentrations and 0.1 N HNO₃ were mixed. A volume of 0.1 ml of the ATP and HNO₃ solutions was assayed with 0.1 ml of enzyme. The enzyme used was DuPont luciferase-luciferin enzyme which was reconstituted with 0.2 M TRIS buffer, 0.01 M MgSO₄. The initial pH of the buffer solution was 8.30 to give a reaction mixture pH of 7.75. Integration time when using the Chem-Glow was 10 seconds, when using the JRB ATP Photometer it was 60 seconds.

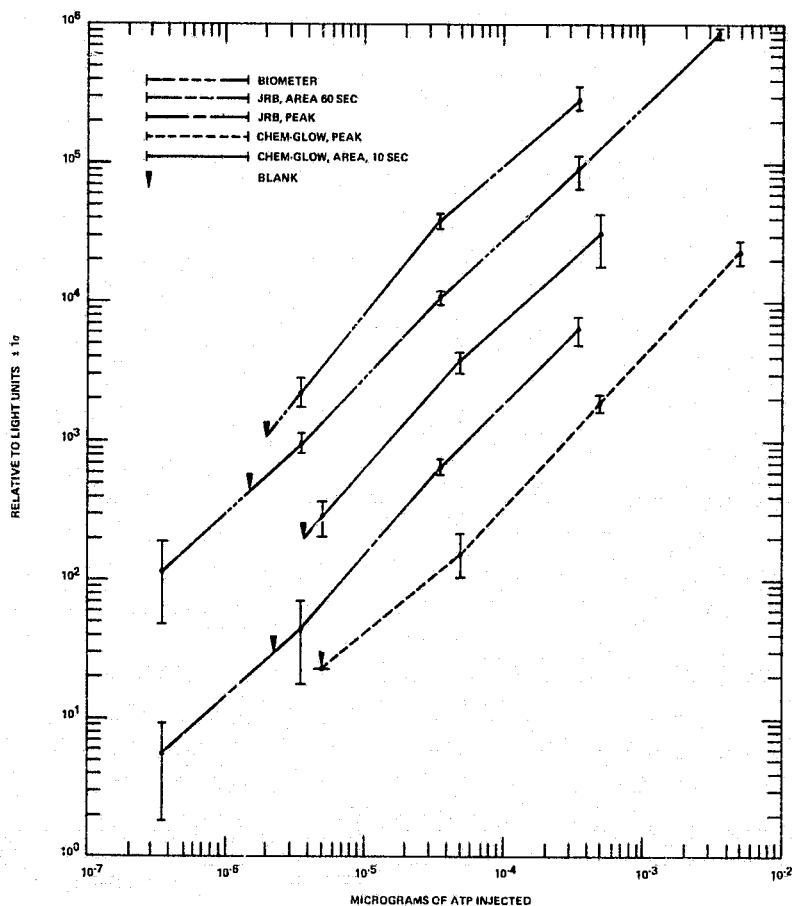


Figure 2-9. Representative ATP Concentration Curve (Because the graphs overlap they have been separated to show linearity. The numerical relationship between the graphs is arbitrary.)

COULTER COUNTER, COULTER ELECTRONICS, INC.

A Coulter Counter is used for high speed particle counting ranging from large particles such as blood cells to those of bacterial origin and smaller. Besides determining the number of particles in a given solution, the counter also measures the volume of the particles. The Model Z_B Counter, the Model P Size Distribution Analyzer, and X-Y Recorder constitute the equipment currently in use in this laboratory.

The operating principle of the counter involves the passage of an electrical current across a small orifice (aperture). When suspended in an electrolyte, particles or cells change the resistance in the path of the current by displacing an equal volume of the electrolytic solution. This results in corresponding current and voltage changes whose magnitude describes the particle volume and whose frequency is proportional to the number of particles in the suspension.

The operation of the Coulter Counter is discussed below to supplement the instruction manual supplied with the instrument. The following procedures have been modified for the applications at GSFC and are not necessarily endorsed by the instrument manufacturers. To operate the Coulter Counter, the three power switches must first be turned on. The switch on the Model Z_B is a pull switch, while the ones on the Recorder and the Model P are of the push variety. After the instrument has warmed up (20 minutes), the storage solution must be rinsed out of the aperture tube. When not in use, the tube is filled with a green cleaning solution called Isoterge, 1/10 in filtered (2 times) saline. To flush out the Isoterge, remove the Tygon tube and the brown brush wire from the Isoterge bottle at the rear of the sample stand and place them in a bottle of freshly filtered (2 times) saline. This saline can be filtered into a vacuum flask using 0.22 μ filters. The reason for filtration is to keep the aperture tube as clean and free from stray particles as is possible.

When the above is completed, open the auxiliary stopcock (the one on the left hand side) while the control stopcock (on top of the aperture tube) is in the open position and wait until all green color disappears from the aperture tube. Before closing the auxiliary stopcock the flushed solution should flow into the two Erlenmeyer flasks at the rear of the sample stand. In addition, remove the plastic container containing the Isoterge from the beaker platform.

All samples of bacterial cells in this laboratory are read after dispersing in normal saline. This saline must be filtered three times into plastic disposable filters or into a very clean vacuum flask. A sample data form is shown in Figure 2-10. A sample of recorded data is shown in Figure 2-11 following the procedure description.

COULTER COUNTER DATA FORM

DATE _____ TECHNICIAN _____
SPECIMEN _____ EXPERIMENT _____
DILUTION _____ VOLUME MEASURED _____
DILUENT _____ STANDARD PARTICLE SIZE _____
SETTINGS 1/AMPLIFICATION _____ 1/APERTURE CURRENT _____
LOWER THRESHOLD _____ UPPER THRESHOLD _____
MATCHING SWITCH _____

READINGS

SAMPLE 1 _____

1. _____

2. _____

3. _____

4. _____

5. _____

\bar{x} _____

σ _____

CV% _____

SAMPLE 3 _____

1. _____

2. _____

3. _____

4. _____

5. _____

\bar{x} _____

σ _____

CV% _____

SAMPLE 2 _____

1. _____

2. _____

3. _____

4. _____

5. _____

\bar{x} _____

σ _____

CV% _____

SAMPLE 4 _____

1. _____

2. _____

3. _____

4. _____

5. _____

\bar{x} _____

σ _____

CV% _____

Figure 2-10. Coulter Counter Data Form

The first sample to read when doing Coulter work is the saline blank. Carefully rinse a new plastic disposable sample container (see Appendix B, Manufacturers and Materials) with the saline. Also, rinse the lower aperture tube by submerging it in the saline filled container. The container is put onto the lowered beaker platform and slowly allowed to rise. The platform will stop with a marginal space left between the aperture tube and the bottom of the beaker. Finally, place at least 12 ml fresh saline in the cup and position it on the platform. The control stopcock should be open (vertical), and the digital display should read zero. Focus and align the microscope so the aperture opening appears as a clear round circle in the center of the field of vision. If any particles appear to be stuck in the aperture window, lower the platform and lightly touch the opening with the cleaning brush until an unimpeded stream of air bubbles rush into the aperture tubes. Then reposition the sample.

The next operation is to set the Model Z_B instrument knobs. The Upper Threshold knob should be turned fully counterclockwise (∞ position). Because the saline represents the blank for further bacterial readings, the 1/Amplification and 1/Aperture knobs are set at the positions necessary for reading bacteria. These are normally a 1/Aperture setting of 1/4 or 0.177 and a 1/Amplification setting of 1/4 or 1/8. The lower threshold is then determined. Lower the threshold to the zero position and slowly raise it while watching the oscilloscope. The oscilloscope should register a pattern which corresponds to the particles remaining in the saline and the electronic noise in the room. A densely colored area should be at the bottom of the pattern, this area should end rather abruptly; only occasional peaks will transcend it. Move the Lower Threshold knob until the top line of the shadow corresponds to the edge of the brightly colored background area. This is the lower threshold value to use for all counts at that particular aperture and amplification settings.

Count the blank by closing the control stopcock. This causes the mercury in the manometer to gradually move up its glass tube until it triggers the start and stop terminals attached to preset points. In the process, the mercury will induce the uptake of a prescribed amount of sample, in this case 0.05 ml. During uptake, the counter should register the background counts on the digital display. To reset the manometer and the counter, reopen the control stopcock.

It is a good idea to take blank counts at the setting which is advisable for the particular organism to be counted. If this is not known, it is advisable to do counts at various aperture and amplification settings. A change in either setting necessitates a readjustment of the lower threshold value. Blanks may

vary from day to day due to different room noise levels and different saline. Thus this procedure must be redone each day or even each half day.

Daily calibrations using particles of known size are necessary as well. For bacterial samples, 0.8 μ latex particles are used. The particles are first diluted 1 drop into 10 ml saline. This suspension is further diluted 1/100 into the final solution which is then placed in a sample disposable container (the blank container may be reused). Place this in an ultrasonic bath, filled with water, and leave it on for 2 minutes; this keeps the particles from aggregating. Align the particle sample under the aperture tube with the control stopcock remaining open. Set the 1/Amplification and 1/Aperture knobs at the appropriate setting for the bacteria to be read. If this is not known exactly, several readings with various combinations of aperture and amplification settings as previously described should be performed. The Lower Threshold knob should be set at the value determined when using the blanks for particular combinations of aperture and amplification settings. The pattern on the oscilloscope should be fairly regular. If this pattern occupies more than 50 percent of the height of the monitor, dilute the particles with more saline. Set the threshold dial at the rear of the Model P on the lower threshold value so that the machine will not attempt to size noise and small contaminants. The Model P should be on a "Stop" at Full-Scale Count Mode and a 512 Range. This means the counter will size 512 particles. The Plot Mode should be on automatic and the pen on the X-Y recorder should be in the up position (unlit).

When this has been accomplished, insert a sheet of graph paper (Coulter provides its own) onto the recorder and align the left-hand side of the sheet with the black line on the surface. The bottom of the sheet should touch evenly on the bottom edge of the plotter. Depress the pen button so that it lights up. The pen should be adjusted so that it is exactly on the zero and the baseline of the paper. If it is not, use the X and Y zero buttons until it is. Likewise, the recorder should stop after reading at 100 on both scales (the edge of the grid), and the gain knob may be used to adjust this.

To read the particle volume after all the indicators are at the appropriate settings, depress the reset button on the Model P. Then push the start button; the plotter should work automatically as soon as the accumulation light goes off. Check that the peak which is recorded on the graph paper does not go beyond the grid and that it started at zero. If not, adjust as before and reset and read again.

In addition, make sure the peak does not rise above the baseline before the appropriate lower threshold window has been reached. Repeat the "reset and start" procedure. Several readings on a particular setting are recommended. Label the peaks on the graph paper as to the aperture amplification and threshold settings. Remember to reset threshold knob at the rear of the Model P when any of the settings are changed.

To count a bacterial sample, first dilute the sample in filtered saline so that the final count will register between 20,000 and 80,000 counts--this is the most accurate range of the counter. Rinse the disposable sample container and the aperture tube with saline before reusing. Pour the bacteria into the cup and align appropriately. The control stopcock should be open, and a fresh piece of graph paper should be inserted. Determine the appropriate amplification and aperture settings by finding the ones which cause the top of the major oscilloscope pattern to come up to half the height of the monitor. Adjust the lower threshold on the Model Z_B and on the rear of the Model P to that previously determined with the blank for these amplifications and aperture settings. To count, close control stopcock. To size, press the reset and start buttons of the Model P. Do not size while counting because this may cause fluctuations in the electrical current. Make sure that the oscilloscope pattern remains the same throughout the procedure. Lower the sample and swirl occasionally to ensure proper dispersing. At least five counts are necessary for accuracy. Similarly, several sizings are appropriate.

A major problem of Coulter Counter usage is clogging of the aperture. Clogging may be detected in any of three ways. An irregularity in the oscilloscope pattern is the easiest indication. A clog may also be seen in the microscope as a small cluster of particles, usually in the lower right-hand corner of the lighted opening. Sometimes a clog will result in stoppage of the mercury flow in the manometer while counting. If a clog should occur, open control stopcock, lower beaker platform, and gently brush the aperture opening with the brush provided. A stream of bubbles should enter the tube. Then replace sample and check that the oscilloscope pattern returns to normal.

After use, the aperture tube must be refilled with the diluted Isoterge solution. The Tygon tubing must be replaced in the green solution along with the brown wire. A sample disposable container filled with Isoterge is positioned on the beaker platform under the aperture tube. The control stopcock must be open. Open the auxiliary stopcock and allow the Isoterge to flow into the tube until the green fluid passes the control stopcock, then shut the auxiliary stopcock and shut off the instrument.

The total bacteria of the original sample is calculated by using the mean value of the counts, less the blank. This value is multiplied by 20 to account for the 0.05 ml volume and by the dilution factor to determine bacteria per ml of the original sample.

Volume measurements are computed by a more complex procedure. Using the 0.8 μ latex particle graph, find the peaks of the aperture and amplification settings employed for the bacterium in question. Compute the volume per channel by measuring the average width of the peak at 50 on the ordinate. Find the window which is the exact center of the peak at 50, and designate this number as T_L . Calculate a threshold factor by the following formula:

$$T.F. = \frac{\text{Particle Volume (V)}}{T_L}$$

$$V = \frac{4}{3} \pi R^3 = .523 D^3$$

where D is the diameter of the particle. Similarly, the center of the bacterial peak is calculated. Multiplying the value of this window by the threshold factor gives the cell volume.

COULTER COUNTER DATA FORM

DATE 4/9/74 TECHNICIAN E.A. KNUST
 SPECIMEN ESCHERICHIA COLI EXPERIMENT PRELIM WITH COULTER
 DILUTION 1:1000 VOLUME MEASURED 1.0 ml
 DILUENT 3% FILT SALINE (RSS) STANDARD PARTICLE SIZE 0.8 μ
 SETTINGS 1/AMPLIFICATION 1/4 1/APERTURE CURRENT 354
 LOWER THRESHOLD 10 UPPER THRESHOLD 2
 MATCHING SWITCH 5

READINGS

SAMPLE 1 <u>SALINE BLANK</u>	SAMPLE 2 <u>E. COLI</u>
1. <u>67</u>	1. <u>73, 793</u>
2. <u>75</u>	2. <u>68, 543</u>
3. <u>66</u>	3. <u>75, 948</u>
4. <u>74</u>	4. <u>68, 188</u>
5. <u>57</u>	5. <u>71, 788</u>
\bar{x} <u>68.2</u>	\bar{x} <u>71.652</u>
σ <u>± 7.3</u>	σ <u>± 3343.7</u>
CV% <u>10.6%</u>	CV% <u>4.7%</u>
SAMPLE 3 _____	SAMPLE 4 _____
1. _____	1. _____
2. _____	2. _____
3. _____	3. _____
4. _____	4. _____
5. _____	5. _____
\bar{x} _____	\bar{x} _____
σ _____	σ _____
CV% _____	CV% _____

Figure 2-11. Sample of Recorded Data from Coulter Counter

SECTION 3

GENERAL LABORATORY MAINTENANCE

RECORD KEEPING

LOG BOOK

Work performed in the laboratory should be recorded each day in a laboratory log book. This record will be useful for inventory control and for future reference.

The log book should include records of each experiment performed and include for each experiment:

- Explicit title of the experiment.
- Date experiment performed.
- Number of mixing tubes used.
- Number of vials of luciferase used.
- Batch date or lot number of each reagent used.

EXPERIMENT REPORT

A daily record of experiments performed by an individual in the laboratory should be maintained, as well as an up-to-date index of experiments. The experiment writeups are kept in individual experiment books and should include:

- Explicit title of experiment (same as that recorded in the log book).
- Batch date or lot number of all reagents used.
- If apyrase was centrifuged previous to experiment.
- Radioactive light standard reading.
- Instrument identification and settings.
- Experiment assignment sheet.

- Hatch chart (Figure 3-1) and graphs.
- Procedure description.
- Results.
- Data tabulation and statistics.
- Conclusions.

Attempt To Lower Blanks By Substituting - 7.5.74
Salts For H₂O

Tube No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0.1N HNO ₃ 0.2ml	xx			xx			xx			xx			xx		
ATP 10 ⁻³ µg/ml in HNO ₃ 0.2ml		xx			xx			xx			xx			xx	
ATP 10 ⁻³ µg/ml in HNO ₃ 0.2ml			xx			xx			xx			xx			xx
H ₂ O 0.2ml	xx	xx	xx												
0.4M NaCl 0.2ml				xx	xx	xx									
0.2M Na ₂ SO ₄ 0.2ml							xx	xx	xx						
0.4M NaNO ₃ 0.2ml										xx	xx	xx			
0.4M Tris pH 7.15 0.2ml													xx	xx	xx
Assay 1. Luciferase 0.25M 0.01M MgSO ₄ pH 8.15			xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
2. Luciferase 0.25M 0.01M MgSO ₄ pH 8.2	xx	xx	xx												
Light units	1.03x10 ⁵	3.65x10 ⁸	3.69x10 ⁶	1.57x10 ⁴	6.24x10 ⁷	6.43x10 ⁵	2.43x10 ⁰	1.19x10 ⁸	1.14x10 ⁶	1.0x10 ⁴	3.96x10 ⁷	3.12x10 ⁷	3.95x10 ⁴	1.96x10 ⁸	2.25x10 ⁶

Figure 3-1. Sample Hatch Chart

GLASSWARE

Experience at the GSFC laboratory has shown that small amounts of ATP and measurable impurities are found in practically every type of solution assayed. Therefore, for accurate analytical work, strict precautions must be taken such as use of sterile, deionized, distilled water; chemically cleaned glassware; or clean, disposable plasticware. Care must be taken to avoid airborne contamination.

Never use glassware that has not been acid cleaned and thoroughly rinsed as ATP will cling to glass. Wash glassware only with phosphate-free soap. If glassware to be used for reagents in the luciferase assay has any phosphate (soap) residue after washing, the residue will interact with the luciferase and give a light reading.

The phosphate-free soap used in the GSFC laboratory is Detergent-1-2-2 Concentrate, distributed by the Water Soluble Products (Chicago, Illinois 60622).

GLASSWARE - HAND-WASH CLEANING

1. Soak and brush the glassware clean with phosphate-free detergent.
2. Rinse at least five times in tap water.
3. Rinse at least three times in deionized, distilled water.
4. Drain, air dry, and store covered.

GLASSWARE - DISHWASHER CLEANING

1. Prerinse glassware.
2. Use dishwasher low-sudsing, phosphate-free detergent.
3. Dishwasher must be attached to distilled, deionized water. Final rinse of laboratory glassware must be in deionized, distilled water.
4. Glassware must be completely dry before storing.
5. Store covered.

ACID CLEANING SLIGHTLY SOILED GLASSWARE

1. Fill with 1.0 N HCl.
2. Leave for at least 30 minutes.
3. Rinse glassware in tap water at least 10 times.
4. Rinse glassware in distilled, deionized water at least three times
5. Drain and air dry.
6. Store covered.

ACID CLEANING VERY SOILED OR STAINED GLASSWARE

1. Prepare dichromate cleaning solution as follows (always add acid to H_2O):

Sodium dichromate $Na_2Cr_2O_7$ - 120.0 g

Tap water - 1000.0 ml

Sulfuric acid concentrated H_2SO_4 - 1600.0 ml

-NOTE-

The mixture should have a red precipitate. Since dilution causes excessive heating, it should be prepared in pyrex glass, not in plastic. Never use the mixture or store it in disposable plasticware as it will dissolve the container. Store it sealed and air tight.

-CAUTION-

It is advisable to wear gloves and goggles when handling dichromate cleaning solution. The acid will eat through your clothes if splashed on them.

2. Clean glassware by hand or by using the dishwasher. Drain.
3. Fill glassware or washing container with dichromate cleaning solution and let glassware soak for 1 hour. If the glassware has a stubborn stain, let it soak overnight.

4. Pour the dichromate solution back carefully into its container for future use.

-NOTE-

The dichromate cleaning solution can be reused many times. The amount of oxidizable materials taken up by the dichromate solution causes it to become green; the amount of water taken up dilutes the solution. Therefore, it can be reused until its cleaning strength deteriorates as signified by a green precipitate.

5. Rinse glassware 10 times in flowing tap water, taking care not to splash it.
6. Rinse the glassware three times in distilled, deionized water or put it in the dishwasher on the last distilled, deionized water rinse cycle. Dry and store the dry glassware covered.

STERILIZATION OF MEDIA

Media is autoclaved at 121° C for 15 minutes to ensure sterilization. Do not cap tubes or flasks tightly because steam must be allowed to penetrate in order to sterilize. After removing from autoclave, media is cooled, capped tightly, and stored at 4° C. A sterilization indicator should always be included to ensure sterilization.

DECONTAMINATION

Contaminated laboratory materials are autoclaved at 130° C for 30 minutes to ensure decontamination before washing.

SECTION 4

MAINTENANCE OF BACTERIAL CULTURES

Storage of stock cultures by freezing on glass beads eliminates the routine problems of frequent subculturing, loss of viability, contamination, and possible genotypic and phenotypic variation (Nagel and Kunz, 1971). The GSFC laboratory has further developed their procedure by including a lyophilization step to increase the storage time of the organisms and to facilitate bead removal from the vial.

MATERIALS

1. Overnight culture of bacteria in mid log phase. When the wavelength, using a Bausch & Lomb Spectronic 20, is set at 540 nm, the culture should be adjusted to give a reading of 0.4.
2. Fresh, sterile, defibrinated sheep or horse blood. Sterility should be verified by supplier.
3. Boro-silicate glass beads (4 mm in diameter) - autoclave 50 in a 10-ml shell vial. They should be allowed to dry after autoclaving because some moisture is retained.

Uniform, viable bacterial cultures can be maintained for subsequent experimentation by coating the surface of glass beads with the organisms. The beads can be stored frozen and then lyophilized, or frozen and stored at -20° C.

PROCEDURE

1. To each vial of beads add equal volumes of broth culture of bacteria and sheep or horse blood. Enough should be added to cover the beads.
2. Mix gently and remove as much liquid as possible with a sterile bulbed Pasteur pipette. Care should be taken to ensure against contamination.
3. Shell freeze the vials with liquid nitrogen while rotating vials.
4. The vials can then be stored at -20° C or lyophilized for longer storage and easier handling when inoculating.
5. The sterility and viability of the beads are tested by removing a bead with sterile forceps and adding to 5.0 ml tripticase-soy broth.

6. The beads, if not lyophilized, should not be allowed to defrost during removal.
7. Growth (turbidity) should be observed within 48 hours.

Glass beads were prepared in this way for several urinary pathogens. For each organism, four growth curves were made by inoculating one glass bead into 5.0 ml trypticase-soy broth. The cultures were grown at 37°C and were not shaken. Figure 4-1 shows the average curve obtained from Proteus mirabilis, \pm one standard deviation for each point. The growth was measured with a Bausch & Lomb Spectronic 20.

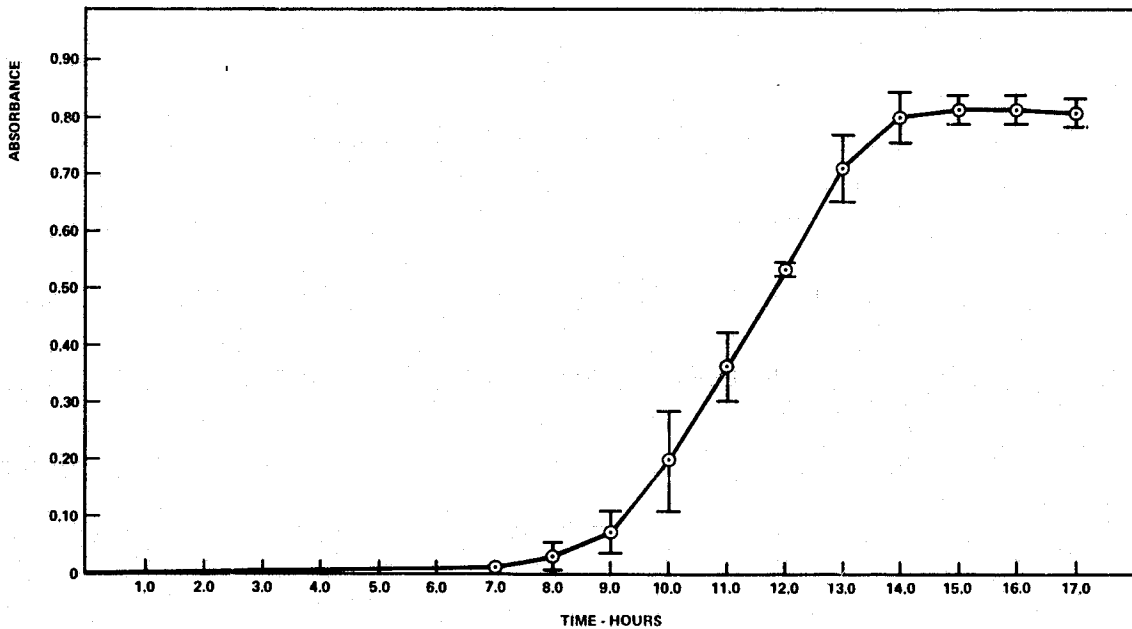


Figure 4-1. Growth of Proteus mirabilis As Measured by Bausch and Lomb Spectronic 20 from Glass Beads, $\pm 1\sigma$

SECTION 5

MEASURING BACTERIA

MICROSCOPIC PROCEDURE

The microscopic procedure for staining and counting total bacteria was developed to enhance the ability to distinguish bacteria from debris when a count of total bacteria is desired. Because adding stain to bacterial cells does not stop them from dividing and does cause clumping, the stain is added just before the counting time, so that little problem with clumping occurs.

The use of zephyrin* to immobilize cells for easier counts will rupture up to 80 percent of the cells, therefore it is not used.

To enhance accuracy, the microscopic counts should be repeated two times by refilling the counting chamber. A minimum of 100 bacteria per count is necessary for reproducibility. Because of the large dilution factor of 10^6 , bacterial counts of 10^8 per ml are necessary for good reproducibility of this system. If it is necessary to count samples with small numbers of bacteria, keep counting squares until at least 100 bacteria are counted and adjust the calculations.

REAGENT

Methylene Blue Stain (Procedure from Bailey and Scott, Diagnostic Microbiology, 1966, p. 322)

Combine:

- 0.3 gm methylene blue
- +30 ml 95% ethyl alcohol

Mix to dissolve.

Add 100 ml distilled H₂O

Filter this solution prior to use.

*Zephyrin solution - a tincture, Benzalkonium Chloride Solution 17% USP U.S.P.H.S. S.S.C. Perry Point, Md. The zephyrin solution is diluted 1:10 in the bacterial solution.

PROCEDURE

Mix the bacterial solution in a ratio of 5:6 with the methylene blue stain. If the bacteria are too numerous to count when this is done, dilute it 1:10 with filtered saline prior to adding the stain.

0.5 ml bacterial solution-diluted if necessary

+0.1 ml methylene blue stain

Vortex well and sit 5 minutes.

-NOTE-

The cells cannot be stored in this state as the stain greatly enhances clumping.

Fill the Petroff-Hausser counting chamber (by Pasteur pipette, syringe, etc.), being careful not to over or under fill, and place in a moist atmosphere (covered petri dish containing moist filter paper) for 5 minutes to allow the cells to settle. Place the chamber, after this period, on the microscope stage and allow it to settle for another 2 or more minutes with the light turned off to decrease any drying effect.

Count 20 of the smallest squares as marked in Figure 5-1. Try to do so quickly as the solution evaporates rapidly. Record numbers on the Microscopic Counting Chamber Data Form. (See Figure 5-2.) Immersion oil may be used if necessary.

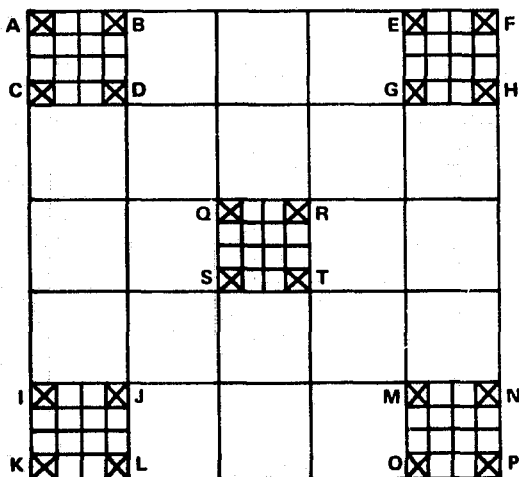


Figure 5-1. Microscopic Counting Chamber

DATA FORM FOR PETROFF-HAUSSER
MICROSCOPIC COUNTING CHAMBER

Experiment Title and Number: _____

Organism: _____

Conditions: _____

Date: _____ Technician _____

Dilution Factor From Original Sample _____

CHAMBER I

CHAMBER II

A _____ K _____

B _____ L _____

C _____ M _____

D _____ N _____

E _____ O _____

F _____ P _____

G _____ Q _____

H _____ R _____

I _____ S _____

J _____ T _____

Total _____ % _____ CV _____ Total _____ % _____ CV _____

Total of Duplicates _____ = (mean) _____

(Mean) _____ $\times 1.2 \times 10^6 \times$ (Dilution factor from original sample) _____

= _____ Bacteria per ml of original sample

Figure 5-2. Data form for Petroff-Hausser Microscopic Counting Chamber

In Figure 5-1, the four outer squares (A through P) of the five designated larger squares are used because they will indicate if there is poor distribution of cells over the whole chamber. If the total number of cells within each of the 20 labeled squares is not approximately the same — 5 per square is the optimal number — poor distribution is indicated and the chamber should be refilled.

Calculations

$$\frac{\text{No. Bacteria}}{\text{No. Squares}} \times \text{Chamber Factor} \times \frac{6}{5} \text{ (Stain Ratio)} \times \text{Dilution Factor} = \text{Bacteria/ml of Stock Solution}$$

where:

No. Bacteria = The total number bacteria counted in all squares

No. Squares = Number of squares counted that the number of bacteria represents (in Figure 5-1 it will be 20)

Chamber factor = 2×10^7 , taking into account the volume of the solution under the cover slip

Dilution factor = Any dilution done to the stock bacteria to facilitate counting prior to the addition of the methylene blue stain

$$\text{Therefore: No. Bacteria/ml of Stock Solution} = \frac{\text{No. of Bacteria}}{20} \times 2 \times 10^7 \times \frac{6}{5} \times \text{Dilution factor}$$

Or:

$$\text{No. Bacteria/ml of Stock Solution} = \frac{\text{No. of Bacteria in 20 small squares}}{1} \times 1.2 \times 10^6 \times \text{Dilution factor}$$

CLEANING OF THE PETROFF-HAUSSER COUNTING CHAMBER

After each microscopic count, clean the counting chamber immediately to avoid allowing the cells to dry. If it is inconvenient to do the cleaning immediately, the chamber can be kept temporarily in the moist atmosphere of the petri dish.

Flood the chamber with distilled water, lift off the cover slip, and gently wipe both sides with a clean lint-free wipe. Tilt the chamber and continue to gently wash with water for a moment longer, then dry with a tissue. Care should be taken in handling, because cover slips are very fragile and easily broken.

Repeat the process again but use 70 percent ethyl or methyl alcohol.

When the counting is finished for the day, gently wash each part of the counting chamber with soapy water (Liqui-Nox or comparable glass washing soap and distilled or continental water), dry, and store wrapped in a lint free wipe.

If soaking the chamber is necessary to clean it, it may be done in a light soapy water or alcohol-water solution. DO NOT allow it to stay submerged for longer than 1 hour. The chamber itself can remain longer but the construction of the cover slip is in two pieces and will start to separate if soaked longer.

AGAR COLONY COUNTING

SPREAD PLATE PROCEDURE FOR AGAR COLONY COUNTS

Procedure

Plating media should be at room temperature, and it is sometimes necessary to shake the excess moisture from the top of the plate. This moisture is usually the cause of contamination. The plates may be dried at 37°C before use to eliminate excess moisture.

The serial dilutions should be in saline, urine, or broth but never in water as this may rupture the bacterial membrane or cause ATP leakage or change in intracellular ATP. If dilutions were made in urine or broth for the experiment these can be used, but do not dilute with these if more dilutions are necessary than used because urine and broth (also other nutrients) will enhance cell division. If this must be done, plate immediately.

Label the plate according to final counts and include calculations on the plate for the 0.1 ml sample. Then with a sterile pipette, pipette 0.1 ml onto agar and spread with a sterilized right angle glass rod immediately.

-NOTE-

It is suggested that, for accuracy, duplicate plates be made on each dilution used. Usually with an overnight culture, the dilutions of 10^{-5} , 10^{-6} , and 10^{-7} are used. This will give a final bacterial count of 10^6 , 10^7 , and 10^8 , since 0.1 ml of the dilutions were used.

Counting Bacteria

In counting the colonies, it is best to count the plate or plates which have 10 to 100 colonies of bacteria on them. It should also be observed whether the bacterial numbers are linear from dilution to dilution. Visually check the

purity of the cultures. Many contaminants can be visually spotted according to morphology of the colonies.

Calculations

In calculating the number of cells on the plate use:

$$\text{No. of colonies on plate} \times \frac{1}{\text{dilution factor}} = \text{Bacteria per ml}$$

Example: 20 colonies on the 10^{-7} plate, made from the 10^{-6} dilution; that is in actuality 2.0×10^8 bacteria per ml.

$$20 \times \frac{1}{10^{-7}} = 2.0 \times 10^8 \text{ bacteria in 1 ml of the original culture.}$$

DROP PLATE PROCEDURE FOR AGAR COLONY COUNTS

The drop plate represents improvements over the spread plate method by allowing the counting of larger numbers of colonies while using fewer media and plates, thus improving accuracy and reproducibility.

The drop plate count is done using a Cooke Engineering Co. microtiter pipette dropper which delivers 0.025 ml in each drop or 1/40 of a ml. Blood agar or trypticase-soy agar plates are used for counting bacteria. To be able to count the optimal number of bacteria, it is necessary to prepare at least four dilutions of the stock desired for counting. The optimal number of colonies per drop is between 20 and 60 colonies. If there are 30 colonies per drop, representing 30 bacteria per drop, there are 1.2×10^3 bacteria/ml.

$$\begin{aligned} 30 \text{ colonies per drop} \times 40 \text{ drops/ml} &= 1,200 \text{ bacteria/ml} \\ &= 1.2 \times 10^3 \text{ bacteria/ml} \end{aligned}$$

If the probable count in the stock can be estimated, such as 1.5×10^8 , then it can be estimated that the dilutions of 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} should be drop plate counted to ensure plating of the optimal dilution for counting.

If there is a total count from the Coulter Counter or a microscopic chamber count, then those dilutions which should be used can be accurately figured. The optimal number of bacteria per milliliter for an accurate plate count is between 8×10^2 bacteria per ml or 20 colonies per drop and 2.4×10^3 bacteria per ml or 60 colonies per drop.

If the total number of bacteria per ml equals 1.2×10^8 according to a Coulter count or a microscopic count, to obtain an accurate plate count, dilute this sample using 10 fold dilutions to 1.2×10^3 and plate. Also, plate the dilutions above and below the estimate to safeguard results. Always make duplicate plates.

Procedure

The technique for using the microtiter pipette is:

1. Fill pipette without air bubbles.
2. Wipe the tip clean.
3. Hold the pipette perpendicular to the plate. A steady hand is necessary.
4. Carefully drop the liquid, a single drop at a time, maintaining equal distance from the plate. Avoid dropping from a distance that would cause splashing.
5. Allow five drops per plate. (This is necessary for each dilution to check accuracy and reproducibility.)
6. Agar should be dry enough to absorb most of the drops in an hour. The cover of the plate should not contain large drops of condensation that will fall on drops and ruin counts by spreading the bacteria. The plates can be predried at 37°C to minimize splashing and eliminate condensation.
7. Do not move plates until drops have dried. Incubate at 37°C , do not invert plates, and read at earliest growth. (Most coliform bacteria are incubated at room temperature overnight to keep from becoming overgrown. Streptococcus faecalis and Pseudomonas are incubated at 37°C .)

The plates should be counted as soon as growth is adequate for visual counting and rechecked later on for slow growing colonies. Each of the five drops for each dilution should be counted and averaged and a coefficient of variation calculated. The counts are recorded on the Crop Plate Count Data Sheet (Figure 5-3). After an average number is obtained for the dilution having between 20 and 60 colonies, it is necessary to calculate the amount of bacteria per milliliter of stock.

Calculation

$$\text{Average No. of colonies} \times 40 \times \text{Dilution factor} = \text{Bacteria per ml of original sample}$$

If the bacteria to count are diluted from the original sample, then that dilution factor must also be taken into consideration.

$$\text{Average No. of colonies} \times 40 \times \text{Dilution factor} \times \text{Dilution factor from original sample} = \text{No. bacteria per ml of original sample}$$

The coefficient of variation should be approximately 13 percent. This is good accuracy and an even lower coefficient can be obtained with patience and practice.

DROP PLATE COUNT DATA FORM

Date: _____ Tech: _____
 Experiment: _____
 Plating Media: _____ Diluent: _____
 Dilution factor from Original Sample: _____

I Organism _____	I Duplicate _____
Dilution _____	Dilution _____
1. _____	1. _____
2. _____	2. _____
3. _____	3. _____
4. _____	4. _____
5. _____	5. _____
Coefficient of Variation: _____ %	Coefficient of Variation: _____ %
Mean: _____	Mean: _____
[_____ × 40 × _____ = _____ bact/ml]	[_____ × 40 × _____ = _____ bact/ml]

II Organism _____	II Duplicate _____
Dilution _____	Dilution _____
1. _____	1. _____
2. _____	2. _____
3. _____	3. _____
4. _____	4. _____
5. _____	5. _____
Coefficient of Variation: _____ %	Coefficient of Variation: _____ %
Mean: _____	Mean: _____
[_____ × 40 × _____ = _____ bact/ml]	[_____ × 40 × _____ = _____ bact/ml]

Figure 5-3. Drop Plate Data Sheet

SECTION 6

REAGENTS

The reagents and materials discussed in this section are those in use at GSFC. Appendix B provides a list of the manufacturers of those materials and reagents included here. When making reagents, reagent grade chemicals of the highest purity available must always be used. Always use distilled, deionized water which is kept refrigerated, and use it only for a 24-hour period. Open bottled water as few times as possible. It is advisable to use disposable plasticware for all situations. Never use glassware that has not been acid cleaned (soaked in 1.0 N HCl for 30 minutes) to remove residual ATP. (See Section 3.)

The reagents should be dispensed as soon as possible and stored in plastic disposable vessels. All reagents are stored frozen (-20°C) in a labeled freezer box. Avoid the use of potassium salts whenever they would come in contact with ATP or luciferase because potassium salts seem to be more inhibitory to luciferase than sodium salts; therefore, pH should be adjusted with HCl and NaOH. Measure volumes exactly with a volumetric flask. (See Appendix C for Dilutions, Exponential Equivalents, and Exponential Dilution Expression.) Every tube must be labeled with contents and date. Prepare a reagent work sheet (Figure 6-1) for each reagent batch.

Reagent Worksheet

Date: _____ Name of Reagent: _____
Technician: _____ Total Volume: _____

Stock Identification: _____

Formula Weight: _____
Diluent Identification: _____

Amount of Stock: _____
Amount of Diluent: _____
Total Volume: _____

Volume of each aliquot: _____
Number of aliquots: _____

Remember: Set aside 2 tubes of aliquots for Quality Control.
Comments:

Figure 6-1. Reagent Worksheet

TRITON X-100

Frequently, a specimen to be analyzed for bacteria contains contaminating non-bacterial cells such as mammalian cells, that is, red blood cells, white blood cells, or epithelial cells. To obtain true bacterial values, these other sources of ATP must be removed prior to bacteria rupture. The nonbacterial ATP is released by Triton X-100(TX), a nonionic detergent, making the ATP accessible to hydrolysis by potato apyrase.

MATERIALS

Triton X-100 (Octyl Phenoxy Polyethoxyethanol)

Sigma Chemical Company

Triton is a trademark of the Rohm and Haas Company

Store at room temperature

DIRECTIONS TO PREPARE 100 ml of 6-PERCENT TX-100 SOLUTION

1. Fill pipette with 6.0 ml TX, exactly to the desired amount, do not overfill (use a pipette filler). Wipe off outside of pipette.
2. Rinse pipette in 94 ml warm H₂O until all TX is dissolved. Swirl gently to mix.
3. Heat TX solution to boiling. It will become cloudy, but will clarify on cooling.
4. Dispense in 5.0 ml amounts.

DIRECTIONS TO PREPARE 100.0 ml OF 10-PERCENT TX-100 SOLUTION

1. Heat 90.0 ml H₂O as above.
2. Add 10.0 ml TX-100 as above.

-CAUTION-

Care should be taken not to swallow any TX-100, as ingestion of TX in rats has been shown to cause subcapsular cysts on the ovaries. If TX should come in contact with skin, it should be washed immediately with volumes of tap water.

APYRASE

Soluble ATP, which contaminates a sample to be assayed for bacterial ATP, can be removed by the addition of apyrase, an ATP hydrolyzing enzyme.

The pH for optimum apyrase activity is 6.0, with the lowest pH for activity being 3.0. The apyrase must be inactivated before extracting the bacterial ATP to be assayed. This is done by lowering the pH, with the extractant nitric acid, to below pH 3.0. The apyrase can be reactivated if the pH is raised again, so care must be taken with the samples containing extracted bacterial or yeast ATP to keep the pH below 3.0.

Apyrase is very stable and extremely active. It contains varying amounts of ATPase, ADPase, and AMPase activities. Because there can be phosphorylating enzymes associated with the luciferase enzyme preparation, the presence of ADP and AMP can also interfere with a quantitation of the amount of ATP when using the luciferase light production reaction. Therefore, each lot number of apyrase should be assayed for its ability to produce a low blank with a high concentration of ATP after a constant time. The concentration of the new lot should be adjusted to give a constant activity, for example, removal of 100 μ g ATP in 15 minutes to two times the blank level.

When using concentration procedures with different lots of apyrase, there are varying amounts of particulates that may clog a filter or interfere as a pellet in the centrifuge tube with extraction and mixing. Therefore, the apyrase, when used in the concentration procedures, is extracted in 0.03 M CaCl_2 , centrifuged or filtered, and lyophilized for storage to be reconstituted to the desired activity upon use.

MATERIALS

Apyrase: 5'ATPase-2.5 units per mg
5'ADPase-0.46 units per mg
5'AMPase-0.03 units per mg

Potato purified-grade 1
Sigma Chemical Company
Store dry below 0°C
2,000 units per vial based on 5' -ATPase activity

DIRECTIONS TO PREPARE 100 ml OF 10 mg APYRASE/ml, 0.03 M CaCl_2

1. 1 g apyrase in 100 ml of 0.03 M CaCl_2
2. Mix gently.

DIRECTIONS TO PREPARE 100 ml OF 40 mg APYRASE/ml, 0.03 M CaCl_2

1. 4 g apyrase in 100 ml of 0.03 CaCl_2
2. Mix gently.

If the apyrase is too cloudy it may be necessary to clarify by centrifuging at 10,000 rpm for 5 minutes.

Discard the precipitate and dispense the supernatant.

PROCEDURE FOR LYOPHILIZATION OF APYRASE

1. When using the Vir Tis Freeze-Dryer, prepare 120 ml 10 mg/ml apyrase, 0.03 M CaCl_2 .
 - Add 1.2 g apyrase to 120.0 ml 0.03 M CaCl_2
 - Stir gently for 10 minutes at 4°C.
2. Filter progressively through millipore Cellulose esters filters of 10 μ , 0.45 μ , and 0.22 μ pore size. Do not use prefilter pads.
3. Pipette 3 ml into 10-ml acid-cleaned shell vials. Stopper and shell freeze with liquid nitrogen. Vials may be stored at -20°C for several weeks or lyophilized immediately. Before lyophilizing, partially open stoppers to allow the moisture to escape and do not allow vials to melt.
4. Prior to placing vials in or on the lyophilizer:
 - Drain liquid from condenser through drain tube in rear.
 - Refrigerate condenser temperature to -50°C.
 - If using only the external attachment points, allow the vacuum to come to 0.2 μ before attaching the vials. However, if the compressor engine is running smoothly the vacuum is usually sufficient.

- If using both the inside and outside attachment points, allow the condenser temperature to lower, place the frozen shell vials inside with partially opened stoppers, replace the lyophilizer top and start the vacuum. Allow the vacuum to reach 0.2μ before using the external attachment points.
5. Place frozen shell vials on external attachment points and turn opening valves slowly to down position. Allow the vacuum to gently pull the vials up. DO NOT FORCE.

If the vials begin to thaw out before they are dry, it is probably due to the stopper being pushed too far, closing it to the vacuum. To correct this:

- close valve
- remove vial and refreeze
- replace as before

From time to time check:

- condenser temperature
- vacuum pressure
- thawing of shell vials

The completion of lyophilization depends on the original amount of liquid and whether or not attachment points on the inside, outside, or both sides of the lyophilizer are used. If the outside attachment points are used, of which there are 39, it takes approximately 4-1/2 hours to complete lyophilization. When the inside is used, approximately 15 hours are required; time to completion increases with the number of vials. When both sides are used, completion time depends on the number of vials attached, however, the outside vials will completely dry more rapidly than those on the inside. Vials are completely dry when they have reached room temperature.

Label and store vials dry and as cold as possible. Shelf life at -20°C is probably several years. To use, reconstitute with 3 ml distilled water or 3.0 ml 0.6 percent TX-100 as required by the procedure used.

CALCIUM CHLORIDE

The soluble, contaminating, nonbacterial ATP in a sample can be removed by adding apyrase, (described in previous section) an ATP hydrolyzing enzyme. Apyrase requires calcium as a cofactor at $5 \times 10^{-3}M$. Within the concentration range of the apyrase used, the calcium concentration must remain $5 \times 10^{-3}M$.

MATERIALS

Calcium Chloride Anhydrous

Analytical reagent
Mallinckrodt Laboratory Chemicals
Formula weight 110.99
Store at room temperature

DIRECTIONS TO PREPARE 100 ml OF 3 M $CaCl_2$ (to be used as a stock for preparation of 0.03 M $CaCl_2$)

1. Bring 33.297 g $CaCl_2$ to 100.0 ml with H_2O .
2. Mix to dissolve.
3. Dispense 2 ml per tube.

DIRECTIONS TO PREPARE 100 ml OF 0.03 M $CaCl_2$

1. Bring 1 ml stock 3 M $CaCl_2$ to 100 ml with H_2O .
2. Mix to dissolve.
3. Dispense 10 ml per tube.

DIRECTIONS TO PREPARE 100 ml OF 0.03 M $CaCl_2$, 0.6-PERCENT TX-100 ml

1. Combine: ● 1 ml stock 3 M $CaCl_2$ ● 10 ml 6-PERCENT Triton X-100
2. Bring to 100 ml with H_2O .
3. Mix to dissolve.
4. Dispense 2 ml per tube.

MALATE BUFFER

Contaminating ATP in a sample to be assayed for bacterial ATP can be bound to surfaces and large molecules such as membrane particles and proteins and can thus be protected from the action of the apyrase.

By dropping the pH of the sample, this bound ATP is released and becomes accessible to the apyrase while the apyrase is still marginally active. Malate buffer at a low pH elutes this bound ATP.

MATERIALS

d-1 Malic acid

Sigma Chemical Company
Molecular weight 134.1
Store at room temperature

DIRECTIONS TO PREPARE 100 ml OF 0.5 M MALATE BUFFER pH 4.25

1. Combine:
 - 6.705 g malic acid
 - 80.0 ml H₂O
2. Mix to dissolve.
3. Adjust pH to 4.25, qs to 100 ml. (qs is used to mean "bring to a final volume of")
4. Dispense 2 ml per tube.

DIRECTIONS TO PREPARE 100 ml OF 0.25 M MALATE BUFFER pH 3.75

1. Combine:
 - o 3.353 g malic acid
 - 80.0 ml H₂O
2. Mix to dissolve
3. Adjust pH to 3.75, qs to 100 ml.
4. Dispense 2 ml per tube

NITRIC ACID (Extractant)

Nitric acid inactivates the apyrase and ruptures the bacterial cells, releasing bacterial ATP. The optimal nitric acid concentration is the lowest necessary for complete ATP extraction. Of the 10 bacterial urinary pathogens tested, 0.1 N HNO_3 was found to be the optimal nitric acid concentration.

MATERIALS

Acid Nitric, Reagent ACS

Fisher Scientific Company
16 Normal, 71 percent by weight
Store at room temperature

DIRECTIONS TO PREPARE 100 ml OF 1.5 N HNO_3

1. Bring 9.37 ml concentrated HNO_3 to 100 ml with H_2O .
2. Dispense in 2.0 ml amounts.

DIRECTIONS TO PREPARE 100 ml OF 1.0 N HNO_3

1. Bring 6.25 ml concentrated HNO_3 to 100 ml with H_2O .
2. Dispense in 1.0 ml amounts.

-NOTE-

1.0 N HNO_3 is usually used at an extracting concentration of 0.1 N and is prepared by diluting 1.0 N HNO_3 stock 1:10 in H_2O .

METHYLENE CHLORIDE (Extractant)

The organic solvent methylene chloride, using the present procedure, does not extract as well as does acetone, may dissolve certain plastics, and the vapor is extremely toxic. It is therefore not the organic solvent of choice. Methylene chloride does, however, have some properties that could make it desirable.

It has a low boiling point, 39.8°C, which may be an advantage in reducing ATP decay that could occur at higher temperatures. In the concentration procedure, using filtration, the methylene chloride may dissolve certain filter membranes better than acetone.

The GSFC laboratory has discontinued further optimization of this extractant. With additional experimentation, the extracting efficiency of methylene chloride may be improved.

MATERIALS

Methylene chloride certified ACS

Also called Dichloromethane CH_2Cl_2
Fisher Chemical Company
Store at room temperature

DIRECTIONS TO PREPARE 100 ml OF 90-PERCENT METHYLENE CHLORIDE

Combine

- 90.0 ml methylene chloride
- 10.0 ml H_2O

-NOTE-

Methylene chloride is insoluble in H_2O and should be vortexed frequently during use. The reagent is not stored, but prepared just prior to use. Because of its high volatility at room temperature, methylene chloride is extremely difficult to pipette or measure accurately. In use with a nonconcentrated procedure, the 10 percent water needed can be supplied by the sample itself.

ACETONE (Extractant)

The organic solvent acetone is used as an extractant in a concentration procedure. Since the solvent is completely removed by heating, the ATP pellet can be resuspended in water for assay. When HNO_3 is used as an extractant, the molarity of the TRIS used for rehydration of the luciferase must be high enough to be able to maintain the optimal light production pH 7.75, in the presence of the nitric acid. Using an organic solvent as an extractant, the molarity of the TRIS can be much lower, since the ATP to be assayed is resuspended in only H_2O . Higher activity is achieved with lower molarity TRIS.

Using this GSFC procedure, acetone is the preferred organic extractant. It is less toxic than methylene chloride and recovers more ATP from the urinary pathogens and yeast tested under the conditions used.

MATERIALS

Acetone, Certified ACS

Fisher Scientific Company
Store at room temperature

DIRECTIONS TO PREPARE 100 ML OF 90-PERCENT ACETONE

Combine:

- 90 ml acetone
- 10 ml H_2O

-NOTE-

This reagent is not stored, but is always prepared just prior to use. Because acetone is very flammable it should not be in use when there are any open flames or burning cigarettes present. In nonconcentration procedures, the 10 percent H_2O can be supplied by the sample.

ADENOSINE TRIPHOSPHATE (ATP)

Measurable impurities or small amounts of ATP are found in practically every type of solution assayed. Therefore, perfectly cleaned glassware or preferably disposable plasticware must be used. (See section on Glassware.) The chemical structure of ATP is shown in Figure 5-2.

MATERIALS

Adenosine 5' Triphosphate from Equine Muscle Disodium Salt, crystalline form

Sigma Chemical Company

Each preweighed vial contains 30 mg ATP, which is 99 percent pure and contains 3.5 H₂O per mole (the amount of H₂O of hydration is variable) when assayed.

Store at -20°C

CALCULATIONS

Calculations for vials of Sigma ATP containing 30 mg of the disodium salt with 3.5 H₂O of hydration per vial to obtain 1000 µg ATP/ml are:

Molecular Weight

$$\text{ATP} = 507.2$$

$$+2 \text{ Na} = 551.2$$

$$+3.5 \text{ H}_2\text{O} = 614.2$$

This is 99 percent pure, therefore

$$\frac{507.2}{614.2} \times 0.99 \times 30 \text{ mg} = 24.530 \text{ mg ATP/vial} = 24,530 \text{ µg ATP/vial}$$

$$24,530 \text{ µg ATP} + 24.53 \text{ ml H}_2\text{O} = 1000 \text{ µg ATP per ml (contents of one vial)}$$

DIRECTIONS TO PREPARE STOCK ATP - 1000 μ g ATP PER ml

1. Dissolve 30 mg (one vial) Sigma ATP in 24.0 ml water. (All traces of ATP are rinsed out of the vial.)
2. Adjust to pH 7.0. (When using Sigma disodium ATP this adjustment is not necessary.)
3. Bring volume to 24.530 ml.
4. Dispense into 1.0 ml aliquots per sterile plastic tube and freeze.

-NOTE-

ATP hydrolyzes on standing and should therefore be thawed no more than 30 minutes prior to use. The more dilute the solution, the greater the loss.

PREPARATIONS OF DILUTIONS

Serial dilutions can then be made to obtain desired concentration of ATP.

1. 1 ml frozen stock = 1000 μ g ATP + 9 ml H₂O = 100 μ g ATP/ml
2. 1 ml 100 μ g ATP/ml + 9 ml H₂O = 10 μ g ATP/ml
3. 1 ml 10 μ g ATP/ml + 9 ml H₂O = 1 μ g ATP/ml
4. 1 ml 1 μ g ATP/ml + 9 ml H₂O = 10⁻¹ μ g ATP/ml
5. 1 ml 10⁻¹ μ g ATP/ml + 9 ml H₂O = 10⁻² μ g ATP/ml
6. 1 ml 10⁻² μ g ATP/ml + 9 ml H₂O = 10⁻³ μ g ATP/ml
7. 1 ml 10⁻³ μ g ATP/ml + 9 ml H₂O = 10⁻⁴ μ g ATP/ml

The chemical structure of ATP is shown in Figure 6-2.

When preparing ATP, each new batch should be tested for activity against a previous batch by assaying the old and new batches under identical conditions.

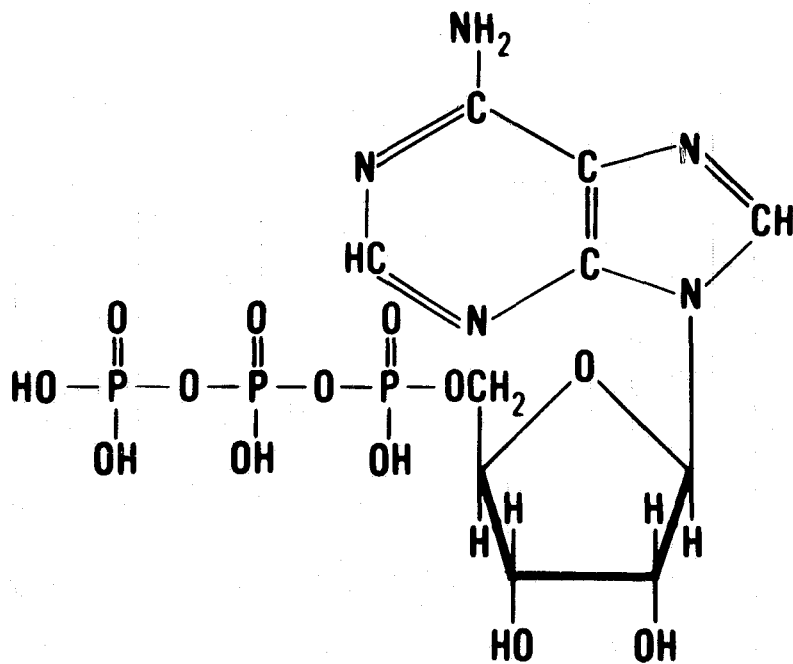


Figure 6-2. Chemical Structure of Adenosine Triphosphate

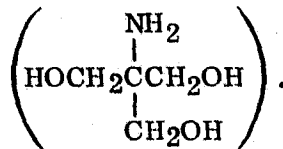
TRIS

The lyophilized luciferase powder is rehydrated in TRIS buffer, containing magnesium, a cofactor in the reaction. One vial of powder is rehydrated in 1.5 ml TRIS-Mg buffer. The pH and concentration of the TRIS is critical.

For optimum light response, the final pH after injection of the sample is 7.75. If the sample contains HNO₃, the pH of the TRIS buffer must be adjusted to compensate for it. The concentration of the TRIS to be used depends upon the relationship between the amount of HNO₃ in the sample and the pH of the TRIS buffer. The lowest concentration of TRIS that will hold the pH effectively at 7.75 is used.

MATERIALS

Trizma Base TRIS (hydroxymethyl) amino methane
Reagent grade.



Sigma Chemical Company
Molecular weight 121.1
Store at room temperature

DIRECTIONS TO PREPARE 100 ml of 2 M TRIS

This concentrated TRIS is used as stock for making other concentrations of TRIS

1. Combine:
 - 24.2 g Trizma Base
 - 80.0 ml H₂O
2. Mix until dissolved. (This may take several hours.)
Do not use heat.
3. Bring dissolved solution to 100.0 ml using a volumetric flask.

-NOTE-

The pH of the 2 M TRIS is not adjusted since it will not maintain a constant pH at this high concentration.

4. Filter through a 0.20 μ millipore filter.
5. Dispense 5 ml per tube.

Diluted TRIS

Various concentrations of the TRIS buffer are used for the different procedures performed at GSFC. The amount of 2 M TRIS for each molarity is:

TRIS Final Molarity	Number ml of 2 M TRIS to give 100 ml	Number ml of Water
0.05	2.5	97.5
0.1	5.0	95.0
0.15	7.5	92.5
0.2	10.0	90.0
0.25	12.5	87.5
0.3	15.0	85.0
0.35	17.5	82.5

If TRIS is to be used to reconstitute luciferase, 0.01 M MgSO₄ is required.

Decrease volume of water by 1.0 ml for every 100 ml to be made and

Add 1.0 ml of 1.0 M stock MgSO₄ per 100 ml of final volume TRIS

The pH of the TRIS, which is dictated by the procedure used, is adjusted with NaOH or HCl before bringing up to final volume.

When using concentration procedures, with 0.005 milliequivalents of nitric acid to be injected (0.2 ml of 0.1 N HNO₃, 1:1 with Na₂SO₄ added to the pellet) see Figure 6-3 for the pH of the TRIS-Mg buffer for luciferase hydration necessary to obtain the optimal light producing pH of 7.75 in the sample-luciferase mixture.

Initial pH of TRIS

0.35 Molar TRIS	8.00
0.30 Molar TRIS	8.10
0.25 Molar TRIS	8.20
0.20 Molar TRIS	8.31

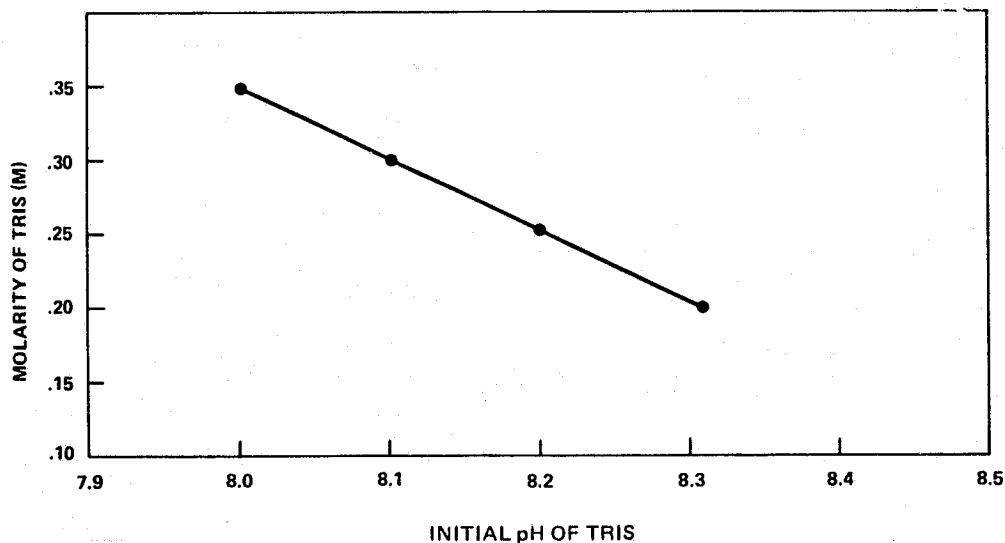


Figure 6-3. Initial pH of TRIS required (for the rehydration of the luciferase) at each molarity of TRIS used to obtain a final pH of 7.75 in the light emitting mixture of HNO_3 extracted sample and buffered luciferase (long centrifuge procedure).

SODIUM SULFATE

Sodium sulfate has recently been substituted for water as the diluent in the centrifuge and nonconcentrated procedures. It has been determined that the sodium sulfate lowers the blank reading by approximately 70 percent, while giving only about a 50 percent loss in activity.

To determine the molarity of the salt to use, the ionic strength of the material to be injected into the luciferase should be equal or higher than the ionic strength of the TRIS-Mg buffer in which the luciferase is rehydrated.

To determine ionic strength

$$\mu = 1/2 (M_1 Z_1^2 + M_2 Z_2^2 + M_3 Z_3^2 + \text{etc})$$

where μ = ionic strength

M = molality of individual ions

Z = valence

MATERIALS

Sodium Sulfate - Certified ACS Anhydrous Na_2SO_4

Fisher Scientific Company

Formula weight - 142.04

Store at room temperature

DIRECTIONS TO PREPARE 100.0 ml OF 0.085 M Na_2SO_4

1. 1.207 g Na_2SO_4
2. Qs (qs is used here to mean "bring to a final volume of") to 100.0 ml with H_2O .
3. Dispense 2.0 ml per tube.

DIRECTIONS TO PREPARE 100.0 ml OF 0.15 M Na_2SO_4

1. 2.103 g Na_2SO_4
2. Qs to 100.0 ml with H_2O .
3. Dispense 2.0 ml per tube.

MAGNESIUM SULFATE

Magnesium is a cofactor required in the luciferase reaction at a concentration of 10^{-2} M. This is added to TRIS buffer to reach a final concentration of 0.01 M MgSO_4 (1.0 ml stock 1 M MgSO_4 plus 99-ml TRIS buffer).

MATERIALS

Magnesium Sulfate Crystal: MgSO_4

Certified ASC Fisher Chemical Company

Formula weight 246.48

Store at room temperature

DIRECTIONS TO PREPARE 100 ml OF 1 M MgSO_4

1. Bring 24.65 g MgSO_4 to 100.0 ml with H_2O .
2. Dispense 1 ml per tube.

FIREFLY LUCIFERASE

The purified mixture of luciferase and luciferin that is used at GSFC is obtained in the lyophilized state from DuPont. Twenty vials of enzyme-substrate powder and one vial of twenty buffer-salt tablets are included in each reagent kit. One vial of enzyme substrate powder contains 0.71 millimoles crystalline luciferin and ≥ 100 units luciferase, where

$$\text{one unit} = \frac{\text{Response to } 1.65 \mu\text{M ATP}}{\text{Response to } 20\mu \text{ Curies } \text{C}^{14} \text{ calibrated light source}} \\ \text{(with an activity of } 10^{-13} \text{ grams ATP/10 } \mu\text{L injection)}$$

The buffer-salt tablets in the DuPont Reagent Kit contain morpholinopropane sulfonic acid (MOPS) 0.01 M at pH 7.4 at 25°C and magnesium sulfate, 0.01 M when rehydrated to 3.0 ml. MOPS is not routinely used in the GSFC laboratory.

Each Reagent Kit from DuPont also contains the reagents and cuvettes (6 50 mm) required for a maximum of 300 ATP assays. This kit is stored at -20°C. It may be stored at higher temperatures if sealed air tight and kept in the dark.

The DuPont manual recommends rehydration of one vial of luciferase in 3.0-ml MOPS buffer, giving a cost per assay of \$0.34. To obtain the sensitivity described in the GSFC procedures, the luciferase is rehydrated in 1/2 this volume, or 1.5-ml TRIS-Mg buffer per vial of luciferase. The same injection volume is used for both concentrations of luciferase, thereby increasing the cost per assay to \$0.67.

A crude preparation of luciferin and luciferase is available from Sigma Chemical Co. at the cost of \$8.00 for approximately 100 assays, or about \$0.08 per assay. The GSFC laboratory is in the process of determining the sensitivity of this unpurified preparation for use in procedures.

The lyophilized enzyme-substrate powder is rehydrated in TRIS buffer, with Mg^{++} added, a cofactor in the reaction. When rehydrating luciferase, always prepare enough to allow 0.1 ml per cuvette, one cuvette at least for each experimental tube to be assayed. If 1.5 ml luciferase or less is required, the TRIS can be added to the luciferase vial itself. Never freeze or vortex this mixture.

After rehydration, the luciferase mixture emits light without the addition of any ATP. This is called inherent light. (See Section 7, Controls). Incubation of

the rehydrated enzyme-substrate powder at room temperature for one hour will reduce the level of inherent light. The inherent light may be due to small amounts of ATP in the enzyme mixture that are not removed during purification. Additionally, when a processed sample containing no ATP is injected into the luciferase there is a light production which limits the sensitivity of the assay and is called the "blank" response.

SECTION 7

LUCIFERASE PROCEDURES

CONTROLS

A series of controls are run with each experiment before an assay is begun. The first is measurement of a radioactive light standard* which is then compared to expected values. A daily record should be plotted to show instrument stability.

Blanks and ATP standards are also run with each experiment, so their values can be used to calculate ATP levels in the unknowns.

The pH is very critical at each step in the procedures described here. The following chart gives acceptable ranges after the addition of each reagent.

Ranges of pH Tolerance

<u>After Addition to Sample of</u>	<u>Acceptable pH Range</u>
Apy-Ca ± TX	5.0 - 7.8**
Malate	4.0 - 4.25
Nitric Acid	1.15 - 1.20
H ₂ O or Na ₂ SO ₄	1.8 - 2.1
Luciferase	7.75 ± 0.08

Replicate measurements at different levels should be performed as a measure of reproducibility. Replication can be measured by replicate injections, replicate mixing tubes, replicate samples etc.

To calculate the percentage of standard deviation of the mean (coefficient of variation) use:

$$\frac{\sigma}{\bar{x}} \times 100 - \text{C.V. } \%$$

*See section on Measurement of Radioactive Light Standards.

**An apyrase control must be run with an experiment if the pH after addition of the apyrase is not within acceptable limits. See section on apyrase control.

For all procedures discussed in this manual, the DuPont luminescence Biometer was used to measure light production. In the discussions which follow:

- Activity is defined as that amount of light units produced by a given amount of ATP under certain chemical conditions;
- Sensitivity is defined as the lowest amount of ATP statistically resolvable above the blank.
- Signal-to-noise-ratio is defined as the activity divided by the blank. It is a measure of the effective or useful sensitivity of the system.

INHERENT LIGHT

After rehydration and before the addition of ATP, the luciferase mixture emits light called inherent light which slowly decays with time after hydration. A check for inherent light must be made before beginning an experiment to ensure the instrument's ability to zero it.

Determination of Inherent Light

1. Place the cuvette in the DRUM of the Biometer and zero the Biometer by pressing the DARK CURRENT switch to the zero position. Hold switch for 3 seconds.
2. Rotate the DRUM 180° to the EXPOSE position, press the COUNT button, and note the digital readout. This is the inherent light of the luciferase.

Determination of Inherent Light Carryover

1. After determination of inherent light, zero the Biometer as before.
2. Press the COUNT button. The digital readout should be no higher than the dark current of the Biometer. A readout higher than the dark current is called carryover light. The presence of carryover light indicates that the inherent light of the luciferase is too great for the instrument to zero. If this occurs, the luciferase should be left at room temperature for 15 minutes and then retested.

BLANKS

The blanks contain the background fluids, such as sterile urine, sterile broth, or sterile saline. The purpose of the blanks is to detect the amount of light produced by the fluid that is not a result of bacteria. The amount of blank light is subtracted from the sample light value. It is important to subtract this blank value in samples that read very low - near the blank value.

ATP STANDARD

1. Add 0.05 ml ATP dilution to either a blank tube or a sample tube. If ATP is added to a sample tube it is called an ATP recovery and must be added when apyrase is inhibited to be measured. The volume of sample must be noted, and the original sample light units must be subtracted.
2. Use known concentration standard ATP value that was injected to calculate the unknown values of ATP/ml in the specimen.

-NOTE-

Rather than adding the ATP for recovery to a sample tube from which aliquots have been removed for injection, a preferable method is to prepare duplicate sample tubes and add ATP instead of the diluent water or Na_2SO_4 . This gives a known volume exactly identical to that of the sample volume. The same can be done for the acetone procedures by using ATP standard solution diluted in the acetone.

APYRASE CONTROL

An apyrase control is run with an experiment if the apyrase pH is not within the acceptable range. This control determines the amount of possible soluble or bound ATP not hydrolyzed that could remain and give falsely high ATP sample readings.

1. Prepare the maximum challenge of ATP (diluted in H_2O) expected in the system.

2. Add apyrase in a ratio to the ATP volume comparable to that used in the procedure.

Wait 15 minutes and assay immediately.

OR

Add nitric acid after the 15 minutes incubation to stop the apyrase activity, dilute, and assay.

This will show how much ATP if any, remains after the apyrase treatment.

NONCONCENTRATED PROCEDURES

NONCONCENTRATED PROCEDURE WITHOUT MALATE BUFFER FOR LUCIFERASE ASSAY OF BACTERIAL ATP

This procedure lacks the sensitivity of the concentration procedures, but it is adequate for samples containing high levels of bacterial ATP. The sensitivity of this procedure is greater than that of the nonconcentrated procedure with malate buffer because of the variable effect of malate buffer on intrabacterial ATP. Since this procedure does not use malate or a similar buffer to help eliminate bound, extracellular ATP, it is suggested that this procedure be used only on pure cultures or when blood cells or tissue cells are not contaminating the specimen.

Procedure

1. Place in a 12-ml centrifuge (17 × 100 mm) tube 0.5 ml sample: urine bacterial culture, etc.
2. Add 0.1 ml 10 mg Apy/ml, 0.03 M CaCl₂ (+0.6 percent TX-100 if contaminating mammalian cells are present).
Vortex well, wait 15 minutes.
3. Add 0.1 ml 1.5 N HNO₃.
Vortex well, wait 5 minutes.
4. Add 4.3 ml H₂O or 0.085 M Na₂SO₄.
Vortex well.
5. Assay by injecting into luciferase rehydrated in 0.25 M TRIS, 0.01 M MgSO₄ pH 8.2.

6. For recovery, add 0.05 ml ATP 10 $\mu\text{g}/\text{ml}$ or 0.5 ml ATP 1.0 $\mu\text{g}/\text{ml}$ (depending on desired accuracy of recovery to remainder of sample).

NONCONCENTRATED PROCEDURE WITH MALATE BUFFER FOR LUCIFERASE ASSAY OF BACTERIAL ATP

The amount of nonbacterial ATP in urines can be quite large in comparison with the bacterial ATP and could include ATP bound to protein or particulates in the specimen that protect it from hydrolysis by the ATPase, potato apyrase. This could be a source of false positive results. Release of the bound ATP, allowing the hydrolysis of all the nonbacterial ATP, occurs if the pH of the specimen is dropped to 3.2, where the binding of ATP by salt linkages is less and the apyrase is still functional. This process can remove all the nonbacterial ATP due to a 10 percent blood contamination. The lowest number of bacteria that can be detected by this procedure is between 5×10^5 and 1×10^6 bacteria per milliliter of specimen.

The addition of malate buffer at pH 4.25 lowers the pH of the solution in the mixing tube to allow bound ATP to be released. The apyrase, which although slowed in activity is still functional at this pH, is able to hydrolyse this newly freed ATP.

This procedure is especially good for samples containing many amorphous crystals and membranes from tissue cells. These crystals are capable of binding soluble ATP and therefore protecting it from the action of the apyrase. Upon the addition of the nitric acid, this ATP would have been released and interpreted as being bacterial ATP.

This procedure uses a small sample volume and a large dilution volume, therefore it lacks the sensitivity of the concentration procedures, but it is suitable for detections of large amounts of bacterial ATP. This direct chemical procedure is recommended for use because of its simplicity when maximum sensitivity is not required.

Procedure

1. Place in a 12-ml centrifuge tube 0.5 ml sample: urine, bacterial culture, etc.
2. Add 0.1 ml 10 mg apyrase/ml, 0.03 M CaCl_2 , (0.6 percent TX-100 if contaminating mammalian cells are present).
Wait 15 minutes, vortexing frequently.

3. Add 0.1 ml 0.5 M malic acid, pH 3.75. Wait 15 minutes, vortexing frequently.
4. Add 0.1 ml 1.5 N HNO₃.
Wait 5 minutes, vortex well.
5. Add 4.2 ml 0.085 M Na₂SO₄ or H₂O.
Vortex well.
6. Assay by injecting 0.1 ml into 0.1 ml luciferase rehydrated in 0.25 TRIS, 0.01 M MgSO₄, pH 8.2.
7. For recovery, add 0.05 ml ATP (10⁻² μg/ml) or 0.5 ml ATP (10⁻³ μg/ml) (depending on desired accuracy of recovery) to the remainder of the treated sample.

CONCENTRATION PROCEDURES

SHORT CENTRIFUGE PROCEDURE FOR PURE SPECIES

This procedure eliminates one centrifugation step and the use of malate buffer, thereby shortening the processing time to less than one hour. It is not guaranteed, however, to remove bound ATP from nonbacterial sources in the sample and therefore is useful when bacteria are being grown in pure culture in known medium rather than with samples containing unknown sources of nonbacterial ATP such as urine or blood.

The sample volume and the apyrase-Ca-TX could be doubled, thereby assaying the bacteria from 2.5 ml of the original sample and making it comparable to the long centrifuge procedure.

Procedure

1. To a 12-ml centrifuge tube add 5.0 ml sample: urine, bacterial culture, etc.
2. Add 1.0 ml 10 mg/ml apyrase, 0.03 M CaCl₂ (0.6 percent TX if mammalian cells are present).
Vortex well.
3. Centrifuge 10,400 RCF × G (8000 rpm using a Lourdes Centrifuge Model A-2, Rotor 1200) at 20°C for 5 minutes, discard supernatant and invert tube on paper toweling to drain for 5 minutes.

4. Add 0.2 ml 0.1 N HNO₃.
Vortex well, wait 5 minutes.
5. Add 0.2 ml 0.15 M Na₂SO₄.
Vortex well.
6. Assay by injecting 0.1 ml into 0.1 ml luciferase rehydrated in 0.25 M TRIS, 0.01 M MgSO₄ pH 8.2.
7. For recovery, add 0.05 ml ATP (1.0 µg/ml) or 0.5 ml ATP (10⁻¹ µg/ml) to the remainder of the treated sample.

LONG CENTRIFUGE PROCEDURE

In order to achieve more sensitivity for the detection of bacteria in biological fluids, various means of concentrating the bacteria could be used. This would also allow the removal of inhibiting factors and permit washing of the bacteria. The bacteria are then extracted and their ATP brought up in a minimal volume. This procedure concentrates the bacteria by centrifugation.

In this procedure, the final concentrated bacterial ATP is suspended in 0.4 ml, of which 0.1 ml is assayed. Therefore, ATP is measured from 2.5 ml of the original sample. In the noncentrifuged procedure, a sample of 0.5 ml is diluted to 5.0 ml and 0.1 ml is assayed. Only 0.01 ml of the original sample is assayed here. The centrifuge procedure concentrates the amount of bacteria detected 250 times more than does the noncentrifuged procedure. However, the centrifuge procedure concentrates the bacterial ATP from the original sample by a factor of 25.

Procedure

1. Place in a sterile 12-ml centrifuge tube 10 ml sample: urine, bacterial culture, etc.
2. Add 0.2 ml 6.0 percent Triton X-100.
Vortex well.
3. Centrifuge 10,400 RCF × G (8000 rpm using a Lourdes Centrifuge Model A-2 Rotor 1200) for 5 minutes at 20°C, discard supernatant and invert tube on paper toweling to drain for 5 minutes.
4. Add 1.0 ml 10 mg apyrase/ml, 0.03 M CaCl₂.
Vortex well.

5. Add 5.0 ml 0.9 percent saline.
Vortex well, wait 15 minutes.
6. Add 1.0 ml 0.5 M malic acid, pH 4.25.
Vortex well.
7. Centrifuge at 10,400 RCF \times G (8000 rpm using a Lourdes Centrifuge Model A-2 Rotor 1200) for 15 minutes at 20°C, discard supernatant and invert tubes on paper toweling to drain for 5 minutes.
8. Add 0.2 ml 0.1 N HNO₃.
Vortex well and wait five minutes.
9. Add 0.2 ml 0.15 M Na₂SO₄.
10. Assay by injecting 0.1 ml into 0.1 ml luciferase rehydrated in 0.25 M TRIS, 0.01 M MgSO₄, pH 8.20.
11. For recovery add 0.05 ml ATP (1.0 μ g/ml) or 0.5 ml ATP (10⁻¹ μ g/ml) to the remainder of the treated sample.

FILTRATION PROCEDURE

A second concentration procedure is being optimized at the GSFC laboratory using filtration rather than centrifugation to concentrate the sample. Reproducible results have been obtained using a presterilized, 2.5 ml capacity disposable filter unit. (Millipore SVGS-025-10, 0.22 μ pore size, 25 mm diameter) in conjunction with a multiple filter unit housing that could be used with pressure or vacuum. This unit has a rack for holding cuvettes to collect the extracted sample and provision for discarding the waste filtrates.

This filtration procedure, when completed, would have a time advantage over the centrifuge procedure, providing results in about half the time. It would allow for processing a larger volume of a given sample. This would be an advantage when assaying a sample with very low levels of bacteria.

Procedures are being tested using nitric acid and using acetone as extractants in conjunction with the filters. TX-100 is being tested in the procedure to decrease the bubble point of the filter to allow faster filtration and more complete removal of wash solutions from the filter unit and the complete recovery of the volume of extracted ATP from the sample.

ORGANIC SOLVENT PROCEDURE

One of the extracting agents that has been found to be optimal at GSFC is an organic solvent, acetone. An advantage of the procedure using acetone is in the assay of ATP from yeast cells. Due to the high concentrations of acid necessary to extract the yeast ATP, a doubling or more dilution of the final sample is necessary to allow assay without inhibition of the luciferase by the acid. No dilution of the sample is necessary in the organic solvent procedure, therefore higher sensitivity results and lower concentrations of yeast cells can be detected.

Procedure

1. Place in 12-ml centrifuge tubes:

Tube a. 10 ml sample: urine, bacterial culture, etc.

Tube b. 10 ml sample: urine, bacterial culture, etc. for ATP recovery.

Tube c. 10 ml background fluid: sterile urine or sterile culture media for blank tube.

2. Centrifuge all tubes 10,400 RCF \times G (8000 rpm using a Lourdes Centrifuge Model A-2 Rotor 1200) for 15 minutes. Decant and discard supernatant. Invert on paper toweling for 5 minutes.

3. Add:

To tube a, 5.0 ml organic solvent 90 percent Methylene chloride or 90 percent acetone.

To tube b, 5.0 ml organic solvent.

To tube c, 5.0 ml ATP 10^{-1} μ g/ml diluted in organic solvent.

Vortex.

4. Place tubes in a heating block* at 90°C for 40 minutes or until no odor of extractant remains. The time should be watched very carefully, as too much heat could cause ATP decay. Remove from heating block and cool.

5. Add:

To tube a, 0.4 ml 0.085 M Na₂SO₄

To tube b, 0.4 ml 10^{-1} μ g ATP/ml diluted in 0.085 M Na₂SO₄

To tube c, 0.4 ml 0.085 M Na₂SO₄

*Heating block is constructed so that the entire length of the tube is heated, subjecting any droplets on the side of the tube to heat.

6. Assay by injecting 0.1 ml into 0.1 ml luciferase rehydrated in 0.05 M TRIS, 0.01 M MgSO_4 , pH 7.75.

SOLUBLE ATP PROCEDURE

This procedure is used to measure the amount of soluble ATP in any biological fluid such as urine. Before beginning the procedure, remove all particulate matter by centrifugation or filtration of the sample as it may interfere with the quantification.

PROCEDURE

1. Assay by injecting 0.1 ml sample (urine) into 0.1 ml luciferase rehydrated in 0.05 M TRIS, 0.01 M MgSO_4 pH 7.75.
2. Urine Blank. To a 12-ml centrifuge tube add 1.0 ml sample (urine).
Add 0.1 ml 10 mg/ml apy, 0.03 M CaCl_2 .
Mix well and wait 15 minutes to hydrolyse soluble ATP.
Assay by injecting 0.1 ml into 0.1 ml luciferase rehydrated as above.
3. ATP recovery or internal standard.
To a 12-ml centrifuge tube add 1.0 ml sample (urine).
Add 0.05 ml ATP 10 $\mu\text{g}/\text{ml}$
Mix well (this gives a concentration of $5 \times 10^{-1} \mu\text{g}$ ATP per 1.05 ml).
Assay by injecting 0.1 ml into 0.1 ml luciferase rehydrated as above.
4. ATP standard solution.
To a 12-ml centrifuge tube add:
1.0 ml H_2O .
0.05 ml ATP 10 $\mu\text{g}/\text{ml}$.
Mix well. (This gives a concentration of $5 \times 10^{-1} \mu\text{g}$ ATP per 1.05 ml).
Assay by injecting 0.1 ml into 0.1 ml luciferase rehydrated as above.
5. Water blank
Assay H_2O by injecting 0.1 ml into 0.1 ml luciferase rehydrated as above.

CALCULATIONS

The basic calculations for all the procedures involve determining the proportion of the original sample assayed; comparing the light units obtained with the light units from a known amount of ATP standard solution. If the proportion of the original sample assayed and the proportion of the ATP standard solution assayed

are the same, or are corrected for, then the unknown sample is in direct proportion to the ratio of the light units from the sample to the light units from the Standard ATP, times the concentration of the standard solution or:

1. Sample light minus blank light = Net sample light
2. ATP standard light minus blank light = Net standard light
3. $\frac{\text{Net sample light}}{\text{Net standard light}} \times \text{Amount of ATP in standard} = \text{Concentration of ATP in sample}$

These calculations can be used to calculate micrograms of ATP per ml of original sample, micrograms of ATP per cell, or bacteria per ml of original sample.

1. $\frac{\text{Sample injection volume}}{\text{Total processed sample volume to be assayed}} \times \text{Original \# ml sampled} = \text{Amount of sample per injection}$
2. $\frac{\text{ATP standard or ATP recovery injection size}}{\text{Final volume of ATP standard or ATP recovery to be assayed}} \times \frac{\text{Standard ATP quantity}}{\text{(Dilution of ATP \times volume added)}} = \text{Amount of ATP injected}$
3. $\frac{\text{Amount ATP injected (step 2)}}{\text{Amount sample injected (step 1)}} = \text{Micrograms of ATP per ml in original sample (This is the factor used to calculate the unknown amount of ATP in your sample.)}$
- 4a. $\frac{\text{Light units of sample minus light units of blank}}{\text{Light units of ATP standard minus light units of blank}} \times \text{Factor calculated in step 3} = \text{Micrograms of ATP per ml in unknown sample}$
- 4b. $\frac{\text{Light units of sample minus light units of blank}}{\text{Light units of ATP recovery minus light units of sample minus light units of blank}} \times \text{Factor calculated in step 3} = \text{Micrograms of ATP per ml in unknown sample}$

5. If starting sample was diluted, to calculate micrograms of ATP in original stock, multiply result of step 4 by the dilution factor.
6. To calculate number of bacteria per ml

$$\frac{\text{Micrograms of ATP per ml}}{\text{Known micrograms of ATP per cell}} = \text{No. bacteria/ml}$$

(Either step 4 or step 5 if sample was diluted)

7. To calculate micrograms of ATP per cell

$$\frac{\text{Micrograms ATP per cell}}{\text{Known number of bacteria per ml of sample}} = \text{Micrograms of ATP per cell}$$

(Either 4 or 5 if sample was diluted)

divided by

(Calculated by drop plate, spread plate, Coulter Counter or microscopic count, etc.)

Experimental Results

The following data and statistics were obtained in the GSFC laboratories. The experiments were performed with each instrument: the DuPont Biometer, Aminco Chem-Glow, and the JRB ATP Photometer. The dotted lines dividing the obtained data indicate the cutoff points above and below which the results are not linear and were not used in linear regression analyses. The range of each instrument is limited by the blank as well as by the instrument saturation.

The first set of data is for ATP concentration curves performed using all three instruments. The same solutions and ATP dilutions were used for all three curves; therefore, the results are comparable.

Water was used to dilute the HNO₃, giving a higher blank than is expected when the diluent is Na₂SO₄. The luciferase was rehydrated in 0.2 M TRIS, 0.01 M MgSO₄, pH 8.31. Because no contaminating mammalian cells were present, a condensed procedure was followed; all steps and reagents were omitted except the final HNO₃ and H₂O.

Title: ATP Curve
 Instrument: Biometer
 Procedure: Analogous to the centrifuge procedure omitting all reagents and steps except nitric acid and the final water, injecting 0.005 meq HNO₃ into 0.1 ml luciferase

$\mu\text{g ATP}$ Injected	CV% (Blank not subtracted)	Light units less blank
3.5×10^{-1}	10%	8.9×10^9
3.5×10^{-2}	17%	9.12×10^8
3.5×10^{-3}	7%	1.09×10^8
3.5×10^{-4}	7%	9.29×10^6
3.5×10^{-5}	13%	1.29×10^6
3.5×10^{-6}	—	1.01×10^6
3.5×10^{-7}	—	6.70×10^5
Blank	0.15%	4.21×10^6

Linear Regression Analysis:

Slope interval, $\beta = 1.06 > 1.02 > 0.98$ and intercept = -1.06×10^1
 when P = 0.05 and N = 15; coefficient of correlation (r) = 9.98×10^{-1}
 F ratio = 2.78×10^3 when table F_{0.95} = 2.69

Title: ATP Curve
 Instrument: Chem-Glow, without integrator
 Procedure: Analogous to the centrifuge procedure omitting all reagents and steps except nitric acid and final water, injecting 0.005 meq HNO₃ into 0.1 ml luciferase

$\mu\text{g ATP}$ Injected	CV% (Blank not subtracted)	Light units less blank
5×10^{-2}	16%	1.98×10^4
5×10^{-3}	6%	1.91×10^3
5×10^{-4}	15%	1.56×10^2
5×10^{-5}	0%	2.30×10^1
Blank	0.2%	2.43×10^1

Linear Regression Analysis:

Slope interval, $\beta = 1.05 > 0.98 > 0.92$ and intercept = -5.54,
 when P = 0.05 and N = 12; coefficient of correlation (r) = 9.95×10^{-1}
 F ratio = 1.07×10^3 when table F_{0.95} = 2.98

Title: ATP Curve
Instrument: JRB ATP Photometer, Area Mode
Procedure: Analogous to the centrifuge procedure omitting all reagents and steps except nitric acid and the final H₂O. 0.2 M TRIS

$\mu\text{g ATP}$ Injected	CV% (Blank not subtracted)	Light units less blank
3.5×10^{-1}	-	Saturated
3.5×10^{-2}	19%	2.96×10^5
3.5×10^{-3}	10%	3.78×10^4
3.5×10^{-4}	16%	2.22×10^3
Blank	0.2%	9.08×10^2

Linear Regression Analysis:

Slope interval, $\beta = 1.02 > 0.93 > 0.83$ and intercept = -6.59,
 when $P = 0.05$ and $N = 9$; coefficient of correlation (r) =
 9.93×10^{-1}

F ratio = 4.86×10^2 when table $F_{0.95} = 3.79$

Title: ATP Curve
Instrument: JRB ATP Photometer, Peak Mode
Procedure: Analogous to the centrifuge procedure omitting all reagents and steps except nitric acid and the final H₂O. 0.2 M TRIS

$\mu\text{g ATP}$ Injected	CV% (Blank not subtracted)	Light Units Less blank
3.5×10^{-1}		Saturated
3.5×10^{-2}	10%	6.16×10^4
3.5×10^{-3}	12%	6.69×10^3
3.5×10^{-4}	32%	4.42×10^2
3.5×10^{-5}	9%	5.7×10^1
Blank	0.8%	2.09×10^2

Linear Regression Analysis:

Slope interval, $\beta 1.02 > 0.89 > 0.76$ and intercept = -5.80,
 when $P = 0.05$ and $N = 12$; coefficient of correlation (r) =
 9.78×10^{-1}

F ratio = 2.16×10^2 when table $F_{0.95} = 2.98$

The following data are for a representative experiment that uses bacteria and was performed on the Aminco Chem-Glow. These data show representative linearity using the long centrifuge procedure.

Title: E. coli suspended in Urine
 Instrument: Chem-Glow Photometer
 Procedure: Long Centrifuge Procedure

Bacteria per ml*	Chem-Glow Light Units Less Blank	$\mu\text{M ATP/ml}$
1.65×10^6	2,800	1.8×10^{-6}
1.65×10^5	340	2.2×10^{-7}
1.65×10^4	40	2.6×10^{-8}
1.65×10^3	5	3.2×10^{-9}

Log phase serial dilutions of E. coli were resuspended in pooled, filtered urine. Microscopic counts equaled viable counts; therefore, there existed a minimum number of dead cells.

Linear Regression Analysis:

Slope interval, $\beta = -1.07 > -1.09 > -1.11$ and intercept = 5.76,
 when $P = 0.05$, and $N = 4$; coefficient of correlation (r) =
 -0.99997.

F ratio = 3.068×10^4 when table $F_{0.95} = 19$.

*Based on a measure of CFU and Total Microscopic Count in the Stock Culture which equals 1.65×10^8 CFU/ml.

The following experimental data show the linearity of the response to standard ATP when diluted in saline, broth, and urine. Results are shown when reagents are allowed to remain in the sample (nonconcentrated) and when they are mostly removed by centrifugation.

Title: ATP Curve
 Instrument: Chem-Glow
 Procedure: Centrifuge Procedure - Long

$\mu\text{m ATP}$ Injected	<u>Light Units less Blank</u>		
	Saline	Broth	Urine
1.1×10^{-4}	62,000	58,000	54,000
1.1×10^{-5}	6,000	6,000	6,500
1.1×10^{-6}	600	550	600
1.1×10^{-7}	<u>58</u>	<u>70</u>	54
1.1×10^{-8}	28	15	<u>7</u>
1.1×10^{-9}	2	4	0

Linear Regression Analysis - Saline:

Slope interval, $\beta = 0.997 > 0.991 > 0.985$ and intercept = -8.71

when $P = 0.05$ and $N = 4$; coefficient of correlation (r) = 9.99×10^{-1}

F ratio = 2.42×10^5 when table $F_{0.95} = 19.0$

Linear Regression Analysis - Broth:

Slope interval, $\beta = 1.08 > 1.02 > 0.96$ and intercept = -8.81

when $P = 0.05$ and $N = 4$; coefficient of correlation (r) = 9.99×10^{-1}

F Ratio = 2.44×10^3 when table $F_{0.95} = 19.0$

Linear Regression Analysis - Urine:

Slope interval, $\beta = 1.05 > 1.01 > 0.97$ and intercept = -8.78,

when $P = 0.05$ and $N = 5$; coefficient of correlation (r) = 9.99×10^{-1}

F Ratio = 3.76×10^3 when table $F_{0.95} = 9.28$

Title: ATP Curve
Instrument: Chem-Glow
Procedure: Nonconcentrated Procedure with Malate Buffer

$\mu\text{m ATP}$ Injected	<u>Light Units less Blank</u>		
	Saline	Broth	Urine
1×10^{-4}	45,000	55,000	52,000
1×10^{-5}	6,500	6,500	5,400
1×10^{-6}	700	600	530
1×10^{-7}	-- <u>61</u>	45	-- <u>44</u>
1×10^{-8}	-- <u>22</u>	-- <u>5</u>	-- <u>9</u>
1×10^{-9}	2	0	4
1×10^{-10}	4	0	9

Linear Regression Analysis - Saline:

Slope interval, $\beta = 1.15 > 1.04 > 0.937$ and intercept = -8.91,

when $P = 0.05$ and $N = 4$; coefficient of correlation (r) = 9.99×10^{-1}

F Ratio = 7.57×10^2 when table $F_{0.95} = 19.0$.

Linear Regression Analysis - Broth:

Slope interval, $\beta = 1.02 > 0.97 > 0.93$ and intercept = -8.67,

when $P = 0.05$ and $N = 5$; coefficient of correlation (r) = 9.99×10^{-1}

F Ratio = 3.13×10^3 when table $F_{0.95} = 9.28$.

Linear Regression Analysis - Urine:

Slope interval, $\beta = 1.02 > 0.98 > 0.94$ and intercept = -8.63

when $P = 0.05$ and $N = 4$; coefficient of correlation (r) = 9.99×10^{-1}

F Ratio = 4.22×10^3 when table $F_{0.95} = 19.0$.

SECTION 8

DETERMINATION OF MICROBIAL SUSCEPTIBILITY TO ANTIBIOTICS

Rapid determination of microbial susceptibility to antibiotics is of great importance in clinical laboratories. There are several standardized tests established which are routinely used by hospital laboratories for this purpose: the broth dilution method for determining the minimum inhibitory concentration (MIC) and the agar diffusion method (Kirby-Bauer) for determining the zone of inhibition. Both the broth dilution and the agar diffusion methods take approximately 48 hours to perform. A rapid method is being developed and evaluated at GSFC and New England Medical Center Hospital (NEMCH) to determine microbial susceptibility to antibiotics in approximately 4 hours. This test uses the firefly luciferase assay for ATP as an indicator of antibiotic effect.

Optimum use of antimicrobial agents to combat infection depends upon the knowledge of the susceptibility of the infecting organism. Although susceptibility can sometimes be predicted from the general behavior of the bacterial species involved, the most reliable approach is generally that of direct measurement of the in vitro interaction between the isolated organism and the drug. Table 8-1 lists concentrations of antimicrobial agents used.

Table 8-1

Antimicrobial Agents Used in Preliminary Studies

Drug	Concentration ($\mu\text{g}/\text{ml}$)
ampicillin	8
penicillin G	0.15 (<i>S. aureus</i>)
penicillin G	8 (others)
nafcillin	0.6
carbenicillin	128
cephalothin	16
tetracycline	6
erythromycin	4
clindamycin	2
gentamicin	6
chloramphenicol	12.5
colistin	8
nitrofurantoin	50
nalidixic acid	12.5
sulfisoxazole	100-300

The availability of a more rapid method of testing bacterial susceptibility would permit prompt selection of the most effective antimicrobial agent.

The procedure developed for determining microbial sensitivity is one in which broth cultures of log phase bacteria (10^6 colony forming units per ml) were grown for 2.5 hours in the presence of different antibiotics. This resulted in the best discrimination between "sensitive" and "resistant" strains. The drug effect was quantitated by calculating the ratio of the slope of the inhibited curve to the antibiotic free growth control. The current interpretation of the ATP index is: $>+0.25$ implies Resistance and $\leq+0.25$ implies Sensitivity.

The effect of twelve antimicrobials on eight different microbial species gave a 94 percent correlation with the standard agar diffusion method (Kirby-Bauer) and broth dilution minimum inhibitory concentrations (MIC).

CALCULATIONS OF ATP INDEX OF INHIBITION

The drug effect on bacterial ATP content is quantitated using the following formula:

$$\text{ATP INDEX} = \frac{B_t - A_o}{A_t - A_o}$$

where:

A = growth control; organism without antibiotic added

B = antibiotic treated organism

t = time of incubation with antibiotic or sterile saline

o = zero time with antibiotic or sterile saline

LUCIFERASE PROCEDURE

METHOD OF INCUBATION

1. Dilute 1000 fold - a fresh overnight broth culture in T-Soy broth to obtain an inoculum of approximately 10^6 CFU/ml.
2. Preincubate 30 min at 37°C .
3. Add 4.5 ml of the overnight culture to Tube A containing 0.5 sterile 0.9% saline (growth control). Add 4.5 ml of the overnight culture to Tube B containing 0.5 ml antibiotic solution in 0.9% saline to yield the final concentration shown in Table 8-1.
4. Assay (see assay method below) 0.5 ml broth culture from Tube (A_o).

5. Incubate Tube A and Tube B for 2.5 hr at 37°C.
6. Assay (see assay method below) 0.5 ml samples from Tube A and Tube B (A_t and B_t).

ASSAY METHOD

1. Place in a sterile 12-ml centrifuge tube (17 × 100 mm) 0.5 ml sample (broth culture).
2. Add 0.1 ml APY-Ca 5.0 mg apy/ml, 0.03 M CaCl_2 . Vortex and wait 15 minutes.
3. Add 0.1 ml 1.5 N HNO_3 . Vortex and wait 5 minutes.
4. Add 4.3 ml sterile deionized H_2O . Vortex.
5. Assay by injecting solution into luciferase rehydrated in 0.2 M TRIS, 0.01 M MgSO_4 pH 8.4.

MINIMUM INHIBITORY CONCENTRATIONS

The minimum inhibitory concentration (MIC) (Ericsson and Sherris, 1971) is run to evaluate the luciferase assay for determination of microbial susceptibility to antibiotics.

PREPARATION OF ANTIBIOTICS FOR MINIMUM INHIBITORY CONCENTRATIONS

Reference standard antibiotic powders to be used for sensitivity testing should be obtained from manufacturers or from standards laboratories. They should be stored at 4°C, under desiccation and in darkness. Stock solutions are prepared by dissolving the antibiotic powders in sterile, distilled water. GSFC stock solutions are prepared to 1,280 µg antibiotic per ml. Not all antibiotics are soluble in water. Some, erythromycin for example, must be dissolved in 95% ethanol according to the specifications of the manufacturer. These stock preparations of antibiotics are sterilized by filtration through a 0.22 µ Millipore filter. The solutions are dispensed in 2.5-ml aliquots using a repeating syringe and stored in individually sealed tubes. They are stored at -20°C and discarded if not used within two weeks after preparation. The stock tubes are never refrozen.

MIC-BROTH DILUTION METHOD

Two-fold dilutions of antibiotics are made in a suitable broth. For each organism tested, a control tube with one ml broth and no antibiotic is included. The organism is grown to logarithmic or early stationary phase of growth in broth. It is diluted to contain 10^4 to 10^5 viable units per ml. One ml of this preparation is inoculated into each tube of diluted antibiotic. The tubes are incubated at 37°C for 16 to 20 hours. The end point is the least amount of antibiotic causing complete inhibition of growth as seen by the naked eye. Care should be taken to note slight amounts of turbidity present caused by the inoculum itself and not by growth of the organism. A standard for determination of complete inhibition may be prepared by inoculating a tube and storing it at 4°C for comparison with incubated tubes.

E. coli ATCC 25922 and S. aureus ATCC 25923 are the MIC standards to be run with each test to check the performance of the test. The test is performed in 30×100 mm nondisposable glass tubes.

KIRBY BAUR - AGAR DIFFUSION METHOD

The agar diffusion method (Ericsson and Sherris, 1971) is used here to evaluate the luciferase assay for determination of microbial susceptibility to antibiotics. The antibiotic discs used in this test should pass a performance standard test. The paper from which the discs are made should have no inherent inhibitory activity or manufacturing residues which could combine with and inactivate the antibiotic. The dye or ink used to code the discs should neither interfere with or potentiate the activity of any antibiotic. Prepared discs are available from manufacturers. The antibiotic content of the discs should give inhibition zones with organisms whose MIC's are within the range of practical therapeutic levels. The diameter of the zones should not exceed 40 mm and should preferably be below 30 mm. Organisms with MIC's at the lower limits of clinical sensitivity should also give a measurable zone of inhibition.

Discs should be stored in sealed water-tight ampoules with a desiccant and at 4°C or below. Before opening an ampoule it is brought to room temperature to prevent water vapor condensation on the discs. The discs from an opened ampoule may be used until the expiration date, if the ampoule is tightly sealed, stored at 4°C , and allowed to equilibrate to room temperature each time before opening. Nine-cm plates are used and are poured to a depth of 4 mm. They must be poured on a level surface to ensure an even depth of medium. Plates are stored at 4°C and dried at 37°C for 30 minutes before use. Mueller-Hinton, blood or chocolate agar plates are routinely used in testing.

The inoculum, an overnight culture, is prepared by diluting in Trypticase soy broth to a concentration that would give a dense, but not confluent growth. This concentration is obtained by adjusting the culture turbidity to compare to a turbidity standard. The turbidity standard is prepared by mixing 0.5 ml of 0.048 M Barium chloride (1.175% w/v Barium chloride with two waters of hydration) with 99.5 ml of 0.36 N H₂SO₄ (1% v/v).

A cotton swab is soaked in this dilution of the culture, and the plate is streaked in four directions to obtain an even and thorough distribution.

The agar surface is dried by incubation at 37°C for 15 minutes. The antibiotic discs are then applied to the surface of the agar using sterile forceps, and are pressed gently into place. No more than 12 discs per plate are used to prevent overlapping of zones. After application of the discs, the plates are allowed to stand at room temperature for 30 minutes. Plates are incubated at 37°C for 18 to 20 hours. In most cases the end-point is obvious, a clearly defined zone around the disc where no growth has occurred. There may be very small pin point colonies within the zone, these should be noted. If full-sized colonies appear within the zone, the culture should be checked for purity and the test repeated. If they still appear, they should be regarded as significant.

The zone size is measured in millimeters and compared with the given sensitivity or resistance of that antibiotic. Since the diameter of the disc is 6.0 mm, only the diameter of a zone greater than 6.0 mm is significant.

SECTION 9

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APPENDIX A

**INFORMATION BULLETINS OF
PHOTOMETRIC INSTRUMENTS FOR ATP ASSAY**

The presentation of these bulletins in no way represents endorsement by the U. S. Government. These are instruments that we have had the opportunity to use in the laboratories at Goddard Space Flight Center.

Du Pont 760 Luminescence Biometer



Key Features

- High sensitivity: 10^{-11} grams of ATP per 10 microliter injection.
- Digital readout direct in ATP or microorganisms.
- Automatic ranging of readout over five decades.
- Unique microsample injection system.
- Purified reagents in convenient kits.
- Disposable microorganism filters.

Contributions

- Reduction of sample size.
- Elimination of culturing: significant reduction of time required.
- Reduction of feedback time lag.
- Labor cost reduction.

Measurement of ATP And Microorganisms Without Culturing

The Du Pont 760 Luminescence Biometer* is a versatile, extremely sensitive photometer specifically designed to provide increased capabilities in research, and routine measurement of bioluminescent and

chemiluminescent reactions. Using the firefly luciferase-luciferin reaction for rapid assay of adenosine triphosphate (ATP), the system offers practical utility for the measurement of ATP from any source, or of any substance which can be coupled to a reaction which involves ATP.

In addition to offering new research capability through sensitive

measurements of 10^{-11} grams of ATP in a 10 microliter sample, the Biometer unlocks the field of rapid measurement of microorganisms. Cell populations can be approximated without culturing, based on the proportionality between the quantity of extractable cellular ATP and the number of cells of a given size class, i.e., bacteria.



Biometer detects light response from firefly reaction.

*U.S. Patent No. 3,359,973; Other Patents Applied For

 **DUPONT** Instruments
REG. U.S. PAT. OFF.

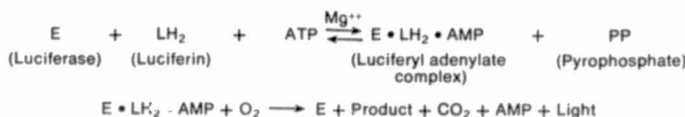
Principle of Operation

ATP Firefly Reaction

When a microsample containing ATP is injected into a suitably buffered reaction mixture of luciferase and luciferin, the peak intensity of the

resulting light flash is directly proportional to the concentration of ATP over more than five decades.

Reference: W. D. McElroy, H. H. Seliger and E. H. White, *Photochemistry*, 10, 153 (1969).



Reagents

Du Pont supplies convenient reagent kits containing purified firefly chemicals for ATP assay. To prepare a reaction mixture, a buffer-salt tablet containing buffer and magnesium sulfate is dissolved in water. Luciferase and luciferin are added to the buffered solution as a premeasured homogeneous powder. The reaction mixture is then dispensed into cuvettes and is ready to use for calibration and assay of ATP.

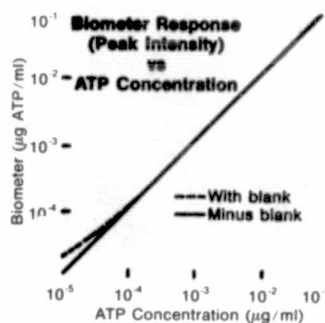
Assay Conditions

Reaction Mixture:[†]

Buffer: Morpholinopropane sulfonic acid (MOPS): 0.01M
 pH: 7.4 at 25°C
 MgSO₄: 0.01M
 Luciferin (crystalline): 0.71 mM
 Luciferase (purified and stabilized): ≥100 units*

Reaction Volume:
 100 microliters

Injection Volume:
 10 microliters



760 Specifications

Size: 15 in. x 16 in. x 7.5 in.

Weight: 23 pounds

Power Requirements:
 105-130 V; 0.2 amps; 50-60 Hz

Photometric Capability:
 3 picolumens, minimum detectable

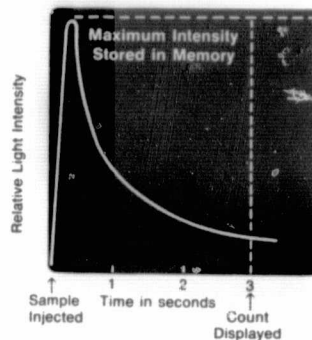
Wave Length Range: 350-600 nm

Output to Optional Recorder:

100 mv ungrounded
 1K source impedance
 0.5 sec. full scale
 2 in./min. chart speed

*1 unit = $\frac{\text{Response to } 1.65\mu\text{M ATP}}{\text{Response to } 20\mu\text{ curies } C^{14} \text{ calibrated light source}}$

†U.S. Patent Nos. 3,164,613; 3,262,940; 3,311,637 and Other U.S. and Foreign Patents Pending



Microorganism Measurements

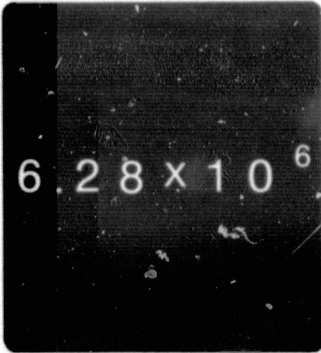
The basis for enumerating microorganisms with the 760 Biometer system, is that the amount of ATP per unit biomass is relatively constant. Consequently, for a given size range or class, such as bacteria, the average amount of ATP per cell can be established.

A study of thirteen representative bacteria species showed a range from 2.2 to 10.3 x 10⁻¹⁰ µg ATP/cell, and indicated that a mean value of 5 x 10⁻¹⁰ µg ATP/cell could be used as an ATP-to-bacteria conversion factor. Furthermore, this factor was found to remain constant across all phases of growth.

Defining the total quantity of living microorganisms (biomass) in a heterogeneous environment by culturing and counting techniques is difficult, time-consuming, inaccurate and sometimes impossible. However, using the quantity of extractable ATP, it becomes practical and meaningful to compare the levels of microscopic life in non-uniform samples. The Biometer system permits measurement of total biomass at the time of sampling rather than under artificially imposed culturing environments.

Potential Applications

C2



Variety of Applications

A wide variety of measurements have been proposed as potential applications for the Du Pont 760 Luminescence Biometer.

In some cases, specific methodology has been developed and usefulness of the application has been clearly demonstrated. However, some of the listed applications are logical extensions of the basic concept and require further exploratory and developmental research.

Extraction Procedures

Since the Biometer is an ATP measuring system, the key step in the operation is to quantitatively extract the ATP from the sample. Some proven techniques include extraction by butanol, perchloric acid, boiling buffer, freezing and thawing, sonic oscillation and differential pressure lysis.

Butanol extraction is recommended for general use. Upon contact with butanol, the cell walls of microorganisms are sufficiently disrupted to allow leakage of the ATP from the cell. Depending on sample source, selective filtration, centrifugation, sample washing or a combination of these techniques may be essential prior to the butanol step.

Following cell disruption, an aqueous phase is introduced to partition the hydrophilic ATP. A microaliquot of the aqueous phase is then injected into the firefly reaction mixture. The resulting light flash is automatically converted to ATP or microorganism concentration per milliliter depending on the instrument calibration.

Microbiology

Measure levels of anaerobic and aerobic bacteria, spores, algae, fungi, yeast, etc., by determining the average amount of ATP per cell and using it as a conversion factor from ATP to microorganism quantity. Measure filamentous and other organisms which cannot be counted by conventional methods.

For example, measure the total biomass consisting of bacteria, epithelial cells, leucocytes, etc., in saliva which cannot be simultaneously measured by conventional techniques. Determine total microfloral activities of rumen liquors. Determine growth rates and effects of various chemical and biological agents on microbial metabolism.

Petroleum Products

Detect the presence of microorganisms growing at the interface of oil and water before slime growth creates a problem—jet fuel, kerosene, diesel fuel, etc.

Urology

Measure total bacteria in urine to quickly screen out negative specimens. Detect L-forms and mycoplasma which cannot be ascertained by normal plating techniques. Rapidly and quantitatively assess antibiotic sensitivity.

Reference: J. S. Ames, "The Adenosine Triphosphate Assay for Antimicrobial Sensitivity," Masters Thesis, University of Wisconsin, 1970.

Hematology/Serology

Measure red and white blood cell numbers, physiological changes, and viability of stored blood. Test for serological immune factors in diseases such as mononucleosis or syphilis.

Cytology

Measure extra- and intracellular ATP pools in tissue cultures to ascertain cell numbers, regeneration times, respiratory rates and the effects of hormones, substrates and pathological agents. Count and measure the viability of sperm cells.

Immunology

Determine the effect of immune sera on tissue or tumor cells by measuring the ATP leakage from the cells. Use the ATP measurement for blood and tissue typing to determine donor-recipient compatibility.

Reference: W. J. Nungester, L. J. Paradise and J. A. Adair, "Loss of Cellular ATP as a Measure of Cytolytic Activity of Antiserum," *Proceedings of the Society of Experimental Biology and Medicine*, 132, 2, 582 (1969).

Physiology

Improve productivity, sensitivity, and specificity of ATP determinations in physiological research. Use the Biometer in studies to elucidate the mechanism of muscle contraction. Measure the photosynthetic production of ATP in plant chloroplasts, and follow oxidative phosphorylation rates in isolated mitochondria.

Potential Applications for the Du Pont 760 Biometer

Fermentation

Monitor fermentation processes to control the production of antibiotics, beverages, biochemicals, enzymes and foods. Increase the yield by better control of inoculum level, nutrient feeding, oxygen levels and harvest timing.

Quality Assurance of Consumer Products

Monitor microorganism contamination levels in process equipment and products in food, dairy, meat, drug and cosmetic industries.

Check the bacterial level to classify raw products before acceptance from vendors. Check product throughout processing utilizing the Biometer to pinpoint bacterial contamination sources in the system. Check end product rapidly and significantly reduce the holding time required.

Waste Water Treatment

Monitor and optimize activated sludge treatment plants to provide better control of waste sludge and oxygen feed rates. Detect poisoning of activated sludge rapidly and prevent the dumping of untreated wastes into the environment. Increase treatment efficiency rather than plant capacity.

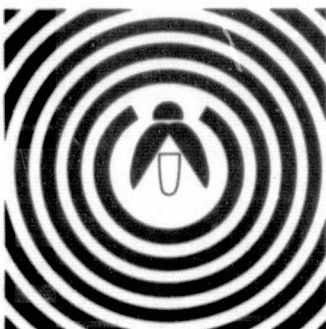
Reference: J. W. Paterson, P. L. Brezonik, and H. D. Putnam, "Sludge Activity Parameters and Their Application to Toxicity Measurements in Activated Sludge," 24th Annual Purdue Industrial Waste Conference, Lafayette, Indiana, May 6-8, 1969.

Biochemical Research

Conveniently measure the ATP/ADP/AMP ratio. Measure and study enzyme and substrate reactions which utilize ATP or which can be coupled either directly or indirectly to a reaction involving ATP, i.e., hexokinase, creatine phosphokinase, apyrase, myokinase, the nucleotide-kinases, mitochondrial oxidative phosphorylation, etc.

Biocides

Monitor biocide effectiveness in recirculating system processes, i.e., paper machines, milling machines, cooling towers, textile sizing and mineral ore leaching. Increase quality and production efficiency, decrease biocide costs and downtime for cleanup.



Soil/Water Ecology

Measure total biomass in soil, fresh and salt water samples as an indication of the ecological state of the environment. Detect life which cannot be measured or interrelated by conventional culturing techniques. Monitor total biomass to determine the levels and effects of chemical, biological, and thermal pollution on the environment.

Reference: O. Holm-Hansen and C. R. Booth, "The Measurement of Adenosine Triphosphate in the Ocean and its Ecological Significance," *Limnology and Oceanography*, 11, 4 (1966).

Virology

Use the ATP content of host cells to follow physiological changes subsequent to viral infection. Monitor ATP release from virus-infected cells as an indicator of replication time and virus titer.

Clean Room Control

Collect and assay microorganisms including spores by filtration of sufficient air to reach the sensitivity level of the Biometer. Monitor hospital operating rooms, pharmaceutical packaging areas, and clean rooms in the space and electronic industries.

Chemiluminescence

Measure chemiluminescent reactions such as the luminol-hydrogen peroxide reaction. Detect and ascertain the significance of chemiluminescence in biological fluids such as urine.

Reference: J. V. Schlegel, G. E. Pipkin and G. N. Shultz, "The Aetiology of Bladder Tumours," 25th Annual Meeting of the British Association of Urological Surgeons, London, June, 1969.

Use the Biometer to measure phosphorescence reactions.

Pharmacology

Measure antibiotic sensitivity by comparing the growth rates of control cultures and broths containing antibiotics. Determine antibiotic activity by titration against standard strains.

Measure cyclic AMP by converting it to ATP, which is then assayed in the Biometer. Purified reagents as supplied by Du Pont are essential to obtain the needed specificity for this assay.

Reference: R. A. Johnson, J. G. Hardman, A. E. Broadus and E. W. Sutherland, "Analysis of Adenosine 3',5'-Monophosphate With Luciferase Luminescence," *Analytical Biochemistry*. (In press)

Application Information

For more information on these and other applications, contact Du Pont Company, Instrument Products Division at:

Wilmington, DE 19898 • Phone: (302) 453-2711 c/r Monrovia, CA 91016 • Phone: (213) 357-2111.

In Europe, Instrument Products Division, Du Pont, 636 Friedberg/Hessen, Postfach 1410, West Germany.



AMINCO CHEM-GLOW PHOTOMETER

For manual use or easily adapts for use with automated systems. The CHEM-GLOW instrument* will detect quantities of ATP down to approximately 10^{-10} molar; sample sizes as large as 1/2-ml can be accommodated with the standard (6 x 50mm) reaction tube. This makes the CHEM-GLOW Photometer a useful analytical tool for a variety of scientific interests.†

applications

Bioluminescence

Detection and measurement of minute quantities of ATP is applicable to many areas of research including: 1) the determination of ATP in *Chlorella*;² 2) bacteria counting^{3,4}; 3) the determination of the viability of stored blood and cell counts⁵; 4) the assay of cyclic AMP¹⁰; 5) assay for flavin mononucleotide.¹¹ 6) Biomass determinations in sewage treatment plants and the effects of microbial cells in the environment.^{12,13}

Chemiluminescence

A method has been developed for the determination of H_2O_2 based on the copper-catalyzed chemiluminescent reaction between luminol and H_2O_2 .⁹ The method has been used also for the determination of bacterial levels.¹⁴

Simplicity of Design

The reaction chamber with photomultiplier assembly is mounted on the top of the microphotometer case. The reaction tube is positioned in front of a mirror, in a ring that rotates around a 931-B photomultiplier tube, so that the sample tube is in front of the photomultiplier tube and under the injection port. This compact arrangement allows the positioning and removal of the reaction tube without exposing the photomultiplier tube to ambient light. The injection port for either sample or substrate employs a rubber septum and guide to direct the injection needle into the center of the sample tube.

Two Models Available

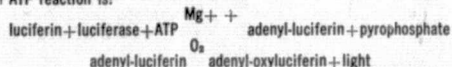
CHEM-GLOW Photometers are available for use with either 6 x 50mm reaction tubes, or 12 x 35mm reaction tubes. By a simple interchanging of the rotating head with an accessory head, either model may be adapted to accept the other size tube.

The solid-state CHEM-GLOW Photometer with fixed 700V photomultiplier tube power supply, regulated in two stages, features three blank-subtract ranges for up to 10 full scales of light signal suppression, and seven sensitivity ranges—100, 30, 10, 3, 1, 0.3, and 0.1. The photometer also has a two position damping switch permitting response to phenomena with life-times down to 5 microseconds.

Located on the back panel are a recorder outlet (set for 50mv, but with a range of 5mv to 60mv) for any slow or fast response strip chart recorder, and a 0.6 volt oscilloscope outlet. Size: 7 3/4" x 8 1/2" x 8" h. Weight: 9 lbs.

* Patent # 3,764,214

† ATP reaction is:



 **AMERICAN INSTRUMENT COMPANY**
DIVISION OF TRAVENOL LABORATORIES, INC.
8030 Georgia Avenue, Silver Spring, Maryland 20910

accessories

Repeating Dispenser

A repeating dispenser accessory mounts on the reaction chamber above the injection port, and permits 0.1ml of substrate from the 5ml syringe to be discharged into the sample tube. Fifty samples can be run before the syringe requires refilling.

Automated System Adapter

An adapter is available which is interchangeable with either size rotating head to permit use of the CHEM-GLOW Photometer with automated systems such as the Technicon Auto-Analyzer[®]. The adapter utilizes a coil-type flow cell.

Recorders

Two models are available. One is a single-pen recorder with a 50 millivolt full scale deflection. The second is a two-pen recorder with one pen having ten times the sensitivity of the other pen; thus if the sample being measured drives the sensitive 5-millivolt pen off-scale, the 50-millivolt pen remains on the recorder chart and the sample is not lost. The 50-millivolt pen matches the CHEM-GLOW Photometer indication. Overall sensitivity is not increased since the measurement is done at a lower decade setting of the microphotometer.

Both models have chart speeds of 2.5, 5, 10, 20, 40, and 80 mm/min. and 285 mm wide charts. Pen speed is less than one second for 99% of full scale. The zero-adjustment is more than 100%; accuracy is $\pm 0.5\%$ and sensitivity is 0.15%. The single pen model has space for a disc integrator if desired.

Standard Light Source

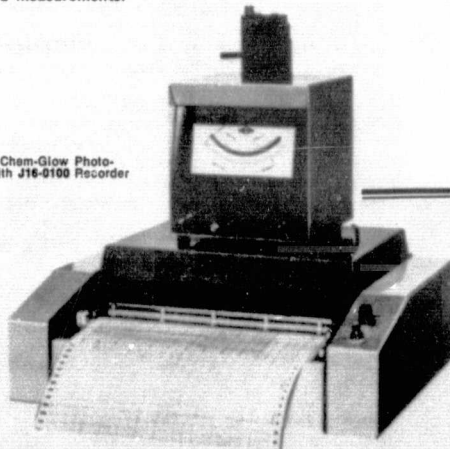
Contains Carbon-14 and liquid scintillator (1 microcurie activity) in a sealed glass vial, 6 x 50 mm.

Integrator-Timer

The Integrator-Timer makes complete and rapid mixing of the sample with the enzyme preparation less critical than when using the peak height method. Even more important are the savings in reagent costs that can be realized by using commercially available luciferin-luciferase mixtures without further purification.

The Integrator-Timer accessory plugs into the oscilloscope outlet of the photometer; this leaves the recorder outlet free if one desires peak height measurements simultaneously with integrated measurements.

J4-7441 Chem-Glow Photometer with J16-9100 Recorder



The Integrator-Timer is a DC analog, RC-type capable of providing precise gating of the input signal for selected time intervals. The integrator circuitry includes switched precision range resistors and a fixed, low-leakage storage capacitor that permit adjustment of the integrator circuitry (characteristic time). Sensitivity ranges are 100, 30, 10, 3, 1, 0.3, and 0.1. Integrator time ranges of 1.0, 3.0, 5.0, 10.0, 20.0, 30.0, and 60.0 seconds can be selected by rotating the selector switch on the front panel.

Integration is begun by depressing the button on the front panel or by remote contact closure. Indicator lamps for clear, accumulate and store modes are used to indicate instrument operation status.

A four digit voltmeter, with approximately 100% over-range, visually displays the time integral. A BCD output connector allows computer or printer compatibility.

In addition to its use with the Chem-Glow Photometer, the Integrator-Timer can be used with the Aminco-Bowman Spectrophotofluorometer, the SPF 125 and the Fluoro-Colorimeter if they employ the Solid-State microphotometer when greater analytical precision, digital readout, and computer or printer compatibility is desired. Size: 8" H, 7 $\frac{1}{8}$ " W x 8 $\frac{1}{2}$ " D. 9 $\frac{1}{4}$ lbs.

Order by Catalog Number

J4-7441 Chem-Glow Photometer—Accepts 6 x 50mm reaction tubes. Complete with solid-state photometer, reaction chamber, rotating head and 931-B photomultiplier tube. Supplied with 1 doz. rubber septums and 1 doz. reaction tubes. 115V, 50/60 Hz.

J4-7441A Chem-Glow Photometer—Same as above, but for 12 x 35mm reaction tubes.

J4-7462 Integrator-Timer—as described. 115V, 50/60 Hz.

J4-7442 Separate Reaction Chamber—Accepts 6 x 50mm reaction tubes. This unit can be connected to any Aminco Photomultiplier Microphotometer to create a Chem-Glow Photometer. Supplied with rotating head, 931-B photomultiplier tube, 1 doz. rubber septums and 1 doz. reaction tubes.

J4-7442A Separate Reaction Chamber—Same as above, but for 12 x 35mm reaction tubes.

J4-7452 Rotating Head of Reaction Chamber—Accepts 6 x 50mm reaction tubes. Interchangeable with head for 12 x 35mm tubes.

J4-7456 Rotating Head of Reaction Chamber—Accepts 12 x 35mm reaction tubes. Interchangeable with head for 6 x 50mm tubes.

J4-7444 Repeating Dispenser—With 5ml syringe.

P1501-605 Automated System Adapter—Interchanges with rotating heads to permit chamber to be used with automated analysis systems such as the Technicon AutoAnalyzer. Includes P403-620 Flow Cell and mirror. If automatic analyzer recorder is used, a J4-8266 Recorder Adapter is required (see below).

J16-0100 Recorder—as described, single pen.

J4-7459 Recorder—as described, two pen.

P403-620 Replacement Coil Flow Cell—For P1501-605 Adapter.

J4-7454 Standard Light Source—as described.

J4-8266 Automatic Analyzer Adapter—Fits between the recorder output of the photometer and recorder used with the Technicon AutoAnalyzer.

P1821-120 Replacement Septums—For all reaction chambers. In packages of one dozen.

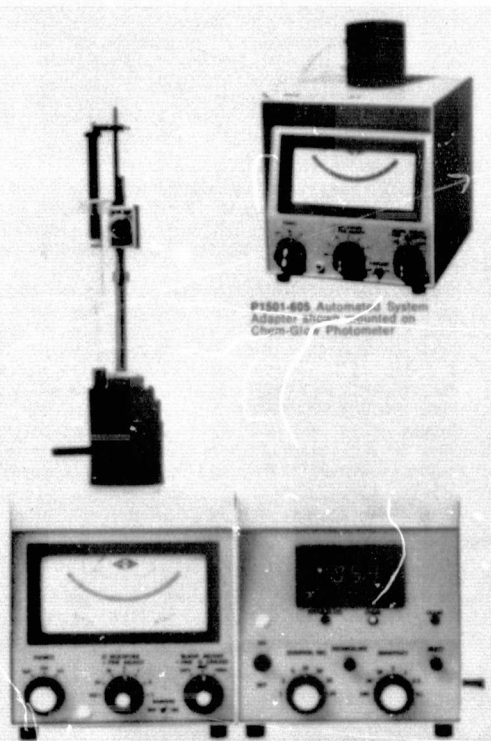
For further details, contact your local representative or



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B2441-B-2M-J-94 Revised September 1974

References

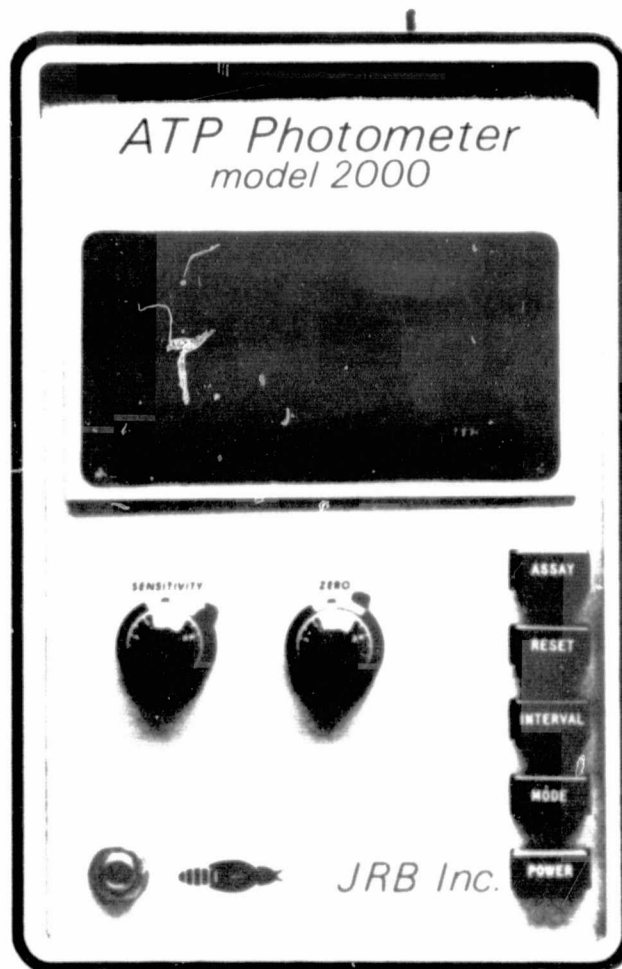
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3. E. W. Chappelle, G. L. Picciolo and R. H. Altland, *Biochem. Med.*, **1**, 252, 1967.
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14. Paper presented at American Society for Photobiology meeting 1974 "Applications of Chemiluminescence to Determination of Bacterial Levels," N. D. Searle.



J4-7441 Chem-Glow Photometer with J4-7444 Dispenser and J4-7462 Integrator-Timer on right.



ATP Photometer



JRB The Model 2000 ATP Photometer

The JRB ATP Photometer is an ultra-sensitive laboratory instrument used for measuring bioluminescent and chemiluminescent reactions. Specifically, the instrument was designed for the measurement of the bioluminescent flash caused by the reaction of Adenosine Triphosphate (ATP) and luciferin in the presence of the firefly luciferase enzyme. Biomass determinations in many different types of media can be rapidly and accurately performed for a wide range of applications.

The JRB Photometer is primarily used for the quantitative determination of ATP by measuring the amount of light given off by its reaction with firefly luciferase enzyme. This bioluminescent reaction is specific for ATP. The reaction rate is related to the concentration of ATP, and one photon of light is emitted for each molecule of ATP which is hydrolyzed.

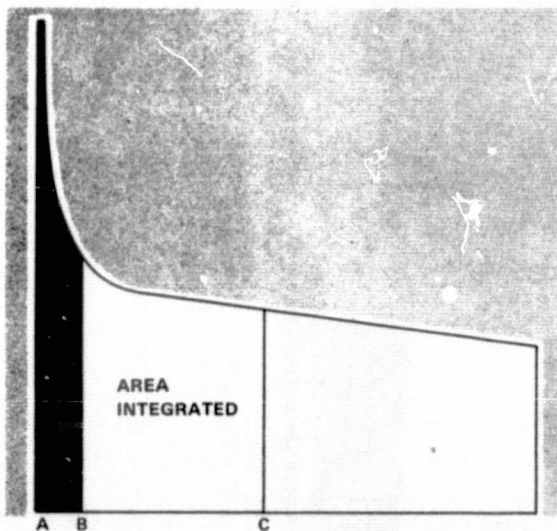


Fig. 1. Light emission response when a sample containing ATP is injected into the enzyme preparation: pt. A, time of injection of sample; pt. B, end of 15-second delay period; pt. C, end of 1-minute light-integration period.

KEY FEATURES:

- DURABLE CONSTRUCTION—ideal for mobile laboratories
- HIGH SENSITIVITY—measures down to 10^{-10} mg ATP (10^{-13} gm). Range: 10^{-9} mg ATP/ml to 1 mg ATP/ml
- VARIABLE SAMPLE VOLUMES—from 10 micro liters to 10 ml
- STANDARD DIGITAL INTEGRATIONS and continuous analog output signal
- TWO STANDARD TIME INTEGRATION MODES—6-second integration ,or 15-second delay + 60-second integration
- DIGITAL PEAK HEIGHT READOUT
- BOTH 115 AND 230 VAC POWER SELECTION
- ACCEPTS ANY CYCLIC RATE POWER SOURCE WITH 48 TO 440 Hz

THEORY

The overall reaction is given by: luciferin (reduced) + ATP + O₂ $\xrightarrow[\text{luciferase}]{\text{Mg}^{++}}$ luciferin (oxidized) + pp_i + AMP + H₂O + h_v.

When a prepared sample containing ATP is introduced into the enzyme preparation, there is an immediate burst of light in the wavelength range of 560 to 580 nm. The intensity of the light decays in an exponential fashion.

The Model 2000 Photometer has the capability of reading both peak height flash or integration of the exponential decay curve.

Light Integration

Most applications of the ATP Photometer use the integration mode. Upon initiation of the start assay switch and mixing of reactants, the JRB unit delays for 15 seconds then measures and integrates the area under the light curve for the next 60 seconds (Figure 1).

Peak Height

The instrument has a peak height measuring mode that is a digital representation of the recorder peak. The unit will record the maximum output that occurs within 3 seconds after initiation of the assay command. The enzyme kinetics kit assessor is required for the peak measuring mode. The counts per minute (CPM) can be compared with a standard calibration curve to determine the absolute quantity of ATP present in the sample. This innovative approach allows for mixing of reactants outside of the light chamber and the use of the cheaper nonrefined enzyme (bringing the cost per sample to 3¢-4¢). Inexpensive disposable scintillation vials can be used as reaction vessels.

SAMPLE PREPARATION

The object of the preparation procedure is to:

- Extract the ATP from the organisms by killing the cells.
- Deactivate the ATP-ase which would degrade the released ATP to Adenosine Di-phosphate and Adenosine Mono-phosphate.
- Separate the ATP solution from cellular and extracellular debris.

The most common procedure is to lyse the isolated cells by boiling in a tris buffer solution (pH7.7). Direct aliquots can be introduced into the boiling tris or, if the level of organisms is low, one can concentrate the cells by filtering. Other standard lysing techniques that have been used are: perchloric acid, butanol, trichloroacetic acid, arsenate buffer and DMSO. Sample preparation and lysing usually takes from 4 to 10 minutes.

BIOMASS DETERMINATION

The biomass determination advantageously utilizes the following characteristics of ATP.

- ATP is found in all living cells.
- ATP is not found associated with nonliving particulate matter.
- ATP is found in a relatively constant concentration in all living cells.
- ATP can be quantitatively assayed in extremely low concentrations by using the chemiluminescent reaction.

In this manner, the ATP Photometer can be used for cell counting, total biomass, and growth and degradation studies.

Biomass may be expressed in many different ways (such as fresh weight, dry weight, volume, etc.), but the most basic physiological parameter would be that of cellular organic carbon. Use of organic carbon to express biomass minimizes the errors involved if one has large vacuolated cells or cells such as diatoms with thick inorganic cell walls or frustules. Extensive studies have shown that all organisms thus far investigated—fresh water and marine bacteria, algae, protozoans, micro-zooplankton and macro-zooplankton—show a fairly uniform amount of ATP when compared to the cellular organic carbon content. Figure 2 shows that in all organisms in the above plant and animal groups the amounts of ATP are equal to about 0.35% of the cellular graphic carbon value. Thus the cellular organic carbon is found by multiplying the ATP value by 286.

- PRECISION TIMER SYSTEM
- LOW ASSAY COST—as low as 4¢ per assay in some cases
- SPEED OF ASSAY—75 seconds (other time frames are available)
- VARIABLE HIGH VOLTAGE CONTROL OF PHOTOMULTIPLIER TUBE
- INTERLOCK SAFETY SWITCHES TO PREVENT ACCIDENTAL PM TUBE OVERLOAD
- DARK CURRENT NULL
- ADAPTABLE SAMPLE HOLDER
- RECORDER JACK 0-10K IMPEDANCE OUTPUT

Total carbon analysis by standard combustion techniques in conjunction with the ATP measurement can thus establish live-to-dead cell carbon ratios.

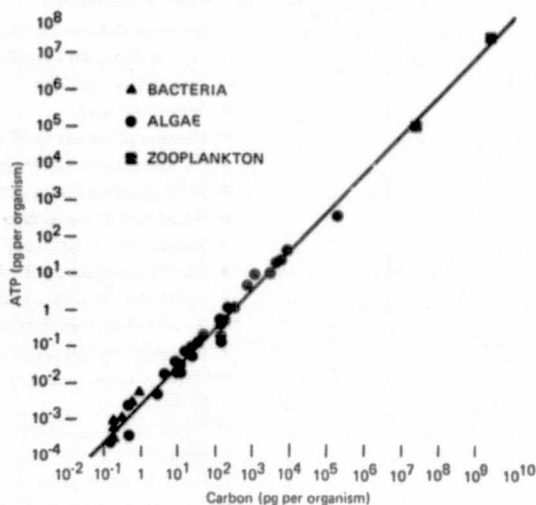


Fig. 2. Cellular contents of ATP as a function of total organic carbon for various unicellular and multicellular organisms.



APPLICATIONS

The ATP luciferin-luciferase reaction has been successfully applied to:

- Biomass determination in ocean profiles
- Eutrophication studies in fresh water
- Food chain studies
- Blood analysis
- Urine and spinal fluid analysis
- Routine bacteria fermenter analysis
- ATP coupled and metabolic studies
- Yeast cell assay in beer and wine
- Activated sludge assay
- Sterile solution control
- Anaerobic bacteria assay in oil-water samples
- Bacterial contamination of dry foods
- Bacterial contamination of prepared foods
- Cellular propagation and degradation (rate) studies
- Dairy products assay
- Microflora assay
- Potable water assay
- Control of biocide additions to open systems
- Cellular enzyme studies

OPTIONAL ACCESSORIES

- Enzyme Kinetics Kit — to study the entire exponential decay curve and peak height measurement
- Analog recorder
- Alternate integration periods for specialized studies

SPECIFICATIONS

Power requirements: Both 115V and 230 VAC \pm 10%
48 to 440 Hz, 24 watts
Size: 7 in. wide by 11 in. high by
11½ in. deep
Weight: 12 lbs.
Recorder output: 0-10V max., 10k impedance

To Place an Order Write:
JRB Inc., P.O. Box 1393, La Jolla, CA 92037
or Call: (714) 459-2601

APPENDIX B
MANUFACTURERS AND MATERIALS

Sigma Chemical Company
P. C. Box 14508
St. Louis, Missouri 63178
314 - 771-5765

Crude Firefly Lantern Extract, Cat. No.
FLE-250

Adenosine-5' -Triphosphate (ATP)
Crystalline disodium salt, Cat. No.
A3127

Apyrase, potato purified, grade 1
Cat. No. A6132

D-L Malic Acid, Cat. No. M0875

Trizma Base, Cat. No. T1503

Triton X-100, Cat. No. T-6878

Sodium Sulfate, Anhydrous, Cat. No.
S-S9627

McGaw Laboratories
Division American Hospital Supply
Corporation
Glendale, California 91201
213 - 246-6521

0.9% Sodium Chloride (Normal Saline)
Cat. No. R1201

Distilled Water, Cat. No. R-1001

E. I. DuPont DeNemours & Co.,
Inc.
Instrument Products Division
Wilmington, Delaware 19898
302 - 774-1000

(Luciferase) Luminescence Biometer
Reagent Kit, Cat. No. 760145

Biometer, Dupont 760

Mallinckrodt Laboratory Chemicals
223 West Side Avenue
P. O. Box 384
Jersey City, New Jersey 07303
201 - 432-2500

Calcium Chloride, Anhydrous,
Cat. No. 4136

Fisher Scientific Company
711 Forbes Avenue
Pittsburg, Pennsylvania 15219
412 - 781-3400

Acetone, Cat. No. A-18

Magnesium Sulfate Crystals, Cat. No.
M-63

Methylene Chloride (dichloromethane)
Cat. No. D-123

Nitric Acid, Cat. No. A-200

Falcon Plastics
1950 Williams Drive
Oxnard, California 93030
805 - 485-8711

Centrifuge Tube 17 X 100 mm,
Polypropylene - uncapped, Cat. No. 2018

Centrifuge Tube 17 X 100 mm,
Polypropylene - with cap, Cat. No. 2059

Millipore Corporation
Bedford, Massachusetts 01730
800 - 225-1380

2.5 ml Filter Unit Cathivex 0.22 μ M
25 mm, Cat. No. SKGS 025 05

Beckton-Dickenson
Stanley Street
E. Rutherford, New Jersey 07070
201 - 939-9000

Needles, 22G, 1 1/2" length
disposable Luer-Lok, Cat. No. 5156

1.0 cc Syringe-disposable tuberculin,
Cat. No. 5602

Scientific Products
Division of American Hospital
Supply Corporation
8855 McGaw Road
Columbia, Maryland 21045
301 - 997-3300

20.0 ml Sample Containers for Coulter
Counter, Cat. No. B3157-7

Detergent 1-2-2 Concentrate (hand
cleaning glassware), Cat. No. C6312-1

Detergent 1-2-2 Lo-Suds (for dish-
washer), Cat. No. C6312-5

American Instrument Company
Div. of Travenol Laboratories, Inc.
8030 Georgia Avenue
Silver Spring, Maryland 20910

Chem-Glow Photometer, Cat. No.
4-7441

Lab-Line Instruments, Inc.
Lab-Line Plaza
Melrose Park, Illinois 60160

Lab-Line ATP Photometer, Cat. No.
9140

Coulter Electronics, Inc.
590 W. 20th Street
Hialeah, Florida 33010
305-885-0131

Coulter Counter

APPENDIX C

REFERENCE DATA

CONVERSION TABLE

Micromoles ATP to Micrograms ATP

10^0	$\mu\text{M} = 551.00 \mu\text{g}$
10^{-1}	$\mu\text{M} = 55.10 \mu\text{g}$
10^{-2}	$\mu\text{M} = 5.51 \mu\text{g}$
10^{-3}	$\mu\text{M} = 5.51 \times 10^{-1} \mu\text{g}$
10^{-4}	$\mu\text{M} = 5.51 \times 10^{-2} \mu\text{g}$
10^{-5}	$\mu\text{M} = 5.51 \times 10^{-3} \mu\text{g}$
10^{-6}	$\mu\text{M} = 5.51 \times 10^{-4} \mu\text{g}$
10^{-7}	$\mu\text{M} = 5.51 \times 10^{-5} \mu\text{g}$
10^{-8}	$\mu\text{M} = 5.51 \times 10^{-6} \mu\text{g}$

Femtograms ATP to Micrograms ATP to Micromoles ATP

10^9	$\text{fg} = 10^0 \mu\text{g} = 2 \times 10^{-3} \mu\text{M}$
10^8	$\text{fg} = 10^{-1} \mu\text{g} = 2 \times 10^{-4} \mu\text{M}$
10^7	$\text{fg} = 10^{-2} \mu\text{g} = 2 \times 10^{-5} \mu\text{M}$
10^6	$\text{fg} = 10^{-3} \mu\text{g} = 2 \times 10^{-6} \mu\text{M}$
10^5	$\text{fg} = 10^{-4} \mu\text{g} = 2 \times 10^{-7} \mu\text{M}$
10^4	$\text{fg} = 10^{-5} \mu\text{g} = 2 \times 10^{-8} \mu\text{M}$
10^3	$\text{fg} = 10^{-6} \mu\text{g} = 2 \times 10^{-9} \mu\text{M}$
10^2	$\text{fg} = 10^{-7} \mu\text{g} = 2 \times 10^{-10} \mu\text{M}$
10	$\text{fg} = 10^{-8} \mu\text{g} = 2 \times 10^{-11} \mu\text{M}$
1	$\text{fg} = 10^{-9} \mu\text{g} = 2 \times 10^{-12} \mu\text{M}$

1 gram (g) = 1000 milligrams (mg)

1 mg = 1000 micrograms (μg)

10^{-15} g = 1 femtogram (fg)

Milliequivalent = Normality \times volume

Final Normality = $\frac{\text{Normality} \times \text{volume}}{\text{Total volume}}$

DILUTIONS

TEN-FOLD SERIAL DILUTIONS

1. One part (ml) original sample + 9 parts (ml) diluent = 1 to 10 dilution of original sample or 10^{-1} dilution of original sample.
2. One part 10^{-1} dilution of original sample + 9 parts diluent = 1 to 100 dilution of original sample or 10^{-2} dilution of original sample.
3. One part 10^{-2} dilution of original sample + 9 parts diluent = 1 to 1000 dilution of original sample or 10^{-3} dilution of original sample, etc.

TWO-FOLD SERIAL DILUTIONS

1. One part (ml) original sample + 1 part (ml) diluent = 1 to 2 (1:2) dilution of original sample.
2. One part 1:2 dilution of original sample + 1 part diluent = 1 to 4 (1:4) dilution of original sample.
3. One part 1:4 dilution of original sample + 1 part diluent = 1 to 8 (1:8) dilution of original sample, etc.

OTHER DILUTION CALCULATIONS

To make a dilution of 1 to 100 (1:100),

1 part original sample + 99 parts diluent

or

1 part original sample + 9 parts diluent = 1:10 dilution of original sample

1 part 1:10 dilution of original sample + 9 parts diluent = 1:100 dilution of original sample

To make a dilution of 1 to 23 (1:23),

1 part original sample + 22 parts diluent

EXPONENTIAL EQUIVALENTS

$$\begin{array}{l}
 1 = 1 \times 10^0 = - \quad - \quad - = 0.1 \times 10^1 = 0.01 \times 10^2 \\
 10 = 10 \times 10^0 = 1 \times 10^1 = 10^1 = 10 \times 10^0 = 0.1 \times 10^2 = 0.01 \times 10^3 \\
 100 = 100 \times 10^0 = 1 \times 10^2 = 10^2 = 10 \times 10^1 = 0.1 \times 10^3 = 0.01 \times 10^4 \\
 1000 = 1000 \times 10^0 = 1 \times 10^3 = 10^3 = 10 \times 10^2 = 0.1 \times 10^4 = 0.01 \times 10^5 \\
 \\
 0.1 = 0.1 \times 10^0 = 1 \times 10^{-1} = 10^{-1} = 10 \times 10^{-2} = - = 100 \times 10^{-3} \\
 0.01 = 0.01 \times 10^0 = 1 \times 10^{-2} = 10^{-2} = 10 \times 10^{-3} = 0.1 \times 10^{-1} = 100 \times 10^{-4} \\
 0.001 = 0.001 \times 10^0 = 1 \times 10^{-3} = 10^{-3} = 10 \times 10^{-4} = 0.1 \times 10^{-2} = 100 \times 10^{-5}
 \end{array}$$

Rules for handling exponents when the numbers have like bases (powers of 10).

1. In multiplication, exponents of like bases are added.

Example:

a. $a^3 \times a^5 = a^{2+5} = a^8$

b. $10 \times 10 = 10^{1+1} = 10^2$

c. $(4 \times 10^4) (2 \times 10^{-6}) = 8 \times 10^{4-6} = 8 \times 10^{-2}$

2. In division, exponents of like bases are subtracted.

a. $\frac{a^5}{a^3} = a^{5-3} = a^2$

b. $\frac{8 \times 10^2}{2 \times 10^{-6}} = \frac{8}{2} \times 10^{2+6} = 4 \times 10^8$

c. $\frac{5.6 \times 10^{-2}}{1.6 \times 10^4} = \frac{5.6}{1.6} \times 10^{-2-4} = 3.5 \times 10^{-6}$

3. Only the numbers of like bases and like exponents can be added or subtracted:

$$(7 \times 10^{-3}) + (6 \times 10^4) = (7 \times 10^{-3}) + (6 \times 10^4)$$

$$(7 \times 10^{-3}) + (6 \times 10^{-3}) = 13 \times 10^{-3}$$

EXPONENTIAL EXPRESSION OF DILUTIONS

1 part of a 1×10^{-5} dilution + 1 part diluent = 0.5×10^{-5} or 5×10^{-6} dilution

1 part of a 5×10^{-6} dilution + 1 part diluent = 2.5×10^{-6}

1 part of a 2.5×10^{-6} dilution + 1 part diluent = 1.25×10^{-6}

etc.

1 part of a 1×10^5 dilution + 1 part diluent = 0.5×10^5 or 5×10^4

1 part of a 5×10^4 dilution + 1 part diluent = 2.5×10^4

1 part of a 2.5×10^4 dilution + 1 part diluent = 1.25×10^4

etc.

RANDOM UNITS

USE OF RANDOM UNITS TABLE

From a universe of A items, to select a random sample of B items, apply the following procedure:

1. Design a method of selecting entries from the table. For example, use the entries in the first line, first column, second line, second column, etc.
2. Each item is assigned a number. If A equals 800, each item would have a number between 001 and 800.
3. Determine an arbitrary scheme of selecting positional digits from each entry chosen in step 1. For example, the first, third, and fifth digits of each entry selected may be used, thereby creating a three digit number for each entry choice.
4. If a number $\leq A$ is formed, choose the corresponding item in the universe for the random sample of B items. If a number $> A$ is formed or if a number is repeated, discard it and use the next acceptable number.

Random Units

Line/Col	1	2	3	4	5
1	10480	15011	01536	02011	81647
2	22368	46573	25595	85393	30995
3	24130	48360	22527	97265	76393
4	42167	93093	06243	61680	07856
5	37570	39975	81837	16656	06121
6	77921	06907	11008	42751	27756
7	99562	72905	56420	69994	98872
8	96301	91977	05463	07972	18876
9	89579	14342	63661	10281	17453
10	85475	36857	53342	53988	53060
11	28918	69578	88231	33276	70997
12	63553	40961	48235	33427	49626
13	09429	93969	52636	92737	88974
14	10365	61129	87529	85689	48237
15	07119	97336	71048	08178	77233
16	51085	12765	51821	51259	77452
17	02368	21382	52404	60268	89368
18	01011	54092	33362	94904	31273
19	52162	53916	46369	58586	23216
20	07056	97628	33787	09998	42698

Acid and Base Concentration Table

	Molecular Weight	Moles per liter	Grams per liter	Percent by weight	Specific Gravity
Acetic acid, glacial	60.05	17.4	1045	99.5	1.05
Acetic acid	60.05	6.27	376	36	1.045
Formic acid	46.02	23.4	1080	90	1.20
		5.75	264	25	1.06
Lactic acid	90.1	11.3	1020	85	1.2
Nitric acid	63.02	15.99	1008	71	1.42
		14.9	938	67	1.40
		13.3	837	61	1.37
Perchloric acid	100.5	11.65	1172	70	1.67
		9.2	923	60	1.54
Phosphoric acid	98	14.7	1445	85	1.70
Sulfuric acid	98.1	18.0	1766	96	1.84
Sulfurous acid	82.1	0.74	61.2	6	1.02
Potassium hydroxide	56.1	13.5	757	50	1.52
		1.94	109	10	1.09
Sodium carbonate	106.0	1.04	110	10	1.10
Sodium hydroxide	40.0	19.1	763	50	1.53
		2.75	111	10	1.11

APPENDIX D

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