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Lumione BL3000 Rapid Microbial Testing System —Rapid Detection of a Single Microorganism in Hours—

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1. Introduction

Microbial testing is a crucial component of quality-control and quality-assurance protocols for a broad range of manufactured products, including pharmaceuticals and medical devices, foods and beverages, cosmetics, and hygiene products. Conventional methods to microbial test rely heavily on culture-based methods, which typically require long periods of time—ranging from several days to several weeks—to yield results. This delay could mean that quality problems involving microorganisms in manufactured products are not detected until after the manufacturing facility has already been cleaned and sanitized, which dramatically complicates the task of identifying and addressing the pathways responsible for the contamination and poses major problems for quality control. Moreover, manufacturers often need to store products for a period before shipping, creating cash-flow problems and other financial difficulties due to storage costs. These challenges became even more acute during the COVID-19 pandemic, which created an urgent global demand for rapid product shipments, and spurred a broad spectrum of proposals for rapid microbial testing—including both direct methods such as solid-state or flow cytometry and indirect methods including immunization methods, nucleic-acid techniques, bioluminescence and fluorescence methods, impedance methods, gas measurement methods, and mass-analysis methods[1]. Today, among various rapid-testing strategies available in the marketplace, the most common method is to combine short-term cultures with high-sensitivity detection (via methods such as bioluminescence, fluorescence, or gas measurement). Because this technique is based on culture methods, it is highly correlated with conventional culture methods, which has the advantage of streamlining the validation[2] process required to establish the trustworthiness of new test methods. However, the need to grow short-term cultures means that this method remains suboptimal for rapid testing; for example, a test requiring 7 days by standard culture methods may require 3 days via short-term methods—a significant improvement in turnaround, but still a far cry from the goal of yielding results in a matter of hours. By contrast, methods such as bioluminescence, cytometry, and nucleic-acid amplification can produce results within hours, but because these methods have lower detection sensitivity—typically at the level of 10-100 CFU (colony-forming units: 1 CFU is the microorganism needed to form a single colony in an agar culture[3], and corresponds to a single microorganism)—they are difficult to control microorganisms at the same level as culture methods.

To address these challenges, we have developed the Lumione® BL3000 (Figure 1), a rapid microbial testing system that measures bioluminescence from adenosine triphosphate (ATP). Because this instrument directly detects ATP in microorganisms without the need to grow cultures, it can produce results in just hours. We have also developed specialized reagents (preprocessing reagent and luminescence reagent) and a measurement apparatus capable of detecting ultra-weak bioluminescence in real time, allowing high-sensitivity detection with an ATP limit of detection below 1 amol. For many types of bacteria, the ATP content of 1 CFU is on the order of 1 amol; thus, the detection sensitivity of our system is approximately 1 CFU, equivalent to that of the most common conventional microbial testing procedure using agar-plate cultures. The Lumione is thus a revolutionary new rapid microbial testing system capable of detecting single microorganisms in a matter of hours.

Fig. 1 The Lumione BL3000 rapid microbial testing system

2. Rapid Microbial Method Using ATP Bioluminescence

2-1. Measurement principles

The ATP bioluminescence method detects light emitted from ATP—a molecule present as an energy source in all living bacteria—via the luciferin/luciferase reaction, which proceeds as follows:

ATP + D-luciferin + O_2

↓ Luciferase Mg^{2+}

AMP + diphosphoric acid + oxyluciferin + CO_2 + luminescence

Because ATP originates from living organisms, the method of ATP bioluminescence avoids the problem of false-positive detection of non-microorganism nanoparticles, a drawback of fluorescence detection and other similar methods. However, the ATP bioluminescence method does require a preliminary step to decompose and eliminate any ATP that may have been introduced by an instrument operator or may be contained in other biological matter present in the sample.

The quantity of ATP contained in microorganisms varies depending on the type of microorganism and the state of its bacteria. For example, yeasts have large bacteria, which contain several hundred amol of ATP per CFU, while small Gram-negative bacteria are known to have roughly 1 amol per CFU[4]. Here, an amol is 1×10^{-18} mol. To convey a sense of the minuteness of this quantity, we note that adding 1/3 teaspoon of salt to Japan's Lake Biwa would change its overall salinity by approximately 1 amol.

We also note that the microorganism detection sensitivity of previous ATP-bioluminescence methods is on the order of 10-100 CFU[3], corresponding to an ATP detection sensitivity not lower than 10-100 amol.

2-2. Microbial testing

The Lumione may be used in two ways: by filtration method or mixing method.

In the filtration method, filtration and recovery are used to enrich the concentration of microorganisms in a sample solution. This method reduces the obstruction of bioluminescence by other solution components, thus yielding higher detection efficiency. However, some samples cannot be filtered or lack sufficient volume to allow filtration.

The mixing method requires only mixing certain reagents into sample solutions. In addition to being an extremely simple procedure, this method also allows measurements of samples that are difficult to filter. However, this method is susceptible to false-negative detection due to obstruction of bioluminescence by other components of sample solutions. Consequently, the mixing method is most appropriate for samples known to have high concentrations of microorganisms—such as when testing the effects of anti-bacterial agents—or for use in conjunction with enriched cultures.

Figure 2 illustrates the procedural flow for testing by filtration method.

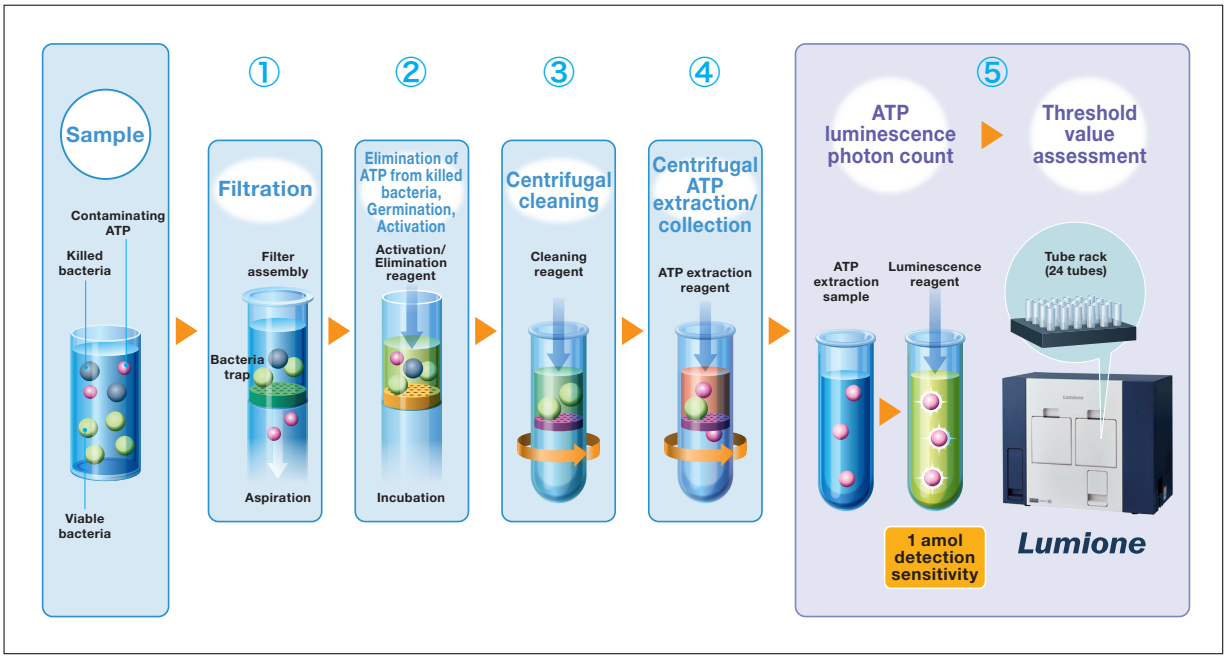


Fig. 2 Testing by filtration method

In filtration method

① A sample prepared by the same methods used in conventional culture tests is introduced into the Lumione filter assembly for aspiration. (For 100 mL of pharmaceutical water using a 100 mL funnel, the filtering process requires about 10 minutes.)

② Next, to decompose any ATP that may have been blended into the sample or that may derive from killed bacteria, as well as to activate spores, an activation/elimination solution—included in the Lumione reagent kit—is added to the filter and the contents are incubated for 40 minutes at 37°C using a block heater.

③ The filter assembly is then cleaned by adding a cleaning solution, which is subsequently removed by centrifugal filtration.

④ Next, the filter is mounted in the Lumione measurement tube, an extraction reagent is added to the filter, and the mixture is allowed to rest at room temperature for 5 minutes, after which the extraction reagent is recovered to the tube by centrifugal filtration.

⑤ The measurement tube is placed in the tube rack and the Lumione measures bioluminescence for 2.5 minutes per sample.

This procedure allows the microorganisms in 100 mL of pharmaceutical water to be detected, at a level equivalent to that of culture-based testing methods, in just 1 hour.

Figure 3 illustrates the procedural flow of testing by mixing method.

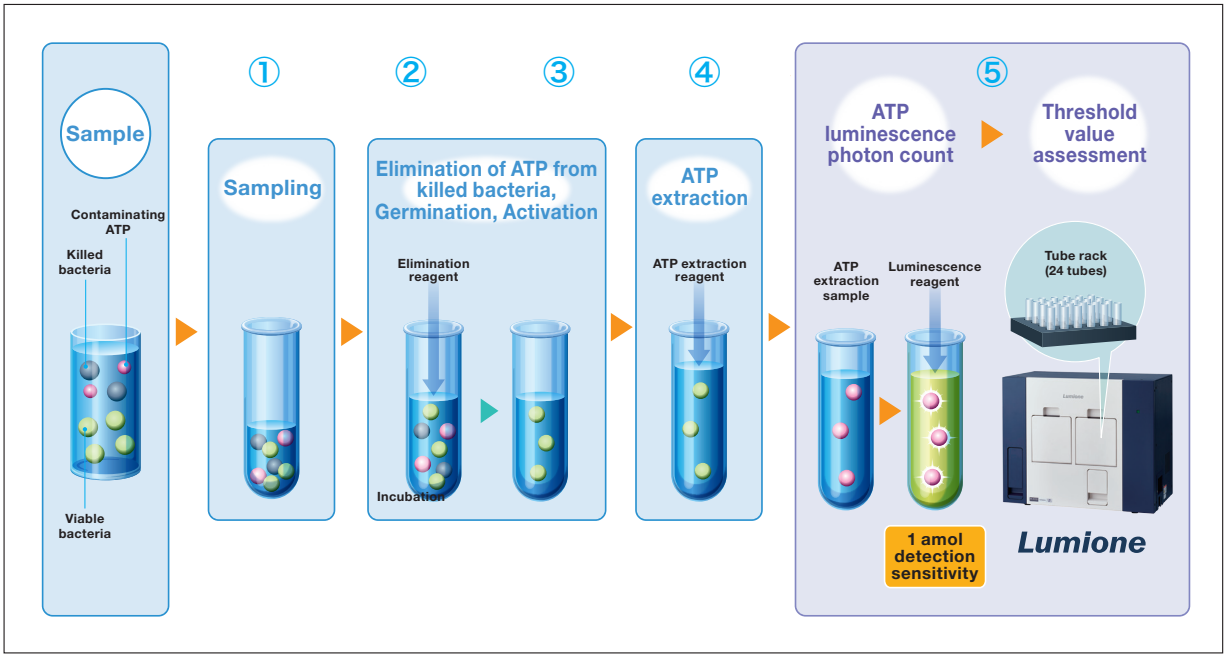


Fig. 3 Testing by mixing method

In the mixing method

① A 10 µL sample prepared by the same methods used in conventional culture tests is dispensed into a sterilized microtube.

② Next, 20 µL of a cleaning solution—included in the Lumione reagent kit—is added to the microtube and the mixture is allowed to rest at room temperature for 30 minutes.

③ Then 30 µL of an ATP extraction liquid is added to the microtube, the contents are stirred, and 50 µL is dispensed into the Lumione measurement tube.

④ The measurement tube is placed in the tube rack and the Lumione measures bioluminescence for 2.5 minutes per sample.

The Lumione measurement rack can accommodate up to 24 measurement tubes. Since the measurement of each tube takes 2.5 minutes, 24 samples can be measured in 1 hour. Also, up to 10 calibration curves can be registered with the instrument, ensuring that ATP quantities are calculated using the calibration curve most appropriate for the liquid composition of the sample being tested.

For both the filtration method and mixing method, the Lumione offers automated pass/fail evaluation, reporting whether or not a given measurement sample is in compliance based on specified warning levels and treatment levels.

3. Key features of the Lumione BL3000

In this section, we describe some key features of the Lumione BL3000.

3-1. High-sensitivity detection

The Lumione features an automated sample dispenser for addition luminescence reagent within a dark chamber from which outside light is blocked. This allows real-time measurement of bioluminescence. Figure 4 shows the results of real-time measurements of ATP bioluminescence, and a calibration curve for low ATP volumes, obtained by the filtration method.

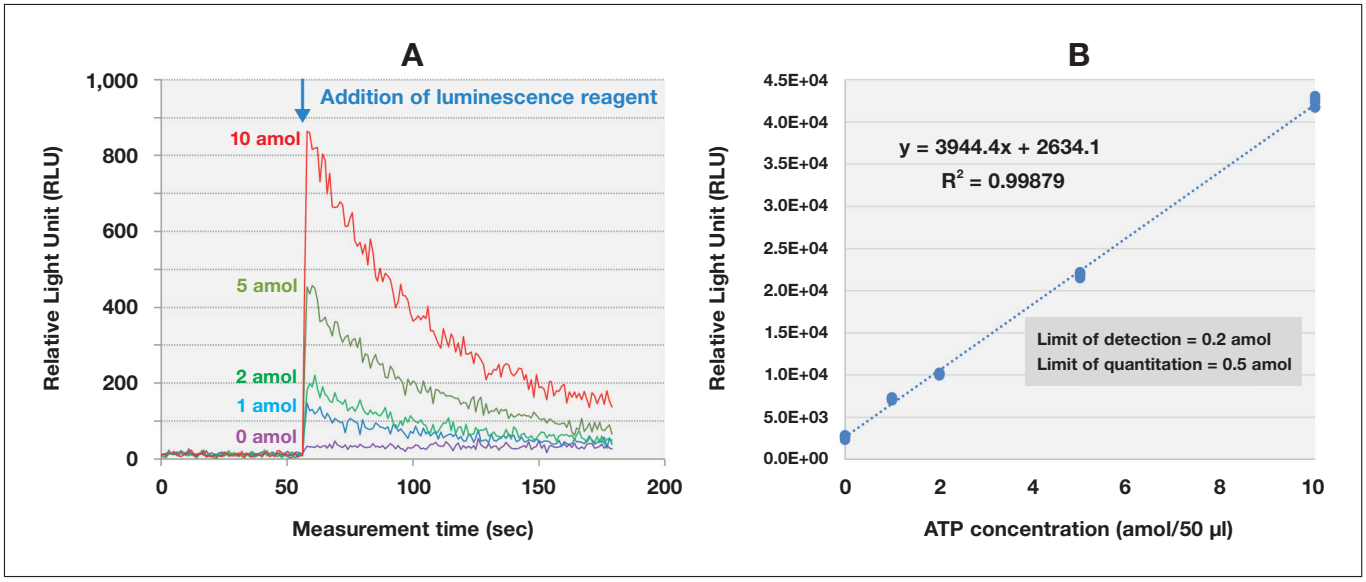


Fig. 4 Real-time measurements of ATP bioluminescence and calibration curve for low ATP volumes (filtration method)

As shown in Figure 4A, ATP bioluminescence via the luciferin/luciferase reaction is strongest immediately after the luminescence reagent has been added, and diminishes rapidly thereafter. The Lumione measures the signal before the reagent is added, accurately capturing the strongest bioluminescence immediately after its addition to ensure stable bioluminescence measurements. Furthermore, we are striving to improve the sensitivity by improving the signal/noise ratio using signal processing technology and the optical system layout using detection the bioluminescence without loss. In addition, in collaboration with reagent manufacturers we have developed specialized reagents to optimize sensitivity for detecting microorganisms at the amol level. We have also optimized the process for manufacturing various sample vessels used in Lumione measurements to prevent ATP contamination, thereby minimizing ATP background.

According to the calibration curve shown in Figure 4B, the limit of detection in this case is 0.2 amol, while the limit of quantitation is 0.5 amol. In our experience, the average limit of detection has been 0.3 amol.

Table 1 lists measured ATP volumes for various microorganisms.

Table 1 ATP content for various microorganisms

Microorganism		ATP content (amol/CFU)	
		Oligotrophic state	Growth phase
Staphylococcus aureus	ATCC 6538	1.1	3.6
Pseudomonas aeruginosa	NBRC 13275	0.3	0.5
Escherichia coli	ATCC 11775	1.2	4.6
Methylobacterium extorquens	NBRC 15911	0.7	1.9
Pseudomonas fluorescens	NBRC 15842	1.1	10
Clostridium sporogenes	ATCC 11437	1.3	8.7
Bacillus subtilis	ATCC 6633	1.6	36
Candida albicans	ATCC 10231	43	260
Aspergillus brasiliensis	ATCC 16404	15	–
Cutibacterium acnes	ATCC 11827	–	3.1

The ATP content of 1 CFU varies depending on the type of microorganism. Even the same microorganism will exhibit lower ATP content in oligotrophic states—in which nutrient sources are lacking—as compared to growth phases with nutrients in abundance. The lowest ATP content of all measured microorganisms is 0.3 amol/CFU for oligotrophic *Pseudomonas aeruginosa*. Because the Lumione's ATP limit of detection is around 0.3 amol, this suggests that our instrument should be capable of detecting a single *Pseudomonas aeruginosa* CFU. A survey of the ATP content of microorganisms reported an average ATP content of 1.5 amol/CFU for relatively small Gram-negative bacteria and 800 amol/CFU for relatively large fungi[4].

These observations indicate that the sensitivity of the Lumione is sufficient to detect a single microorganism.

3-2. Wide dynamic range

Figure 5 shows an example of the dynamic range achievable via the mixing method.

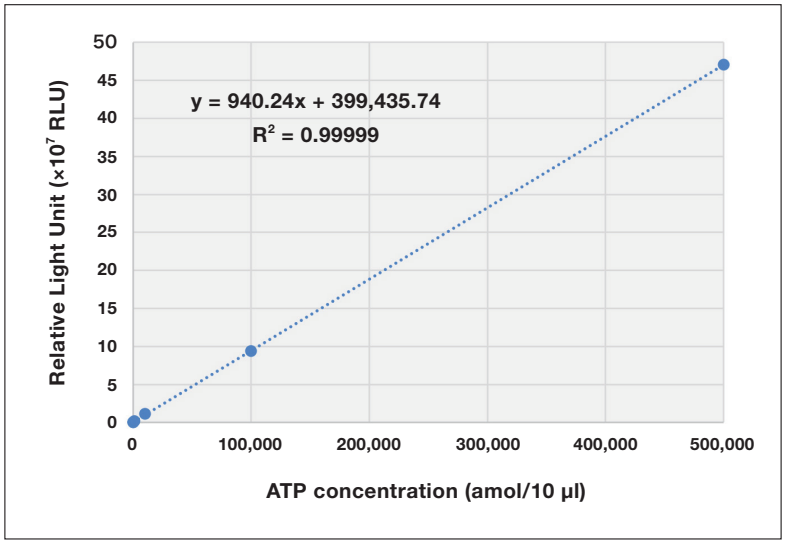


Fig. 5 Dynamic range

Over the entire range of ATP content from 0-500,000 amol, we find excellent linearity, with a correlation coefficient of 1.00. For effectiveness tests of antibacterial agents used in hygiene products, this wide dynamic range is sufficient to ensure accurate testing over five orders of magnitude without the need to dilute samples—promising a major reduction in manpower requirements.

3-3. Software feature to support data integrity

In recent years, improper use of test data in various industrial sectors has become a significant social problem, and data integrity—measures to prevent data tampering or forging and to secure the completeness and accuracy of data throughout the data lifecycle by objective standards—has become an important aspect of quality control.

In addition, CSV (Computerized System Validation) is required for measuring instruments used in pharmaceutical manufacturing. In Japan, the Ministry of Health, Labour and Welfare (MHLW) has issued ER/ES guidelines. In the U.S., the Food and Drug Administration has issued CFR 21 Part 11. In the EU, it is stipulated in Chapter 11 (Annex 11) of the EU Pharmaceutical Affairs Regulation.

The Lumione software incorporates a number of provisions to support data integrity, as detailed in Table 2.

Table 2 Primary software provisions to support data integrity

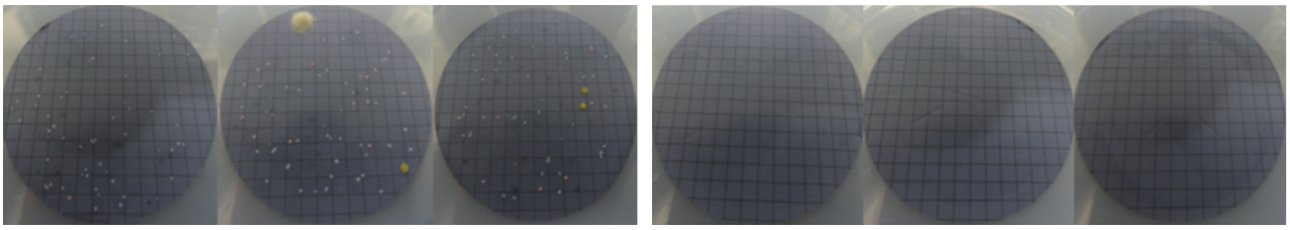
Item	Spec
User authentication	Only pre-registered users who correctly enter an ID and password may use the system
Access control	The control operation of the inspection equipment can be restricted for each registered user unit
Audit trail	The following events are automatically recorded in an audit trail: • Creation, modification, or deletion of user accounts • User login/logout • Creation, modification, or deletion of instrument settings, including measurement parameters • Creation, modification, or deletion of data Audit trail entries include the following information: • ID of user responsible for operation • Date and time of operation • Type (creation, modification, or deletion) and entry of operation
Data backup	Data and audit trails must be able to be backed up

4. Measurement Examples

For a pharmaceutical water used in the manufacture of medicines, Figure 6 compares the results of microbial tests conducted by conventional culture method and by filtration method on the Lumione.

	Method	Result	Mean
Tap water sample 1	Lumione	430 amol	460 amol
		490 amol	
	Culture	77 CFU	79 CFU
		81 CFU	
Tap water sample 2	Lumione	1000 amol	1200 amol
		1400 amol	
	Culture	65 CFU	66 CFU
		67 CFU	
Tap water sample 3	Lumione	560 amol	530 amol
		500 amol	
	Culture	66 CFU	67 CFU
		67 CFU	

	Method	Result	Mean
Purified water sample 1	Lumione	0.1 amol	0.1 amol
		0.0 amol	
	Culture	0 CFU	0 CFU
		0 CFU	
Purified water sample 2	Lumione	-0.2 amol	-0.1 amol
		0.0 amol	
	Culture	0 CFU	0 CFU
		0 CFU	
Purified water sample 3	Lumione	-0.1 amol	0.0 amol
		0.0 amol	
	Culture	0 CFU	0 CFU
		0 CFU	



Tap water sample 1 | Tap water sample 2 | Tap water sample 3 | Purified water sample 1 | Purified water sample 2 | Purified water sample 3

Fig. 6 Results of microbial tests for pharmaceutical waters

We tested three samples of tap water used at a pharmaceutical manufacturing plant, as well as three samples of purified water used at the same plant, using both conventional agar-plate methods and ATP detection via filtration method on the Lumione. For all three tap water samples, the conventional method found colonies, and the Lumione detected ATP. For all three purified-water samples, the conventional method did not find colonies, and the Lumione did not detect ATP. This indicates that Lumione testing via filtration method is capable of replicating test results obtained via conventional agar-plate culturing.

We next present a case study involving ATP measurements to test the effectiveness of a disinfectant. In these types of tests, various quantities of a disinfecting agent are added to solutions containing fixed concentrations of a known microorganism and the reduction in microorganism concentration is measured to gauge the effectiveness of the disinfectant. Because these tests involve high microorganism concentrations, they do not necessarily require high-sensitivity detection. Thus, for Lumione measurements we use the mixing method shown in Figure 3 due to the simplicity of its preprocessing requirements.

Figure 7 presents the results of disinfectant effectiveness tests performed on a disinfecting agent provided by a customer. We prepared disinfectant solutions with concentrations of 0, 0.1, 1, and 10 ppm, then added *Candida albicans* at a concentration of 7.8×10^4 CFU/mL. After 4 hours, for testing via the culture method, we prepared a dilution series, grew cultures on an agar plate, and measured the number of viable bacteria, while for testing via the Lumione, we measured ATP by the mixing method. In each case, we computed log reduction ratios quantifying the reduction in microorganism concentrations.

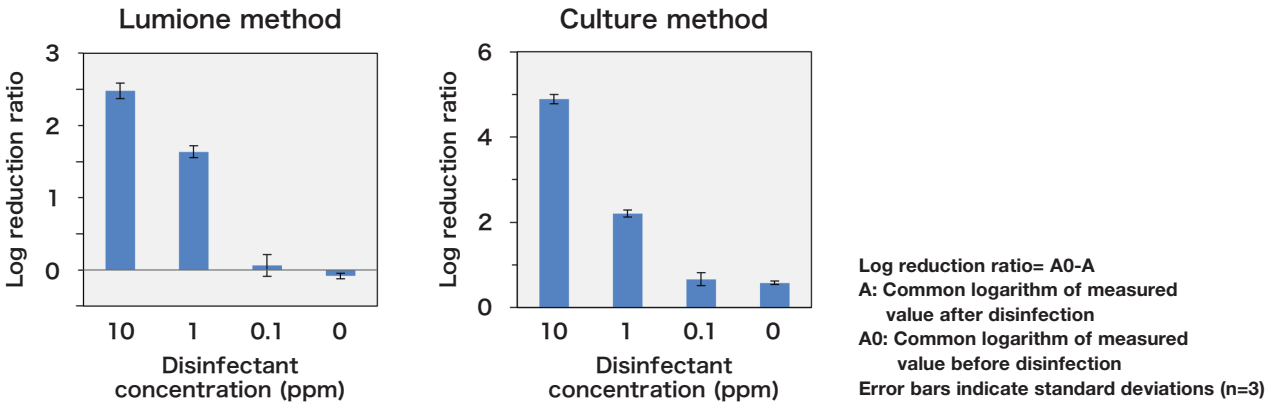


Fig. 7 Comparison of disinfectant effectiveness test results using the Lumione and the culture method

Our culture-based and Lumione tests yielded similar results for log reduction ratios for the various concentrations of disinfectant solutions. For culture tests, the concentration of each agar-plate culture (Petri dish) must be adjusted to ensure an appropriate number of colonies (between 30 and 300 CFUs), requiring preparation of two to three test samples for each disinfectant concentration—or a total of six to nine samples for the full set of n=3 concentrations. In contrast, the high dynamic range of the Lumione—spanning five orders of magnitude—eliminates the need to prepare multiple samples of varying bacteria concentrations. In the case of n=3, lumione testing requires preparing just three samples, a significant reduction in manpower.

5. Conclusions

The Lumione rapid microbial testing system yields results in a matter of hours at a high sensitivity of 1 CFU, thereby offering a number of key practical advantages:

- For water-handling equipment at pharmaceutical plants—reducing the time required to resume operation after periodic testing will increase uptime rates.
- Shortening testing times will reduce the risk of "make-to-stock."
- Microorganism controls at intermediate stages of pharmaceutical-manufacturing processes will improve risk management and enhance quality.
- When irregularities occur, minimizing the extent of their impact and preserving the environment in which they arose will facilitate rapid identification of the causes.
- Shorter testing times for disinfectants and anti-bacterial agents will improve efficiency in the development process.

In addition to promoting adoption of ATP testing in various industrial sectors—including pharmaceuticals and medical devices, foods and beverages, cosmetics, and hygiene products—we anticipate that the field of regenerative medicine will create significant new demand for microbial testing. In future work, we hope to develop new applications in which improved preprocessing methods eliminate ATP effects originating from samples, thus expanding the range of applicability of ATP-based microbial testing.

1. "Lumione" is a registered trademark of Hitachi High-Tech Corporation in Japan.

References

1) Rapid Microbial Methods <G4-6-170>, Japanese Pharmacopoeia, 17th Edition, General Information (2021) (in Japanese).

2) Validation of Analytical Procedures <G1-1-130>, Japanese Pharmacopoeia, 17th Edition, General Information (2021) (in Japanese).

3) Kikkoman Biochemifa Company, Lucifell HS Set User's Manual (2011) (in Japanese).

4) N. Hattori *et al*., *Analytical Biochemistry*, **319**, 287-295 (2003).

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