Dr Eric Bagur
*Bacteriological Quality Control Manager,
Bristol-Myers Squibb*

Concurrent Evaluation of both Compendial and Rapid Methods (ATP Bioluminescence) for Monitoring Water Quality in Pharmaceutical Manufacturing

Much has been published elsewhere about the limitations of the compendial microbiological methods and the needs of current Pharmaceutical manufacturing, in particular related to the adoption of PAT as a quality tool[1-4]. Rapid Microbiology is seen by many as a tool for addressing a number of these limitations[5,6]. Rather less has been published in terms of case histories from users demonstrating how to apply rapid methods to solve specific problems arising from the use of traditional microbiology. This paper addresses this gap by presenting studies based on experience gained from utilising a rapid method based on ATP bioluminescence (the PallchekTM Rapid Microbiology System).

Water Analysis – Limitations of the current method

Historical practice for monitoring water systems has been based on the heterotrophic plate count. In this method:

▲ Water samples are filtered through 0.45µm membranes

▲ The membranes are plated in duplicate onto R2A agar and incubated (5 days, 30°C)

▲ Colonies are counted after 5 days and the count is then compared with the alert and action limits established for the water system being monitored (see Figure 1).

> *The delay between obtaining an OOS result and taking corrective action is a major problem*

The compendial method has the advantages of being relatively easy to perform because of the low materials required and a moderate to low cost. The major disadvantages of this method are the time delay between performing the test and obtaining the results and the possibility that stressed organisms may not be detected.

The delay between obtaining an OOS result and taking corrective action is a major problem. It may cause underestimation of the real contamination level, firstly because additional growth may occur in the water system during the incubation period (particularly of stressed organisms, which may be slow growers). Underestimation of contamination level may occur resulting in inappropriate, and thus ineffective, corrective action.
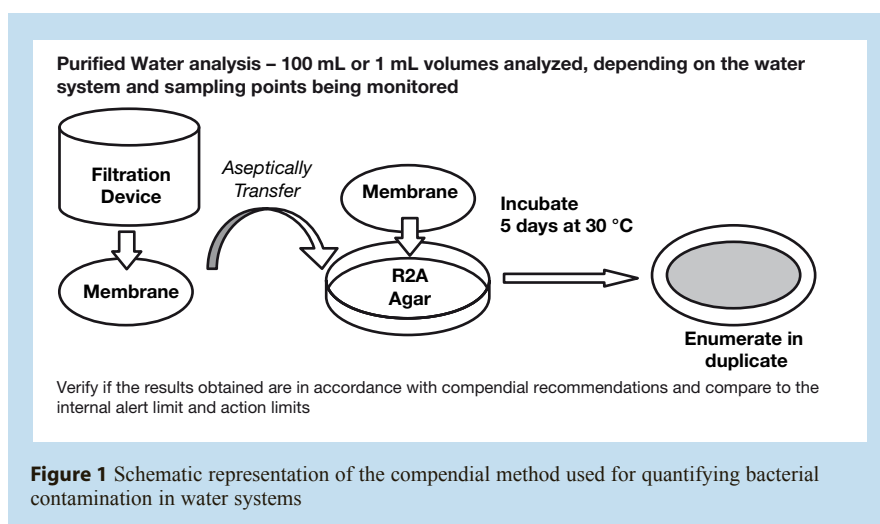

Purified Water analysis – 100 mL or 1 mL volumes analyzed, depending on the water system and sampling points being monitored

Filtration Device → **Membrane**

Aseptically Transfer → **Membrane** →

R2A Agar → **Incubate 5 days at 30 °C** → **Enumerate in duplicate**

Verify if the results obtained are in accordance with compendial recommendations and compare to the internal alert limit and action limits

Figure 1 Schematic representation of the compendial method used for quantifying bacterial contamination in water systems

Figure 2 The Pallchek™ Rapid Microbiology System

42 sample points from production loop and 30 sample points from distribution loop.

Table 1 shows action levels, which are based on the compendial methods. These are based on the historical data.

The water quality monitoring action level established for the two plants are different. They were fixed at different levels, according to comparability, between produced and distributed purified water quality and historical monitoring data.

ATP Bioluminescence as a rapid method

Adenosine triphosphate (ATP) bioluminescence is a widely-used technology, e.g. in the Food industry, as a quality monitoring tool[7,8]. It is a well accepted technology and already accepted as a sensitive method for water testing[9] having gained regulatory acceptance for the release of pharmaceutical

Water systems studied

Two distribution water systems at the Guyenne plant (existing plant) and two at the Gascogne plant (new plant) were both monitored in this study.

In the old plant, a total of 27 sample points are routinely analysed, including eight sample points from production loop and 19 sample points from the distribution loop.

In the new plant, a total of 72 sample points are routinely analysed, including

Purified Water	Control Frequency	Action Level at Guyenne (Existing Plant)	Action Level at Gascogne (New Plant)	European Pharmacopoeia recommended maximum level
Production System	Daily	15 cfu/mL 1500 cfu/100 mL	1 cfu/mL 100 cfu/100 mL	100 cfu/mL 10000 cfu/100 mL
Point of Use (Manufacturing)	Weekly	15 cfu/mL 1500 cfu/100 mL	2 cfu/mL 200 cfu/100 mL	100 cfu/mL 10000 cfu/100 mL
Point of Use (Washing)	Monthly	15 cfu/mL 1500 cfu/100 mL	15 cfu/mL 1500 cfu/100 mL	100 cfu/mL 10000 cfu/100 mL

Table 1 Specifications for water monitoring in the Gascogne and Guyenne plants

Purified Water Filtration

Step 1 – cell lysis
Add 150 µl extractant

Pall Microfunnel™

Membrane → **15 Sec** → **Membrane**

Measure ATP bioluminescence

Pallchek™ luminometer reading

Step 2 – bioluminescence detection
Add 100 µl Bioluminescence Reagent

The result obtained is compared to the threshold limit (both expressed in RLU) and correlated to the corresponding value in cfu.
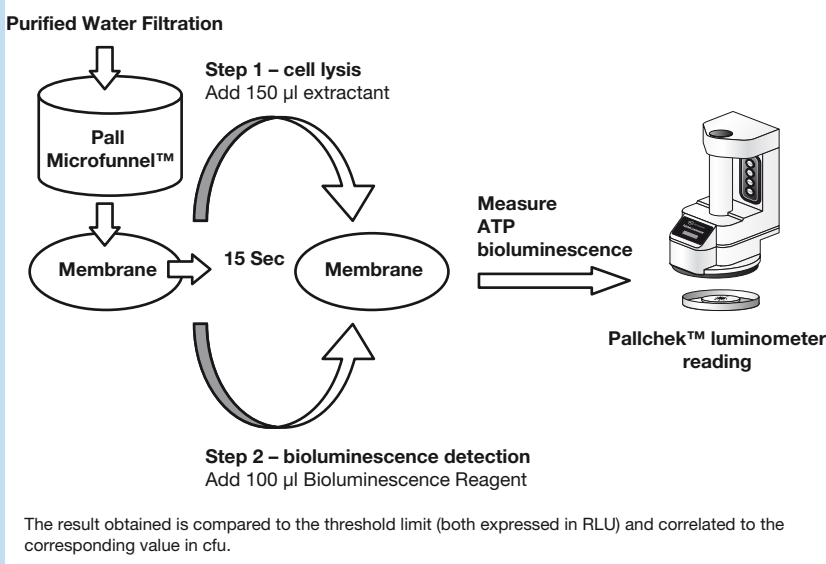
Figure 3a Direct measurement of ATP bioluminescence: main steps in the protocol

products as well as for monitoring pharmaceutical water[9].

Quantitative ATP bioluminescence measurements can be made within minutes. However, these measurements are limited in terms of detection sensitivity. This is because they directly measure the ATP content of any microorganisms in a sample. This limits sensitivity to around 100-1000 cfu.

Qualitative and semi-quantitative measurements can be used to demonstrate the presence or absence of very low levels of contaminating microorganisms within 24 hours (qualitative measurements) or to estimate the level of contamination (semi-quantitative measurements). Further details of these methods are provided in the Methods and Materials section.

Materials and Methods

Test system description

The technology evaluated was the Pallchek™ Rapid Microbiology System (see Figure 2 on page 60).

The system consists of a handheld luminometer that detects microbial contamination based on measurement of ATP. The luminometer seals onto an aluminum test plate, to create a light-tight measuring system using a flexible gasket that surrounds the measuring chamber. During a measurement, this gasket is sealed against the aluminum plate using vacuum, provided by a vacuum pump within the luminometer.

When performing a test, samples are placed onto disposable plastic trays which fit onto the aluminum test plate. Reagents are added to the sample to enable ATP measurement.

The first reagent is an extractant, which lyses any microbial cells present in the sample to release intracellular ATP. The second reagent, added after the lysis step, is a luciferin-luciferase substrate/enzyme reagent, incorporating material extracted from the firefly, *Photinus pyralis*. This enzyme/substrate reaction generates photons in proportion to the level of ATP present in the sample analysed. Photons emitted by the sample are measured using the Pallchek™

> *The luminometer seals onto an aluminum test plate, to create a light-tight measuring system using a flexible gasket that surrounds the measuring chamber*

luminometer. This instrument contains a photo-multiplier and associated circuitry for photon detection and amplification. Results are displayed on a liquid crystal display on this luminometer as Relative Light Units (RLU). One RLU represents a count of one photon per second.

Microbial cells contain relatively constant levels of ATP. This level depends on the type of microorganism, cell size, nutrient conditions, phase of the growth cycle and stress. Therefore any direct relationship between microorganism count and RLU reading must be demonstrated using standardised samples. This point is covered in more detail in the Results and Discussion section.
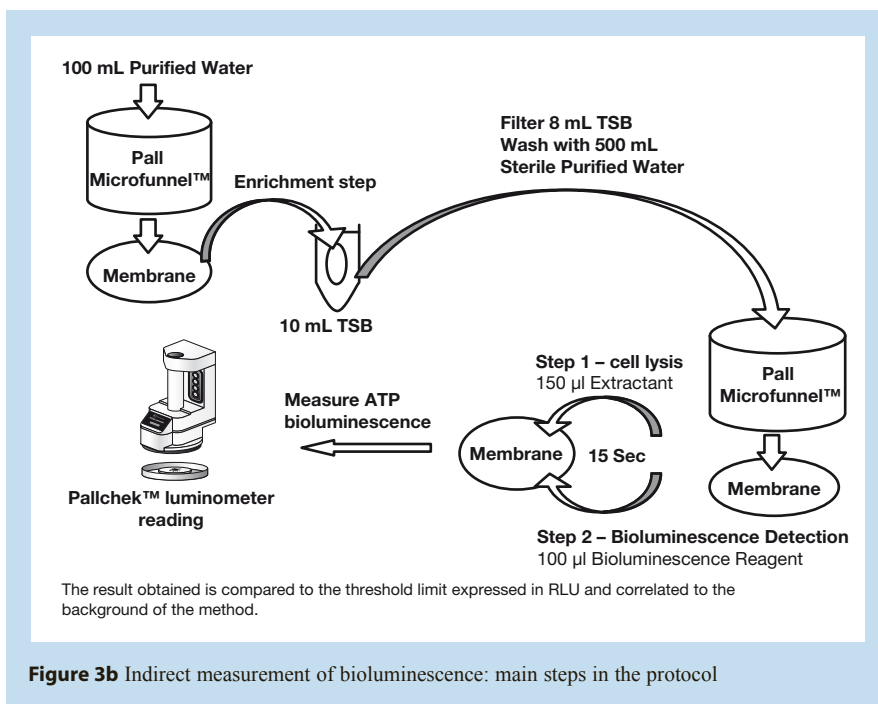

Figure 3b Indirect measurement of bioluminescence: main steps in the protocol

Normally, the system is used in conjunction with sample collected using membrane filtration. For a sample concentrated on membrane, measurements can be made in two main ways:

▲ *Direct measurement of ATP* in which a sample is collected and immediately analysed using the PallchekTM Rapid Microbiology System. This method has a detection limit of around 100-1000 cfu, based on the amount of ATP typically contained in bacterial cells and the limit of detection achievable with the reagents

and luminometer used. This method provides a result about one minute after sample filtration. Figure 3a (on page 60) shows the main steps in this method.

▲ *Indirect measurement of ATP* in which the filtered sample is inoculated into 10mL of liquid culture media and incubated overnight (enrichment step). Following incubation, 8mL of the liquid culture media is analysed. This method can detect down to one cfu and provides results within 24 hours. Figure 3b, shows main steps in this method.

Assay Number	RLU Reading at ATP Concentration (Dilution) of:						
	7.25×10^{-7} 0	7.25×10^{-8} 10^{-1}	7.25×10^{-9} 10^{-2}	7.25×10^{-10} 10^{-3}	7.25×10^{-11} 10^{-4}	7.25×10^{-12} 10^{-5}	7.25×10^{-13} 10^{-6}
1	4.2×10^7	3.9×10^6	4.2×10^5	3.2×10^4	4.0×10^3	4.5×10^2	6.8×10^1
2	4.5×10^7	3.8×10^6	4.1×10^5	3.9×10^4	4.2×10^3	4.2×10^2	7.1×10^1
3	4.0×10^7	3.8×10^6	3.7×10^5	3.5×10^4	4.0×10^3	4.3×10^2	6.9×10^1
4	4.3×10^7	3.6×10^6	3.8×10^5	4.2×10^4	4.3×10^3	4.5×10^2	7.5×10^1
5	4.1×10^7	4.0×10^6	4.0×10^5	4.0×10^4	4.4×10^3	4.1×10^2	6.8×10^1
Average	4.2×10^7	3.8×10^6	4.0×10^5	3.8×10^4	4.2×10^3	4.3×10^2	7.0×10^1
RLU average without background	4.2×10^7	3.8×10^6	4.0×10^5	3.8×10^4	4.1×10^3	3.8×10^2	1.3×10^1
Standard Deviation	1923538.4	148324.0	20736.4	4037.3	178.9	17.9	2.9
CV	4.6	3.9	5.2	10.7	4.3	4.1	4.2

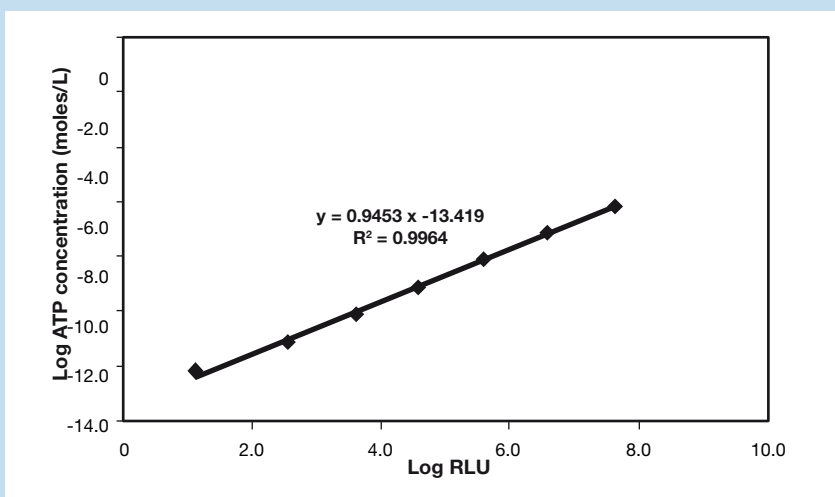**Figure 4a** ATP Correlation Curve Data

Figure 4b ATP Correlation Curve constructed from using the data in **Figure 4a**

Using samples collected by membrane filtration has several advantages. These are:

- The membrane can be washed with appropriate solutions to remove any sample components that might interfere with detection.
- Any microorganisms present in the sample are concentrated on the membrane surface.
- This maximises the amount of light signal proximal to the photomultiplier tube in the luminometer.
- Contact between the reagents used for ATP measurement and any microorganisms present in the sample is maximised.

Preliminary Study

As with any new technology, demonstration of system capability and Performance Qualification are critical components of the successful introduction of Rapid Microbiology technology. This section describes how this was undertaken for the PallchekTM Rapid Microbiology System. The steps covered in this section are:

- System suitability tests, including background measurements and confirmation of reagent performance
- Correlation of RLU with ATP
- Correlation of RLU with cfu
- Threshold value determination

(i) System suitability tests

To ensure that the system is functioning correctly, System Suitability Tests were performed at the beginning of every measurement session, following the supplier's recommendations. These tests were as follows:

- **Background measurements** – Demonstration of consistent, low, background readings minimises the possibility of recording false positive results. Background is measured in two stages, first for the instrument and second for the reagent.

To determine instrument background, the luminometer is placed on the aluminum test plate supplied with the instrument and a background reading taken. This should be ≤ 20 RLU.

To determine the reagent background, a plastic sample tray is placed on the aluminum test plate. 150 µL of extractant is added to this tray, followed by 100 µL of reconstituted bioluminescence reagent. A measurement is then taken within five seconds. The reading obtained should be < 80 RLU.

- **Confirmation of reagent performance** – This test acts as a positive control demonstrating that a specific concentration of ATP gives a reading within defined, expected, limits.

A plastic holder is placed on the aluminium test plate. 100 µL of a 10^{-9} M solution of ATP standard (produced using the manufacturer's ATP Correlation Kit) is added to the sample holder, followed by 100 µL of reconstituted bioluminescence reagent and the reading is taken.

The reading obtained is typically between 10^5 RLU and 10^6 RLU when the Pall High Sensitivity Reagent Test Kit is used.

(ii) Correlation of RLU with ATP

The production of an ATP Correlation Curve is a basic procedure used for operator training and comparison of user measurements with those obtained by the manufacturer during system validation. The procedure also allows the user to identify any drift in system performance and to qualify and compare the performance of new batches of reagents with those previously utilised.

In this method, six serial, ten-fold, dilutions of the ATP standard solution supplied by the manufacturer are made. For each of these dilutions, the RLU values are measured five times. The data obtained are used to construct a graph by plotting logarithmic values of ATP

| Stock culture dilution	RLU Reading						RLU corrected for back-ground	CFU (average of 3 replicates)
	Test 1	Test 2	Test 3	Test 4	Test 5	Average		
10^{-1}	1.6×10^5	1.8×10^5	1.5×10^5	1.4×10^5	1.6×10^5	1.6×10^5	1.6×10^5	$4.6 \times 10^6*$
10^{-2}	1.9×10^4	2.4×10^4	2.1×10^4	1.9×10^4	2.0×10^4	2.1×10^4	2.1×10^4	$4.6 \times 10^5*$
10^{-3}	4.4×10^3	5.4×10^3	3.7×10^3	4.5×10^3	4.8×10^3	4.6×10^3	4.5×10^3	$4.6 \times 10^4*$
10^{-4}	4.2×10^2	5.5×10^2	5.7×10^2	5.1×10^2	4.8×10^2	5.1×10^2	4.9×10^2	$4.6 \times 10^3*$
10^{-5}	1.2×10^2	1.1×10^2	1.2×10^2	1.3×10^2	1.0×10^2	1.2×10^2	9.8×10^1	$4.6 \times 10^2*$
10^{-6}	5.6×10^1	5.6×10^1	5.4×10^1	4.4×10^1	4.6×10^1	5.1×10^1	3.3×10^1	4.5×10^1
10^{-7}	4.6×10^1	3.5×10^1	3.4×10^1	2.8×10^1	4.3×10^1	3.7×10^1	1.9×10^1	4.0
10^{-8}	3.6×10^1	5.5×10^1	3.7×10^1	3.9×10^1	5.2×10^1	4.4×10^1	2.6×10^1	$4.0 \times 10^{-1}*$

* Extrapolated cfu reading

Figure 5a ATP bioluminescence for *Pseudomonas aeruginosa* ATCC 9027

concentrations on the x-axis and logarithmic values of RLU readings on the y-axis.
An example is presented in Figures 4a and 4b on pages 61 and 62 (see Results and Discussion).

(iii) Correlation of RLU with cfu

The second main step in system qualification is correlation of RLU readings and cfu measurements. The two main objectives of this study are characterisation of sample background and establishment of the relationship between RLU readings and cfu measurements for specific relevant microorganisms. The method used is as follows:

- ◢ Control samples are inoculated with microorganisms
- ◢ These samples are serially diluted in ten-fold steps, to produce samples covering a range of microorganism concentrations from roughly 10^1 to 10^8 cfu
- ◢ The RLU reading of each serial dilution is measured five times
- ◢ In parallel, samples are plated onto agar and incubated. These samples are used to determine the number of cfu for each sample analysed

Figure 5b Graph of data based on **Figure 5a**
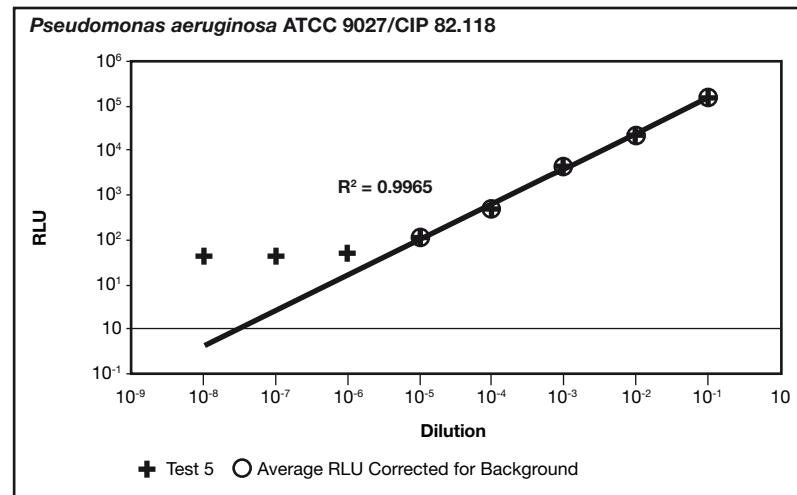
Estimated Background Threshold 4.4×10^1

	RLU Value	Dilution	Extrapolated CFU value
Detection limit (3 x background)	1.3×10^2	10^{-5}	460
Quantification limit (10 x background)	4.4×10^2	10^{-4}	~ 4000

- ◢ A correlation curve is created by plotting the logarithmic values of the cfu count (from the agar plates) on the x-axis and the logarithmic values from the RLU measurements on the y-axis.

Stock culture dilution	RLU Reading						RLU corrected for back-ground	CFU (average of 3 replicates)
	Test 1	Test 2	Test 3	Test 4	Test 5	Average		
10^{-1}	3.1×10^5	2.8×10^5	3.0×10^5	3.1×10^5	2.9×10^5	3.0×10^5	3.0×10^5	$8.36 \times 10^{6*}$
10^{-2}	2.8×10^4	3.4×10^4	3.2×10^4	3.0×10^4	2.9×10^4	3.1×10^4	3.1×10^4	$8.36 \times 10^{5*}$
10^{-3}	7.3×10^3	8.7×10^3	8.2×10^3	7.9×10^3	8.3×10^3	8.1×10^3	8.1×10^3	$8.36 \times 10^{4*}$
10^{-4}	7.0×10^2	6.8×10^2	7.1×10^2	6.5×10^2	6.7×10^2	6.8×10^2	6.7×10^2	$8.36 \times 10^{3*}$
10^{-5}	1.2×10^2	1.3×10^2	1.6×10^2	1.4×10^2	1.3×10^2	1.4×10^2	1.2×10^2	$8.36 \times 10^{2*}$
10^{-6}	9.7×10^1	7.4×10^1	1.0×10^2	8.6×10^1	7.8×10^1	8.7×10^1	7.0×10^1	8.3×10^1
10^{-7}	3.2×10^1	1.6×10^1	2.9×10^1	2.7×10^1	2.3×10^1	2.5×10^1	8.4	8.0
10^{-8}	1.6×10^1	3.4×10^1	2.8×10^1	1.9×10^1	2.5×10^1	2.4×10^1	7.4	1.0

* Extrapolated cfu reading

Figure 6a ATP bioluminescence for *Strapylococcus aureus* ATCC 6538

In addition, five control samples (no microorganisms present) were tested, to determine background.

Representative correlation curves are shown in Figures 5a (page 62), 5b (page 63) and 6a and 6b (page 64).

(iv) Threshold determination

The detection limit for ATP bioluminescence was set at three times the background level. The detection limit represents the RLU value above which RLU readings can be considered different from background.

The quantification limit for ATP bioluminescence was set at 10 times the background level. The quantification limit represents the RLU value above which RLU readings give an accurate estimation of the number of microorganisms present in a sample.

The RLU values established for the detection and quantification limits were

Estimated Background Threshold : 2.5×10^{-1}

	RLU Value	Dilution	Extrapolated CFU valve
Detection limit (3 x background)	7.5×10^1	10^{-6}	83
Quantification limit (10 x background)	2.5×10^2	10^{-5}	> 1000

Figure 6b Group of data drawn from **Figure 6a**

converted to the corresponding cfu values using the correlation curve between RLU and cfu just described. This conversion provided the number of microorganisms that corresponds to these two RLU values.

In accordance with the International Committee for Harmonisation's recommendations, a threshold limit was fixed at five times the background for all data generated using the different microorganisms tested. This corresponds to at least 1000cfu.

In accordance with the International Committee for Harmonisation's recommendations, a threshold limit was fixed at five times the background for all data generated using the different microorganisms tested

Water system monitoring

This section describes the methods used for water system monitoring. It covers:

- Sample background measurement
- Semi-quantitative measurement procedures based on direct ATP measurement
- Qualitative measurement procedures, based on indirect ATP measurement.

As mentioned previously, the method used for monitoring depended on the quality of the water being monitored.

(i) Sample background measurement

Sample background is measured for uncontaminated control water samples that have been demonstrated to contain no microbial contamination using compendial methods. For this study, control samples used for background measurement were representative of the water production systems being monitored.

These samples provided measurements that define the threshold RLU value above which readings indicate the presence of microbial contamination. The method used to analyse these samples is shown in Figure 7 on page 66. The threshold limit set based on the RLU readings obtained for these samples was five times the background RLU measurements obtained.

The RLU values obtained for these blank control samples should be ≤ 150 RLU when filtration of the samples is performed using Pall® GN-6 Metricel® membranes. Other
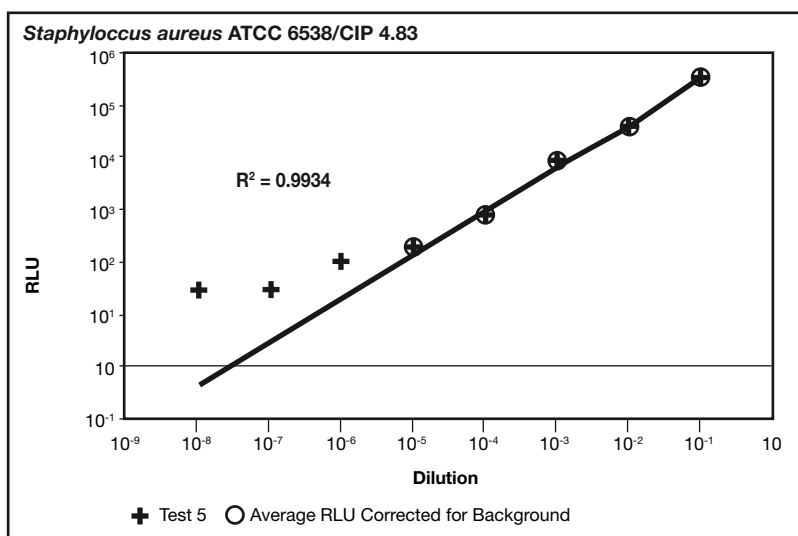
membranes may give different background values. In this case, the threshold value must be adjusted accordingly.

Several tests were performed, with different membrane washing volumes, to set up the lowest and reproducible RLU sample background value.

Blank controls are repeated during each routine test in order to reevaluate the threshold limit according to environmental conditions of testing.

Sample Volume	Case 1	Case 2	Case 3	Case 4
1000 mL	–	+	+	+
100 mL	–	–	+	+
10 mL	–	–	–	+
Contamination Level Estimated	< 1 CFU/mL <100 CFU/ 100 mL	< 10 CFU/mL < 1000 CFU/ 100 mL	< 100 CFU/mL < 10000 CFU/ 100 mL	< 1000 CFU/mL < 100000 CFU/ 100 mL

+ = RLU value > Threshold Limit (Contamination > 1000 cfu/sample volume)
– = RLU value < Threshold Limit (Contamination < 1000 cfu/sample volume)

Table 2 Direct Measurement based on different sample volumes: Interpretation of Data

(ii) Semi-quantitative procedure based on direct ATP measurement

This method was utilised when historical data demonstrated that some microbial contamination was likely to be present in water samples. In this procedure, water samples are collected and immediately analysed, providing a result within about one minute after sample filtration. This procedure is shown schematically in Figure 8 on page 66.

The main steps in this method are:

▲ Water sample collection and filtration of three aliquots of this sample. The volume of these samples is based on the expected microbial contamination level (see Table 1).

▲ Immediate measurement of ATP bioluminescence values for these samples.

▲ Comparison of the RLU reading obtained against the threshold limit determined previously. This reading is reported as presence (+) or absence (-) of contamination, based on a RLU reading either above or below the threshold value previously determined.

Table 2 summarises the relationship between sample volume, presence (+) or absence (-) result obtained and the contamination range (number of cfu contained in the sample) that can be inferred, based on correlation of RLU to cfu. Note that this table is based on a study performed during methods development, using pure ATCC cultures, not with actual contaminated samples from the water systems studied. The objective of this table is to demonstrate how actual data can be ascribed probable contamination levels, based on RLU measurements.

The advantage of direct measurement is that a real time result (data within 30 minutes) can be achieved and the method is fast and simple to perform. In addition, analysis of several sample volumes allows rapid
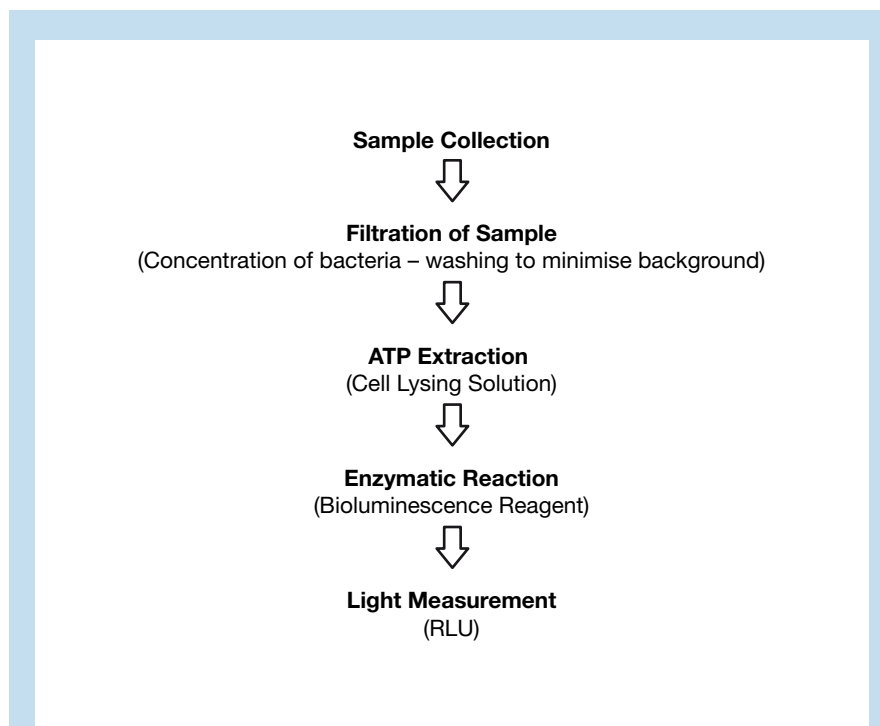
Sample Collection

⇩

Filtration of Sample
(Concentration of bacteria – washing to minimise background)

⇩

ATP Extraction
(Cell Lysing Solution)

⇩

Enzymatic Reaction
(Bioluminescence Reagent)

⇩

Light Measurement
(RLU)

Figure 7 Direct reading protocol used for samples where some contamination expected

estimation of contamination level and quickly identifies an OOS result, thus minimising the possibility of recording false positive or negative results.

The main disadvantage is sensitivity, limiting the method to water systems where there is typically a relatively high level of microbial contamination (> 1000 cfu/sample) present. For systems that deliver water quality at or near to few cells, typically 'Water for
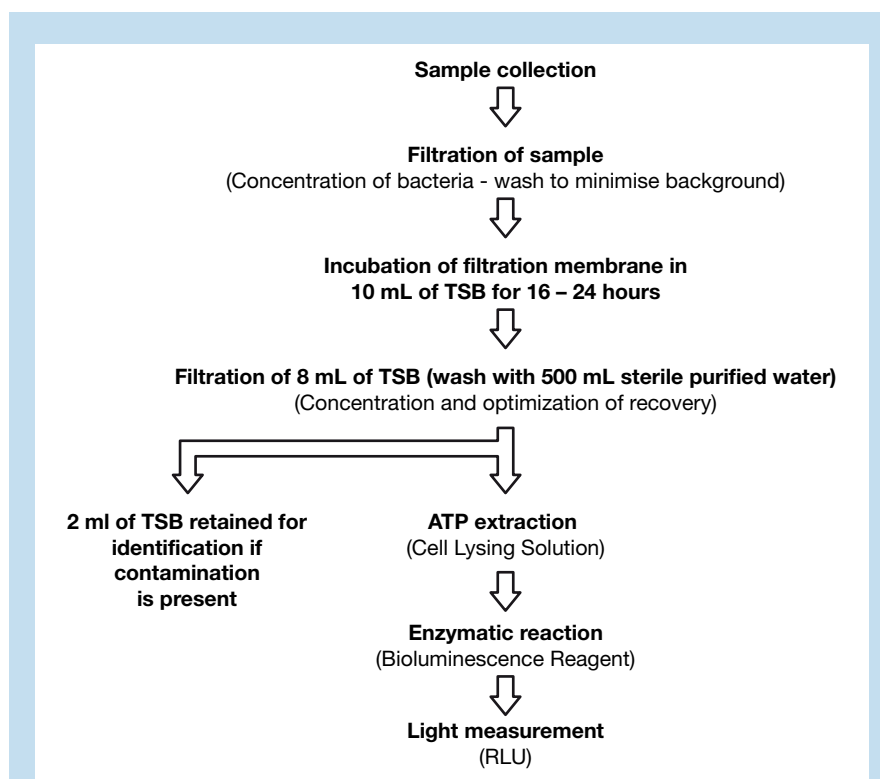

Sample collection

⇩

Filtration of sample
(Concentration of bacteria - wash to minimise background)

⇩

**Incubation of filtration membrane in
10 mL of TSB for 16 – 24 hours**

⇩

Filtration of 8 mL of TSB (wash with 500 mL sterile purified water)
(Concentration and optimization of recovery)

**2 ml of TSB retained for
identification if
contamination
is present**

ATP extraction
(Cell Lysing Solution)

⇩

Enzymatic reaction
(Bioluminescence Reagent)

⇩

Light measurement
(RLU)

Figure 8 Indirect reading protocol in the case of samples with no contaminants, or with minimal contamination expected

Injection' standards or highly 'Purified Water' systems, a different approach, described in the next section, is required.

(iii) Qualitative procedure based on indirect ATP measurement

Where the expected microbial contamination level in water samples is very low (0-100 cfu/100mL) semi-quantitative direct measurements are not suitable. This is because the RLU reading in this very low contamination range is at or below the quantification limit established during system qualification. A different approach must be used to determine if water quality is within acceptable limits.

The indirect method, described in Figure 3b, is a presence/absence method that measures ATP based on an enrichment step. This enrichment step involves membrane filtration of the sample and overnight incubation of the analysis membrane in liquid media. This step promotes microbial growth and thus amplification of the ATP bioluminescence signal obtained.

In this method, a water sample is collected and diluted twice in ten-fold serial dilutions, to provide 100mL volumes of undiluted samples and of samples at dilutions of 1/10 and 1/100. Each dilution is then filtered using a 47mm diameter analysis membrane. These membranes are aseptically transferred into 10mL volumes of liquid media. After overnight incubation, 8mL samples of this media are filtered through 47mm diameter analysis membranes. The ATP bioluminescence value of these membranes is measured, as described previously.

The background value of the method is established by analysing several control samples uninoculated with bacteria. Using the same approach as described for the semi-quantitative method, a threshold limit is set based on the mean background reading obtained. This threshold limit is set at five times the background.

A RLU result higher than the threshold limit represents a positive result (+) and means that the sample analysed contains at least 1 cfu. A RLU result lower than the threshold limit represents a negative result (-) and means that sample contains less than 1 cfu.

The data in Table 3 on page 67 summarises the possible range of results achievable after 24 hours of incubation. This table demonstrates

how the pattern of presence (+) or absence (-) results obtained for the three different dilutions can be used to estimate the contamination level in a sample, in the range of < 1 cfu/100mL to > 100 cfu/100 mL. Note that this table is based on a study performed during methods development, using pure ATCC cultures, not with actual contaminated samples from the water systems studied. The objective of this table is to demonstrate how actual data can be ascribed probable contamination levels, based on RLU measurements.

This method is very successful for a water system where very low microorganism contamination levels are expected.

The advantages of this method is that it provides a much more rapid alert compared to the compendial method, particularly when the compendial method is performed using R2A agar, where it is not possible to obtain results in less than five days.

Results and Discussion

Figure 4 shows a representative correlation curve of ATP concentration versus RLU readings. This demonstrates a very good correlation coefficient (r^2) of 0.9964 between the two sets of data. As discussed in the Materials and Methods section, construction of ATP correlation curves represent a very good training tool for this type of ATP bioluminescence method. They also allow the user to assess any drift in instrument or reagent performance, to compare reproducibility of system performance between different operators, reagents and instruments and to assess method ruggedness and robustness by deliberately varying critical aspects of the method, such as sample and reagent volumes. Reproducibility, ruggedness and robustness are critical factors (identified with others) that are recommended to be part of new technology assessment in United States Pharmacopeia[8] and European Pharmacopoeia[9].

Figures 5 and 6 show representative data that demonstrate the correlation between RLU and cfu for two microorganisms, *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027). Note that these curves are for pure cultures. They may not be representative of data obtained using natural isolates from a manufacturing environment, since environmental stresses may well reduce the amount of ATP present in any recovered micro-organisms.

Sample Volume	Case 1	Case 2	Case 3	Case 4
1	–	+	+	+
1/10	–	–	+	+
1/100	–	–	–	+
Contamination Level Estimated	< 1 CFU/100 mL	< 1 – 10 CFU/100 mL	< 10 – 100 CFU/100 mL	< 100 – 1000 CFU/100 mL

+ = RLU value > Threshold value (Contamination < 1 cfu/sample volume)
– = RLU value < Threshold value (Contamination < 1 cfu/sample volume)

Table 3 Indirect measurement of several dilutions of sample: interpretation of data

Representative data obtained from microbiological monitoring of the water systems in the BristolMyersSquibb plants at Gascogne and Guyenne are presented in Tables 4 and 5. Note that this part of the study was performed using the ATP bioluminescence method in parallel with the compendial method. Sampling methods and sampling times were different for both compendial method and bioluminescence method. In addition, tests were performed during validation of the loop (just after sanitisation or before the start of production) which makes it likely that any microorganisms present were subjected to additional stress.

Despite the differences between the compendial and alternative methods, the results obtained with both were comparable (see Tables 4 and 5). These data show that the direct measurement method is better for the evaluation of contaminated samples with an action level of around 100cfu/mL. For the direct method, a sample volume of 100ml would be more appropriate to determine if the contamination level was above or below an alert level of 10cfu/ml, which is a more stringent alert level than the actual one (20cfu/mL).

Results obtained with the ATP bioluminescence method (using the indirect measurement of ATP) show a ten fold difference compared with the results obtained using the compendial method. The difference may be due to differences in sampling and testing conditions between the two methods but may also reflect the fact that ATP bioluminescence can detect stressed bacteria that may not be detected using the compendial method. In any case, these data demonstrate good overall correlation between the compendial method and the ATP bioluminescence method, namely:

▲ Gascogne plant: when the compendial method gives a result of > 10cfu, the bioluminescence method gives a result between 1-10cfu. From analysis of three

Sampling Point	Compendial Method Result cfu/100 mL	Direct ATP bioluminescence 1000 mL	Direct ATP bioluminescence Result cfu/100 mL	Indirect ATP Bioluminescence Dilution 1	Indirect ATP Bioluminescence Dilution 1/10	Indirect ATP Bioluminescence Dilution 1/100	Indirect ATP Bioluminescence Result cfu/100 mL
A1	16	–	< 100	+	–	–	1 – 10
B2	20	–	< 100	+	–	–	1 – 10
A1	1	–	< 100	–	–	–	< 1
B2	0	–	< 100	–	–	–	< 1
A1	0	–	< 100	–	–	–	< 1
B2	11	–	< 100	–	–	–	< 1
A1	0	–	< 100	–	–	–	< 1
B2	19	–	< 100	+	–	–	1 – 10
A1	1	–	< 100	–	–	–	< 1
B2	7	–	< 100	–	–	–	< 1
A1	1	–	< 100	–	–	–	< 1
B2	19	–	< 100	+	–	–	1 – 10

Table 4 Gascogne purified water system with an action level established at 100 cfu /100 mL

Sample Number	Sampling Point	Compendial Method Result cfu/mL	Direct Method 1000 mL	Direct Method Result cfu/mL	Indirect Method Dilution 1	Indirect Method Dilution 1/10	Indirect Method Dilution 1/100	Indirect Method Result cfu/mL
1	Entry to recirculation loop	16	+	> 1 cfu	+	+	-	< 0.1–1 cfu
2	Entry to recirculation loop	33	+	> 1 cfu	+	+	+	> 1 cfu
3	Entry to recirculation loop	72	+	> 1 cfu	+	+	+	> 1 cfu
4	Entry to recirculation loop	29	+	> 1 cfu	+	+	+	> 1 cfu
5	Entry to recirculation loop	13	+	> 1 cfu	+	+	-	< 0.1–1 cfu

• Compendial method and bioluminescence rapid method were not performed in parallel using the same sample.

Table 5 Guyenne purified water system with an action level established at 100 cfu/mL

different sample volumes, the ATP bioluminescence method can demonstrate whether the contamination level is above or below an alert level of 10cfu/100ml.

▲ Guyenne plant: the results from indirect measurement of ATP are not well-suited to contamination level of samples.

This study was an evaluation of the bioluminescence method, not a validation of the method. The results obtained helped to determine the appropriate analysis method, based on the contamination level of each water system. This study also shows that wild microorganisms do not behave in the same way as pure cultures and that the analysis method must be suited to the actual contamination level of any samples analysed.

Conclusions

The Pallchek™ Rapid Microbiology System is a sensitive method for detecting microbial contamination. To obtain consistent and meaningful results, it is critical that:

▲ Accurate and aseptic techniques are used during the preparation, storage and use of reagents.

▲ Test procedures are standardised and followed exactly the same way every time that a test is performed.

▲ System suitability and other appropriate control experiments are performed.

▲ Successful operator training and continuing competency are confirmed using techniques such as the ATP Correlation Curve.

If these steps are followed, the method can be quickly implemented in routine Quality Control analysis.

This method allows simple interpretation of the results obtained and application of productive and fast tests. Application of the system as a water quality monitoring tool allows several important rapid actions to be implemented in a manner far more compliant with the objectives of PAT than is possible with the compendial methods.

These actions include:

▲ Resampling and analysis of purified water when an alert, action or specification limit is exceeded, allowing proactive corrective action to be taken.

▲ Quick negation or confirmation of an OOS result.

▲ Early alert to manufacturing to take the water system out of use as a safety measure, based on a valid OOS result (and to sanitise it as a precaution).

▲ Rapid analysis of subsequent water samples during system restart after a process excursion.

References

1. Miller, M. (2005). The impact of Process Analytical Technology, cGMP's for the 21st Century and other regulatory and compendial initiatives on the implementation of Rapid Microbiological Methods. Encyclopedia of Rapid Microbiological Methods, Volume I, pages 195-295. Published by Davies Healthcare International Publishing LLC. ISBN 1-930114-85-0.

2. Johnson, R. (2007). A "PAT" on the back for Rapid Microbiological Methods. European Pharmaceutical Review, Issue 4, pages 84 – 88

3. Newby, P.J., G. Dalmaso, S. Lonardi, B. Riley, P. Cooney and K. Tyndall (2004). The introduction of qualitative rapid microbiological methods for drug product testing. Pharmaceutical Technology, Sept 1 issue, pages 6-12.

4. Riley, B.S. (2006). Rapid Microbiology Methods in the Pharmaceutical Industry. American Pharmaceutical Review 7 (2): 28-31.

5. Dostalek, P. and T. Branyik (2003). Prospects for Rapid Bioluminescent Detection Methods in the Food Industry – a Review. Czech Journal of Food Sciences 23 (3): 85-92.

6. Stanley, P.E. (2005). Bioluminescence and Chemiluminescence Applications. Rapid Microbiology. A review of bioluminescent ATP techniques in rapid microbiology. Journal of Bioluminescence and Chemiluminescence 4: 375-380

7. American Society for Testing of Materials (2002). D 4012-81. Volume 11.01. Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water.

8. United States Pharmacopoea 31/National Formulary 26 (2008). General Chapter <1223> 'Validation of Alternative Microbiological Methods.

9. European Pharmacopoeia 5th Edition, Supplement 5.5 (2006). 5.1.6. Alternative Methods for Control of Microbiological Quality pp. 4131-4142.

Dr. Eric Bagur

Dr. Eric Bagur is bacteriological quality control manager at Bristol-Myers Squibb, France.