

LuminUltra has made several advances in ATP monitoring technology that have solved the problems that have plagued 1st Generation ATP products, while still maintaining rapid and easy-to-use methods. The result is a line of 2nd Generation ATP test kits that can be used to for microbial monitoring of almost any fluid samples, from drinking water to industrial process water, chemical products, and wastewater.

Test Kits Tailored to Specific Applications

LuminUltra initially developed its first protocol for the measurement of in ATP wastewater samples. This has traditionally been a major challenge since wastewater samples are laden with interferences, inhibitors, and represent one of the most concentrated microbial populations that one can find in in any application. Developing a reagent system advanced enough to minimize and/or neutralize inhibitors present in wastewater samples while accurately measuring all intra-cellular and extra-cellular ATP content allowed for the expansion of this 2nd Generation ATP technology into much cleaner samples. As such, LuminUltra has identified a number of additional applications for its line of test kits such as drinking water, cooling water, fuels, and specialty chemical products (e.g. latex polymers, admixtures, paint).

Quantitative Sampling

While ATP ‘pen’ devices are the most common format due to their portability and convenience, LuminUltra decided to deviate slightly from this format to focus on accuracy rather than over-simplification. Pen devices – whether swabs or ribbed dipsticks – provide inaccurate incorporation of the sample into the test. Microbial floc, clumps, slime masses, and filaments can be excluded from water picked up

by ribbed dipsticks or swabs; they can also pick up ‘clumps’ of biomass that skew the result of the test. Failing to pick up and subsequently detect these components, which can be indicative of serious problems, is a serious drawback.

In addition, dipsticks only sample approximately 50µL of a fluid sample. This small sub-sample size may not provide a true representation of the entire sample. LuminUltra has developed its protocols to ensure precise sampling, whether it is 1mL, 10mL, 100mL or more, depending on the protocol used.

Complete Extraction/Recovery of ATP

Several wastewater samples were assayed using LuminUltra’s QuenchGone21 Wastewater (QG21W) test-kit for total ATP (tATP). In conjunction, the samples were also assayed using three competitor ATP test-kits currently on the market. Refer to Table 1 for the test-kit comparison on wastewater samples.

Table 1: ATP Extraction Comparison on Wastewater Samples

Sample #	LuminUltra QG21W tATP (ng/mL)	Competitor 1 (% QG21W tATP)	Competitor 2 (% QG21W tATP)	Competitor 3 (% QG21W tATP)
1	711	35%	60%	64%
2	152	15%	20%	99%
3	549	3%	20%	48%
4	153	6%	8%	44%
5	205	6%	42%	26%
6	99	68%	58%	62%
7	1914	13%	22%	40%
8	1740	16%	24%	30%
9	3978	0%	3%	1%
10	4693	0%	1%	2%
AVG	-	16%	26%	42%

In all tests, LuminUltra’s QG21W total ATP protocol recovered significantly more ATP than the competing ATP tests. LuminUltra’s

advanced ATP protocol uses dilution and complex reagent formulations to minimize light quenching/inhibition, maximize intra-cellular ATP recovery (i.e. cell lysis), and stabilize both released intra-cellular ATP and extra-cellular ATP.

Several industrial water samples were assayed using LuminUltra's QuenchGone21 Industrial (QG21I) test-kit for total ATP (tATP). In conjunction, the samples were also assayed using three competitor ATP test-kits currently on the market. Refer to Table 2 for the test-kit comparison on industrial samples.

Table 2: ATP Test-Kit Comparison on Industrial Water Samples

Sample #	LuminUltra QG21I tATP (pg/mL)	Competitor 1 (% QG21I tATP)	Competitor 2 (% QG21I tATP)	Competitor 3 (% QG21I tATP)
1	2760	1%	7%	93%
2	2062	1%	9%	79%
3	1983	41%	26%	67%
4	1794	71%	36%	85%
5	2569	47%	36%	86%
6	2181	67%	24%	95%
7	403	42%	37%	55%
8	171	113%	72%	105%
9	310	27%	42%	69%
10	338	34%	38%	63%
AVG	-	44%	33%	80%

In nearly all tests, LuminUltra's QG21I total ATP protocol recovered significantly more ATP than the competing ATP tests. While the competing methods performed better on the process waters tested, there appeared to be a modest relationship between extraction efficiency and total bioburden, indicating that the competing extraction reagents struggled significantly with increased loading.

LuminUltra's Quench-Gone Aqueous (QGA) kit, which is designed for samples with low suspended samples, utilizes a filtration protocol to concentrate microorganisms while removing inhibition and extra-cellular ATP. The microorganisms retained on the filter membrane are lysed and intra-cellular ATP is recovered. As mentioned previously, some competing products utilize extraction reagents which are not strong

enough to lyse all cells resulting in only partial ATP recovery. For comparison, the QGA protocol was performed on various samples using LuminUltra's UltraLyse™7 extraction reagent alongside two competing extraction reagents. Refer to Table 3 for the results from this comparison.

Table 3: Comparison of UltraLyse 7 to Two Competing Extraction Reagents

Sample #	LuminUltra QGA (pg/mL)	QGA w/ Competitor 1 Extraction Reagent (% QGA ATP)	QGA w/ Competitor 2 Extraction Reagent (% QGA ATP)
1	1.5	5%	18%
2	1.1	8%	35%
3	0.25	8%	4%
4	9.72	1%	0%
AVG	-	6%	14%

For all samples, UltraLyse 7 recovered significantly more ATP compared to the competing extraction reagents. The synergistic combination of surfactants and ATP stabilization components make UltraLyse 7 and all other formulation of UltraLyse, superior to competing extraction reagents.

Quantitative Measurement of Extracellular ATP

Differentiating between intra-cellular ATP and extra-cellular ATP is necessary not only to get an accurate measurement of microbial activity, but also to assess microbial health in certain applications. Most 1st Generation ATP tests measure the entire ATP content of a sample via Total ATP, thus providing users with an inflated estimate of the living population. Some ATP tests kits provide users with protocols to measure extra-cellular ATP, although extra-cellular ATP can exist in many forms, most of which are only able to react with Luciferase in its 'free' form.

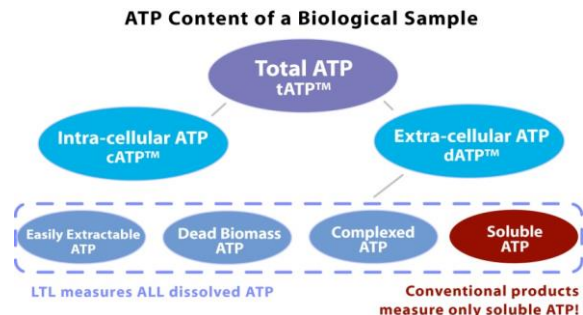


Figure 1 – The Various Forms of ATP

Extra-cellular ATP is often bound to cellular debris or complexed with other components present in a sample, such as heavy metals, cationic treatment polymers, and other inert substances. These complexed ATP molecules are unavailable to react with luciferase and as such are not included with performing an extra-cellular ATP measurement. Therefore, to get a true measurement of extra-cellular ATP, it is important to stabilize all extra-cellular ATP – free and complexed – so that it is available to react with luciferase. LuminUltra has developed a buffer (LumiSolve™) which not only re-solubilizes complexed ATP but also stabilizes all soluble ATP to obtain the most accurate measurement of extra-cellular ATP. In addition, LuminUltra's extraction reagents (UltraLyse) contain components that extract ATP from cells, re-solubilize complexed extra-cellular ATP, and stabilize ATP from all sources (intra- and extra-cellular) to provide the most accurate measurement of total ATP.

Mitigating Interferences

The method by which a sample is processed plays a big role in ATP recovery as well as the neutralization of inhibitory agents. 1st Generation ATP protocols simply require users to swab surfaces or dip a ribbed pen into a water sample. The ATP is then extracted from the microorganisms caught on the swab or ripped dipstick and the entire extract is then added directly to a luciferase reagent to be assayed. While this method is very easy, it is not very accurate. The combination of very small sample volumes and incomplete ATP extraction due to

mild releasing agents may result in lower than actual results (false negatives). In addition, if any quenching or inhibitory agents are still present after sample processing, RLU results will be even lower. Once ATP is extracted, it is important to immediately stabilize this ATP to ensure it does not complex or degrade resulting in an underestimation of the samples ATP content. LuminUltra has developed its protocols and reagents to overcome all of these issues. Several formulations of a proprietary extraction reagent (UltraLyse) have been developed and are used depending on the required potency of the sample being tested to ensure maximum ATP recovery (i.e. to achieve complete cell lysis). Various filtration and/or dilution steps are utilized to minimize the concentration of quenching and inhibitory agents so that they have no effects on the luciferase reaction. Finally, LuminUltra's reagent system contains various components that neutralize inhibitory agents and stabilize ATP so it does not degrade or bind to other components in the sample.

Biocides

To demonstrate the effectiveness of the 2nd Generation ATP measurement, several common industrial biocide solutions were prepared in LumiSolve and spiked to contain 1ng/mL of ATP. These samples were assayed with LuminUltra's luciferase/luciferin reagent (Luminase™). A control was performed by spiking LumiSolve, without biocide, to contain 1ng/mL of ATP. Table 4 shows the results from these biocide inhibition tests.

Table 4: Inhibitory Effects of Biocides on Luminase

Biocide (ppm Active Ingredient)	RLU	% Control RLU
Control (No Biocide)	19715	-
Isothiazolone (CMIT/MIT, 1000ppm Active Ingredient)	19654	100%
Methylene bis(thiocyanate) (MBT, 1000ppm Active Ingredient)	19173	97%
DBNPA (1000ppm Active Ingredient)	18946	96%
Bronopol (1000ppm Active Ingredient)	19298	98%

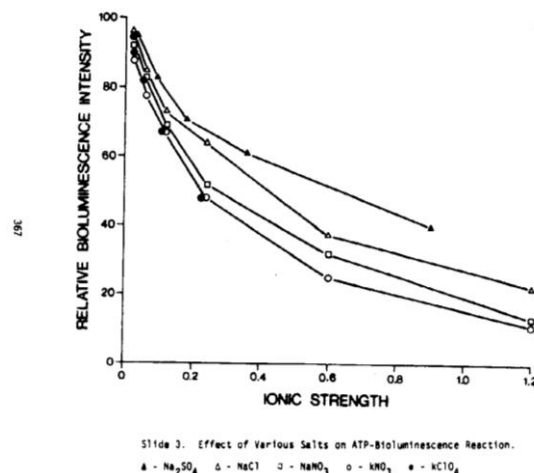
Ingredient)		
Phenol (100mg/L Active Ingredient)	17519	89%
Sodium Hypochlorite (15mg/L FAC)	17425	88%

The results indicate that even when biocides are present at very high concentrations, Luminase is not significantly inhibited and complete ATP recovery is observed. That is, the RLU readings for the biocide-ATP solutions were similar to the control ATP solution. In most cases, biocides are not present at the high concentrations tested during this validation, indicating that Luminase would be resistant to biocide levels present in real-world samples.

Salinity

It is commonly known that the total dissolved solids (TDS) concentration of an assay solution can present problems for enzymatic reactions and other microbiological quantification methods.

The following graph (Afghan et al., *An Improved Method for Quantitative Measurement of ATP and Its Application to Measure Microbial Activity in Natural Waters, Activated Sludges, and Sediments*, 2ND Bi-Annual ATP Methodology Symposium, 1977, SAI Technology Company) demonstrates that with increasing ionic strength (salt concentration), the ATP assay is severely impacted in terms of measurable signal. Once you get into the 20,000 ppm of TDS range and above, at least 50% loss of light output can be expected, which creates a severe false negative.



To demonstrate that LuminUltra's products are not impacted in this way, several salt (Sodium Chloride) samples were prepared in reverse osmosis-purified water and processed using LuminUltra's Quench-Gone Organic Modified (QGO-M) protocol. After the samples were processed, the diluted extracts were spiked to contain 1ng/mL of ATP. The ATP spiked diluted extracts were assayed to check for inhibition (i.e. ATP spike recovery). As a control, a sample of reverse osmosis-purified water with no salt added was processed using LuminUltra's Quench-Gone Organic Modified protocol and its diluted extract was spiked to contain 1ng/mL of ATP. Table 5 contains the results from these high salinity inhibition tests.

Table 5: Inhibitory Effects of Dissolved Solids (Salinity) on Luminase

Salt Concentration	RLU1	RLU2	% Control RLU
Control (No Salt)	20,306	21,594	-
0.01% NaCl Solution	22,306	22,999	108%
0.1% NaCl Solution	20,744	23,621	106%
1% NaCl Solution	21,068	20,950	100%
5% NaCl Solution	21,325	21,897	102%
10% NaCl Solution	19,308	21,128	96%
15% NaCl Solution	19,476	22,511	100%
20% NaCl Solution	20,171	19,605	95%
30% NaCl Solution (Saturated)	19,958	19,532	94%

The results indicate that when samples with high dissolved solids are processed using LuminUltra's QGO-M protocol, inhibition due to the dissolved solids is minimized. As such, these

components do not have any negative effects on Luminase. The ATP spiked into the diluted extracts of the high salinity samples was completely recovered and results were similar to ATP recovery for the control sample. LuminUltra's QGO-M method utilizes several steps during which inhibitory agents are minimized or neutralized, including a sample filtration step during which a sample is filtered through a glass microfiber filter. This essentially traps all microorganisms on the filter membrane while allowing soluble inhibitors to pass through the filter. Any inhibitors that remain on the filter after sample filtration are washed away using a specialized solvent (LumiClean™) which also assists in cell lysis. ATP is not soluble in LumiClean and therefore remains on the filter to be extracted after the LumiClean filter wash step.

Organics

Organic molecules can also pose problems in the firefly luciferase assay. To check this, several metal working fluid (MWF) solutions were prepared in reverse osmosis-purified water and processed using LuminUltra's Quench-Gone Organic Modified (QGO-M) protocol. After the samples were processed and diluted extracts obtained, the diluted extracts were spiked to contain 1ng/mL of ATP. The spiked solutions were assayed to check for inhibition (i.e. ATP spike recovery). As a control, a sample of reverse osmosis-purified water with no salt added was spiked with ATP and processed using LuminUltra's Quench-Gone Organic Modified protocol in the same way. Table 6 contains the results from these organic solution inhibition tests.

Table 6: Inhibitory Effects of Organics on Luminase

Organics Concentration	RLU1	RLU2	% Control RLU
Control (No Organics)	20,306	21,594	-
0.01% MWF Solution	21,208	22,589	103%
0.1% MWF Solution	20,281	23,328	103%
1% MWF Solution	21,315	26,030	113%
5% MWF Solution	23,531	21,477	107%
10% MWF Solution	22,523	25,619	115%

15% MWF Solution	22,679	26,611	118%
20% MWF Solution	24,288	22,863	112%

These results indicate that when samples with high dissolved organics content are processed using LuminUltra's QGO-M protocol, inhibition due to the dissolved organics is minimized. As such, these components do not have any negative effects on Luminase. The ATP spiked into the diluted extracts of the MWF solutions was completely recovered and results were similar to ATP recovery for the control sample. Again, the filtration and LumiClean filter-wash steps assist in minimizing and neutralizing the dissolved organics present in a sample allowing for accurate measurement of ATP concentration in the sample.

Metal Ions

Certain heavy metals are known to be especially inhibitory to the luciferase enzyme. Two metal salt solutions ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were prepared in ultrapure water and spiked to contain 1ng/mL of ATP and analyzed to measure ATP recovery. A control was also performed by spiking ultrapure water to contain 1ng/mL of ATP. Each solution was diluted and assayed until inhibition was eliminated (< 20% difference between dilutions). Table 7 contains the results from these biocide inhibition tests.

Table 7: Inhibitory Effects of Heavy Metals on Luminase

Dilution	1000ppm $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ RLU	1000ppm $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ RLU	Control RLU
1	140378	22724	149121
1/2	70427	20405	71232
1/4	35360	24330	36214
1/8	18282	14621	18240
1/16	8668	7339	9011

The results indicate a 1000ppm $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ solution is not inhibitory to Luminase, since ATP recovery was similar to the control ATP solution. While there was inhibition present in a 1000ppm $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ – not all of the ATP spike was recovered – this inhibition was almost

completely removed when the solution is diluted by ¼. LuminUltra's protocols often utilize at least 1/10 dilutions during sample processing, indicating that a sample with 1000ppm CuSO₄·5H₂O would not be inhibitory to Luminase after being processed.

A number of references exist that describe comprehensive experiments that were performed to identify ATP assay inhibitors; these include:

- Velazquez et al., *Quenching and Enhancement Effects of ATP Extractants, Cleansers, and Sanitizers on the Detection of the ATP Bioluminescence Signal*, Journal of Food Protection, Volume 60, Number 7, 1997, pp. 799-803.
- Chu et al., *Using ATP Bioluminescence Technique for Monitoring Microbial Activity in Sludge*, Biotechnology and Bioengineering, Volume 75, Number 4, 2001, pp. 469-474.
- Tobin at al., *An Improved Method for the Determination of ATP in Environmental Samples*, Water Research, Volume 12, pp. 783-792.

Quantitative Reporting

1st Generation ATP monitoring products force users to record and work with RLUs (relative light units) rather than converting results into actual ATP concentrations. This can be problematic, as RLU readings can be dependent on a number of other factors beyond the actual ATP concentration in the sample. These include:

- Luciferase reagent activity may vary between batches;
- Luciferase reagent activity decreases with age;
- Luciferase reagent stability is highly dependent on storage conditions (eg. room-temperature stored product will lose activity faster than refrigerated product);

- Luminometer performance may change over time or if left on for extended periods;
- Ambient temperature may drift over the course of ATP analyses

ATP standards have not been a common part of conventional ATP test kits because most ATP standards are unstable and/or expensive. LuminUltra has developed liquid-stable ATP standards (UltraCheck™) that are stable at use concentrations for years, even at room temperature. Use of an ATP standard allows users to convert RLU results into ATP concentrations, effectively taking into consideration all factors that may affect light output/detection from the luciferase assay. In addition, UltraCheck RLU values provide an immediate measure of enzyme activity and therefore an indication of the remaining useful life of Luminase.

Summary

In the context of its comparison to 1st Generation ATP testing methods, 2nd Generation ATP tests have the following advantages:

1. **Sampling Accuracy** – Ensuring a known quantity that is sufficiently large to meet sensitivity requirements significantly improves accuracy and precision of the method.
2. **Complete Extraction** – 2nd Generation methods achieve 100% ATP extraction, whereas competing products only extract and measure a small fraction of the total.
3. **Resistance to Interferences** – The biggest difference that sets LuminUltra apart from 1st Generation ATP test kit providers is that all other ATP testing products are adaptations from those used in food and hygiene applications and for that reason are often distorted by interferences (i.e. chemicals, metals, etc.). Rather than following the same path, LuminUltra re-invented this concept so that it would be free of interferences and

therefore suitable for use in fluid applications.

4. **Quantitativeness** – All 2nd Generation ATP test kits come with an ATP standard (UltraCheck 1) that converts instrument outputs into ATP concentrations and subsequently into approximations of microbial counts. This not only puts results on a more easily understood basis, but it also accounts for instrument quality, enzyme activity, temperature, and several other factors. Standardizing RLU results using the UltraCheck 1 standard enables LuminUltra's ATP tests to be used with nearly any instrument that uses a photomultiplier tube.
5. **Compensation for Dead Cells** – 2nd Generation methods can isolate living microorganisms from dead ones. Conversely, competing ATP test methods usually do not differentiate, and in the rare cases that they do they require two separate tests (doubling the cost per test).
6. **Application-Specific** – The 2nd Generation line of ATP test kits includes kits designed for specific types of samples rather than a “one size fits all” solution, ensuring that samples are processed according to their specific and unique characteristics to ensure the most accurate results.

LuminUltra's 2nd Generation ATP testing solution offers enhanced sensitivity and greater accuracy when compared to 1st Generation ATP testing products. When customers examine the total cost of ownership, LuminUltra's solution provides customers with significantly more opportunities for cost savings and improved control. In water management, for example, sensitivity limitations of 1st Generation testing would not reveal problem situations until the system had become grossly contaminated due to their lack of sensitivity. For any water or wastewater application, 1st Generation ATP testing products are unsuitable in helping to meet the high standards and expectations placed on today's system managers.

LuminUltra's 2nd Generation ATP testing solution provides superior information, thereby allowing for better operating quality and minimized risk of system instability, equipment failure, or human health impacts.