FUELSNAP

Rapid ATP Test System

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2 FUEL TESTING

Microbial growth in hydrocarbon and bio fuel systems arises because of the impossibility of keeping storage facilities sterile and the inevitable presence of water from condensation. The only remedy is preventative maintenance. Preventative maintenance is only feasible and cost effective if it is low cost and conducted with minimal disruption to operations and can be done safely without exposing the technician or engineer to the fuel or any of the substance contained in fuel additives.

The gradual introduction of biofuel into the Aviation Industry will heighten the need for testing in that sector. Several different types of test kits are commercially available for use as monitors of microbial contamination in fluids where bacteria and fungi are present. The hazards of such contamination in food processing and the medical and industrial fields have been well documented and test kits expose early signs of contamination. Typically, specific tests are needed for each industry and those for fuels are a further development of these.

2.1 NECESSITY FOR CHANGE

For some time the focus has been on identifying specific species of micro-organisms living in fuel and attempting to quantify their numbers. These tests have been largely related to specific organisms because of their particular activities in fuel. The number of species capable of living in fuel has, until recently, been limited by the ability of fuel to hold a water droplet sufficient in size to support the microbial habitat. Fuel sources and structures have however recently changed dramatically, and so has the profile of organisms found therein. While the introduction of biofuels is critical to the energy future of every country the related changes in chemical structure meant a change in the way the water distributes and sits in fuel.

The growth of micro-organisms in fuel is inhibited by a number of factors, but a key issue promoting their growth is the availability of water.

An important element is the size of water droplets, measured and expressed as water activity[1] (a_w). Most bacteria won't grow at <0.95$a_w$but some will thrive at =0.75a_w. Many fungus species grow and survive =0.6a_w.[2]The addition of highly hygroscopic biofuels dramatically increases water levels and the water droplet sizes. While traditional hydrocarbons will contain water droplets up to a level of 200 ppm, biofuels hold up to 1500 ppm and the measured water activity tends to start as 0.75a_w to ensure optimal fuel quality. This creates an environment where more species of bacteria and fungus can flourish.

[1]Water activity indicates the amount of water in the total water content which is available to micro-organisms. Each species of micro-organism (bacteria, yeast and mould) has its own minimum aw value below which growth is no longer possible.

[2]Fuel and fuel system microbiology By Frederick J. Passman

Added to this, the ability of microbes to survive and grow in legacy fuels is also inhibited by the lack of suitable nutrients and the pH balance. However, modern biofuels add various extra nutrients which create a more and more ideal environment for organisms to flourish.

The identification process of these microbes is very difficult because the composition of the fuels we use continuously changes and therefore the microbe's environment also changes. This allows a wider and wider range of organisms to inhabit fuel. Finding appropriate tests is further complicated by their complex nature where cannibalism, parasitism and symbiotic relationships are the rule.

2.2 MEASURING ACTIVITY RATHER THAN QUANTITY

Traditional methods focus on quantifying the number of microbes present in specific volumes of fuel. These methods however pose a number of issues mathematically. CFU counts rely on the principle of colonies growing in specific environments. Any possible change in this environment can alter the profile of the organisms capable of surviving in the specific conditions in the fuel tank. Alternative approaches rely on the law of averages that certain microbes will always be present.

Both methods aim to establish a low, medium and high base line.

The risk of a low base line lies in the potential for the conditions where microbes cannot grow and multiply too rapidly change to a positive environment allowing even as little as one or two organisms to rapidly multiply and form larger colonies.

While the growth curve for each organism is different, the risk lies in setting a mid-level that might be too close to the point where growth accelerates 10 fold with costly consequences.

Rather than attempting to determine a statistical average of microbial numbers, it is possible to monitor change in activity using ATP technology.

Increased activity = increased numbers

By measuring activity, it is not only possible to monitor change but also to trend and predict. A measure of activity rather than quantity provides a trend with a time line. A trend allows us to eliminate anomalies and forecast a time frame for action. A trend also allows us to monitor remedies and adjust them accordingly to maximize effectiveness.

2.3 MONITORING CHANGE

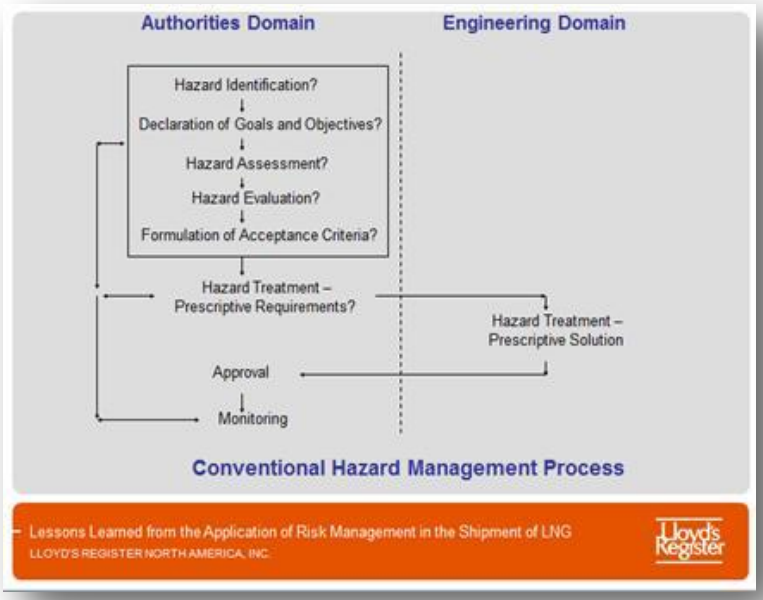
We need to be able to monitor change. The only reliable method to determine the impact of all organisms is the ability to monitor change. What we need to know is how these colonies survive, and when do they start to multiply forming new colonies, how rapidly a colony grows to the point of critical mass before it breaks up.

We should also be able to distinguish if are dealing with fungal or bacterial infestation. The ability to distinguish between the two allows us to determine the risk levels between microbial induced corrosion, and fungal matting of slime.

2.4 MEASURING THE RATE OF CHANGE

Testing forms a key part of any risk management model. If it cannot be tested, it cannot be measured; if it cannot be measured it is unable to be controlled. The engineering environment changes constantly and so does the environment where microbes live. When samples are taken there are many factors which can make testing inaccurate. Living organisms respond to UV exposure, changes in temperature and lack of water; some may die while others thrive. Accuracy and therefore assessment of risk generally suffers if samples older than 24 hours are tested.

Estimating the risk on a universal profile such as the number of cells, or specific types of organisms, or colonies based on a very small sample from a very large tank, can result in a high margin of error. Water moves and redistributes itself constantly through a fuel tank as temperature changes occur either due to position to the sun, thermal radiation, or altitude etc. The accuracy of these tests are further complicated by biofuels that tend to stratify with these temperature changes.



The best solution is for an accurate but simple test to be provided where any engineer can quickly take a measurement, anywhere in the world. With one that tracks all living organisms in a fresh sample, decisive and timely action can be taken to avoid the cost of failure and shutdown. To eliminate anomalies the engineer should be able to upload the results to a central database where it can immediately be compared against historical data. In this way, false positives can be avoided and it becomes possible to predict problems before they become serious; in other words, he can take charge of and manage the risk to his systems.

With sufficient and simple data based on time and location, the engineer can also identify the source and push risk management down the line, protecting all the assets in his system and putting remedial cost back onto suppliers.

3 WHY ATP TESTING?

Adenosine Tri-Phosphate (ATP) Testing provides a practical, rapid and easily repeated solution to the difficulty of testing fuel for microbial activity in hydrocarbons.

The relationship between the amount of ATP on the sample and the Relative Light Unit (RLU) result reading on the Luminometer is simple:

High contamination = Large amount of ATP = More light produced in reaction

= High RLU reading

The RLU reading is directly proportional to the amount of ATP collected from the sample. A high RLU reading indicates a large amount of ATP at the test location. This in turn indicates the presence of contaminants.

Less ATP results in less light output during the bioluminescent reaction and consequently, a lower RLU reading.

4 KEY FACTS ABOUT ATP TESTING

- ATP TESTING METHODS DETECT LEVELS IN INDIVIDUAL LIVING CELLS.
- ATP DOES NOT DIFFERENTIATE MICROBIAL TYPES BUT DETECTS THEM ALL EVEN AT LOW LEVELS.
- ATP IS QUICKLY PRODUCED AND QUICKLY DISAPPEARS FROM THE CELL
- ATP IS MADE ‘ON-DEMAND’ AS IT IS ‘THE ENERGY’ MOLECULE IN A CELL
- ATP IS ONLY PRESENT WHEN THE CELL IS USING OR TRANSFERRING ENERGY SO IT MEASURES VIABLE ACTIVITY ONLY
- DEAD CELLS CONTAIN NO SIGNIFICANT LEVELS OF ATP
- ATP LEVELS CHANGE WITH THE LIFE-CYCLE OF THE CELL SO THE CORRELATION WITH CFU’s AND OTHER SCALES ARE PURELY THEORETICAL
- ATP LEVELS CHANGES WITH CELL TYPE AND CAN APROXIMATE THE NUMBER OF CELLS UNDER SOME CONDITIONS
- ATP DETERMINES THE PRESENCE OF CELLS AT LOW LEVELS AND IS VERY SENSITIVE
- DORMANT CELLS SUCH AS SPORES DOES NOT PRODUCE ATP AND QUICK INCUBATION IS REQUIRED

5 BACKGROUND TO ATP TEST SYSTEMS

ATP testing has been introduced as a measure of fuel testing for a number of years now and carries approvals under ASTM D7463 Developed by Sub Committee: D02.14 and ASTM D7687 - 11 Standard Test Method for Measurement of Cellular Adenosine Triphosphate in Fuel, Fuel/Water Mixtures, and Fuel-Associated Water with Sample Concentration by Filtration.

ATP is a chemical produced by living cells and found in almost every environment where organic matter is present. ATP is the energy transfer molecule used as a starter by enzymes in catalyst reactions.

While ATP is mainly associated with living cells it exists as free ATP (fATP) and cellular ATP (cATP). ATP can thus be used as a basic indicator for the presence of organic matter. By inducing cell lysis in ATP, it can be used as a more accurate measure to quantify microbial mass.

ATP reacts with a light emitting pigment called Luciferin. Luciferin is found in fireflies and is responsible for the glow in the dark phenomena. Luciferin produces light when it is combined with an enzyme and ATP. This reaction, and the intensity of this reaction, depends on the level of ATP present, and can be measured with an adequately sensitive Luminometer and expressed in RLU's (Reflective light units).

The RLUs measured will equate directly to the level of viable organic matter present.

6 THE FUELSNAP™ ANALYSER

FuelSnap contains the world's first liquid-stable bioluminescence reagent. The single liquid reagent provides unprecedented accuracy and reproducibility.

The FuelSnap™ ATP monitoring system is a simple, easy to use, handheld instrument fully integrated with the FuelTrend™ Software platform allowing for extensive tracking and reporting on current and historical data. This system uses state-of-the-art photodiode technology and is designed to be simple and user-friendly. This revolutionary palm-sized instrument is an extremely sensitive, accurate and affordable fuel monitoring system.

It is based on a platform widely used by the largest food processors in the world, hospitals, restaurants, supermarkets and other manufacturing industries where rapid detection of contamination is crucial. FuelSnap™ allows companies quickly to determine the cleaning efficiency and hygienic status of surfaces and water, validates HACCP/SSOP programs, ensures food safety, and leads to improved product quality and reduction in costs.

Used with the FuelSnap™ test pen, extremely low levels of contamination can be detected in just 15 seconds. The FuelSnap™ monitoring system provides companies with a quick and easy way to monitor fuel quality, help ensure business continuity and reduce the risk and costs.

FuelSnap Analyser - Key Features:

- Light and user-friendly
- Simple robust design
- Results in 15 seconds
- Sensitive: detects down to 0.1 femtomole of ATP
- 20 programmable test plans
- 250 programmable locations per test plan
- Location name identification on screen
- Programmable user identification
- Stores 2000 results
- FuelTrend data analysis software to receive and analyse data from Analyser
- Self-calibrating
- Removable protective inner pocket for easy cleaning
- Low voltage (2 x AA batteries runs 1000's of tests)
- Low maintenance
- Integral protected USB connector
- Large easy-read display screen
- Easy navigate 7 -button keypad
- IP65 compliant (water resistant) and Shock Resistant

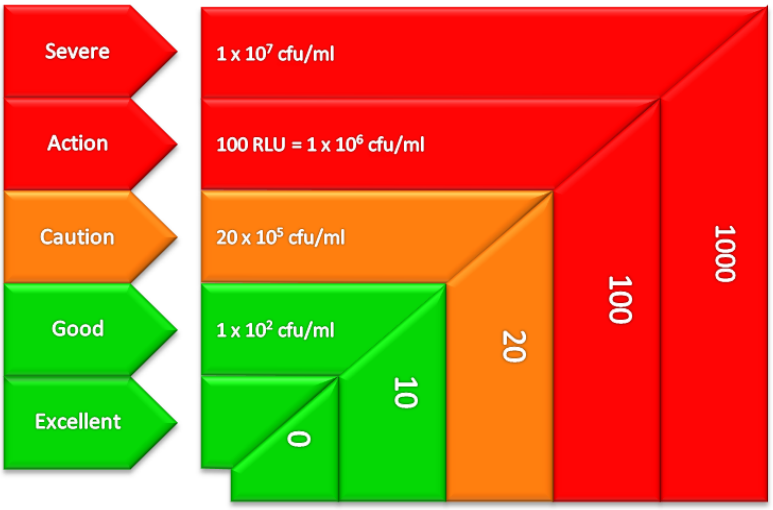
FuelSnap Test Pen - Key Features:

The FuelSnap™ pen is a user-friendly, self-contained ATP water testing device, used in conjunction with the FuelSnap™ Analyser. This small testing device is easy to use, economical, and gives real time results. The honeycomb-shaped dipper collects 100µl of fuel ensuring consistent sample collection. FuelSnap™ uses a liquid-stable reagent in place of freeze-dried enzymes, giving superior reproducibility and sensitivity.

- All-in-one, low-cost, one-shot testing device
- Simple. quick, and easy to use
- Results in seconds
- Unique liquid-stable reagent provides unprecedented accuracy and reproducibility
- 12-month shelf life at refrigerated temperature (2-BOc)
- 4-week shelf life at room temperature (21°c)
- Tolerant to temperature abuse

FuelSnap™ Readings

FuelSnap displays a number at the end of the test, which indicates to the operator whether contamination is unacceptable (red), acceptable but needs monitoring (amber), or clean (green).



7 SUMMARY

- Hitherto tests for fuel contamination have been complex, expensive, take a long time to conduct and are not generally very accurate.
- Adenosine Tri-Phosphate (ATP) is present in all living cells. The FuelSnap Analyser quickly and cheaply detects to a high degree of accuracy the presence of ATP in a fuel sample, and therefore of living cells. This in turn is a measure of the extent of microbial and fungal contamination. A further test can distinguish between microbial and fungal cells.
- The FuelSnap Analyser is lightweight, hand-held and easy to use. It uses cheap one-shot "test pens" which give a result within a few seconds. The Analyser records every test internally and the data can be downloaded onto a database. This allows the recording of detailed historical records, which can then be analysed for trends and predict maintenance requirements.

ANNEXES

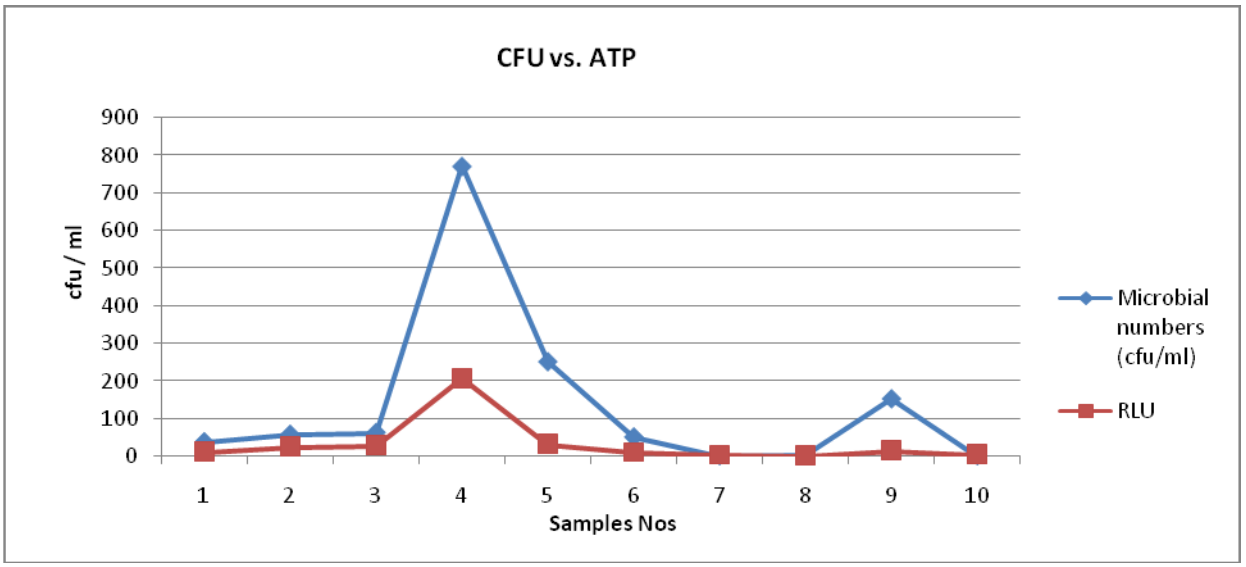
TEST KITS COMPARISON TABLE

		Conventional Colony Forming Unit (cfu) Tests			**Immunoassay**	**ATP**	
		Microb-monitor$_2$	**IP385**	**EASICULT COMBI**	**FUELSTAT *resinae***	**HY-LiTE Jet A1 Fuel Test**	**FuelSnap Rapid ATP**
Detection Limit and Sensitivity sufficient for	FUEL	✓	✓	No (not applicable)	✓	✓	✓
	WATER	✓	✓	✓	✓	✓	✓
Measured Parameter		Quantitative assessment of cfu	Quantitative assessment of cfu	Semi-quantitative assessment of cfu	Assesses negligible, moderate and heavy levels of a compound associated with growth of *H.resinae*	Quantitative assessment of ATP	Quantitative assessment of Total ATP
Assessment method		Count number of colonies (or compare to chart)	Count number of colonies	Assess number of colonies by comparison to chart	Visual observation of test and control lines on test paddle	Numerical instrument reading (RLU)	Numerical instrument reading (RLU)
Can be used for Fuel & Water		✓	Fuel (can be modified for water)	No, water phase only	✓	✓ (Manufacturer recommends testing both phases together)	✓ (Manufacturer recommends testing both phases together)
Direct/indirect test for fuel		Direct	Direct	N.A.	Use aqueous extractant	Use aqueous extractant	Direct with measured dipper
Time for Result		1 day (Heavy) to 4 day (Up to 6 day if not incubated at 28°C–30°C)	3-5 d	2-5 d	10 mins	<10 mins	<1 min
Suitable for field use		Yes	No, Laboratory Test	Yes	Yes	Yes	Yes
Transport Restrictions		None	N.A.	None	None	None	None
Shelf life/conditions		1 year ambient (2 years refrigerated)	N.A.	9 months ambient	18 months ambient	1 year refrigerated	12 months refrigerated 8 days ambient temperature

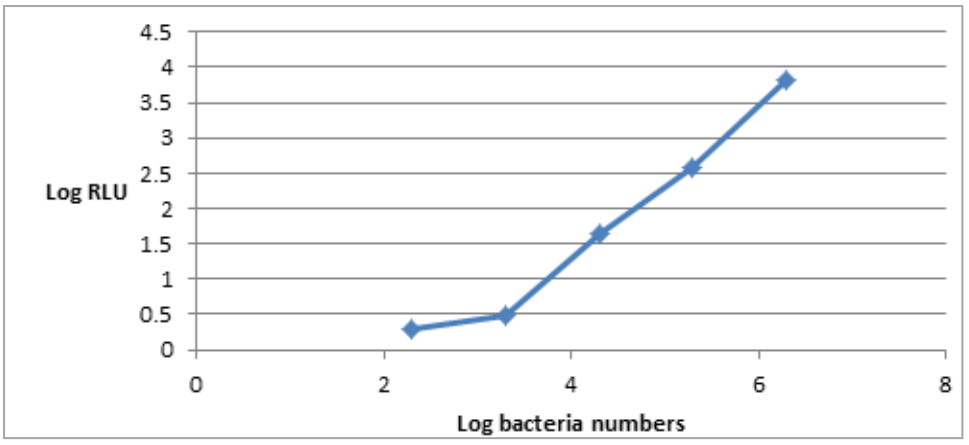
DETECTS:	Bacteria	✓	✓	✓	No	✓	✓
	SRB & other anaerobes	No	Only with modification to protocol	No	No	Yes	Yes
	Yeasts	✓	✓	✓	No	✓	✓
	All moulds	✓	✓	✓	No	✓	✓
	H. resinae	✓	✓	✓	✓	✓	✓
	Mould mycelium	✓	✓	✓	✓ (*H. resinae* only)	✓	✓
	Mould spores	✓	✓	✓	No	No	No (incubation module required)
Can be used to validate effective biocide treatment as per IATA Guidance (after 10 days/5 flight cycles/when biocide fuel has been burnt) *		✓	✓	✓ Underestimates when trace of biocides present	✓ May show positive result for contamination for up to 3 days after treatment if fuel not burnt	✓ A special protocol may be needed in certain cases	✓
Sampling		Clean bottle & equipment	Clean bottle & equipment	Clean bottle & equipment	Clean bottle & equipment	Clean bottle & equipment	Clean bottle & equipment
Special Disposal Considerations		In disinfectant	N.A.	In disinfectant	None	None	None
Cost of Analyser eqpt.							<£1000
Cost of each test							<£5

FUELSNAP RLU vs. CFU (Colony Forming Units) COUNT

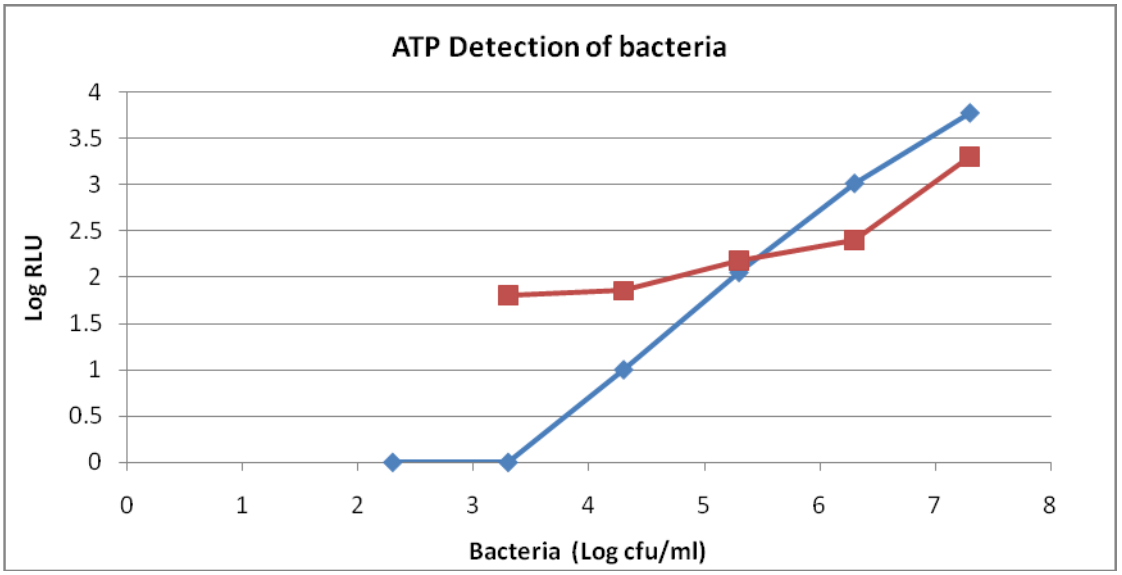
Typical data shows RLU result from the rapid ATP test for various samples plotted against Microbial counts (cfu/ml):



- FUELSNAP detects all sources of ATP in the sample i.e. organic , dissolved and particulate or microbial ATP
- FUELSNAP FREE detects dissolved ATP in the sample and indicates the level of organic matter in the sample
- Subtracting the result of Total from Free ATP is used to estimate microbial ATP



COMPARISON OF HY-LITE ATP (ASTM D7463) AND ATP



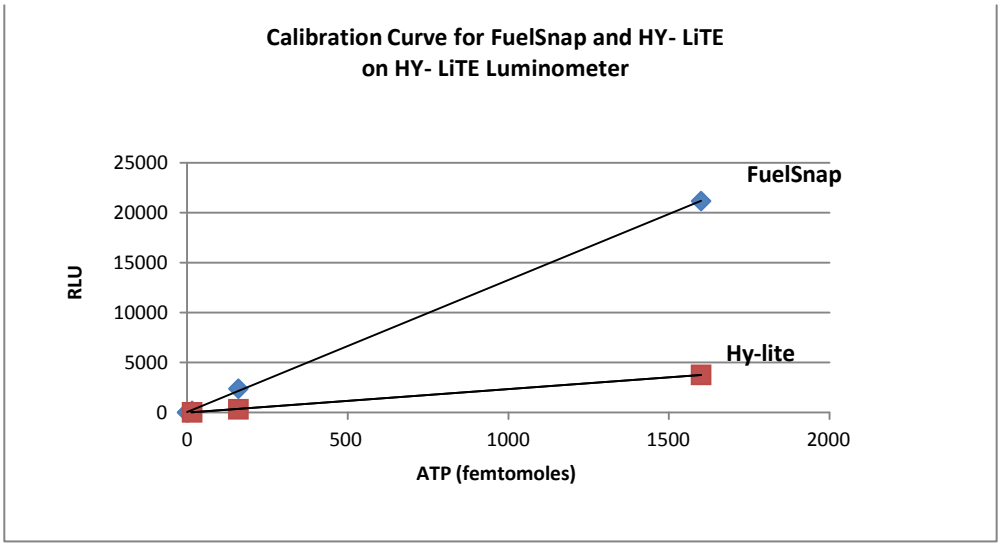
Bacterial nos / ml	HyLite	FuelSnap
	RLU	RLU
10000	70	54
100000	150	440
1000000	250	3650
10000000	2000	6500

Please note these measurements are approximate, and represent average values

- Microbial enumeration methods are highly variable in themselves.
- ATP content of microbes varies depending on size, type and health of the microbe.
- ATP is also present in organic matter and the ATP test cannot differentiate between different sources of ATP
- Accordingly, the correlation between two highly variable methods needs careful examination and interpretation.
- The ATP method provides a rapid qualitative estimate of microbial numbers only.
- The unit of measurement is RLU which is unique to each instrument reagent system ;
    - HyLite RLU is not the same as FuelSnap RLU
    - HyLite RLU scale is not linear with respect to microbial numbers because of the reagent formulation.
    - FuelSnap RLU is linear with respect to microbial numbers

COMPARISON OF THE PERFORMANCE OF FUELSNAP ASSAY ON MERCK HY-LITE® LUMINOMETER

	HY- LITE Sampling Pen	FuelSnap
Blank Value	37.3 RLU	26.9 RLU
Sensitivity / limit of detection	13.51 femtomoles	1.59 femtomoles
Light generated (RLU) per femtomole ATP	2.37	13.2



The FuelSnap system generates more light for a given amount of ATP and typically will give about 5x higher RLU values than the HY-LiTEpen. When implementing a more sensitive system the Pass, Caution and Fail thresholds should be increased in order to maintain the same hygiene standards. Maintaining the same thresholds will result in a tightening of standards.In general, the thresholds measured by FuelSnap should be adjusted by a factor 5.

8 ACCURACY OF THE SYSTEMS

A big potential issue with any equipment is the introduction of noise through the electronic circuitry. Noise picked up in the system will restrict the ability of any system to measure the low levels of microbial activity.

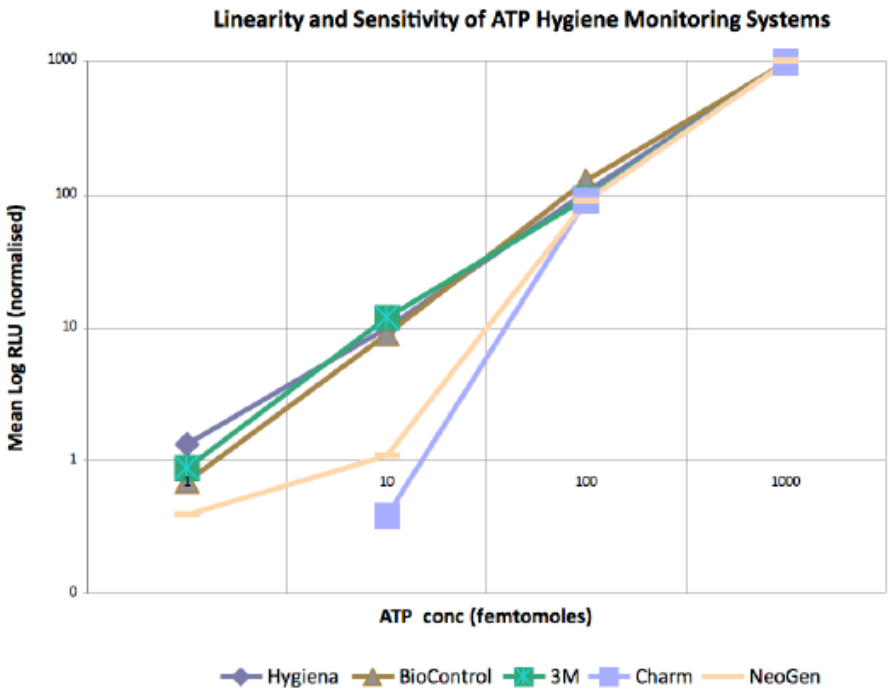
ZERO has to mean ZERO

The EnSure handset supplied by Hygiena has proven to be the most reliable handheld luminometer for the test

8.1 Linearity

The linearity graph shows a straight-line relationship between RLU and ATP. ***y = mx+ c*** where 'y'= RLU and 'x'= ATP, and both increase in a constant predictable way. The straighter the line, the better the linearity, the better the precision and more reliable the detection.

This is particularly important at low ATP levels at low RLU values. Linearity is described by the term Correlation Coefficient (r) which shows how well the data approaches the perfect fit i.e. r = 1.000



8.2 SENSITIVITY

Sensitivity is defined as the Limit of Detection (LoD). It is the smallest amount detectable above the background noise of the system. The smaller the LoD the more sensitive the system.

Background noise is the signal detected by the systems in the absence of ATP that can come from both the instruments (as electrical interference) and the reagent swab devices (as chemical interference from impurities).

Signal – Background Noise = True meaningful result

A low background noise means a clear signal with little interference that enables the detection of the lowest amount of sample i.e. maximum sensitivity.

The graph shows the limit of detection (LoD) for each ATP test system