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Sterility Test: Comparison of sensitivity of rapid microbiological methods based on ATP bioluminescence with compendial plate count method. Establishment of a scale of sensitivity - Transition from qualitative microbiological *method to* quantitative method

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ABSTRACT

Three known methods of sterility analysis are compared: The traditional method of counting colonies on agar plates and two alternative bioluminescent methods for determining the cell concentration by the value of adenosine triphosphate with and without adenylate kinase as an enhancer. All three considered methods are used as quantitative methods. The scale of sensitivity of quantitative methods selected. The number of microorganisms expressed in colony-forming units is used as a measure of sterility (non-sterility). Colony-forming Unit is determined by compendial plate count method which is consist of counting the colonies on agar plates. The natural unit of the scale is one colony. Bioball™ granules containing a fixed Colony-forming Unit of certain microorganisms were used as a reference standard for non-sterility. The minimum detectable number of microorganisms expressed in Colony-forming Unit was taken as the sensitivity of the method. On the scale of sensitivity, the methods were arranged in descending order as follows: 1) Adenylate kinase-enhanced ATP bioluminescence method (the most sensitive), 2) Plate-Count Method, 3) ATP bioluminescence method. The sensitivity of Plate-Count Method was chosen as a unit, it is also chosen as a reference method.

Keywords: Sterility test, rapid microbiological method, ATP, adenylate kinase, bioluminescence, quantitative method.

1. INTRODUCTION

USP <71> Sterility Tests states: "These Pharmacopeia procedures are not by themselves designed to ensure that a batch of product is sterile or has been sterilized. This is accomplished primarily by method suitability of the sterilization process or of the aseptic processing procedure." "Sterility Tests" is based on the visual observation of turbidity in liquid culture media after minimally 14-days incubation. The test uses two types of media, tryptic soy broth (TSB) and fluid thioglycolate medium (FTM) incubated at 20-25°C and 30-35°C, respectively [1]. The sterility test is a qualitative test. Its result is expressed in variables of binary type: (yes / no), (+/-) or (0/1).

The current sterility testing method has a good track record, but there has been much discussed about its subjectivity and duration of the test. Turbidity is subjective and relies on human interpretation. There are alternate sterility methods which can provide faster and objective results [2]. Several alternative methods for rapid microbiology testing are available and are based on different biologic principals; solid-phase cytometry, nucleic acid amplification, flow cytometry etc.,[3]. Among them, the Firefly Adenosine Triphosphate (ATP) bioluminescence method has been very popular since 1947 [4]. The reaction proceeds according to the scheme [5]:

Luciferin + ATP + O_2 (Firefly Luciferase + $MgCl_2$)→Oxyluciferin + AMP + PPi + CO_2 + LIGHT emission 560nm

Where PPi is inorganic pyrophosphate. ATP molecule (507.18 DA) functions as the primary energy source for all living cells including microorganisms. Energy is generated via hydrolysis of phosphate groups in the ATP molecule. ATP is a useful marker of cellular contamination. The ATP based cell detection

luminometry realized in for example Pallchek™ Rapid Microbiology System [6].

Adenylate kinase is another useful marker of cellular contamination. Adenylate kinase is a macromolecule (24142.65 DA), it presents in mitochondrial and myofibrillar compartments in the cell [7,8]. Adenylate kinase catalyze the reversible reaction of the conversion of adenosine diphosphate (ADP) into ATP and adenosine monophosphate (AMP) with a reaction equilibrium constant close to unity [9]:

2ADP + (Adenylate kinase + $MgCl_2$) <-> ATP + AMP

It is clear that's the excess of ADP shifts the equilibrium to the ATP formation. AK makes it possible to multiply the amount of cells ATP. Thus, the intensity of the luciferin-luciferase luminescence can be increased many times over. The analytical method of ATP bioluminescence amplified by adenylate kinase (AK method) is based on this principle. In the current work sterility test by AK method was performed on the Celsis luminometer and Celsis® AMPiScreen system [10]. The design of the luminometers makes it possible to obtain numerical values of the luminescence. Their scale is graded in Relative Light Units (RLU). The scale is linear for a specific range of ATP. RLU results are compared to a blank baseline RLU result and any sample with a signal-to-noise ratio of greater than three (i.e., 3 x blank baseline) is recorded as "Positive" based on validation. The sample results are classified as either "Negative", "Positive", or "Overload." Negative results indicate that the product's ATP levels are below the baseline ("0"). Positive or Overload results indicate that the product's bioburden levels are at least three-times the baseline and are both considered positive ("1"). In practice, numeric data is not used in binary mode. Currently, both ATP and AK are used as quality binary analytical methods for sterility testing [2, 4, 5, 11, 12]. USP <1223> defines the limit of detection (LOD) for qualitative microbiological method as the "lowest number of microorganisms in a sample that can be detected under the stated experimental conditions. Due to the nature of microbiology, the limit of detection refers to the number of organisms present in the original sample before any dilution or incubation steps; it does not refer to the number of organisms present at the point of assay" [3, 13]. It must be emphasized that the standard qualitative test, unlike the presented direct method, includes a growth-enrichment period, during which sample is dispensed into standard microbiological culture medium and incubated for few days. Two qualitative methods compared using binary statistical approach. Binary outcomes are input in a Receiver Operating Characteristics table representing the number of paired samples having positive/negative results for the alternate ATP-bioluminescence test and the compendial test. A one-sided McNemar's X^2 test with continuity correction is used to determine whether a statistically significant difference exists between the two methods [11, 14].

To do not to be confused with LOD of a qualitative microbiological method, here we are talking about the limit of sensitivity (LOS) of quantitative ATP and AK methods. LOS refers to the properties of a luminometer and the way ATP is detected, including signal amplification. LOS is defined as the smallest number of microorganisms (Colony Forming Units) in a test sample that can be detected under specified experimental conditions. The aim of the presented work is to establish a numerical scale for the sensitivity of sterility methods using generally accepted standards.

2. MATHERIALS AND METHODS

Sterile distilled water prepared in laboratory. Fluid Thioglycollate Medium (FTM), Tryptic Soy Broth (TSB) Dehydrated Cultured Media, and Tryptic Soy Agar Dehydrated Cultured Media from Hardy Diagnostic. ATP disodium salt hydrate standard from Sigma. Standards of microorganisms of the predetermined concentrations from Bioball [15]. Analytical balance OHAUS®. Incubators: 22.5°C ± 2°C, 32.5°C ± 2°C. Vacuum manifold. Biological Safety Cabinet. PALL micro funnels – GN6 membrane 0.45 µm. Eppendorf Centrifuge 5415D and Eppendorf Centrifuge Tubes 1.5 mL. Vortex Genie II. Celsis automatic luminometer and Celsis® Ampiscreen Reagent Kits for the Celsis AMPiScreen method (AK method) [14, 16]. Pallchek Luminometer and Reagent Kits for the Pallchek™ Rapid Microbiology System (ATP method) [6, 16]. Plate-Count Method in particular Surface-Spread Method, is described in [17].

Standard solutions of the microorganisms were prepared according to manufacturer [6] with modifications. Stock standard solution (30 CFU per 1 ml) was centrifuged at 500 g for 10 minutes. Supernatant containing water soluble possible contamination (ATP, enzymes) was discharged. Pellets of all alive bacteria was reconstituted in the same volume, diluted gradually by water to make a set of standard solutions of different concentrations. The concentration of the standards was checked by Plate-Count Method.

The second microorganism standard was prepared as follow: Corresponding growing media 10 ml (appendix 1) was inoculated (10 CFU) by standard of challenged microorganisms and incubated for 4 days at the corresponding

temperature (appendix 1) [1, 17]. The new enriched solution was used as the second standard. The concentration of microorganisms in the second standard was, again, estimated by Plate-Count Method. Plates was inoculated with 10^3 – 10^6 times diluted second standard, because, according to preliminary studies, after amplification the concentration of microorganisms increased up to 10^6 CFU per 0.1ml. The following microorganisms was used: *Aspergillus brasiliensis* (Asp.), *Pseudomonas aeruginosa* (Ps.), *Bacillus subtilis* (Bac.), *Staphylococcus aureus* (St.), Escherichia Coli (E. Coli), *Candida albicans* (Ca.) and *Propionibacterium acnes* (Prop. Ac.).

Each experimental point is the average of five measurements. Standard deviations were calculated. The lines were constructed using the Least Squares Method. Correlation coefficients (r) were calculated.

3. RESULTS AND DISCUSSION

3.1. Determination of a working range of the ATP luminometers:

Working range was determined using ATP standard solutions. The series of solutions of ATP was prepared to build the functional dependence RLU - concentration of ATP Each experimental point is the average of five measurements. The line is constructed using the Least Squares Method. (Figure I). This dependence is linear in range RLU from 0 to 3.5E+06. Correlation coefficient – r ≥ 0.999. Above 3.5E+06 RLU both luminometers do not give numerical results but show "overload". Steep of the correlation curve for Celsis is 32443 and the numerical working range is from 0 to 108 pMol ATP. Steep of the correlation curve for Pall is 4 60701 and its numerical working range, respectively, equal to from 0 to 8.0 pMol ATP (it is 14 times less than for Celsis).

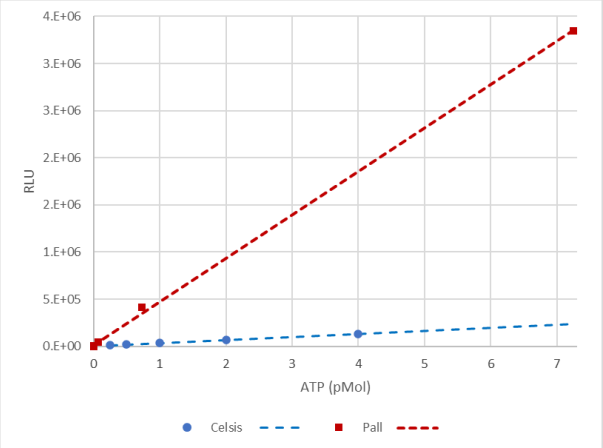


Figure - I: Calibration curves for two luminometers: Celsis and Pall.

3.2. AK method. Direct sensitivity determination by using microorganisms' standards.

Series of water solutions of different concentrations of microorganisms was prepared using BioBall - a small water-soluble ball containing a precise number of microorganisms [6]. Results for Asp., Ps., Bac., St., E. Coli, Ca., and Prop. Ac. are presented in Figure II. Iven at 0.5 colony forming units (CFU) per 0.1ml, the RLU was between 300000 and 700000 RLU and blank was 1000 RLU, it is mean that the LOS of all 6 microorganisms was much less than 0.5 CFU per 0.1ml .

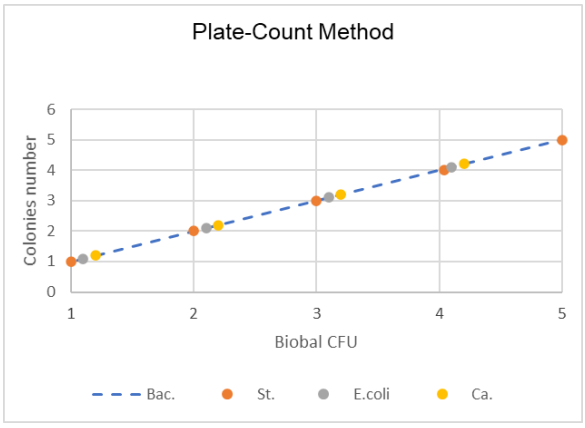


Figure II - A: Dependence of the test result on the reference concentration of microorganisms in the sample in CFU units. (A. Plate-count method)

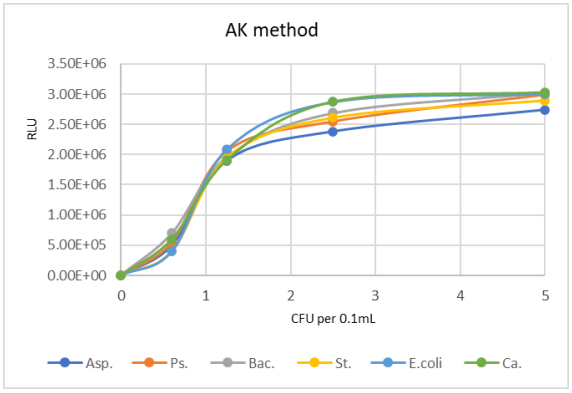


Figure II – B: Dependence of the test result on the reference concentration of microorganisms in the sample in CFU units. (B. AK method)

The more detailed data at low concentration of microorganism presented for Propionibacterium acnes in Figure III (a, b). LOS of AK method could be graphically determined as an intersection point of horizontal dushed line (blank) and RLU curve, or it can be defined analytically as the intersection of the blank line and the line approximating the experimental points. Figure III (b). The blank was established as three times of average noise signal, in the presented work it was 1000 RLU. As can we see, the LOS Propionibacterium acnes for AK method (Celsis) is 0.05 CFU per 0.1ml, it is 20

times less than for Plate Counting Method. Bioluminescent method without AK amplification (ATP method) is not sensitive enough to detect biocontamination of 40 CFU and less. So, it is necessary to have more concentrated samples of microorganisms.

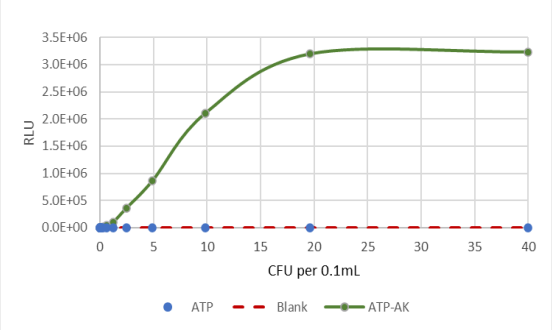


Figure III - A: Comparison of the AK method (solid line) and the ATP method (blue circles). CFU-RLU dependence for Propionibacterium acnes. (A. - general dependence; The horizontal dashed line is a 3 times blank baseline)

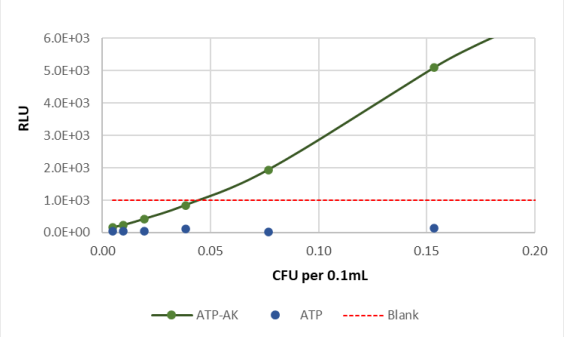


Figure III – B: Comparison of the AK method (solid line) and the ATP method (blue circles). CFU-RLU dependence for Propionibacterium acnes. (B. - dependence at low concentrations of the microorganism. The horizontal dashed line is a 3 times blank baseline)

3.3. ATP method. Estimation of LOS using secondary standards:

The BioBall standards was used to prepare more concentrated second standard solution of microorganisms. The stock was prepared by inoculation corresponding growth media with 0.1ml of 10 CFU standard and subsequent incubation for 4 days at corresponding temperature (22.5 or 32.5 C). The concentration of microorganisms increased dramatically (about 10 million times) see Table1. This enriched solution, was diluted gradually by media to have a set of the second standard solutions.

Table – 1: The stock solution concentrations after enrichment; 4 days of incubation; 10ml media; 10 CFU inoculation with etalon microorganisms; control by Plate-Count Method.

Microorganisms	Number of colonies per 100µL. 10000 times diluted solutions.	Estimated CFU/100 µL of enriched solutions
Bacillus Sub.	30 ±3	3.00E+05
Candida alb.	30 ±3	3.00E+05
Staphyllococus aur.	50 ±5	5.00E+06
Pseudomonas aer.	50 ±4	5.00E+06

The solutions were tested and the RLU versus CFU relationship is shown in Figure IV. Each experimental point represents the mean of five measurements. The RLU - CFU dependence is approximated by lines. Lines are drawn using the least squares method. As we can see, LOS for Ps. is about 500 CFU, this is the minimum LOS among the tested microorganisms.

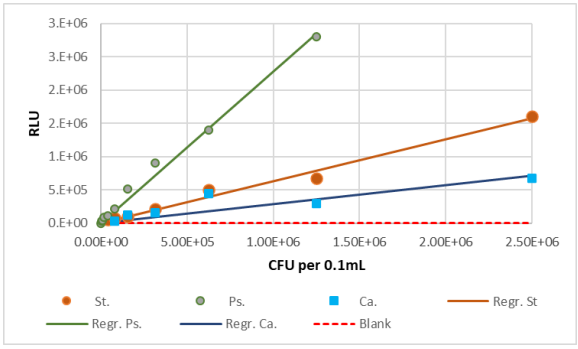


Figure IV A, CFU-RLU dependence ATP method. Pallchek™ Rapid Microbiology System. (B - dependence at low concentrations of the microorganisms. The horizontal dashed line is a 3 times blank baseline)

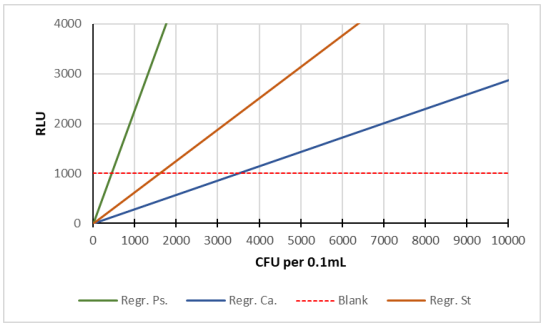
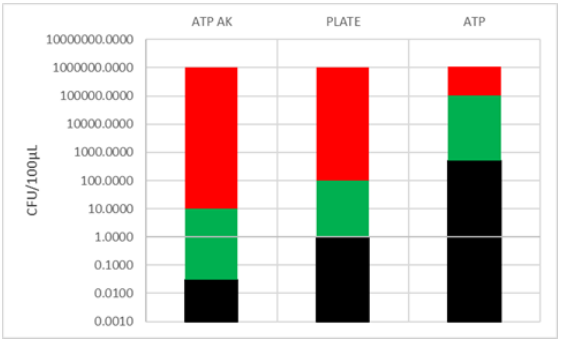


Figure IV – B: CFU-RLU dependence ATP method. Pallchek™ Rapid Microbiology System. (B. - dependence at low concentrations of the microorganisms. The horizontal dashed line is a 3 times blank baseline)

The available data allow us to place the three considered methods on the sensitivity scale. It is natural to take the limit of sensitivity of the method of counting colonies on plates as a unit of the scale. The sensitivity limit of each method corresponds to the lower border of the green bar (Figure V). The upper border of the green bar is

the limit of the linear area of dependence of Relative Light Units (bioluminescence method) or the Number of Colonies (Plate-Count Method) on the number of microorganisms. The red area is the saturation area. The green area can be expanded with the red area by diluting the samples.

Figure V. Comparison of the methods for sensitivity limits and quantitative ranges. Black zone - the presence of cells is not detected. Green zone - the presence of cells is quantified. Red zone - cells are present in an excess (overload).



It is interesting to compare our results with ones in [14]. Using an indirect multi-step method and binary statistical analysis, the authors estimate the LOD AK as 0.079 CFU / ml, which is equal to 0.0079 CFU / 100 µl. Although our LOS score is higher, our results are taken directly from the experiment.

3.4 Transition from qualitative microbiological method to quantitative method:

The sensitivity of the AK method is so high, and it seems that it can be improved to 0.01 CFU, that we can talk about a transition from a qualitative to a quantitative method of analysis. The most important criterion is the sensitivity limit of the method. Saturation is not a problem as the samples can always be diluted. Sample preparation becomes the most important stage of the analysis. Differential centrifugation can be used to prepare samples from aqueous solutions. If oil solutions or creams are analyzed, then vacuum filtration should be used, followed by luminescence analysis on a wet filter, as in the case of Pall, but in this case, the filter is processed not with two, but with three reagents, including the ADP solution for AK-amplification. For **Appendix 1**.

quantitative analysis, standard calibration curves of the CFU - RLU relationship are required for each microorganism or group of microorganisms. Since, in the general case, the CFU - RLU dependence is not linear it has S-shape (Figure II and III), the working region of microorganisms' concentrations will have to be divided into 3 sections: initial, middle, and final. For the middle section linear regression can be used, and for the extremes, parabolic.

4. CONCLUSION

The scheme for the quantitative analysis of samples for sterility is shown in Figure VI. The samples are analyzed by the AK method. The analysis result is presented as the number of microorganisms (CFU) in the sample. If the analysis does not detect the presence of microorganisms (CFU <LOS), then the culture medium (TSB and FTM) is inoculated with the test material and incubated for 2 or more days at the appropriate temperature. The new rich solution is tested again. The result of the second test, again, is presented as the number of microorganisms in the sample. The number can be 0 (sterile) or n <LOS (non-sterile, but the number of microorganisms in the original sample is less than the LOS).

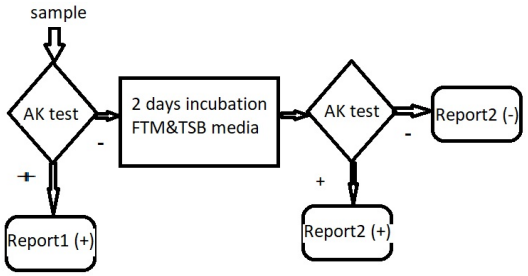


Figure - VI. The scheme for the quantitative analysis of samples for sterility.

If the sensitivity of the method is enough to detect one single cell, it will be possible to completely abandon the incubation of samples in a nutrient medium to increase the concentration of microorganisms.

Acknowledgements

The author is grateful to Dr. Wayne Dehaven for participating in the discussion of the work.

The following microorganisms were tested (Biomerieux Bioball SingleShot)

Organism	Strain	Corresponding Strains	Description	Media	Incubation Temp (°C)
Escherichia coli (cell)	NCTC 12923	NCIMB8545, CIP53.126, NBRC3972, WDCM00012	Gram negative, facultative anaerobe	FTM	32.5°C ± 2°C

Aspergillus brasiliensis (spore)	NCPF 2275	CIP1431.83, IMI149007, NBRC9455, WDCM00053	fungus	TSB	22.5°C ± 2°C
Clostridium sporogenes (spore)	NCTC 12935	NBRC14293, NCIMB12343, CIP100651, DSM1446	Gram positive, anaerobe	FTM	32.5°C ± 2°C
Staphylococcus aureus (cell)	NCTC 10788	CIP4.83, NCIMB9518, NBRC13276, FDA209, WDCM00032	Gram positive, facultative anaerobe	FTM	32.5°C ± 2°C
Pseudomonas aeruginosa (cell)	NCTC 12924	NCIMB8626, CIP82.118, NBRC13275, WDCM00026	Gram negative, facultative anaerobe	FTM	32.5°C ± 2°C
Candida albicans (cell)	NCPF 3179	CIP48.72, NBRC1594, WDCM00054	yeast	TSB	22.5°C ± 2°C
Bacillus subtilis subsp. Spizizenii (spore)	NCTC 10400	CIP52.62, NCIMB8054, NBRC3134	Gram positive, preferential aerobic	TSB	22.5°C ± 2°C
Propionibacterium acnes (cell)	DSM 1897	CIP 53.117; DSM 1897; JCM 6425; KCTC 3314; NCTC 737	Gram positive, aerotolerant anaerobe	FTM	32.5°C ± 2°C

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