Determination of the Limit Of Detection for rapid microbiological method based on ATP bioluminescence by Direct Method. Comparison of the Limits Of Detections for the Celsis® Rapid Detection System with using the AMPlScreen™ assay and The Pallchek™ Rapid Microbiology System.

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Introduction

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" [1].

The purpos of the presented work is to compare sensitivity of two luminometers and LODs of two bioluminescence methods using a set of standard solutions contained different and predetermined amount chalenged analytes/ microorganisms. The minimal amount of microorganisms which can be detected was considered as a LOD of the method. Actually it is direct method of LOD determination the results show a difference in sensitivity of the compared bioluminescent methods. It must be emphasized that that the standard LOD test [3], unlike the presented, includes a growth-enrichment period, during which sample is dispensed into standard microbiological culturemedium and incubated for few days. After incubation sample placed into a luminometer. LOD is calculated indirectly using statistical approach and logistic regression [3]. The amount of microorganisms is expressed in the Colony Forming Units (CFU). It is determined by counting a colonies on an agar plates after the plates inoculation and incubation for =<5 days at a corresponding temperature.

Metods

The standards of microorganisms of the predetermined concentrations (Bioball [6]) was used to determine the LOD of Cellsis instrument [4] with using the adenilate kinase-amplifed bioluminescence (AK) method [3, 4]. AK method was compared with the Bioluminiscent Method without the adenilate kinase-amplification wich is used in the Pallchek™ Rapid Microbiology System [5]. The results of measurements was expressed in Relative Light Units (RLU). Stock solutions of the microorganisms were prepared according to manufacturer - Bioball [6] than they were diluted gradually by water to make a set of solutions of

different concentrations. The concentration of stock solution was checked by Plate-Count Method [2] it was always equal to the data of manufacturer. The minimal concentration of microorganisms which gives RLU 3 times more than average background value was considered as LOD. The background value was the RLU of water or growing media. In case when LOD exeed the stock solution concentration, the concentration of microorganisms was amplified by inoculation (10 CFU) of corresponding growing media 10 ml (TSB or FTM) by microorganisms from stock solutions and incubation for 4 days at the corresponding temperature (yeast and mold 20-25 grad and bacterias 30-35 grad C). The new enriched solution was used as the stock solution for LOD determination. The concentration of microorganisms in the stock solution was estimated by Plate-Count Method [2]: The plates was inoculated by microorganisms from the stock solution (100mkl) and incubation for =<5 days at a corresponding temperature.

Results

1. Determination of a working range of the instruments.

Working range was determined with using ATP standard solutions. The series of of solutions of ATP was prepared to build the functional dependence RLU - concentration of ATP (Pic.1). This dependance is linear in rangr RLU from 0 to 3.5E+06.

Coefficient of linear regression (a) for Celsis is 32443 and the working range is from 0 to 108 pMol ATP. PallCheck has the coefficient a=460701 and its working range, respectivly, equal to from 0 to 8.0 pMol ATP (it is 14 times less than for Celsis).

Pic.1

2. Direct LOD Determination by using BioBall.

Series of water solutions of differen concentrations of microorganisms was prepared usind BioBall a small water-soluble ball containing a precise number of microorganisms [6]. The results for Propionibacterium acnes are presented in Pic.2 (A and B). As can be clear from theese pictures the lowest detectable number of Propionibacterium acnes in a sample (LOD) is no more than 0.1 CFU.

Pic.2

The results for Bacillus Sub., Candida alb., Staphyllococus aur., Pseudomonas aer., Aspergillus bras. and E.Coli. are presented in Pic.3. It is visible, that the lowest detectable number of this six microorganisms in sample (LOD) is less than 1.0 CFU. If the interpolation between 0 CFU and the fist point close to 0 is taken in acount, LOD is roughly estimated as 0.2 CFU.

Pic.3

So, it can be concluded, that lowest number of microorganisms in a sample that can be detected by Celsis under the stated experimental conditions is not exeed 1 CFU and lay in gange 0.1 - 0.2 CFU per sample.

These results can't be directly compared with PallChak ones because the minimal amount of microorganisms that can be detected with Pall was not be reached by using BioBall standards. It can be only concluded that LOD more than 80CFU/100mkl (Pic.2 B)

3. Estimation of LOD of Bioluminescent Method without the adenylate kinase-amplification (PallChak).

The BioBall standards was used to prepare more concentrated satrting solution of microorganisms (stock). The stock was prepared by inoculation corresponding growth media with 100 mkl of 10 CFU standard and subsequent incubation for 4 days at corresponding temperature (20-25 or 30-35 C). The concentration of microorganisms increased dramaticaly (about 1milion times). This enriched

solution, was diluted gradually by media to have a set of solutions of different concentration. The solutions was tested and the dependence RLU vs Degree of Dilution (DOD=1/(how many times the starting solution was diluted)) is presented in Pic.4. The picture shows that at the relative concentration equal to 0.001 the RLU is significant more than background (0). This dilution of stock solution correspond to LOD.

Because the concentration of stock solution was about 1000,000 CFU or more, it is impossible to count such a big number on the plate, so, the stock solution was diluted and the set of solutions of different concentrations was tested by Plate-Count Method. If number colonies on a plate was in range 5 - 100 CFU, the corresponding to it solution was choozed and concentration of stock solution was calculated as CFU of the solution divided by Degree of Dilution. (For example: diluted 100,000 times stock solution (DOD=1E-04) exhibit the 50 colonies on a plate, so the stock solution concentration is equal: 50 CFU / 1E-04 = 5000,000 CFU per sample (100 mkl)). Results are presented in Tab.1.

Pic.4 Dependence relative concentration of stock solution (DOD) vs RLU PallChek per sample (100 mkl).

Table 1. The stock solution concentrations after enrichment.

4 days of incubation, 10ml media, 10CFU inoculation. Plate-Count Method .

M-O	CFU/100mkl
Bacillus Sub.	3.0E+05
Candida alb	3.0E+05
Staphyllococus aur.	5.0E+06
Pseudomonas aer.	5.0E+06

Discussion and conclusion

Puting together data from Pic.4 and Table 1, it can be concluded that PallChak roughly estimated LOD for Staphyllococus aur. is equal 5E+06 * 0.001 = 5000

CFU and for Pseudomonas aer. and Candida alb. LOD =300 CFU. So that, LOD of Bioluminescent Method without the adenylate kinase-amplification lay in range 5000. - 300. CFU per 100mkl. Bioluminescent Method with AK amplification (Celsis) give us the LOD in range 0.1 - 0.2 CFU per 100mkl.

It is interesting to compare our results with ones in [3]. Using inderect method and statistical aproach they calculated the LOD for the Bioluminescent Method with AK amplification as 0.079 CFU/ml it is equal to 0.0079 CFU/100mkl. This result is almost 10 times les than our one. But [3] used growth-enrichment step at wich sample is dispensed into standard microbiological culture medium and incubated for 18-24 hours. After incubation an aliquote of the enrichment culture was tested in luminometer. It is clear, that the concentration of microorganisms into a sample is increased multiple times (100 or 1000 or 1000,000), but that concentration was not determined and was not known. In this way it is impossible to compare our results directly to [3] ones.

The growth-enrichment step with following by concentration of microorganisms estimation was used by us to prepare new stock solution of high concentration (homemade standard) for LOD direct determination in case of Bioluminescent Method without AK amplification (PallChek). It was necessary only because the standards with high concentration of a microorganisms are missing.

Celsis instrument with using AK amplification is about 50,000 - 1500 times more sensitive then PallChek. This significant difference in LOD's is, obviously, because of the AMPIScreenTM assay the amplification of ATP by AK is used.

The applied by us direct method for LOD determination is a simple and natural one particular to Adenilate Kinase-amplified bioluminescence metod. Used in [3] indirect method LOD determination is more abscure and more complex. In case of bioluminescence metod without the Adenilate Kinase-amplification the determination of LOD is more complex because it takes an addition step (growth-enrichment step). This step also reduces the accuracy of the direct LOD determination because the homemade standards are used. Nevetherless method remain simple and natural.

References:

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