

**Statistical analysis for the Validation of Rapid Microbiological Method with using Adenosine triphosphate bioluminescent assay and Adenilate Kinase-Amplified bioluminescent assay. Analysis of Paired Dichotomous Data by Fisher's Exact Probability Test, Exact Binomial Test, Mid-p Binomial test, McNemar's test and McNemar's test with Yetes' correction.**

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**Keywords:** Fisher Exact Probability Test, Exact Binomial Test, Mid-p Binomial, McNemar's test; Chi-square test; paired dichotomous data; 2×2 contingency table; Adenosine triphosphate bioluminescent assay, Adenilate Kinase-Amplified bioluminescent assay, Validation procedure for a new Rapid Microbiological Method.

In course of carrying Validation procedure for a new Rapid Microbiological Method [1][2] a Dichotomous Data are collected. Adenosine triphosphate (ATP) bioluminescent assay assess microbiological quality of pharmaceutical products using a presence/absence test method. Two sets of Dichotomous Data are collected. The first data set is the data obtained by the new method, the second data set is the data obtained by the reference method. The data was analysed by 5 statistical methods and results were compared. On basis of the analysis was made the conclusion about which Microbiological Method and which statistical analysis are preferable.

## **MATERIALS AND EQUIPMENT**

Incubators- 22.5°C ± 2°C and 32.5°C ± 2°C; Celsis® Advance II; Celsis® Ampiscreen Reagent Kits; PALLChek™ luminometer; PALL bioluminescence reagent kit – PALL part#7142; PALL sample holders; Vacuum manifold; Biological Safety Cabinet (BSC); Eppendorf BioPur pipette tips; PALL microfunnels – GN6 membrane 0.45 microns; Refrigerator; Freezer; Eppendorf Centrifuge 5415D; Eppendorf Centrifuge Tubes 1.5 mL; Bioballs (Biomerieux); Fluid Thioglycollate Medium (FTM); Tryptic Soy Broth (TSB); PC HP Windows10, Free software “LibreOffice” version 6.0.0.3.

## **Method**

**Basic ATP bioluminescence method** [1][4] includes two steps. First one it is cell membrane lysis step. The second step it is the light emission enzymatic reaction :

1. Cells + (Membran destroyer) --> ATP(I) + (ADK + citosol components + cell debris)
2. ATP(I) + O<sub>2</sub> + Luceferin(red.) + Luceferase + Mg<sup>2+</sup> ----> [Luceferrin-Luceferase] + ATP(I) + O<sub>2</sub> + Mg<sup>2+</sup> -----> Luceferin(ox.) + AMP + PP<sub>i</sub> + CO<sub>2</sub> + Light + Luceferase

Where:

ATP(I) – Intracellular adenosine triphosphate.

Membran destroyer – the reagent which destroys cell membranes and releases citosol components, commonly it contains Trichloroacetic acid and detergent.

Luceferin(red.) - reduction form of Luceferin.

Luceferin(ox.) - oxidized form of Luceferin

Luceferase -enzyme

[Luceferrin-Luceferase] – enzyme -substrate complex

AMP – adenosine monophosphate

PP<sub>i</sub> – diphosphate ion.

Light - quantum of light.

ADK - enzyme Adenilate kinase [5].

It is known (and we are proofed it lab.) that the number of light quanta in this reaction linearly depends on the number of ATP. Also, an ATP is direct proportional to the number of cells in the sample.

**ADK - amplified luminescent method** [5][6] includes one addition step it is increasing the ATP number by enzymatic reaction of cytosolic ADK and ADP reagent:

1. Cells + (Membran destroyer) --> ATP(I) + (ADK + citosol components + cell debris)
2. 2ADP + ADK <-- [ADK]--> ATP(II) + AMP+ ADK
3. [ATP(I)+ATP(II)] + O<sub>2</sub>+ Luceferin(red.) + Luceferase +Mg<sup>2+</sup> ----> [Luceferrin-Luceferase]+  
+ ATP(I) + O<sub>2</sub> + Mg<sup>2+</sup> -----> Luceferin(ox.)+AMP+PP<sub>i</sub> + CO<sub>2</sub> + Light +  
+ Luceferase

Where:

ADP – adenosine diphosphate – the added reagent.

[ADK] - enzyme-substrat intermediate complex

ATP(II)- adenosine triphosphate the product of ADP conversion.

[ATP(I)+ATP(II)] – total number of ATP.

If ADP is taked in excess and time of reaction is fixed, the ATP(II) directly proportional to ADK. ADK is directly proportional to cells number. So, in both methods the light output is proportional to the number of cells in the sample. Light output is measured by the instrument's photomultiplier unit and recorded in Relative Light Units (RLUs). The results are compared to baseline RLU values and are classwified as “negative” or “positive” based on the signal-to-noise ratio. A negative result indicates that the product's bioburden levels are below the assay's limit of detection whereas positive results indicate that the product's bioburden levels are at or above the test's limit of detection.

## Experiment Design

In purpose of validation of a new method, the two methods was compared, the new and a reference one [1]. An outcome of the tests is a binary, dichotomous data (+, - ; yes, no, presence or absence a biocontamination in the sample) they are may be presented in 2x2 contingency table (Table 1.). The equivalence of two test methods that detect microbiological contamination was evaluated by comparing the rate of positive and negative results obtained

from identical samples. The methods were: Rapid Adenilate Kinase-amplified (AK) ATP bioluminescence method which is used in **Celsis®** instrument (new method)[7] and ATP bioluminescence method without AK which is used in Pallchek™ instrument (reference method)[8]. The second reference method was the compendial method: Visual inspection of the 10ml test-tubes of growth media for turbidity after 0-7 days of inoculation.

Table.1 2x2 contingency table [9]. a,b,c and d are represents total number of the corresponding outcomes, for example "a" is equal to a number of samples for which both methods gives positive result, "b" is equal to a number of samples for which the New method gives the positive result and the Reference method gives the negative result. The table is used in Binomial Test and McNemar Test.

		Reference method		
		'+'	'-'	
New method	'+'	a(+,+)	b(+,-)	a+b
New method	'-'	c(-,+)	d(-,-)	c+d
		a+c	b+d	a+b+c+d

Both ATP bioluminescence methods occurs in two phases: off-line phase of sample preparation and culture incubation, and the on-board phase of the bioluminescence assay. Each phase has a number of user-defined parameters that are determined during method validation. Parameters considered in the off-line phase include incubation time and temperature, volume of product to be sampled, culture media volume, and type of culture media necessary to neutralize product (drag) and support growth. The off-line phase is a common phase for all the considered methods.

**Off-line phase include:** Inoculation of 10ml of liquid growth media, dispensed into 10ml glass test-tubes, with challenged microorganisms (Bioballs (Biomerieux))[10] to yield concentrations of 10.0, 1.0 and 0.1 CFU/100ml. Cultures was incubated at 30-35 or 20-25 C, depends on kind of media and microorganisms [3] (see Appendix#1) for 0 to 7 days period. After incubation the 10 ml test-tubes of growth media was visually inspection for turbidity, it is Compendial test which give us the first set of dichotomous data (turbid +/-not turbid -) also samples was tested on Celsis and Pall instruments. Fiftin (or 18) pairs of assessment were conducted for each organism. These consisted of three different inoculum levels and five replicates (6 replicates for Propionibacterium acnes) at each concentration.

## Celsis® & Pallchek™ On-board phase

**Celsis:** 1. Remove culture vessels from the incubator and transfer into Biological Safety Cabinet (BSC). 2. Vortex vessel minimally 30 seconds to homogenize. 3. Transfer 1.5 mL of homogenized sample into labeled sterile Eppendorf tube using aseptic technique. 4. Centrifuge using Eppendorf Centrifuge 5415D. 5. Decant supernatant into container for later handling as biowaste. 6. Add 100 µL sterile water and vortex. Incubate at 32.5°C ± 2°C up until testing with Celsis®. 7. Follow Celsis® manual for adding samples to Advance II reader. Samples are tested in duplicate. 8. Negative cut-off is calculated internally in software as three-times the media control. On-board phase for AK method has a critical parameter, it is ADP conversion period, during which microbial adenilate kinase catalyses the conversion of reagent-supplied ADP to ATP. This reaction is linear for as long as ADP is present and can be used to increase the signal-to-noise ratio of the assay. The default parameters for most applications is 40 minutes.

**PallChek:** 1. Pour 20 ml of sterile water into the Pall microfunnel and Filter 2. Add 10 mL of enriched media sample (i.e., the ENTIRE test tube) and filter through the Pall microfunnel. 3. Pour 100 ml of sterile water into the microfunnel and filter [3] 4. Run Pallchek Test on the filter following instructions below 5. Place a new sample holder on the aluminum plate using new set of sterile forceps 6. Remove and discard the lid to the filter funnel. Carefully collapse the filter funnel by squeezing the sides of the cup together and discard the cup 7. Using the sterile forceps, remove the filter from the microfunnel and place it on the sample holder. Discard the bottom of the filter funnel 8. Add 150 µL (0.15 ml) of extractant to the filter using Micropipetor 100-1000 µL and Biopur tip 100-1000 µL and spread with sterile spreader. Leave the spreader on the test plate. 9. Within 15 seconds, add 100 µL (0.15 ml) of reagent mix to the filter using Micropipetor 20-200 µL and Biopur tips 20-300 µL and spread with the sterile spreader-discard spreader. 10. Test within 5 seconds and record results 11. Remove sample holder and discard. Set aside forceps for cleaning. Use a new set of sterile forceps for the next test. 12. Threshold for CONTAMINATION is set at 3.0E+03.

For each kind of microorganisms test results was summarised by all three concentrations and paired data was placed in electronic table for calculation (Table 5).

## Statistical analysis

**Binomial test** [9][11]. In statistics, the binomial test is an exact test of the statistical significance of deviations from a theoretically expected distribution of observations into two categories. One common use of the binomial test is in the case where the null hypothesis is that two categories are equally likely to occur (such as a coin toss). A binomial test can be used, where  $b$  (Table 1) is compared to a binomial distribution:  $PDF(b,n,p) = C^n_b P^b (1-P)^{n-b}$  [COMBIN(n,b)\*POWER(p,b)\*POWER((1-p),(n-b))] with size parameter  $n = b + c$ , integer variable  $b$  from 0 to  $n$  and  $p = 0.5$ . Effectively, the exact binomial test evaluates the imbalance in the discordants  $b$  and  $c$  (Table 1). In this case, the Null Hypothesis ( $H_0$ ) is :  $p_b = p_c = 0.5$ . The goal is to calculate the  $p$ -value (or asymptotic significance) using  $2 \times 2$

contingency table and Binomial distribution. A p-value  $\leq 0.05$  (0.05 is the level of significance) indicates strong evidence against the null hypothesis, so the null hypothesis should be rejected. The traditional advice has been to use the exact binomial test when  $b + c < 25$ .

$P(b) = \text{MIN}(\text{CDF}(b), 1 - \text{CDF}(b-1))$  where CDF - Cumulative Distribution Function

With using Microsoft Excel notation the above formula expressed as follow:

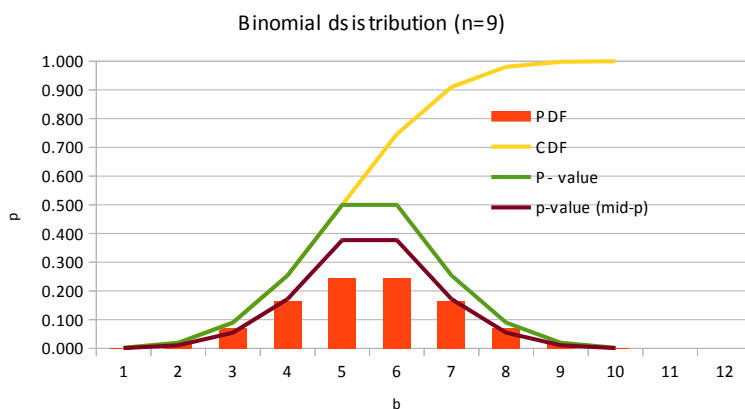
$P(b) = \text{IF}(n=0, 1, \text{IF}(b=0, \text{BINOMDIST}(b, n, 0.5, 1), \text{MIN}(\text{BINOMDIST}(b, n, 0.5, 1), 1 - \text{BINOMDIST}(b-1, n, 0.5, 1))))$

In this expression we set  $P(b) = 1$  if  $n=0$  and  $P(b) = \text{CDF}(b)$  if  $b=0, n \neq 0$  to escape error marks.

**Mid-p binomial test** is calculated by subtracting half the probability of the observed  $b$  from the exact P-value. The reason to introduce Mid-p binomial test is to lower p-value (exact-p-value). When  $b + c < 6$  then, according to [5], the exact p-value exceeds the common significance level 0.05. But the mid-p correction is not found to exceed the nominal significance level [5].

$\text{mid-P}(b) = \text{MIN}(P(b) - \text{PDF}(b)/2, 1 - (P(b) - \text{PDF}(b)/2))$

Picture 1. Binomial distribution, Probability Distribution Function (PDF), Cumulative Distribution Function (CDF), P-value, p-value (mid-p).



An interesting observation when interpreting Binomial test is that the elements of the main diagonal (2x2 table) do not contribute to p calculation. Thus, the sum  $b + c$  can be small and statistical power of the test described above can be low even though the number of pairs  $a + b + c + d$  is large.

**McNemar's test** [9][11]. In statistics, McNemar's test is a statistical test used on paired data. It is applied to  $2 \times 2$  contingency tables (Table 1) with a dichotomous trait, with matched pairs of subjects, to determine whether the row and column marginal frequencies are equal (that is,

whether there is "marginal homogeneity"). The null hypothesis of marginal homogeneity states that the two marginal probabilities for each outcome are the same, i.e.  $p_a + p_b = p_a + p_c$  and  $p_c + p_d = p_b + p_d$ . Thus the null hypotheses is  $H_0: p_b = p_c$ .

The McNemar test statistics is:

$$\chi^2 = \frac{(b - c)^2}{b + c}$$

Under the null hypothesis, with a sufficiently large number of discordants (cells b and c),  $\chi^2$  has chi-squared distribution with 1 degree of freedom. If the  $\chi^2$  result is significant, this provides sufficient evidence to reject the  $H_0$ , in favor of alternative hypothesis that  $p_b \neq p_c$ , which would mean that the marginal proportions are significantly different from each other. For random variable Y defined as  $Y = X^2$  where  $X \sim N(0, 1)$  it is Normal distribution with  $\mu = 0$  and  $\sigma = 1$ ,

$$PDF_Y(y) = \frac{1}{\sqrt{2\pi}} y^{-\frac{1}{2}} \cdot e^{-\frac{y}{2}}, \quad CDF_Y(y) = \int_{-\infty}^y PDF(y) dy, \quad Y = X^2 \sim \chi^2$$

The derivation of the formula for PDF is in appendix#2.

There are two ways to compute a p-value from a contingency table using McNemar approach. First is to use McNemar test statistic, the second way is to use Yates' correction test statistics

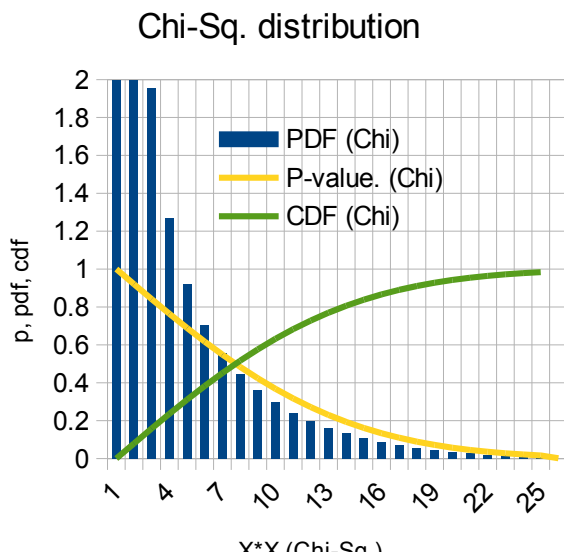
$$\chi^2_{Yates} = \frac{(b - c + 1)^2}{b + c}. \quad \text{p-value in both cases is calculated as follow:}$$

$$P\text{-value} = 1 - CDF_Y(y), \quad Y = X^2 \sim \chi^2$$

The  $\chi^2$  distribution and p-value curve are presented in Picture 2. It should be noted that

$$\chi^2_{Yates} > \chi^2 \Rightarrow p\text{-value}(Yates') < P\text{-value}.$$

Both corrections (Mid-p Binomial and Yates') decrease the p-value. The Yates' continuity correction is designed to make the chi-square approximation better. With large sample sizes, the Yates' correction makes little difference. With small sample sizes, chi-square is not accurate, with or without the correction [13][11][12].



Picture 2. Chi-Squared distribution.

### Fisher Exact Probability Test [9][11].

Fisher test is traditionally used with relatively small samples but, it is valid for all sample sizes. The test is the best choice as it always gives the exact p-value.

Logic of Fisher test. Let we have a Set-1 of total N elements consisted of two kinds of elements ( K (success or "+") and K'("-") ). Let construct a Set-2 of elements randomly collecting n elements from Set-1 . What is the probability to have in the Set-2 composition of exactly k (+) of (K) and n-k (-) of (K'). This probability (P(X=k) is equal to total number of way to make the Set-2 from Set-1 divided by total number of way to make set of n elements from N ones.

$$P(X=k) = \frac{\text{Combin}(K, k) * \text{Combin}((N-K), (n-k))}{\text{Combin}(N, n)}$$

$$\frac{(K! * (N-k)! * (N-n)! * n!) / (k! * (K-k)! * (n-k)! * (N-K-n+k)! * N!)}{(*)}$$

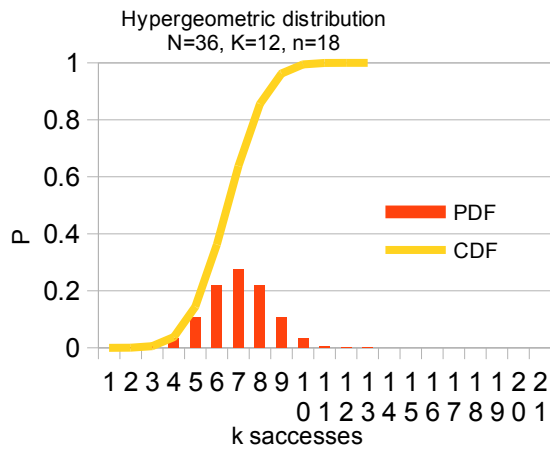
where: Combin(K,k) is how many ways to collect k elements (A) from K. Combin(N,n) is the total number of ways to make the set of n elements from N.

Combin((N-K), (n-k)) is how many ways to complete the Set-2 of sample by elements (K) from the Set-1.

This conditions (\*) characterize the hypergeometric distribution (Picture 3).

A random variable X follows the hypergeometric distribution if its probability distribution function (pdf) is given by (\*) where N is the Set-1 size, K is the number of success states in the Set-1, n is the number of draws (i.e. quantity drawn in each trial), k is the number of observed successes, Combin(a,b) is a binomial coefficient. So we can rewrite the equation (\*) using Microsoft Excel notation as follow:  $P(X=k) = \text{HYPGEOMDIST}(k, n, K, N)$  (\*\*)

Picture 3. Hypergeometric distribution



Let put the data into 2x2 table:

Table 2. The table for the Fisher Exact Probability Test.

	'+' k	'-' n-k	n
Set-1	K-k K	N-K-(n-k) N-K	N-n N

Let rename the elements of the table:

Table 3.

	'+' A	'-' B	A+B
Set-1	C A+C	D B+D	C+D N

Now the formula for  $P(X=k)$  looks nice:  $P(X=k) = \frac{\text{COMBIN}((A+C),A) \cdot \text{COMBIN}((B+D),B)}{\text{COMBIN}(N, (A+B))} =$

$$\frac{(A+C)! (A+B)! (B+D)! (C+D)!}{A! B! C! D! N!}$$

To make connection with the 2x2 table used in Binomial Test and McNemar Test (Table 1) let write the equivalences:

$$k = (\text{new test has positive results}) = (+, -) + (+, +) = a+b = A$$

$$n-k = (\text{new test has negative results}) = (-, +) + (-, -) = c+d = B$$



$K-k = (\text{reference test has positive results}) = (+,+) + (-,+)=a+c = C$

$N-K - (n-k) = (\text{reference test has negative results})=(-,+)+(-,-)=b+d =D$

$K=a+c+a+b=2a+c+b;$

$n=c+d+a+b= N-n; n=N/2; \quad .$

Now we can put our test results into Table 1 and use it to calculate p-value with using as for Binomial Test, McNemar Test and for Exact Fisher tests.

### 1) Binomial test.

$P(b) = \text{IF}(n=0,1,\text{IF}(b=0,\text{BINOMDIST}(b,n,0.5,1),\text{MIN}(\text{BINOMDIST}(b,n,0.5,1),1-\text{BINOMDIST}(b-1,n,0.5,1))))$  where  $n=b+c$ . (+)

### 2) mid-p Binomial test;

$P(b) = \text{IF}(n=0,1,\text{IF}(b=0,(\text{BINOMDIST}(b,n,0.5,1)-\text{BINOMDIST}(b,n,0.5,0))/2),\text{MIN}((\text{BINOMDIST}(b,n,0.5,1)-\text{BINOMDIST}(b,n,0.5,0))/2),1-\text{BINOMDIST}(b-1,n,0.5,1)-\text{BINOMDIST}(b,n,0.5,0)/2)))$  where  $n=b+c$  (++)

### 3) McNemar test

$P\text{-value} = \text{IF}(n=0,1,1-\text{CHISQDIST}((b-c)^2/n,1,1))$  where  $n=b+c$  (+++).

### 4) Yates' correction

$P\text{-value}(\text{Yates' correction}) = \text{IF}(n=0,1,1-\text{CHISQDIST}((b-c)^2+2*\text{abs}(b-c)+1)/n,1,1))$  where  $n=b+c$  (++++)

### 5) Fisher Exact Probability Test

$P\text{-value} = \text{MIN}(\text{HYPGEOM.DIST}(a+b,n,a*2+b+c,n*2,1),1-\text{HYPGEOM.DIST}(a+b,n,a*2+b+c,n*2,1))$  Where  $n= a+b+c+d$  (++++)

To use Microsoft Excel or LibreOffice Calc, the 2x2 contingency table need to be transformed to a single row, and put it into electronic spreadsheet. Than the one-site p-value may be calculated using the formulas above, see Table 4.

Table 4. A fragment of LibreOffice Calc electronic spreadsheet. Each row correspond to one microorganism. The second column described which methods are compared and how long the samples was incubated. One-site p-values are in the cells are on the intersection of Name of test column and row. Last column shows if the Null hypothesis is acceptable (Yes) or not (No).

M.O.	Method & Incubation time	a	b	c	d	b+c	(b-c)	(b-c)*(b-c)	"N"= a+b+c+d	Binomial	McNemar	McNemar Yates'	Mid-p Binomial	Fisher	N <sub>0</sub> is TRUE. YES/NO
Propionibacterium acnes	Celsis/Pall 4 days	5	2	0	11	2	2	4	18	0.25	0.157	0.034	0.125	0.36	YES
Propionibacterium acnes	Celsis / Visual (1Day)	5	1	0	12	1	1	1	18	0.5	0.317	0.046	0.25	0.5	YES
Staphylococcus aur.	Celsis/Pall (4 days)	8	0	2	5	2	2	4	15	0.25	0.157	0.034	0.125	0.36	YES
Clostridium spor.	Celsis/Pall (4 days)	9	0	0	6	0	0	0	15	1	1	1	1	0.64	YES
Clostridium spor.	Celsis / Visual (4days)	9	0	0	6	0	0	0	15	1	1	1	1	0.64	YES
Aspergillus bres.	Celsis/Pall filtr. (4days)	6	0	0	9	0	0	0	15	1	1	1	1	0.64	YES
Aspergillus bres.	Celsis/Visual (4days)	5	0	0	10	0	0	0	15	1	1	1	1	0.65	YES
Bacillus Sub.	Celsis/Pall (4 DAYS)	8	1	0	6	1	1	1	15	0.5	0.317	0.046	0.25	0.5	YES
Bacillus Sub.	Celsis/Visual (4DAYS)	8	0	0	7	0	0	0	15	1	1	1	1	0.64	YES
Candida alb.	Celsis/Pall (4 days)	4	0	0	11	0	0	0	15	1	1	1	1	0.66	YES
Candida alb.	Celsis/Visual (4days)	4	0	0	11	0	0	0	15	1	1	1	1	0.66	YES
Pseudomonas aer.	Celsis/Pall (4days)	4	0	0	11	0	0	0	15	1	1	1	1	0.66	YES
Pseudomonas aer.	Celsis/Visual (4days)	4	0	0	11	0	0	0	15	1	1	1	1	0.66	YES
E.Coli	Celsis/Pall (4days)	7	1	1	6	2	0	0	15	0.75	1	0.48	0.5	0.64	YES
E.Coli	Celsis / Visual (4days)	7	0	0	8	0	0	0	15	1	1	1	1	0.64	YES
Propionibacterium acnes	Celsis / Visual (1hour)	0	0	5	13	5	5	25	18	0.031	0.025	0.007	0.016	0.02	NO
Propionibacterium acnes	Visual/ (Visual +4 days)	0	0	6	12	6	6	36	18	0.016	0.014	0.004	0.008	0.01	NO

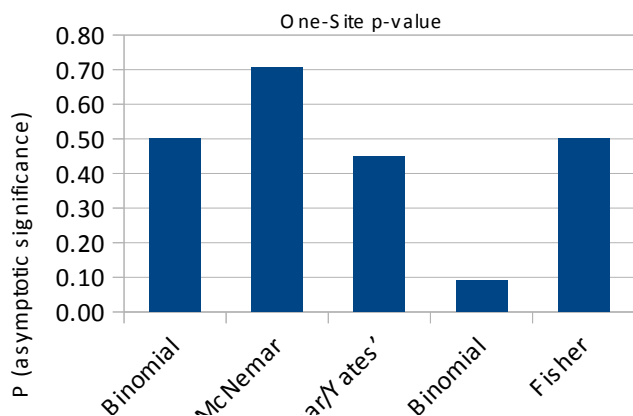
Except two special cases (Table 4)  $N_0$  hypothesis can't be rejected. The two lowermost rows in Table 5 shows these two very special cases: First one - the slow growing microorganism – Propionibacterium acnes incubated only 1 hour and then the samples were tested on Celsis and visually. Celsis reveals presence of biocontamination because the instrument has very low Limit of Detection (LOD) [15], but visual test – not. The second case – the samples of Propionibacterium acnes visually compared after 1 hour and 4 days incubation. In that both cases the  $N_0$  should be rejected. The gray cells in the table - it is not relabel results because  $b+c \leq 5$  and it is considered as low [9][11]. So, the exact Binomial and Fisher tests are preferable [9]. With small sample sizes, chi-square (McNemar's) test is not accurate, with or without the correction [14].

In the Picture 4 summed data of testing samples of 8 microorganisms by two methods (Celsis and Pall) are presented. All 5 statistical tests give us  $p\text{-value} > 0.05$ , so the  $N_0$  couldn't be rejected. As we can see, Mid-p Binomial test gives as the p-value lower than all other ones. It looks like the mid-p correction of the Binomial test should be used with precaution and only with a good reason. In our previous work [15] it was shown, that Celsis instrument and

method is about 1000 times more sensitive than Pall's (Poll's LOD = 1000\* Celsi's LOD), but the off-line procedure amplifies concentration of microorganisms in sample about 1000,000 times. So even if two methods differ 1000 times in their sensitivity the binary test results (+/-) will be the same. Actually, after amplifying of the microorganisms concentration the very different binary tests such as Rapid Bioluminescence, Compensial Visual and Compensial Plate Count tests will give us, usually, the same results. The 1000,000 times amplification guarantees detection if the microorganisms were presented in sample at beginning. It is the strong leveling effect[15]. The 2 x 2 contingency tables look, also, very similar because almost all numbers concentrate in main diagonal a(+,+) , d(-,-) and b+c number is, usually, low. So, the best choice in this situation is to use the Binomial Test or Fisher Exact Probability Test to calculate exact p-value.

Picture 4.

Celsis/Pall, 8 microorganisms, 4 days incubation, N=123, b+c=7, (b-c)=1



The hypothesis of equivalence was tested using receiver operating characteristics (ROC) to determine accuracy, precision, specificity and sensitivity, using 70% as the acceptance criteria. Accuracy measures the closeness of the results obtained by the Celsis and reference methods:  $(a+d)/(a+b+c+d)$ . Precision measures the degree of agreement between both test methods where the Celsis indicates positive:  $a/(a+b)$ . Specificity is the ability of the Celsis to detect the entire range of microorganisms that were present in the test. Specificity measures the agreement of both methods when the reference method indicates a negative test sample:  $d/(b+d)$ . Sensitivity measures the ability of Celsis to identify the same positives as those identified by the reference method:  $a/(a+c)$ . The ROCs summed outcomes are presented in Table 5, which is a right part of full electronic table.

Table 5. Receiver Operating Characteristics comparing Celsis and reference methods of detecting microbial contamination.

M.O.	Method & Incubation time.	Accuracy ((++)+(-))/N	Precision (+ +)/((++)+(+-))	Specificity (-)/((+)-+(-))	Sensitivity ((+)((++)+(-+))
Propionibacterium acnes	Celsis/Pall (4days)	0.89	1	1	0.71
Propionibacterium acnes	Celsis / Visual (1Day)	0.94	1	1	0.83
Staphylococcus aur.	Celsis/Pall (4days)	0.87	0.8	0.71	1
Clostridium spor.	Celsis/Pall (4days)	1	1	1	1
Clostridium spor.	Celsis /Visual (4days)	1	1	1	1
Aspergillus bres.	Celsis/Pall (4 days)	1	1	1	1
Aspergillus bres.	Celsis/Visual (4 days)	1	1	1	1
Bacillus Sub.	Celsis/Pall (4DAYS)	0.93	1	1	0.89
Bacillus Sub.	Celsis/Visual (4DAYS)	1	1	1	1
Candida alb.	Celsis/Pall (4days)	1	1	1	1
Candida alb.	Celsis/Visual (4days)	1	1	1	1
Pseudomonas aer.	Celsis/Pall (4days)	1	1	1	1
Pseudomonas aer.	Celsis/Visual (4days)	1	1	1	1
E. Coli	Celsis/Pall (4days)	0.87	0.88	0.86	0.88
E. Coli	Celsis / Visual (4days)	1	1	1	1

The table shows the equivalence of the methods. And it is not surprising because of mentioned above the leveling effect.

Despite the the strong leveling effect of the amplification of the microorganisms, the using modern rapid and high sensitive test method has an advantages. The first advantage of the Celsis reveals in some special cases as has been shown above. Second veri important advantage to use sensitive instrument and method it is work with smal aliquot of samples. For example, PallCheck analisis requaered to use all the volume of a sample (10ml), it is destructive test. For Celsis analisis we may use only 10mkl aliquot of sample to reach the same precision. So, the sample, pratical, still intact and the test may be repeated. And finally, the rapid test save us time and money.

## Appendix#1

Table. Microorganisms, medias and temperature of incubation. FTM and TSB media – Becton Dickenson (BD)

Organism ( <i>Bioballs</i> ( <i>Biomerieux</i> ))	Strain	Corresponding Strains	Description	Media	Incubation Temp (°C)
<i>Escherichia coli</i> (cell)	NCTC 12923	NCIMB8545, CIP53.126, NBRC3972, WDCM00012	Gram negative, facultative anaerobe	FTM	32.5°C ± 2°C
<i>Aspergillus brasiliensis</i> (spore)*	NCPF 2275	CIP1431.83, IMI149007, NBRC9455, WDCM00053	fungus	TSB	22.5°C ± 2°C
<i>Clostridium sporogenes</i> (spore)	NCTC 12935	NBRC14293, NCIMB12343, CIP100651, DSM1446	Gram positive, anaerobe	FTM	32.5°C ± 2°C
<i>Staphylococcus aureus</i> (cell)*	NCTC 10788	CIP4.83, NCIMB9518, NBRC13276, FDA209, WDCM00032	Gram positive, facultative anaerobe	FTM	32.5°C ± 2°C
<i>Pseudomonas aeruginosa</i> (cell)**	NCTC 12924	NCIMB8626, CIP82.118, NBRC13275, WDCM00026	Gram negative, facultative anaerobe that preferentially uses aerobic respiration	FTM	32.5°C ± 2°C
<i>Candida albicans</i> (cell)**	NCPF 3179	CIP48.72, NBRC1594, WDCM00054	yeast	TSB	22.5°C ± 2°C
<i>Bacillus subtilis</i> subsp. <i>Spizizenii</i> (spore)	NCTC 10400	CIP52.62, NCIMB8054, NBRC3134	Gram positive, preferential aerobic respiration	TSB	22.5°C ± 2°C
<i>Propionibacterium acnes</i> (cell)*	DSM 1897	CIP 53.117; DSM 1897; JCM 6425; KCTC 3314; NCTC 737	Gram positive, aerotolerant anaerobe	FTM	32.5°C ± 2°C

\* Standard Deviation <4.5 cfu; \*\* Standard deviation <3.5 cfu;

## Appendix#2 Chi-squared distribution. Derivations of the PDF.

Def. Random variable Y as  $Y = X^2$ , where  $X \sim N(\mu, \sigma)$  (Gauss)  $PDF_X(x) = \frac{1}{\sqrt{2\pi} \cdot \sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}}$ ,  
 choose  $X \sim N(0,1)$ ,  $PDF_X(x) = \frac{1}{\sqrt{2\pi}} e^{-\frac{x^2}{2}}$ .

Then, for  $y < 0$ ,  $P(Y < y) = 0$  and for  $y \geq 0$ ,  $P(Y < y) = P(X^2 < y) = P(-\sqrt{y} < X < \sqrt{y}) =$

$$CDF_X(\sqrt{y}) - CDF(-\sqrt{y}) = CDF_X(\sqrt{y}) - (1 - CDF_X(\sqrt{y})) = 2CDF_X(\sqrt{y}) - 1$$

remember:  $PDF_Y(y) = \frac{d}{dy} CDF_X(y)$

$$PDF_Y(y) = 2 \frac{d}{dy} CDF_X(\sqrt{y}) - 0 = 2 \frac{d}{dy} \int_{-\infty}^{\sqrt{y}} \left( \frac{1}{\sqrt{2\pi}} \right) e^{-\frac{x^2}{2}} dx =$$

$$= \frac{1}{\sqrt{2\pi}} y^{-\frac{1}{2}} \cdot e^{-\frac{y}{2}}$$

$Y = X^2 \sim \chi^2_1$

where  $\chi^2_1$  is chi-squared distribution with one degree of freedom.

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