Validation of the Celsis AMPiScreen® Amplified ATP Bioluminescence Method for Rapid Detection of Contamination in a Betamethasone Suspension

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ABSTRACT: This performance qualification was performed to show the comparability of two qualitative methods, run side by side, to assess the microbiological quality of a non-filterable opaque suspension. Comparability was measured by observing the degree of agreement between the two methods with the protocols performed repeatedly on different lots of the same product with different concentrations of inoculum. The accuracy, precision and specificity of the alternative method was expressed in terms of relative rates of false positive and false negative results between the Celsis AMPiScreen® method and the pharmacopoeial method using a standardized, low-level inoculum. The data were analyzed using a receiver operating characteristic table (ROC) using an acceptance criteria of 70% for each attribute. Accuracy measures the closeness of the results obtained by Celsis AMPiScreen® method and the traditional plate count test method. Precision measures the agreement among both test methods where the Celsis AMPiScreen® indicates a positive. Specificity measures the agreement of both methods when the plate count test method indicates a negative. Based on these studies it was determined that the Celsis AMPiScreen® assay provides results in 24 hours that are equivalent to the pharmacopoeial test method for detecting microbial contamination in a product matrix.

KEYWORDS: Rapid Microbiology, Celsis Advance, AMPiScreen, Microbial Limits Testing, Equivalence Verification

Introduction

In this study, the equivalence of two microbiological test methods was evaluated by comparing the rate of positive and negative results obtained using the Celsis Advance® Luminometer and Celsis AMPiScreen® (AMPiScreen) assay kit to the rates obtained by the compendial plate count method on identical samples. The degree to which the two methods agreed when performed repeatedly on different lots of the same product was measured. The accuracy, precision, and sensitivity of the AMPiScreen method (alternate method), expressed in terms of the relative rates of false-positive and false- negative outcomes using a standardized low-level inocula, were computed and tested against an acceptance criteria of 70%. This performance qualification was based on the United States Pharmacopoeia 32 General Chapters 1223 “Validation of Alternative Microbiological Methods”[1] and European Pharmacopoeia 6.0, Chapter 5.1.6 “Alternative methods for control of microbiological quality”[2].

Methods

Overview of the Celsis rapid detection system using AMPiScreen®.

The Celsis rapid detection system using AMPiScreen is a test system that utilizes microbial enzymes and ATP bioluminescence technology to rapidly assess the microbiological quality of pharmaceutical products using a presence/absence test method. The standard test includes a short growth-enrichment period, during which product is inoculated into standard microbiological culture medium and incubated for 18-24 hours prior to assay. After incubation, an aliquot of the enrichment culture is transferred into a cuvette and placed into the Advance Luminometer, where the bioluminescence assay, enhanced with linear enzyme-mediated ATP amplification is carried out automatically. Light output is measured by the photomultiplier tube and reported as Relative Light Units (RLUs).The results are compared to baseline RLU values and instrument controls and classified as either, “Negative”, “Positive”, or “Overload” based on

indicate that the product's bioburden levels are below the test's limit of detection. Positive or Overload results indicate that the product's bioburden levels are above the test's limit of detection. The residual volume of enrichment culture is retained for additional testing should the need arise.

AMPiScreen Assay

The AMPiScreen test method occurs in two phases; an *off-board* phase that addresses sample preparation and culture incubation, and an *on-board* phase that addresses the automated bioluminescence assay. Each of these modules has a number of parameters that must be defined by the user during the method validation phase. Among the parameters to be considered in the off-line module are the incubation time and temperature, the volume of product to be sampled, the volume of culture media, and the type of culture media to be used to neutralize product and support growth. The AMPiScreen method is sufficiently flexible that virtually any combination can be used, provided that the neither the product nor media possess significant levels of ATP, nor the selected conditions satisfy compendial requirements. The On-board parameters include the signal integration period and the ATP amplification period. The default signal integration period is 1 second, though this can be increased to as much as 10 seconds given that the background signal is sufficiently low. Another critical parameter of the *on-board* phase is the ATP amplification period, during which microbial enzymes catalyze the conversion of 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 parameter for most applications is 40 minutes.

In this study, 1mL of betamethasone was transferred into 25mL of TAT broth and inoculated with challenge organisms to yield concentrations of 10, 1.0, and 0.1 CFU/mL. The TAT culture media contains azolectin and polysorbate 20 to neutralize the inhibitory effects of the product. Cultures were incubated at 30-32˚C for a 24-hour period. After incubation, antifoam reagent and glass beads were added to the culture and agitated on a linear shaker for 30 minutes at room temperature.

Two, 50μL aliquots of the sample were transferred into duplicate cuvettes and placed into the Advance Luminometer, where the bioluminescence assay enhanced with linear ATP amplification was automatically carried out using the following steps:

a. Injection of LuminAMP™ reagent

b. Addition of LuminEX® extraction reagent

c. Incubation period (ATP conversion cycle)

d. Addition of LuminATE® reagent

Results were displayed as relative light units (RLUs). Samples that exhibited mean RLU values 3 times the baseline controls were scored as positive.

Plate Count Assay

One mL aliquots of betamethasone were transferred into tubes containing 25mL of molten TAT+ (bacteria) or SDA+ medium. The SDA+ medium was supplemented with 0.005% lecithin and 0.04% polysorbate 20. TAT+ medium was supplemented with 0.015% agar. Bacterial cultures were incubated 30-35˚C for three days. Yeast and mold cultures were incubated for 5 days at 20-25˚C.

Organisms and culture conditions

The organisms evaluated in this study are the recommended compendial organisms specified in the Total Viable Aerobic Count method chapters of USP/EP/JP[3,4]. The culture media and incubation conditions used to support growth were determined for each organism, based upon the conditions needed neutralize the product and achieve optimal growth as specified in the USP/EP/and JP compendia. Organisms, ATCC identification and growth conditions used in each series are given in Table 1. Organisms were obtained as lyophilized Bioballs™ from bioMerieux. Bioballs™ were dissolved in sterile saline to yield an initial concentration of 10 cfu/mL and serially diluted in saline to the final concentration.

Table 1. Microorganisms and culture conditions

Organism	Media		Incubation Temperature (°C)
	AMPiScreen	Plate Count	
Aspergillus brasiliensis ATCC 16404	TAT	SDA+	20-25
Escherichia coli ATCC 8739	TAT	TAT +	30-35
Staphylococcus aureus ATCC 6538	TAT	TAT +	30-35
Pseudomonas aeruginosa ATCC 9027	TAT	TAT +	30-35
Bacillus subtilis ATCC 6633	TAT	TAT +	30-35
Candida albicans ATCC 10231	TAT	SDA+	20-25

TAT, (Tryptone,-Azolectin-Tween)
TAT+, TAT broth solidified with 0.015% agar
SDA+, Sabouraud dextrose agar supplemented with lecithin (0.005%) and polysorbate 20 (0.04%)

Experimental Design

For each microorganism in the test panel, the product was inoculated at three different concentrations (10 CFU, 1 CFU and 0.1 CFU) and assayed for the presence of microorganisms using with both methods: Celsis AMPiScreen and the compendial plate count method. Tests were performed on three different batches of Betamethasone suspension. Each concentration was tested 5 times (5 replicates) by each method. The results were expressed as positive (+ = presence of microorganism) or negative (- = absence of microorganism). A one sided McNemar's test was used to test for a statistically significant difference between the rates of positive outcomes observed using the Celsis AMPiScreen assay and those observed using the plate count method. If the rates of positive outcomes between methods were not found to be significantly different, the hypothesis of equivalence would be tested using a receiver operating characteristic table[5] to determine accuracy, precision, and specificity, using 70% as the acceptance criteria. The results were summarized according to the following format:

	Plate Count Method	
AMPiScreen™	Positive (+)	Negative (-)
Positive (+)	(++) A	(+-) B
Negative (-)	(-+) C	(--) D

where:

The compendial method is the reference method.

A is the number of times that the result obtained was positive with both methods (true positive).

D is the number of times that results obtained was negative with both methods (true negative). B is the number of times when a positive response is observed with the AMPiScreen method and negative with compendial (false positive).

C is the number of times where the AMPiScreen method shows a negative response and the compendial a positive response. (False negative).

Since the experimental data from each test method are binary, reflecting the presence or absence of an organism in the sample matrix, logistic regression can be used to estimate the limit of detection for each method.[7] The inoculum concentration (Log10(CFU/mL)) was used as the predictor variable and the binary result as the response variable. Two-sided 95% confidence intervals were computed on the predicted probability of response and the log value producing the largest upper confidence limit less than or equal to 0.1 (10%) was selected as the limit of detection.

Results

Fifteen pairs of assessments were conducted for each organism. These consisted of three different inoculum levels and five replicates at each concentration. In all 45 tests were conducted for each test method. The results are presented in Table 2.

Table 2. Comparison of outcomes by AMPiScreen™ and plate count method.

0.1 cfu	Test 1		Test 2		Test 3		Test 4		Test 5		Total Positive	
Organism	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC
A. brasiliensis	-	-	-	-	+	-	-	-	+	-	2	0
C. albicans	-	-	-	-	+	-	-	-	-	-	1	0
P. aeruginosa	-	-	-	-	+	-	-	-	-	-	1	0
E. coli	-	-	-	-	-	-	+	-	-	+	1	1
S. aureus	-	-	-	-	-	-	-	-	-	-	0	0
B. subtilis	+	+	-	-	-	+	-	-	-	-	1	2
Subtotals											6	3

1.0 cfu	Test 1		Test 2		Test 3		Test 4		Test 5		Total Positive	
Organism	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC
A. brasiliensis	+	+	-	+	-	-	-	+	+	+	2	4
C. albicans	+	+	+	+	+	-	-	-	-	+	3	3
P. aeruginosa	-	+	+	+	+	+	+	-	+	-	4	3
E. coli	+	+	+	+	+	+	+	-	+	+	5	4
S. aureus	-	-	+	-	+	+	+	-	-	-	3	1
B. subtilis	+	+	-	-	+	-	+	+	-	-	3	2
Subtotals											20	17

10.0 cfu	Test 1		Test 2		Test 3		Test 4		Test 5		Total Positive	
Organism	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC	ATP+	PC
A. brasiliensis	-	+	-	+	+	+	+	+	+	+	3	5
C. albicans	+	+	+	+	+	+	+	+	+	+	5	5
P. aeruginosa	+	+	+	+	+	+	+	+	+	+	5	5
E. coli	-	+	+	+	+	+	+	+	+	+	4	5
S. aureus	+	+	+	+	+	+	+	+	+	+	5	5
B. subtilis	+	+	+	+	+	+	+	+	+	+	5	5
Subtotals											27	30

A one sided McNemar's test[5] was used to test the null hypothesis that the percentage of positive outcomes obtained by the plate count method was equal to, or better than those obtained using the AMPiScreen method (Table 3). The X^2 statistic obtained from the data was 0.190 and the one-sided p-value was 0.3318. By conventional criteria, this value is not considered to be statistically significant, thus failing to reject the null hypothesis.

Table 3. Comparison of the probabilities of detecting a contaminated sample by method.

Probability of Detection	Concentration (CFU/mL)			
	10	1	0.1	All
AMPiScreen	0.90	0.67	0.20	0.59
Plate Count	1.00	0.57	0.10	0.56
Difference	-0.10	0.10	0.10	0.03
p-value	0.20	0.41	0.29	0.51

The hypothesis of equivalence was tested using a receiver operating characteristic (ROC) table to determine accuracy, precision, and specificity, using 70% as the acceptance criteria. This specification is based on expected results for each concentration, taking into account the probability of growth of the microorganisms at different concentrations of inoculum and the variability linked to very low inoculation levels.

Accuracy measures the closeness of the results obtained by the Celsis AMPiScreen method and the plate count method:

$$(A+D) / (A+B+C+D).$$

Precision measures the degree of agreement among both test methods where Celsis AMPiScreen indicates positive: A/(A+B). Precision is also called the "positive predictive value" of a test.

Specificity is the ability of the Celsis AMPiScreen to detect the entire range of micro-organisms that may be present in the sample under test. Specificity measures the agreement of both methods when the plate count method indicated a negative test sample: D/(B+D). Specificity measures the ability of Celsis AMPiScreen to identify the same positives as those identified by the conventional method. These measures of agreement may also be expressed in terms of false positive and false negative rates through the following equations:

$$\text{Specificity} = D / (B+D) = TN / (FP+TN) = 1\text{-}FP / (FP+TN)$$

$$\text{Precision} = A / (A+B) = TP / (TP+FP) = 1\text{-}FP / (TP+FP)$$

$$\text{Accuracy} = (A+D) / (A+B+C+D) = (TP+TN) / (TP+FP+FN+TN) = 1\text{-}(FN+FP) / (TP+FP+FN+TN)$$

$$\text{Sensitivity} = A / (A + C) = TP / (TP+FN)$$

A = (++) = TP = True Positives
B = (+-) = FP = False Positives
C = (-+) = FN = False Negatives
D = (--) = TN = True Negatives

Receiver Operating Characteristics tables for the paired outcomes are presented in Table 4. Accuracy of the AMPiScreen method for testing low level contamination of Betamethasone Suspension was determined to be 75.6%. Precision was 70.5%, specificity was 74.0%, and sensitivity was 77.5%. Each of these attributes satisfied the 70% acceptance criteria for demonstrating equivalence.

Table 4. Receiver Operating Characteristics (ROC) table for the AMPiScreen method.

cfu	A (+,+)	B (+,-)	C (-,+)	D (-,-)	Specificity	Precision	Accuracy	Sensitivity
0.1	1	5	2	22	81.5%	16.7%	76.7%	33.3%
1.0	13	7	4	6	46.2%	65.0%	63.3%	76.5%
10.0	27	0	3	0	00.0%	100.0%	90.0%	90.0%
All	41	12	9	28	70.0%	77.4%	76.7%	82.0%

Accuracy	((41+28) / (41+12+9+28))*100 = 76.7%
Precision	(41/(41+12))*100 = 77.4%
Specificity	(28/(28+12))*100 = 70.0%
Sensitivity	(41 / (41+9)) *100= 82.0%

The limit of detection (LOD) was calculated using repeated measures logistic regression on data generated from 6 different species and three concentrations of inocula. The response variable was whether or not a sample was determined to be positive by the test method. The continuous predictor variable was the concentration of organisms in the inoculum (Log_{10}(CFU/mL). The limit of detection was selected as the value with the highest concentration of cells such that we may be 97.5% confident that there is less than a 10% chance of detecting an organism. A single LOD has been computed to apply to all organisms.

Figures 2 and 3 present the inverse interpolation for the limit of detection on the log scale for each test method. The dashed red line represents the 95% one-sided upper confidence limit on the probability of detection estimated by the logistic regression. The solid curved line is the predicted probability of detecting an organism. The horizontal line is at 0.1 (10%) and the vertical line is situated at the dilution level, at which the confidence limit reaches 10%.

For the AMPiScreen method, the Log_{10} value of the LOD was 1.85, or 0.014 CFU/mL. For the place count method, the Log_{10} LOD value was - 1.1, or 0.079 CFU/mL. The probability of detecting contamination at 1 CFU/mL was identical for both methods. The smaller LOD value for the AMPiScreen method indicates that this method is able to detect lower levels of contamination in the suspension product than the plate count method.

Figure 2. Logistic Regression Curve for Probability of Detection for AMPiScreen Method

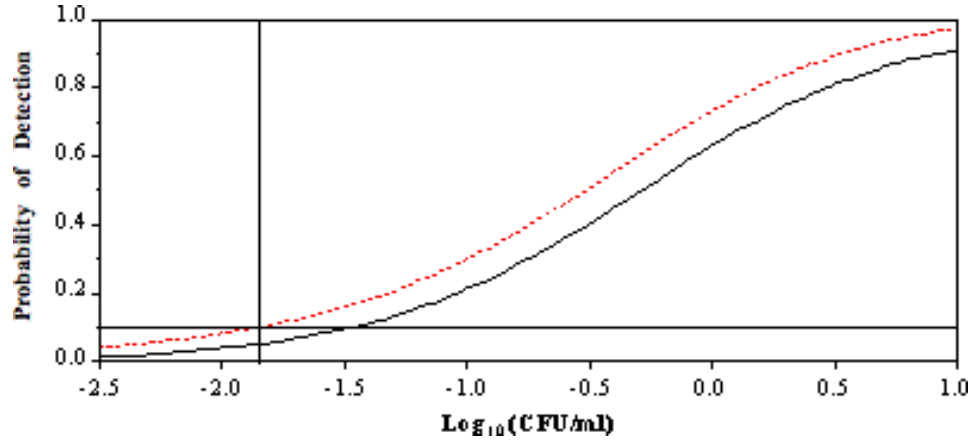
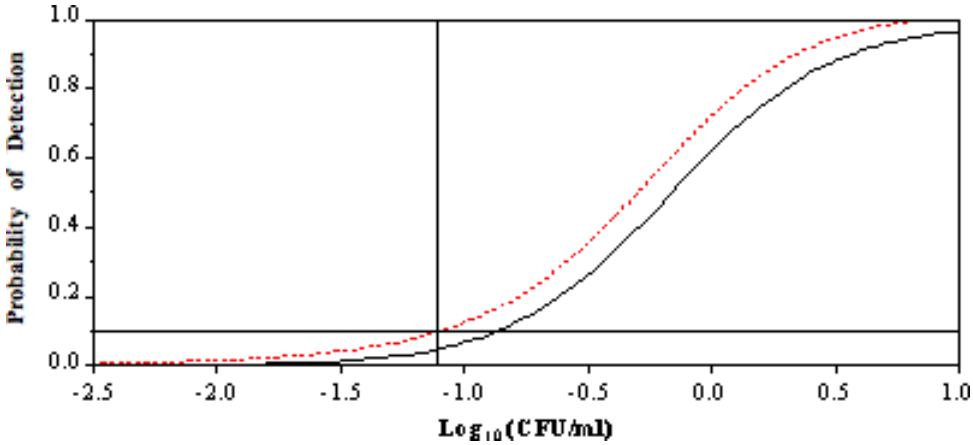


Figure 3. Logistic Regression Curve for Probability of Detection for Plate Count Method



Conclusion

This study determined that the Celsis AMPiScreen method satisfied the acceptance criteria required to demonstrate equivalence to the traditional plate count method for screening a non-sterile solution for microbial contamination. The McNemar's test concluded the Celsis AMPiScreen method was not statistically superior to the plate count method for detecting contaminated samples. As such, the hypothesis of equivalence was testing using a Receiver Operating Characteristics Table using an acceptance criterion of 70%. Each of the critical method attributes, accuracy, precision, specificity, and sensitivity, were found to meet or exceed the 70% acceptance criteria. The limit of detection for the AMPiScreen method was 5- fold smaller than that for the plate count method, indicating that the AMPiScreen method is able to detect lower levels of contamination than the plate count method.

As such, the Celsis AMPiScreen method was found to be equivalent to the compendial plate count method for detecting low concentrations of microorganisms in a suspension product after a 24-hour enrichment period.

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