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Poster Presentations

How to validate challenging parameters of a quantitative rapid method in Pharma Industry? Two Customer Examples

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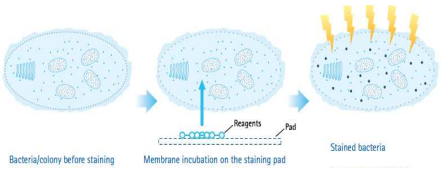
Introduction

The interest of Pharmaceutical Industry to implement methods which allow faster results and release of their products is very high. For the majority of customers the validation of Rapid Microbiological Methods (RMM) according to EP 5.1.6 is still challenging. Especially the parameters that require advanced statistical knowledge like accuracy, precision and linearity could prevent customers from completing their validation and using of the method in routine. Additionally validation work can take a lot of resources.

The following customer examples of global Pharmaceutical companies provide two different approaches how to implement and validate a RMM method for the application of Rapid Bioburden Testing.

Both studies were performed with the Milliflex® Quantum, a fluorescence-based technology for filterable samples.

The principle of the fluorescence detection is based on an enzymatic reaction. The fluorogenic substrate used is a non–fluorescent viability marker that is cleaved by nonspecific intracellular enzymes resulting in a fluorescent product [1]. Accumulation of fluorescence inside cells is an indicator of microbial metabolism activity and membrane integrity. The dye is diluted in a staining buffer allowing cell membrane permeabilization and thus passive dye introduction into cells, maintaining the cells viability.



Validation Principles

Milliflex® Quantum method is validated as an alternative method to the compendial test of bioburden for pharmaceutical products (Microbiological examination of Non Sterile Products, EP 2.6.12, USP <61> or JP 4.05) according to Guidelines EP 5.1.6, USP <1223> and PDA TR 33, Validation of Alternative Microbiological Methods [5], [6], [7], [8].

We consider here the Performance Qualification done by the user, considering that a primary validation has been carried out by the supplier (EP 5.1.6 gives a very comprehensive matrix with the respective rules of Supplier and User in the validation process)

As Milliflex Quantum is a **quantitative method**, the validation of a Rapid Microbiological Method comprises the following parameters:

- LOQ /LOD
- Precision
- Linearity/ range
- Specificity
- Robustness/ Ruggedness
- Suitability
- Equivalence

Methodology

To perform the validation experiments, the alternative method (Milliflex® Quantum) is compared to the traditional method by membrane filtration (Milliflex®), using materials and equipment provided by the supplier.
As the method is to be validated for its intended use, to demonstrate the specificity of the method, experiments were done with culture collection strains (ATCC) but also in-house isolates, some of which had been stressed (heat or starvation).
Validation studies require statistics because they include experiments which provide data that must be analyzed and interpreted. In particular, quantal tests related to the sensitivity of the method (LOQ/ LOD), precision and accuracy require a good level of statistical knowledge and methods.
Then, we focus on these parameters because they are considered as challenging for customers and, additionally, specificity is also treated, because it is key for the performance and justify the choice of an alternative method.

In both customer cases, Milliflex® Quantum is validated as an alternative global bioburden method with a reduced incubation time of 72 H instead of 5-7 days. In customer case 1 the Milliflex® Quantum is used as a qualitative test for LOD (using MPN) and as a quantitative test for Specificity.

Validation Parameters and Statistical Methods

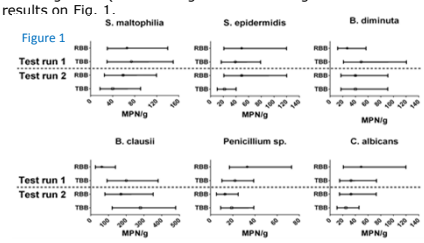
customer 1 [2]

Limit of Detection and (Quantification)

LOD and LOQ are the lowest number of microorganisms that can be respectively detected or accurately counted. LOQ was not in the scope as counting of colonies are done by an operator in both cases.

Test by MPN: inoculation of rinsing solution with 50, 5, 0.5 and 0.05 CFU . Alternative method (RBB) and compendial one (TBB) tested in parallel, two runs of each 10 replicates per concentration and microorganism (15 microorganisms including 7 in-house isolates). See results on Fig. 1.

Figure 1



Specificity

The specificity of a method is defined as the potential to detect a broad range of microorganisms.

Remark: False positive rate is also to be tested , with a great number of blank test repetitions.

In this example, the specificity is demonstrated with 15 microorganisms including 7 representative stressed in-house isolates.

Test: 2 test runs of 10-100 CFU in 100mL with 3 replicates per microorganism. For some strains, the sample size is increased to reach the required test power. The CFU count for alternative test should not be statistically different as compared to the compendial test. For each run a 2-sample t-test at a confidence level of 95% is performed. (p-value must be >0.05). Finally, a test power calculation is used to demonstrate non inferiority, with a limit of $\geq$0.8. See results Table 1.

Table 1

Species	Sample Size RBB/TBB	RBB mean +/- SD [CFU]	TBB mean +/- SD [CFU]	p-value	Test Power
Pseudomonas aeruginosa	6/6	33 +/- 3	40 +/- 7	0,06	0,99
Staphylococcus aureus	5[1]/6	53 +/- 4	50 +/- 6	0,42	1,00
Bacillus subtilis	8[1]/9	36 +/- 4	45 +/- 4	0,00	1,00
Escherichia coli	9/9	23 +/- 5	20 +/- 4	0,25	0,93
Burkholderia cepacia	6/6	76 +/- 12	72 +/- 7	0,53	0,99
Stenotrophomonas maltophilia	6/6	33 +/- 7	31 +/- 6	0,59	0,82
Bacillus idriensis	6/6	21 +/- 2	22 +/- 4	0,51	0,97
Bacillus clausii	9/9	54 +/- 13	53+/- 14	0,67	0,79[3]
Staphylococcu epidermidis	6/6	33 +/- 5	33 +/- 6	0,92	0,92
Staphylococcus warneri	9/9	38 +/- 8	37 +/- 8	0,80	0,87
Pseudomonas stutzeri	6/6	48 +/- 7	41 +/- 8	0,13	0,95
Kocuria rhizophila	6/6	79 +/- 7	77 +/- 9	0,64	1,00
Penicillium sp.	6/6	88 +/- 12	90 +/- 6	0,72	1,00
Aspergillus brasiliensis	6/5[1]	46 +/- 7	48 +/- 2	0,52	0,99
Candida albicans	9/9	32 +/- 7	35 +/- 8	0,45	0,90

customer 2

Accuracy and linearity

Note: Staphylococcus aureus(ATCC 6538) is treaded as an example but each microorganism from culture collections and isolates can be tested by the same methodology.

Test: a stock solution (Vt) is spiked with 8 BioBall® of 550 CFUs (4400 CFU), 4 dilutions are created from Vt with volumes: V1=36 mL, V2=32.4 mL, V3=18 mL, V4=0.72mL

Each volume is complemented to 36 mL and then fully tested with Rapid method (RMM) and Compendial (CM) i.e., 18 X1 mL for each.

- To demonstrate accuracy, the total number of observed CFU are pooled over all dilutions for each method separately (X_{RMM} and X_{CM}) and the likelihood ratio test is calculated:

$$LRT = 2[X_{RMM}(\ln X_{RMM} - \ln(X_{RMM}+X_{CM})/2) + X_{CM}(\ln X_{CM} - \ln(X_{RMM}+X_{CM})/2)]$$

LRT larger than 3,841 indicates a difference in detection. See results in Table 2. [3],[4]

- To demonstrate the linearity
  A regression curve is established for each method separately with Expected versus Observed counts on dilution (See Figure 2). Expected counts are derived from the total counts (X_{RMM} and X_{CM}) proportionally to the dilution volumes. Linearity is obtained when the regression line does not deviate from the line y=x.

Precision

Candida albicans (ATCC 10231) is treaded as an example but each microorganism from culture collections and isolates can be tested by the same methodology.

Test: create a low and high spiked dilution over multiple days that is analyzed by multiple analysts (8 runs of 4 samples for each method). Calculate Repeatability (%) and Intermediate Precision (%) via a random effects ANOVA model (see ICH Q2(R1)). Results in Table 3 & Table 4. [3]

Discussion of Results

Limit of Detection

As demonstrated in Fig. 1, in all cases the 95% confidence intervals of the traditional and the rapid bioburden test do overlap, no significant difference regarding the LOD of any of the test strains is observed demonstrating adequate LOD for the alternative method.

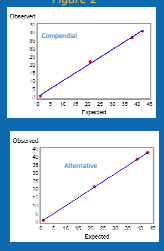
Specificity

As we can see in Table 1, after exclusion of some outliers [1] with the Grubb's test, the specificity is fully demonstrated for 14 stains. For Bacillus subtilis, the Rapid method is tested against the Traditional one by a non-inferiority test at a confidence level of 95% with a boundary of 70%. (EP 2.6.12 and USP <1227>showing that the difference is acceptable.

Table 2

Dilution V_1		Dilution V_2		Dilution V_3		Dilution V_4	
AL	CM	AL	CM	AL	CM	AL	CM
34	47	38	38	26	22	0	1
42	47	38	40	20	20	1	2
38	42	25	45	19	30	1	1
35	40	41	35	20	14	1	0
42	41	33	42	24	17	0	1
37	42	42	40	21	25	1	1
46	46	33	51	29	22	2	0
33	42	42	28	27	20	0	1
39	46	34	48	18	16	2	0
48	41	48	26	20	26	2	2
41	40	30	44	26	22	1	0
40	37	34	27	25	20	1	0
51	42	40	41	20	26	1	0
37	46	39	38	17	18	0	1
41	48	29	33	26	31	1	1
43	34	39	41	25	17	1	4
47	40	41	40	20	15	0	1
46	46	33	40	18	23	2	2

Figure 2



Accuracy & linearity

After pooling total number of observed CFU for each method (Table 2), the Likelihood Ratio Test is calculated: LRT=0,43 < 3,841. In conclusion, no significant difference is observed between accuracy of both methods. For the linearity, further investigations on the regression curve and associated slopes demonstrate perfect linearities of both methods.

Precision

Table 3

Run	Compendial				Milliflex Quantum			
Dilution	V_1 19,6		V_2 78,2		V_1 19,6		V_2 78,2	
	Mean (CFU)	STD	Mean (CFU)	STD	Mean (CFU)	STD	Mean (CFU)	STD
1	14,3	1,71	63,3	8,68	15,5	3,32	61,8	4,72
2	17,5	3,87	63,5	5,00	16,3	3,95	72,0	8,12
3	17,8	2,99	72,3	8,02	17,5	3,79	71,5	12,07
4	18,3	4,03	75,0	9,06	19,5	6,81	67,5	12,12
5	18,8	3,86	66,0	5,00	19,5	2,38	66,5	7,59
6	15,0	2,94	66,0	2,16	15,0	6,68	66,5	6,61
7	19,3	3,10	60,5	21,70	20,5	4,43	81,5	8,10
8	21,3	2,87	73,3	6,80	22,3	3,40	68,0	3,56

Per method concentration and run: 4 samples

Table 4

Dilution	V_1 19,6		V_2 78,2	
	Compendial	Alternative	Compendial	Alternative
Averages	17,75	18,25	67,47	69,41
Repeatability (%)	18,3	25,2	14,8	12,1
Intermediate Precision	20,3	26,0	15,1	13,4

After calculation of repeatability and intermediate precision (see Table 4) with data available on Table 3, we demonstrate that, as CV-repeatability satisfies the criteria based on Poisson and the total variation is less than 30% for both methods, the precision is fully acceptable for the alternative method.

Summary

With the right validation set-up and statistical approach it can be demonstrated that RMM methods provide the same performance as traditional microbiological methods adding additional advantage like time-to-release to the routine testing. Moreover, the customer examples show that statistical requirements of the EP 5.1.6 chapter can be overcome through the use of relevant tools.
However, resource and time for validation can be limited using statistical support or service offer from method suppliers.

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[6] Council of Europe, EDQM, European Pharmacopoeia, Ph. Eur. 9,2, EP 2.6.12, EP 5.1.6.
[7] PMDA, Japanese Pharmacopoeia, JP 17th edition, 4.05.
[8] Parenteral Drug Association, PDA TR 33 (Rev 2013).

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Evaluation of a Rapid Microbiological Method for filterable raw materials in Pharma Industry

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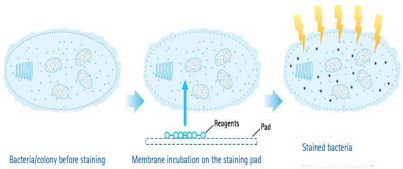
Introduction

The study was performed as part of a Microbiological Quality Control project dealing with rapid microbiological methods (RMM) at Merck Healthcare, a biopharmaceutical company focused on the areas of oncology, neurodegenerative diseases and endocrinology.

The Milliflex® Quantum System and Aim

The principle of fluorescence detection is based on an enzymatic reaction. The fluorogenic substrate used is a non-fluorescent viability marker that is cleaved by nonspecific intracellular enzymes, resulting in a fluorescent product [1]. The viability marker exhibits bright green fluorescence, which is measureable at wavelengths between 480 and 490 nm (Fig. 1) [2]. The dye is diluted in a staining buffer, allowing cell membrane permeabilization and, thus, dye introduction into cells [1] [3]. Accumulation of fluorescence inside cells indicates microbial metabolic activity and membrane integrity. The aim of the study was to validate the Milliflex® Quantum rapid microbiological method for the examination of filterable raw materials that are common in microbiological quality control in the pharmaceutical industry. The effects of raw materials on growth were also of particular interest.



Materials & Methods

The causative microorganisms, Staphylococcus aureus, Bacillus subtilis, Candida albicans and Aspergillus brasiliensis, as well as the isolate, Staphylococcus epidermidis, were used for the validation. It was assumed that microorganisms, which are detected during microbiological quality control, would be stressed. This stress can occur through starvation, heat, dehydration or disinfectants. To simulate these conditions and induce spore formation, microorganisms were exposed to 60 °C (Fig. 2), or starvation in the case of B. subtilis. The target was to obtain an inoculum around 50 % compared to frozen stock solution [4]. Validation was achieved through one of two approved methods: inoculation during the last rinsing step, or direct inoculation of the raw material buffer solution. In the event of inhibition, the first method was used to obtain measureable results. Evaluation followed after three days. In parallel to the Milliflex® Quantum method, the traditional technique was performed with an incubation period of five days. Finally, the two methods were compared and assessed.

Discussion

Results & Prospects

As expected, the bacteria reacted sensitively to low pH, and their growth was inhibited, while the mold and yeast were mostly unaffected. Orange oil, peach aroma and orange aroma had the greatest inhibitory effect on all microorganisms except A. brasiliensis, which was only verifiably inhibited by orange aroma. The inhibition of the growth of P. aeruginosa and B. subtilis by sodium saccharin was expected at this concentration [5]. Nicotinamide had an inhibitory effect on B. subtilis and P. aeruginosa, but surprisingly not on S. aureus [6]. In comparison to the other organisms, stressed B. subtilis seemed to be highly sensitive to changes in pH and other conditions. In most cases, the Milliflex® Quantum System did not show significant differences in received values, since the statistical threshold was just one unit below 0.05. The high values in the recovery rates were due to the afore-mentioned inhibitory effects of the raw materials (Fig. 3, Orange oil). The low numbers of colony-forming units (CFU) also resulted in greater recovery rates. A. brasiliensis was not measurable after five days, thus determination of recovery rate and t-value were not feasible. However, evaluation via the Milliflex® Quantum System was possible.

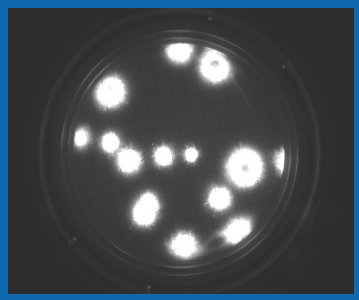
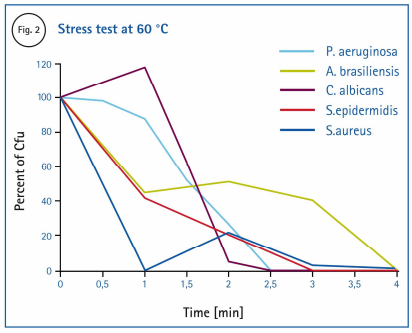


Fig. 3

Raw Materials	A. brasiliensis		C. albicans		B. subtilis		P. aeruginosa		S. aureus		S. epidermidis	
	Recovery Rate [%]	T-test	Recovery Rate [%]	T-test	Recovery Rate [%]	T-test	Recovery Rate [%]	T-test	Recovery Rate [%]	T-test	Recovery Rate [%]	T-test
Orange aroma	n.e.	n.e.	100.69	0.87	94.83	0.74	52.94	0.20	73.68	0.34	100.00	1.00
Ascorbic acid	n.e.	n.e.	102.70	0.88	88.75	0.30	119.35	0.59	102.60	0.91	100.00	1.00
Dexpanthenol	n.e.	n.e.	92.41	0.10	100.00	1.00	79.37	0.06	113.11	0.07	117.07	0.31
Dexpanthenol	n.e.	n.e.	96.67	0.56	87.01	0.52	86.11	0.19	89.29	0.39	100.00	1.00
Glycerol 85% PHA Prod	n.e.	n.e.	90.00	0.39	106.19	0.18	78.95	0.18	117.81	0.44	118.10	0.06
Glycerol 85% PHA Prod	n.e.	n.e.	122.69	0.19	119.10	0.26	90.32	0.10	96.72	0.90	103.57	0.88
Glycine PHA Prod	n.e.	n.e.	102.96	0.87	96.49	0.92	81.82	0.50	108.93	0.71	127.93	0.10
Metformin hydrochloride/Magnesium stearate 0.5 %	n.e.	n.e.	81.06	0.07	119.10	0.26	74.19	0.42	110.00	0.75	106.06	0.86
Sodium dihydrogen phosphate dihydrate PHA Prod	n.e.	n.e.	95.29	0.65	90.91	0.57	100.00	1.00	79.55	0.35	100.96	0.96
Nicotinamide PHA Prod	n.e.	n.e.	102.44	0.81	111.86	0.40	106.25	0.77	112.50	0.55	112.62	0.28
Orange oil	85.71	0.65	250.00	0.40	100.00	1.00	117.39	0.71	103.51	0.92	94.59	0.83
Peach aroma 501.079C	n.e.	n.e.	98.63	0.71	110.29	0.47	n.e.	n.e.	104.88	0.80	102.44	0.92
Pyridoxine hydrochloride PHA Prod	n.e.	n.e.	108.03	0.17	108.62	0.63	104.00	0.78	115.00	0.39	100.00	1.00
Sodium saccharin	n.e.	n.e.	101.65	0.92	89.47	0.72	107.14	0.83	80.00	0.09	107.69	0.53
Thiamine chloride hydrochloride PHA Prod	n.e.	n.e.	101.37	0.92	109.68	0.10	80.00	0.25	74.19	0.11	118.75	0.16
Thiamine chloride hydrochloride PHA Prod	83.33	0.68	95.71	0.67	101.56	0.92	77.78	0.23	90.91	0.59	113.25	0.45



Summary

The Milliflex® Quantum method represents a rapid alternative method for the quantification of contaminants in filterable products. The system has been successfully validated for three raw materials: glycerol, pyridoxine hydrochloride and thiamine chloride hydrochloride. This rapid microbiological method allows pharmaceutical manufacturers and quality control laboratories to address contamination events sooner, avoid line shutdowns, reduce storage costs and enable the earlier release of products to the market.

References
[1] Baumstummler, A. et al. (2009). Milliflex® Quantum: A Fluorescence-based Platform for the Rapid Detection of Contaminants in Filterable Products (Poster).
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A new smart incubator that is detecting and enumerating industry – relevant microorganisms in real time guarantees complete data integrity

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INTRODUCTION

Tight control of microbial environment surrounding health and personal care products is key for pharmaceutical industry. Quality and safety of final product is ensure through microbial monitoring of raw materials especially water, manufacturing environment and manufacturing process with in process and final product testing. Despite a number of benefits, alternative microbiology methods are poorly implemented and microbial controls are still performed using compendial petri dishes methods with operator-visual enumeration. These human-based tests are highly prone to individual mistakes (mis-labeling, mis-dilution, mis-counting, mis-reporting, loss of data ..) globally questioning the company data integrity policy. Here we present an innovative system, the EviSight™ compact, an integrated biomonitoring instrument able to incubate and read petri dishes in a real-time manner. This system is designed to be compliant with 21 CFR 11 requirements ensuring complete data integrity. The performances of the technology were challenged and demonstrated by 4 different and independent pharmaceutical industries through 3 main applications (90 mm and contact petri dish analysis, filter based analysis). Results obtained by these beta sites allow to establish statistically-relevant trueness, precision and equivalence of this method.

MATERIALS AND METHODS

• EVISIGHT™ COMPACT PRESENTATION

The EviSight™ Compact system is a smart incubator that automatically analyses commercial petri dishes for microbial detection and enumeration without colony staining or opening of the culture disposable.

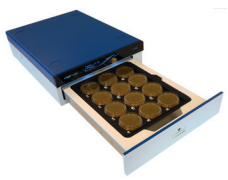


Figure 1

Thanks to its interchangeable tray (figure 1), the EviSight™ Compact system can be used with a wide range of disposables: 90 mm / 55 mm / contact plates for filter-based (water and filterable products) and direct agar (environmental monitoring, product control ..) analysis.

Figure 2

With the first version, up to 4 readers can be stacked and connected to the same computer, offering greater incubation and processing capacity in a minimum of space (figure 2).
Each reader can be run individually allowing disposable and set temperature (from 20 to 35°C) flexibility.

• STRAINS AND DISPOSABLES

Test microorganisms included in the study were sourced from culture collection and commercial calibrated strains.

- BioBall Multishot (bioMérieux):
  Aspergillus brasiliensis ATCC 16404, ref. **56011**
  Bacillus subtilis ATCC 6633, ref. **56012**
  Candida albicans ATCC 10231, ref. **56013**
  Pseudomonas aeruginosa ATCC 9027, ref.**56017**
  Staphylococcus aureus ATCC 6538, ref. **56019**

- Culture collection:
  Methylobacterium extorquens NBRC **15911**
  Pseudomonas fluorescens NBRC **15842**

Additional in-house microorganisms from the different pharma industries collection.

The different disposables used are listed in the table 1:

	PRODUCT	REFERENCE	SUPPLIER
Culture media 90 mm	R2A	43551	bioMérieux
	TSA	43011	bioMérieux
	TSA 3P w. neutralizers	43811	bioMérieux
	Sabouraud 3P	43814	bioMérieux
Culture media 55 mm	R2A	AEB523487	bioMérieux
Culture media Contact plates	Count-Tact 3P	43691	bioMérieux
	Count-Tact SDA 3P	43812	bioMérieux
White plain filters	Cellulose acetate filters	11106-47-ACN	Sartorius
	PVDF filters	HVWP047S6	Millipore
	MicroFunnel filters	516-8963	Pall

Table 1: Disposables used in the study

• EVISIGHT™ COMPACT EVALUATION

Filtration applications were tested using R2A plates with the 3 different filters spiked with 3 microorganism concentrations (5, 25 and 50 CFU/test), each condition tested in five replicates.90 mm and contact plate applications were tested with direct inoculation of 3 microorganism concentrations (5, 25 and 50 CFU/test), each condition tested in five replicates.
Incubation temperature were set according to pharmacopoeia. Maximum incubation times were set until optimal growth for easy detection by human eye.

• STATISTICAL ANALYSIS

EviSight™ compact performances were established from results obtained by four pharmaceutical industries using suitable statistical tests. EviSight™ compact enumeration was compared to operator-visual enumeration.

RESULTS AND DISCUSSION

• GROWTH KINETIC AND "GO BACK IN TIME" FEATURE

The EviSight™ compact system runs a high magnification technology enabling detection of microorganisms in their early stages of growth when they form microcolonies that are invisible to the naked eye (between 30 and 250 µm). As a real-time incubator and analyzer EviSight™ compact pictures the incubated plates every 30 minutes and powerful algorithms interpret microorganisms (micro)colonies growth. The system then deliver, for each plate incubated, a growth curve kinetic as illustrated in figure 3.

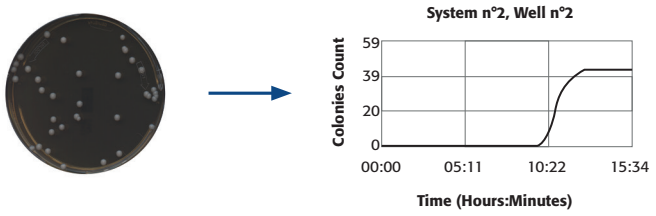


Figure 3: *C.albicans* growth curve

As the system record the successive images of each plate, it's possible to look microbial growth at different times of interest (before colonies swarming, pigmentation...).
The figure 4 illustrates this "go back in time" feature for a swarming mold.

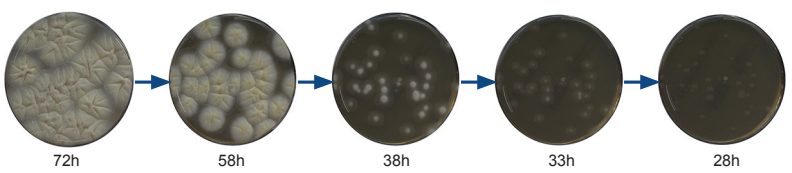


Figure 4: *A.brasiliensis* growth on contact plate monitored at different incubation times

• EVISIGHT™ COMPACT TRUENESS

The enumeration results from EviSight™ compact and manual were compared using a boxplot graphical representation (figure 5).
This allows to visualize distribution characteristics of a quantitative variable.

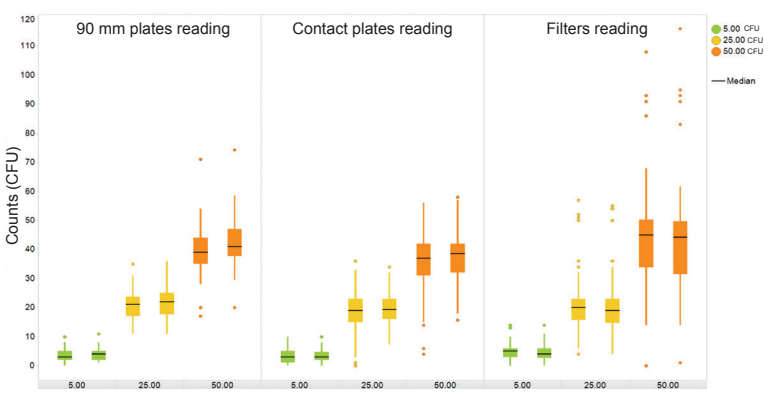


Figure 5: Boxplots of microorganisms counts obtained with EviSight™ compact (left side of the pair) and manually (right side of the pair)

EviSight™ and manual methods show similar enumerations

• PRECISION

For both EviSight™ and manual enumeration, for each concentration level, repeatability is expressed thought standard deviation and coefficient of variation (table 2). Statistical comparison of repeatability variances obtained with both enumeration methods was performed using the Fischer test (alpha = 5%) for each concentration level.

		EviSight™ compact repeatability estimation			Manual Count repeatability estimation			Fischer test	
Application	Target conc (CFU)	Mean	std dev	CV (%)	Mean	std dev	CV (%)	p value	Conclusion
90 mm plates	5	3.33	1.7	51.9%	3.58	1.8	49.8%	0.4278	NO STATISTICALLY SIGNIFICANT DIFFERENCE
	25	21.14	4.6	21.8%	21.98	4.7	21.2%	0.4703	NO STATISTICALLY SIGNIFICANT DIFFERENCE
	50	39.92	8.8	22.1%	42.20	9.3	22.0%	0.3683	NO STATISTICALLY SIGNIFICANT DIFFERENCE
Contact plates	5	3.28	1.9	57.8%	3.23	1.9	58.6%	0.5	NO STATISTICALLY SIGNIFICANT DIFFERENCE
	25	18.35	4.6	25.3%	19.34	4.4	23.0%	0.3451	NO STATISTICALLY SIGNIFICANT DIFFERENCE
	50	35.77	6.0	16.8%	37.03	5.2	14.2%	0.1005	NO STATISTICALLY SIGNIFICANT DIFFERENCE
Filters	5	4.86	2.9	60.2%	4.42	2.7	60.9%	0.2988	NO STATISTICALLY SIGNIFICANT DIFFERENCE
	25	21.07	5.1	24.3%	20.69	5.1	24.5%	0.4728	NO STATISTICALLY SIGNIFICANT DIFFERENCE
	50	44.73	7.4	16.4%	44.05	6.9	15.7%	0.3335	NO STATISTICALLY SIGNIFICANT DIFFERENCE

Table 2: Precision estimates and statistical comparison of EviSight™ compact and manual enumerations

Precision is demonstrated as all repeatability variances were shown to be statistically comparable between EviSight™ compact and manual enumeration.

• NUMERATION EQUIVALENCE OF EVISIGHTTM COMPACT AND MANUAL COUNTS.

Results obtained from EviSight™ compact and manual numeration, for all concentration, were plotted together and a linear regression was performed (figure 6).

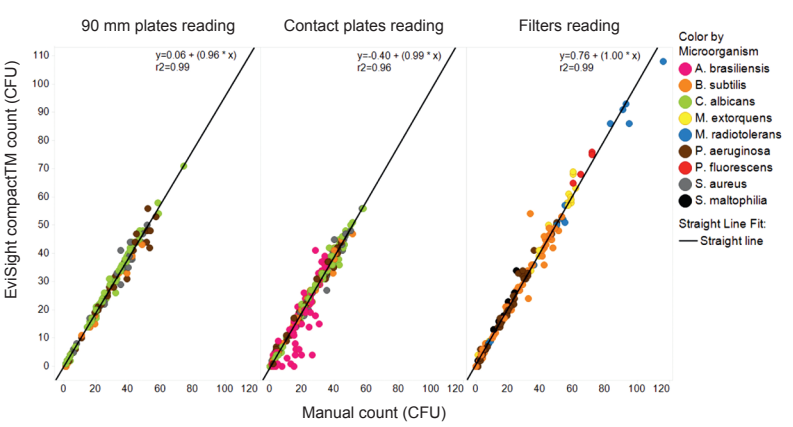
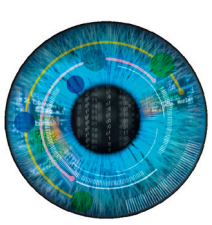


Figure 6 - Linear regression of Evisight enumeration results versus mean of manual plate counts

All regression analysis show a coefficient of determination r^2 greater than 0.9, a slope of the line of the best fit lying between 0.8 and 1.2 and a y-intercept close to zero.
As there is no statistical difference of count between both methods, EviSight™ compact and manual enumerations are demonstrated equivalent.

CONCLUSIONS



Health and personal care products are closely reviewed by regulatory agencies as they involve patient / customer safety. In particular, microbial monitoring of raw materials, manufacturing environment and products is critical. Petri dishes traditional microbiology with operator-visual enumeration is still a reality in pharmaceutical industry but subject to human mistakes hard to justify. EviSight™ compact system provides you an automated analysis of your petri dishes and microbial enumeration in a real-time fashion. Performances of this system were demonstrated in terms of trueness, precision and equivalence to visual enumeration with in-house real-life pharmaceutical industry studies. Moreover, the real-time monitoring of microorganisms growth allow to generate growth curve and analyze previous images, a valuable tool for investigations.

REFERENCES

• USP 40 - NF 35 - Chapter <61>, Chapter <1231>, Chapter <1223> and official monographs on water

• PE 9 - Version 9.3 - Chapter 2.6.12, Monographies «eau pour préparations injectables» et «eau purifiée», Chapter 5.1.6

• JP 17 - Chapter 4.0.5, Chapter G8, Chapter G4

. PDA Technical Report 33

Development of R2A Media Optimized for Pharmaceutical Grade Water and Evaluation of Automated Water Testing Using the EviSight™ Compact

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INTRODUCTION

R2A 3P® is a line extension of the existing 3P® product range. Therefore, this is an irradiated double wrap product, with flexible storage (2-25°C), extended shelf-life (6 months), and moisture/dehydration management. Based on R2A medium developed by Reasoner and Geldreich, R2A 3P® detects microorganisms that dwell in highly stressed environments, such as pharmaceutical water systems, and can be used for stressed microorganisms requiring low nutrient media for optimal growth.

The formulation and performance standards of R2A 3P® were designed to be compliant to the Pharmacopoeia recommendations [EP 9, JP 17, and USP 40] for R2A water testing. Additional regulatory guidance and peer-reviewed publications were referenced for product development and validation. The growth performance of the newly developed R2A 3P® was compared to commercially available media and included both Pharmacopoeia and environmental isolates. The EviSightTM Compact automated incubation and enumeration instrument uses high-magnification digital imaging technology for detecting colonies at early stages of growth (20-50 µm). Pictures are taken every 30 minutes. The system is designed to deliver data integrity and full traceability. This study evaluated R2A 3P® using the EviSight Compact and Pharmacopoeia isolates after standard filtration at minimum and maximum incubation times to determine feasibility for automated water testing.

MATERIALS AND METHODS

• TESTED PRODUCTS

The R2A 3P® media developed at bioMérieux and other culture media used in this study are detailed in Table 1.

bioMérieux Item No.	Name	Description
M1065	R2A	Non-irradiated control
421986	R2A 3P®	Irradiated; 2-25°C storage; 6 month shelf-life
NA	Media A*	Irradiated; 2-8°C storage; 3.5 month shelf-life
NA	Media B*	Irradiated; 2-8°C storage; 4 month shelf-life

Table 1: Culture Media used in this study *Commercialy available R2A media

• LIBRARY OF TESTED MICROORGANISMS

A total of 70 microorganisms were used in this study and were sourced from commercially available and internal library collections and included wild isolates obtained from pharmaceutical manufacturers.

• GROWTH PROMOTION TESTING

Growth promotion was evaluated with the panel of 70 strains. The microorganisms were prepared using a fresh cell suspension or BioBall® format. A target of <100 cells were used for all microorganisms. The cells were either applied directly to the test media using a spiral plater or were filtered through a 0.2 µm PES membrane. Performance testing was run in duplicate for both stressed (1 day at 2-8˚C in sterile purified water) and non-stressed organism testing.

Plates were incubated at 30-35˚C for USP/PE recommended and environmental organisms and at 20-25˚C for JP recommended organisms for 3 to 7 days. Colony counts were performed using an automated colony counter. Acceptance criteria for microorganism recovery was between 50 – 200% compared to non-irradiated R2A controls.

• EXUDATION

R2A 3P® was evaluated for condensation/ exudation inside and outside the plate and packaging over the shelf-life of the product using the qualitative and quantitative scale outlined in Table 2.

Acceptance criteria was established to be ≤2 on the lid and ≤1 in the sleeve.

Exudation score	Description
0 – Dry	No fog, condensation or droplets
1 – Condensation	Moist lid or with humidity area, no separate drops and/or droplets size < 1mm
2 – droplets	Separate droplets size ≥ 1 mm, and ≤ 4 mm
3 – Drops	Several big separate drops
4 - Water	No separate drops, puddle of water in the lid

Table 2: Exudation scale

• DEHYDRATION

The newly developed R2A 3P® media was designed with a deeper fill volume (25 mL) to withstand extended incubation periods required for retrieval of stressed/slow growing organisms.

Dehydration was assessed over the shelf-life of the product using the scale shown in Table 3.

Acceptance criteria was established to be ≤10% of minor shrinkage/cracks of total samples tested.

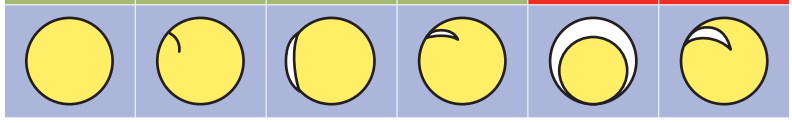
1 - Fully Intact	2 - Meniscus break	3 - Retraction from side ≤2mm	3 - Cracking ≤2mm	4 - Retraction from side >2mm	4 - Cracking >2mm
					

Table 3: Dehydration scale

• EVISIGHT™ COMPACT

Growth of Pharmacopoeia microorganisms (after standard filtration) on the EviSight Compact instrument (Figure 1) was evaluated at minimum and maximum incubation time on R2A 3P.

Two filter types were used to determine feasibility and included:

- Millipore™ - 0.45µm polyvinylidene fluoride (PVDF)
- Pall® - 0.2µm polyethersulfone (PES)

Figure 1: EviSight™ Compact Instrument, Single incubator

RESULTS AND DISCUSSION

• GROWTH PERFORMANCE – STANDARD INOCULATION AND FILTRATION WITH STRESSED ORGANISMS

Growth promotion was evaluated on R2A 3P® and commercially available irradiated R2A culture media with a total of 70 microorganisms.

Growth promotion using membrane filtration was evaluated on R2A 3P® and commercially available irradiated R2A culture media with a total of 11 stressed microorganisms.

Figure 2 clearly illustrates R2A 3P® having better growth performance as compared to commercially available irradiated R2A media. R2A 3P® has the highest growth recovery rate without recorded failures for all microorganisms tested, whatever the inoculation method.

The growth promotion results for the Pharmacopeia microorganisms (Table 4) show acceptable (50-200%) recovery for all tested media with R2A 3P® and Media B showing slightly better performance compared to Media A.

A total of 62 environmental isolates were tested for growth promotion. Only R2A 3P® was able to successfully support 100% recovery within acceptable limits. Table 5 details the isolates that R2A 3P® was able to detect compared to other commercially available R2A media. The results indicate the importance of including a wide-range of water and environmental microorganisms in product development.

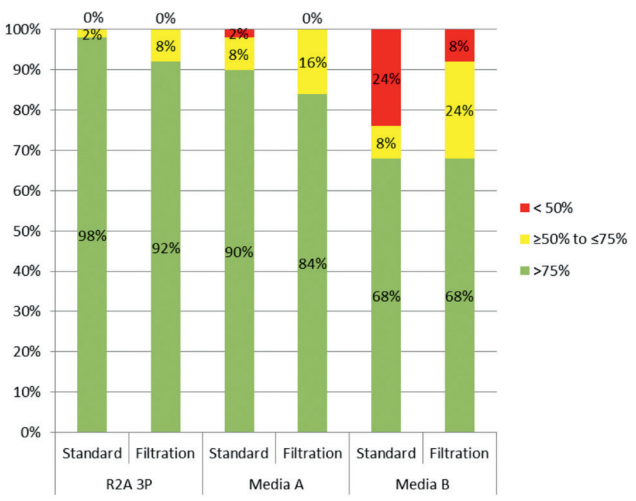


Figure 2: Growth performance consolidated recovery rate of R2A 3P® other irradiated R2A media, direct inoculation and Filtration with stressed organisms

RESULTS AND DISCUSSION CONTINUED

• TESTED PRODUCTS

Organisms	Temp °C	% Recovery Rate		
		R2A 3P®	Media A	Media B
% Recovery of Pharmacopoeia Organisms at 50-200%		100%	100%	100%
% Recovery of Pharmacopoeia Organisms at >75%		100%	70%	100%

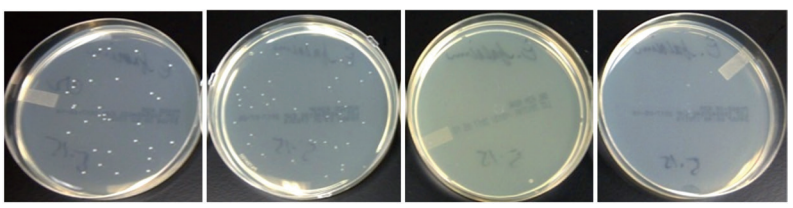
Table 4: Pharmacopoeia organism recovery, R2A 3P® vs. other irradiated R2A media

Organisms	% Recovery Rate		
	R2A 3P®	Media A	Media B
Aeromonas media API 1212102	102	96	24
Aeromonas sobria 201402043	106	94	42
Bacillus licheniformis 201402030	154	88	27
Brevundimonas vesicularis 201402044	110	113	0
Chryseobacterium daecheongense API 1310029	76	93	0
Corynebacterium striatum 201401014	148	55	39
Cupriavidus pauculus API 1310032	86	82	42
Enterococcus faecium 201402002	117	11	14
Iodobacter fluviatilis API 0606196	117	110	0
Ralstonia insidiosa API 0512080	107	104	0
Ralstonia pickettii 201509004	145	106	0
Sphingomonas leidyi API 1310041	152	131	0
Sphingomonas leidyi API 1310044	162	112	0
Sphingomonas paucimobilis 201310007	123	112	0
Virgibacillus pantothenticus 201402040	84	82	1

Table 5: Environmental organism recovery at ≤50%, R2A 3P® vs. other irradiated R2A media

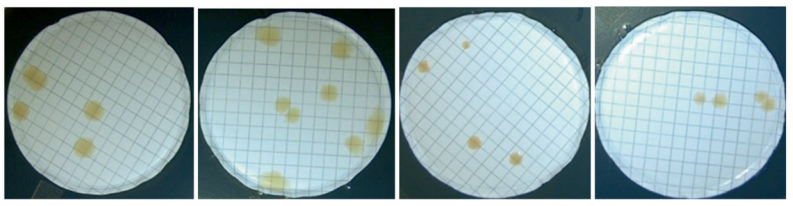
To further illustrate growth and recovery:

- **Figure 3** is an example of an environmental isolate on control and tested media,
- **Figure 4** shows equal or better performance of R2A 3P® compared to non-irradiated R2A control, with the filtration method.



Non-irradiated R2A R2A 3P® Media A Media B

Figure 3: *Enterococcus faecium* 201402002 after 2 days incubation at 30-35°C



Non-irradiated R2A R2A 3P® Media A Media B

Figure 4: *Pseudomonas aeruginosa* ATCC 9027 after 3 days incubation at 30-35°C

• EXUDATION

During stability shelf life testing, results from condensation/ exudation monitoring show that R2A 3P® conforms to the required qualitative and quantitative scoring scale: ≤1 for sleeves and ≤2 for plate lids (Figure 5).

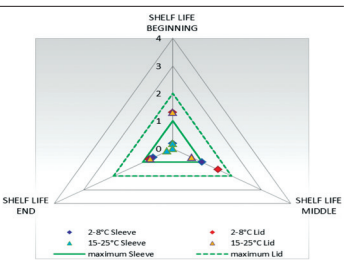
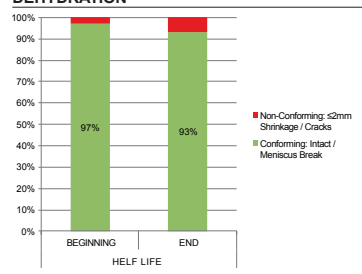


Figure 5: R2A 3P® exudation sleeve and plate lid scoring over shelf life

• DEHYDRATION



Stability testing for dehydration with extended incubation time during and up the end of total product shelf life show that R2A 3P® meets the required standards for optimal culture medium integrity at ≤10% of minor shrinkage/ cracks of total samples tested (Figure 6).

Figure 6: Dehydration evaluation for R2A 3P® over shelf life

• EVISIGHT™ COMPACT

The EviSight Compact feasibility testing with R2A 3P® show comparable results to manual reading using both PVDF and PES filter types (Table 6). The results using the 0.2 µm PES filter from Pall showed the least variation between minimum and maximum reading time points. The results support that R2A 3P® is compatible with EviSight™ Compact. Additional studies are being performed including mixed cultures to fully validate the media for automated water testing.

Organism	Filter	Min / Max Incubation	Colony Forming Units	
			Manual	EviSight
Pseudomonas aeruginosa ATCC 9027	Millipore	36 hours	48	51
		72 hours	48	54
	Pall	36 hours	48	40
		72 hours	48	45
Bacillus subtilis ATCC 6633	Millipore	36 hours	41	41
		72 hours	41	41
	Pall	36 hours	38	36
		72 hours	38	36
Pseudomonas fluorescens NBRC 15842	Millipore	72 hours	65	64
		5 days	65	67
	Pall	72 hours	27	27
		5 days	27	27
Methylobacterium extorquens NBRC 15911	Millipore	5 days	23	12
		7 days	28	28
	Pall	5 days	35	34
		7 days	35	34

Table 6: EviSight™ Compact vs. manual enumeration results on R2A 3P® at minimum and maximum incubation

CONCLUSION

As a premium 3P® culture medium with flexible storage (2-25°C), R2A 3P® was carefully designed and developed for optimal performance with validation of consistent proven product performance up to the end of total shelf life (6 months).

The newly developed R2A 3P® showed equal or greater growth performance as compared to non-irradiated R2A and commercially available irradiated R2A media. R2A 3P® was the only tested medium able to successfully support growth recovery of 70 microorganisms. The results illustrate the importance of inclusion of environmental and stressed microorganisms during product development.

R2A 3P® is compatible with EviSight™ Compact allowing users to validate automated water testing with full data integrity and traceability.

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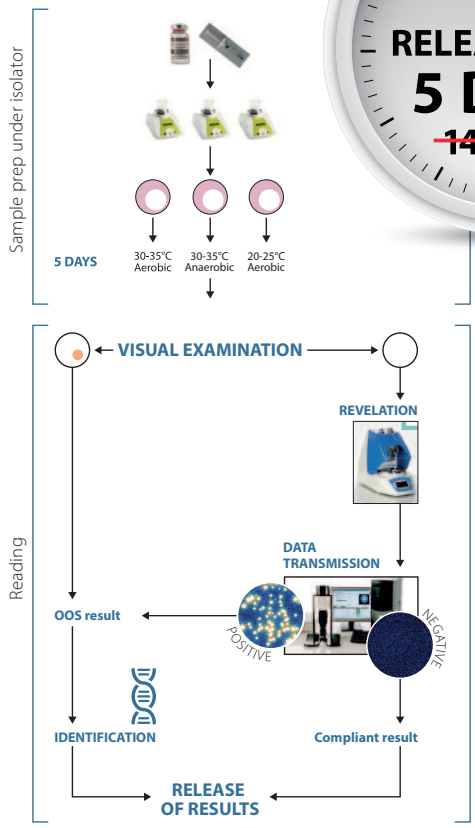
Rapid Sterility Testing

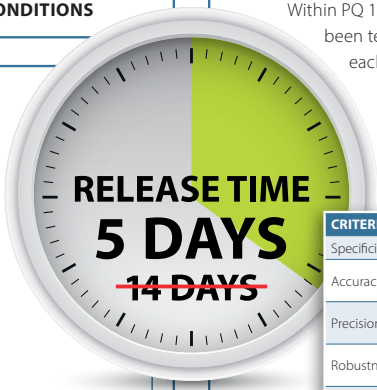
Release your finished product in only 5 days under GMP

Key benefits of Rapid Sterility Test

- **RELEASE YOUR BATCH IN 5 DAYS**
  – three times faster than the compendial method
- **SAVE STORAGE COSTS**
- **INCREASE THE QUALITY OF YOUR ANALYSIS**
  – objective computer assisted reading
- **IDENTIFICATION OF CONTAMINATION POSSIBLE**
- **GUARANTEED GMP AND C-GMP CONDITIONS**

Flow chart Rapid Sterility Test





System validation and method validation

Method validation consists of different phases:

PQ 1: validation of the system without product matrix

PQ 2-1: suitability test on the product

PQ 2-2: comparability of both alternative and compendial methods on the product

Within PQ 1, criteria of Qualitative Method have been tested and proven to be compliant for each criterium.

Validation results

Successful validation of PQ1 phase criteria

CRITERIA	RESULT	STATUS
Specificity	Each microorganism was detected	Compliant
Accuracy	Equivalent to the compendial method	Compliant
Precision	Equivalent to the compendial method	Compliant
Robustness	Variations applied to the system had no impact	Compliant
Ruggedness	Variations applied to the system had no impact	Compliant
Limit of detection (LOD)	Equivalent to the compendial method, alternative method superior for Propioni-bacterium acnes and *Micrococccus luteus*	Compliant

Validation results have proven that RST method is equivalent to the compendial method.

Why Confarma?

- 14 years of experience with sterility testing
- Experience with cell-therapy products
- Product specific validation
- Routine Testing
- Consulting
- Implementation support on your site
- Training

Regulations

- Ph. Eur. 5.1.6
- USP <1223>
- TR#33

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Validation of a Rapid Sterility Test – Implementation Strategy and Perspective from a Contract Service Laboratory

C. Fromm, S. Gärtner, Dr. T. Meindl, Labor L+S AG, Germany

Abstract

Microbial contamination in sterile pharmaceutical manufacturing remains a great risk to patient safety. Thus, examination for sterility is often the final criterion for a batch approval.
In line with the development of new, complex pharmaceuticals with short half life (ATMPs, proteins, etc.) the compendial 14-day test according to Ph. Eur. 2.6.1 and USP <71> is not appropriate. Thus, especially for these compounds, alternative rapid methods need to be implemented.
The Development and use of these alternative methods is well supported by relevant authorities (e. g. PEI, FDA).

One of the most important prerequisites for approval is an appropriate validation of the alternative method, showing equivalence to the compendial test method.

Since there is no single technology suitable for any sample matrix, various technologies are available for various matrices. For samples not containing natural ATP, ATP bioluminescence measurement by means of Charles River's Celsis® AMPiScreen technology is well established. For samples containing ATP, a suitable established method can be BioMerieux BacTAlert® system which uses another ubiquitous cellular marker.

Goal of this project was to demonstrate the equivalence of an alternative, rapid method for sterility testing to the compendial, pharmacopeial method on a broad spectrum of sample matrices. With this alternative methods, a broad spectrum of pharmaceuticals (and medical devices) can be tested for sterility fulfilling all requirements of the respective authorities and saving at least 50 % of time for test performance.

Validation Strategy

Well accepted validation requirements for alternative methods are described in:

- Ph. Eur. 5.1.6: Alternative methods for control of microbiological quality
- USP <1223>: Validation of Alternative Microbiological Methods
- PDA TR No 33: Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods

Based on these sources a validation strategy was developed comprising of the following parameters:

Parameter	Ph. Eur. 5.1.6	USP <1223>	PDA TR no. 33	Interpretation
Specificity	yes	yes	yes	Detection and identification of all tested microorganisms
Detection limit	yes	yes	yes	Equivalence to the traditional method
Precision	yes	repeat precision	no	Precise results of the alternative method
Correctness	yes	no	no	Correct results of the alternative method
Robustness	yes	yes	yes	No influence of deliberately incorporated variations
Insensitivity	no	yes	yes	No influence of unavoidable variations
Equivalence or comparison test	yes (making use of products)	yes (without product)	yes (making use of products)	Equivalence to the traditional method with use of various products

Results

Statistical backbone

Results were evaluated using a χ^2 strategy. The acceptance criterion was calculated to be $\chi^2 < 3.841$ (critical value for the 5 % significance level).

Detection Limit

The detection limit was determined by testing both methods in parallel.
To reach statistical safety the following experiments have been performed (in total 720 independent experiments)

Method	Number of positive results	Number of negative results	Total
Alternative method	203	157	360
Standard method	199	161	360
Total	402	318	**720**

The calculated χ^2 value of 0.090 is < 3.841; thus, the acceptance criterion was fulfilled

Equivalence test

In the presence of various product matrices (parenteral solution, electrolyte solution, product to be dissolved, oily product, product containing dye) the equivalence of the compendial and the alternative method was tested.

Results:
Prerequisite method suitability test: no interferences such as falsely positive or falsely negative results were determined

Comparative results:

Method	Number of positive results	Number of negative results	Total
Alternative method	174	114	288
Standard method	174	114	288
Total	348	228	**576**

Calculated χ^2 value: 0.00 is < 3.841, acceptance criterion was fulfilled.

Summary/conclusions

A straight forward validation strategy fulfilling international accepted regulatory requirements is suggested.
A successful strategy in three steps was demonstrated:

1. General method validation without products (compare both methods); broad scale general equivalence study
2. Method suitability test using products (alternative method only); general system suitability test
3. Equivalence test using products (compare both methods); matrix specific validation, showing system suitable for a broad range of samples with different matrices

Greater than 1500 tests were needed to demonstrate the equivalence of the alternative and the compendial method. Since this broad scale approach of the study was performed, for any suitable matrix a compendial suitability test of the samples matrix has to performed only. Thus, based on the preliminary work, a product specific implementation of the alternative method can be performed in the same time as for the compendial method.

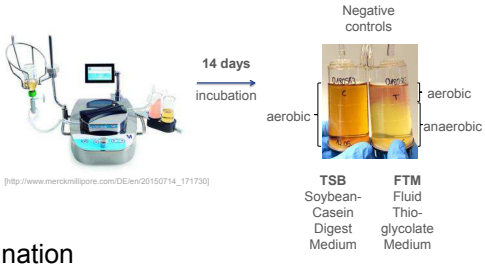
Methods

In this project, an ATP bioluminescence technology (Charles River's Celsis® AMPiScreen) was performed. This method detects microbial derived ATP in the sample via an automated luciferin-luciferase enzyme reaction system.
The underlying principle of the examination is in accordance to Ph. Eur. 2.6.1 and USP <71>, since membrane filtration and direct inoculation can be used. In the project, membrane filtration was performed. The only difference to the compendial evaluation by the human eye is an automated detection by means of ATP bioluminescence.

Membrane filtration

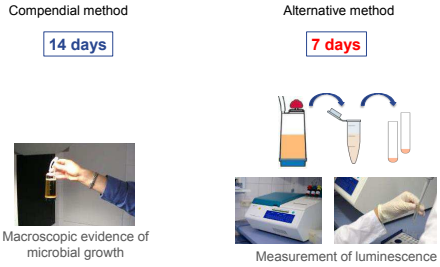
Process steps of classic membrane filtration based sterility testing (Ph. Eur. 2.6.1 and USP <71>):

1. Product filtration
2. Transfer of incubation media, followed by incubation
3. Visual examination after 14 days

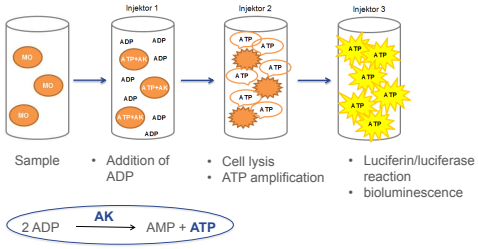


Examination

Replacement of visual inspection by two technicians by automated, audit trail controlled examination.



Principle of AMPiScreen Technology:



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- European Directorate for the Quality of Medicines & Health Care. Pharmeuropa: Ph.Eur.Chapter 2.6.1(2014) Sterility.
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- Parenteral Drug Association PDA (2013) Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods. Technical Report No.33 (Revised2013).
- European Directorate for the Quality of Medicines & Health Care. Pharmeuropa: Ph. Eur. Chapter 5.1.9 (2014) Guidelines for using the test for sterility.

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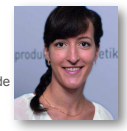

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Microbiological methods applied during the post marketing quality surveillance of products dispensed by Belgian pharmacists

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Association of Pharmacists Belgium (APB) – Brussels, Belgium.

Introduction

The Laboratory of the Association of Pharmacists Belgium (APB) is assigned to carry out the post marketing surveillance of all medicinal drugs dispensed in Belgian community pharmacies. With over 1000 analyses each year, the APB Laboratory supports the pharmacist in its legal responsibility to dispense medicines of high quality. APB conducts also quality improving programs for community pharmacists, including a study on purified water used in pharmacies.

Methodology

Various chemical and biological tests are performed on both raw materials and finished products. For the microbiological analysis of pharmaceutical products all current pharmacopoeial methods are used.

Non-sterile products

- Determination of total aerobic microbial count (TAMC) & total combined yeasts/moulds count (TYMC) (Ph. Eur. 2.6.12)
- Tests for specified microorganisms (Ph. Eur. 2.6.13)

Sterile products

Sterility testing using membrane filtration or direct inoculation (Ph. Eur. 2.6.1)

Endotoxin testing of injectables

Qualitative (gel-clot) or quantitative (chromogenic) (Ph. Eur. 2.6.14)

Applications

POST MARKETING QUALITY SURVEILLANCE

An example of a specific study is the analysis of dietary supplements. Compared to medicines, products such as dietary supplements are differently regulated and controlled. Systematic analysis of these products such as for medicines can reveal prominent quality defects.

- The Biology Platform of the APB Laboratory evaluated seven medicines and eight food supplements, containing anthranoid laxatives. The acceptance criteria of the Ph. Eur. 5.1.8 category B were applied. All the medicinal products complied, while for one food supplement TAMC (>10^4 CFU/g) and TYMC (>10^2 CFU/g) were exceeded as well as the bile-tolerant gram-negative bacteria count (MPN >100 CFU/g). For a second food supplement only the number of bile-tolerant gram-negative bacteria was unacceptable (>100 CFU/g).
- In a second study 80 teas were sampled and analysed over a period of five years. Six of them demonstrated non-conformities. Three of them did not comply with the acceptance criteria of Ph. Eur. 2.9.40 – uniformity of dosage units. Three other samples demonstrated non-conformities at a microbiological level: TAMC and TYMC were exceeded and/or *Escherichia coli* was present.

QUALITY IMPROVING PROGRAMS

Purified water is used for the reconstitution of finished products and/or during compounding in pharmacies. In Belgium purified water is commercially available under different packaging forms and routine practices differ strongly between pharmacies. We are listing current practices in Belgium, in order to analyse the corresponding microbiological water quality. Our goal is to formulate best practices for the proper use of purified water for all Belgian pharmacists.

Conclusions

Complementary to European programs by health authorities, the APB Laboratory provides a unique system of quality control of the products dispensed in Belgian community pharmacies. In this context, microbial enumeration and identification tests, sterility testing and endotoxin assays are particularly important in the achievement of our mission.

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Evaluation of MALDI-TOF Mass Spectrometry for Identification of Yeasts Commonly Found During Environmental Monitoring

Prasanna D. Khot, Ulrich Herber and Christine E. Farrance

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ABSTRACT

Background The performance of MALDI-TOF mass spectrometry (MS) is less understood for yeast isolates commonly found during environmental monitoring in manufacturing processes.

Methods Yeast isolates, identified to the species level by sequencing of the ITS2 ribosomal region, were tested for identification using the Bruker Biotyper MALDI-TOF MS. A supplemental custom yeast MALDI-TOF MS library was developed at Charles River Laboratories (CRL). Concordance between sequence-based identification (ID) and that generated by the MALDI-TOF was evaluated using score thresholds recommended by the manufacturer, and score thresholds internally validated at CRL, with and without CRL's supplemental MALDI library. Additionally, operational performance was evaluated using data generated from a six month period of testing (13-March to 14-September 2017).

Results When sample spectra were analyzed with the supplemental custom yeast MALDI-TOF MS library the number of isolates correctly identified to the species level increased significantly. Some of the isolates, not identified by the CRL threshold, had good quality spectra implying potential for library development. Library entries were constructed to represent the diversity of organisms encountered in our laboratory which has further improved the identification rate for these organisms.

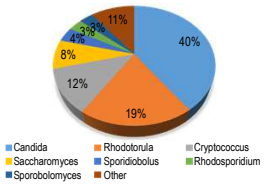
Conclusions These results show that MALDI-TOF MS can be an effective platform for identification of yeast commonly found during environmental monitoring, and supplementing the library with the diversity of isolates relevant to manufacturing processes is critical to improve performance.

BACKGROUND

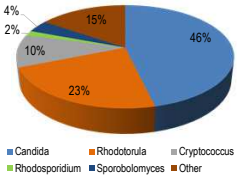
MALDI-TOF mass spectrometry has assumed a vital role in microbial identification spanning the fields of clinical microbiology and environmental monitoring. Although identification of yeasts commonly found in clinical settings by this technology is mainstream, its performance is less understood for yeast isolates commonly found during environmental monitoring in manufacturing processes. The goal of this study was to evaluate performance of MALDI-TOF MS for yeasts commonly found during environmental monitoring.

STUDY COHORTS

Fitness-for-use cohort by genus (n=152)



Operational performance cohort by genus (n=1145)



CONCLUSIONS

- With Bruker's MALDI-TOF MS library and score thresholds, identification rates were 59% (90/152) to species (score ≥2.0), 11% (16/152) were low confidence scores (≥1.7 to <2.0), and 29% (44/152) were not a reliable ID (<1.7) (Figure 1).
- Identification rates improved to 82% (124/152) to species (score ≥1.75), and 17% (26/152) were not a reliable ID (<1.75), when CRL's supplemental MALDI-TOF MS library and score thresholds were applied (Figure 1).
- Evaluating Operational performance of MALDI-TOF MS revealed a significant difference in the identification rates, 53% versus 20% No ID rate between using Bruker-only library and CRL's supplemental library, respectively (Figure 2).
- These results show that MALDI-TOF MS is an effective platform for identification of yeast commonly found during environmental monitoring, but supplementing the library with the diversity of isolates relevant to manufacturing processes (Table 1) and optimizing score thresholds is critical to improve performance (Figure 1 and 2).

MATERIALS AND METHODS

Study isolates
Fitness-for-use: Yeast isolates (152) representing 66 unique species and 19 unique genera were selected based on their frequency of occurrence of testing at Charles River Laboratories (CRL). Reference species identification of these isolates was performed by sequencing the ITS2 portion of the ribosomal RNA gene region.

Operational performance: Yeast isolates (n=1145) tested at CRL\Accugenix laboratories from 13-March to 14-September 2017.

MALDI-TOF mass spectrometry
Isolates were cultivated as pure cultures at 32°C on Sabouraud Dextrose Agar or Trypticase soy agar and tested by MALDI-TOF MS at 24 to 48 hours of growth. The direct smear method was attempted first, if No ID, then followed by formic acid/acetonitrile extraction method recommended by Bruker Daltonics. Mass spectra were acquired for two spots per isolate. Data were collected between 2,000 and 20,000 m/z in linear positive ionization mode using the Real Time Classification Biotyper 4.0 software with Bruker Daltonics library version 5989 and version 6903 for the Fitness-for-use study and Operational performance, respectively.

MALDI-TOF MS CRL library development
Isolates that failed to identify and other isolates of the same species were used to generate MALDI-TOF MS library entries as per the propriety GMP compliant procedures at Charles River Laboratories. A supplemental MALDI library database containing 41 and 101 entries was developed for Fitness-for-use study and Operational performance, respectively.

Data analysis
The spot (out of two) resulting in higher score value was used for analysis. Concordance was calculated after applying scoring thresholds (2.0-3.0 for high confidence identification and 1.7-1.999 for low confidence identification) as per Bruker's recommendation and using Charles River Laboratories' internally validated thresholds (≥1.75 for species-level and <1.75 for No identification). In addition, concordance and performance were calculated with and without Charles River Laboratories supplemental MALDI library entries in the reference database.

RESULTS

Figure 1. Accuracy and performance of MALDI-TOF MS for yeast identification (n=152)
CRL supplemental Yeast library n=41 entries and Bruker library version 5989

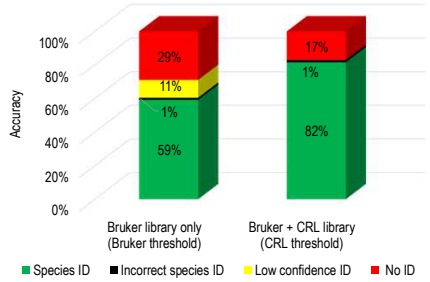


Figure 2. Operational Performance of MALDI-TOF MS over six-months (n=1145)
CRL supplemental Yeast Library n=101 entries and Bruker library version 6903

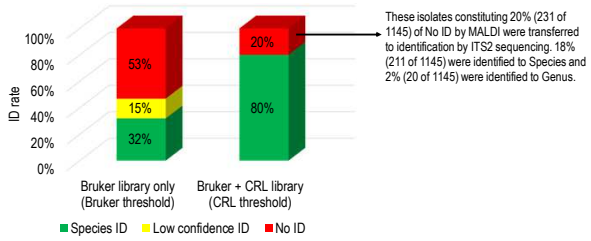


Table 1. Number of species by genus added to the CRL library to improve coverage (n=101)

Table 1.

Genus	No. unique species	No. of library entries
Candida	21	33
Cryptococcus	10	13
Rhodotorula	8	17
Rhodosporidium	4	6
Sporobolomyces	4	5
Pseudozyma	3	3
Sporidiobolus	2	3
Trichosporon	2	2
Bullera	1	3
Bulleribasidium	1	1

Table 1. continued

Genus	No. unique species	No. of library entries
Citeromyces	1	1
Coniochaeta	1	1
Cystobasidium	1	1
Debaryomyces	1	1
Filobasidium	1	1
Hannaella	1	1
Komagataella	1	2
Lecythophora (Coniochaeta)	1	1
Saccharomyces	1	2
Wickerhamomyces	1	4

Simpler RMM Validation for Environmental Monitoring Using Current USP Ch <1223> Requirements

Serge Ohresser, Jamie Brosseau, David Jones, Rapid Micro Biosystems, Lowell, MA 01854, USA

Introduction

The question asked by many pharmaceutical microbiologists and regulators alike is whether the use of an automated colony counter should be considered as an alternative microbiological test method and subject to full method validation. As the process is automation of the incubation and reading of a traditional microbiological method, it may be subject to a more limited verification. Based on these discussions, a case will be made for method verification and not an alternative method validation strategy, a position justified by the USP40/NF35 General Informational Chapter <1223> *Validation of New Microbiological Testing Methods* and industry practice as found in the 2013 PDA Technical Report 33 (Revised) *Evaluation, Validation and Implementation of Alternative and Rapid Microbial Methods*.

Regulatory and Compendial Guidance for the Validation of Alternative Microbiological Test Methods

What is the USP position on alternative microbiological methods?

USP40/NF35 General Notices 6 Testing Practices and Procedures provides guidance of the use of automated and alternative test methods. 6.20 Automated Procedures states: *"**Automated and manual procedures employing the same basic chemistry are considered equivalent**."* Furthermore, 6.30 Alternative and Harmonized Methods and Procedures states: *"Alternative methods and/or procedures may be used if they have advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other specialized circumstances"*.

Why should the Growth Direct ™ System NOT be considered an alternative method?

USP40/NF35 <1223> states:

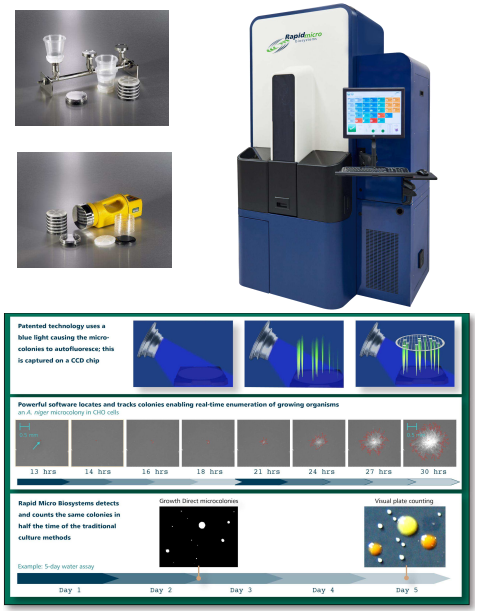
"*There are commercially-available enhancements to growth-based methods that allow colonies on solid media to be read more quickly, with substantially less incubation time, than is possible using only the unaided eye. In the implementation of these enhanced methods for the detection of colony growth, **only the detection capability of the method requires verification***."

This statement supports the view that the Growth Direct™ System is not an alternative method requiring method validation.

PDA Technical Report No. 33 (Revised) dated September 2013, states the following:

"*Some alternative or rapid technologies may be considered automated traditional or compendial microbiological methods, especially when the results are in colony-forming units (CFU). These technologies may be qualified for their intended use without the need for demonstrating certain method validation requirements as specified in Section 5.0 of the Technical Report. **For these technologies, at least accuracy and precision assessments should be performed.***"

The Growth Direct Technology



The Growth Direct™ System is an automated rapid microbial enumeration platform suitable for in process product testing, environmental, and water monitoring that integrates digital imaging, robotic cassette handling, incubation, and software control. Samples are prepared and loaded into the incubators. Cassettes are removed from the incubator every four hours and illuminated by blue light. The green auto fluorescence from the microorganism is then captured by a camera to build up an image time series that differentiates growing micro-colonies from debris. Post imaging, the cassettes are returned to the incubator by the robotic system.

The membranes employed in the system are 0.45 micron, mixed cellulose ester as used for compendial testing. The membranes are stained black to quench the auto fluorescence of the cellulose esters and the underlying media that may inhibit the auto fluorescence of the captured microorganisms. The membranes are placed on standard compendial microbiological growth media used for drug product, environmental monitoring, and water testing. The media cassettes are incubated at the recommended temperatures for times customized to the local facility flora.

Table 2: Standard Media and Incubation Conditions used for Routine Microbial Enumeration in the Pharmaceutical Industry

Microbiological Culture Media	Target Microorganisms	Referenced Methods	Incubation Conditions
Soybean-casein digest agar	Total aerobic microbial count	USP <61> Microbiological Examination of Non-sterile Products: Microbial Enumeration Tests and USP <1116> Microbiological Control and Monitoring of Aseptic Processing Environments	30-35°C for 3 to 5 days
Sabouraud dextrose agar	Total combined yeast and mold count	As above	20-25°C for 5 to 7 days
R2A agar	Waterborne bacterial count	USP <1231>Water for Pharmaceutical Purposes and AWWA/APHA Standard Methods	30-35°C for 48 to 72 hours

Recommendations for the Method Verification of the Growth Direct™ System

Prior to performing the method verification, a standard IOQ protocol would be completed to verify the performance of the equipment.

The method verification approach was to plate 6 replicates of representative microorganisms, as defined in USP40/NF35 <61> with representative environmental organisms in the countable range of <100 CFU per membrane. Enumerate the CFUs using the Growth Direct™ System and continue to incubate the test units for a total 3-5 days for bacteria and 5-7 days for fungi. Post incubation, multiple experienced microbiologists (3) count the colonies using the standard visual inspection procedure to minimize counting error. This testing is conducted in place of the typical Performance Qualification.

Results

The data below verifies the enumeration accuracy of the GD system and vision algorithms for the EM application using organisms defined in the USP and found in the environment. Standard Growth Promotion studies verify the nutritive properties of the media during incoming QC.

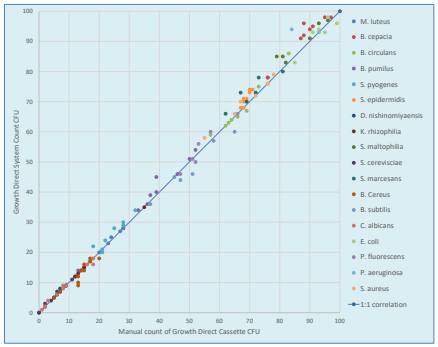


Figure 1: Verification of Enumeration Accuracy by The Growth Direct Software

Method Suitability Tests

The method suitability testing for EM must be met prior to routine testing. This requirement is independent of method validation or verification.

Cassettes used for air, surface, and personnel monitoring in a pharmaceutical facility, contain neutralizing agents for commonly used disinfectants. The recovery of microorganisms especially from facility and equipment surfaces with residual disinfectant needs to be validated. The acceptance criteria for the disinfectant residue neutralization should be between 50% and 200% recovery.

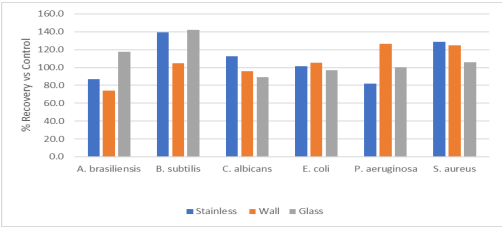


Figure 2a: Microbial recovery after sampling coupon surfaces coated with a Biguanide

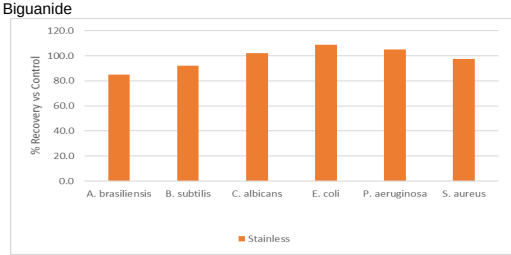


Figure 2b: Microbial recovery after sampling stainless steel coated with Vesphene

Conclusions

Following the USP guidance chapters, the Growth Direct™ System meets the conditions stated in USP <1223> and the industry practice document PDA Technical Report No. 33 that it is an automated system for the incubation and enumeration of the compendial microbial plate count method. As such, the system only requires verification of the counting method and method suitability to be performed.

The verification of the system colony enumeration and the method suitability for environmental sample testing was performed successfully.

References

1 USP 1223 USP40/NF35 General Informational Chapter <1223> *Validation of New Microbiological Testing Methods*
2 USP 61 USP40/NF35 *Microbiological Examination of Non Sterile products: Microbial Enumeration Tests*
3 TR33 2013 PDA Technical Report 33 (Revised) *Evaluation, Validation and Implementation of Alternative and Rapid Microbial Methods*
4 A. Cundell and D. Jones 2017 PDA Journal of Technology, In Press. *Method Verification Requirements for an Advanced Imaging System for Microbial Plate Count Enumeration*