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Validation of the Growth Direct System for Microbial Environmental Monitoring and Define Optimal Incubation Conditions

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Summary

The validation of the Growth Direct system is described for the automated incubation and enumeration of microbial colonies on TSA LP80 and TSA LP80HT media plates. The analytical validation strategy and data generated are given to demonstrate the technology is validatable following the requirements of USP <1223> for microbial recovery. Single and serial temperature incubations were evaluated to optimize microbial detection. Data is provided to demonstrate equivalence to the current compendia test method for environmental monitoring.

1. Introduction

Pharmaceutical environmental monitoring (EM) testing is a significant percentage of the workload in the QC microbiology department. Each EM sample test is divided into 2 phases: first sampling/setting up the test and secondly reading and recording the test result(s). The second phase involves a highly trained analyst performing the very repetitive task of data collection (colony counting) and collation for which they may be overqualified. A reduction in the analyst involvement on the EM testing effort through automation would be a benefit to the department in that other higher value activities could be performed by the same person. The Growth Direct™ (GD) system is an automated colony counter, London et al (1), that can be linked into a bi-directional LIMS system to upload sample work-lists and download results thus automatically removing these time-consuming, laborious tasks from the analyst workload while improving data security.

Incubation conditions for the EM test have been under discussion over the last few years in terms of temperature and incubation strategy, whether a single temperature or serial produces “better” results, Gordon et al (2), Symonds et al (3), Sage et al (4). For single temperature protocols the risk to detection of mold due to inhibition at 30-35°C has been introduced with the recommendation to move to 25-30°C. Gordon et al (2). Moldenhauer (5) has proposed a validation strategy to define the optimal conditions for each monitored site. For the study performed in this paper a serial incubation strategy was employed.

The paper describes the validation of the Growth Direct system as an automated colony counter applied to Pharmaceutical EM testing.

Growth Direct Technology

The Growth Direct System for rapid microbial enumeration comprises two automated incubators, robotic sample transport systems, an advanced imaging system, two servers (one for system control, the other for image analysis) and associated hardware and staging required for the handling of up to 679 Growth Cassette™ products. The Growth Cassette products are plastic contact plate style cassettes with specific mechanical and optical features that facilitate the automated handling and imaging process. The Growth Cassette products incorporate standard media depending on the application. For EM two main media options are available, TSA LP80 and TSA LP80HT. Both media are standard formulations used in the pharmaceutical industry. The Growth Direct EM test method requires the presence of a black mixed cellulose ester membrane, 0.45-micron pore size, to improve the signal to noise ratio for the detection system. The presence of the membrane is the only difference to traditional methods and the influence its presence has on organism recovery is the basis of this study.

During the incubation phase, images of each cassette are taken at intervals of 4 hours, allowing organisms and debris that are naturally fluorescent under the excitation blue light (450-490nm) of the imager to be detected in the green (520-560nm) spectrum. The images are recorded by a

Charged-Coupled Device (CCD) camera. Analysis of the behavior of objects over the incubation time by proprietary growth rules of the vision analysis software allows the Growth Direct System to distinguish and enumerate the growing objects from the background and debris, London et al (1). At the end of an assay, the system reports the number of growing objects found on the surface of the membrane in the cassette. The technology is defined as a Quantitative Enumeration method and was validated as such.
The imaging method does not harm the cells, and as such is a non-destructive method. The micro-colonies can grow into visible colonies for use in subsequent microbial identification.

Validation Rationale

The validation of the Growth Direct System and dedicated consumables was performed in accordance with a subset of the requirements of PhEur 9.2; 5.1.6 (6) and USP 38/NF33 Ch <1223> (7). Guidelines from USP Ch <1116> (8) were followed as well for the EM testing.
The data presented in this report supports the verification of the system's performance as an "automated compendial test". The test requirements for "automated compendial" are described in USP Ch <1223> (7), the PDA TR33 technical report (9) and the article by Jones et al 2018 (10).
The parameters required for the analytical verification of the automated compendial test are precision and accuracy as defined in TR33 (9). Growth promotion of the media is required to show equivalence to current compendial media formulations. Method suitability is shown by running the new method in parallel to the control and demonstrating equivalent performance and verification of disinfectant neutralization.
Changing to a rapid microbial method, allowed an evaluation of incubation conditions to determine optimal recovery in minimum time to result, TTR.
This paper describes the approach used and data generated to validate an EM test on the Growth Direct System. The standard validation process was used, Installation and Operational Qualification (IQ, OQ) to verify hard and software identification and performance, Performance Qualification (PQ) testing growth promotion and software detection accuracy and finally Method Qualification (MQ) when used in the manufacturing environment in routine mode.

Installation, Operation Qualification and Performance Qualification (IOPQ)

IOQ testing focused on the software performance to verify the correct steps and sequences and bidirectional LIMS connectivity to the Growth Direct showing correct transfer of data in each direction.
The PQ phase of the validation utilized microorganisms to qualify colony count accuracy of the detection system software as required by USP <1223>. Organisms were presented in a neutral buffer such as Fluid A, buffered saline, or Peptone broth. The USP/EP organisms and organisms from the environment collection were used as examples of the spectrum required to be detected using the EM media TSA LP80 and TSA LP80HT (Table 1). The IOQ validation is described in more detail in Nguyen et al (11).

Table 1 USP/PhEur test organisms for use in the PQ

Test Microorganism	ATCC Number	Incubation Temperature	Incubation Duration
Bacillus subtilis	6633	30-35°C	≥48 hours
Staphylococcus aureus	6538	30-35°C	≥48 hours
Escherichia coli	8739	30-35°C	≥48 hours
Pseudomonas aeruginosa	9027	30-35°C	≥48 hours
Candida albicans	10231	30-35°C	≥48 hours
Aspergillus brasiliensis	16404	30-35°C	≥48 hours
Mixed organisms S. aureus, C. albicans	6538 & 10231	30-35°C	≥48 hours
EM *Staphylococcus epidermidis*		30-35°C	≥120 hours
Bleach stressed *B. subtilis* spores		30-35°C	≥120 hours

System Count Accuracy

The results for the enumeration accuracy are shown in Figure 1. A very good 1:1 correlation is seen between the manual (mean of 3 analysts) and system count for the test organisms that passed the acceptance criteria for Growth Direct cfu counts to be >90% of the manual count 90% of the time, Fowler et al (12).

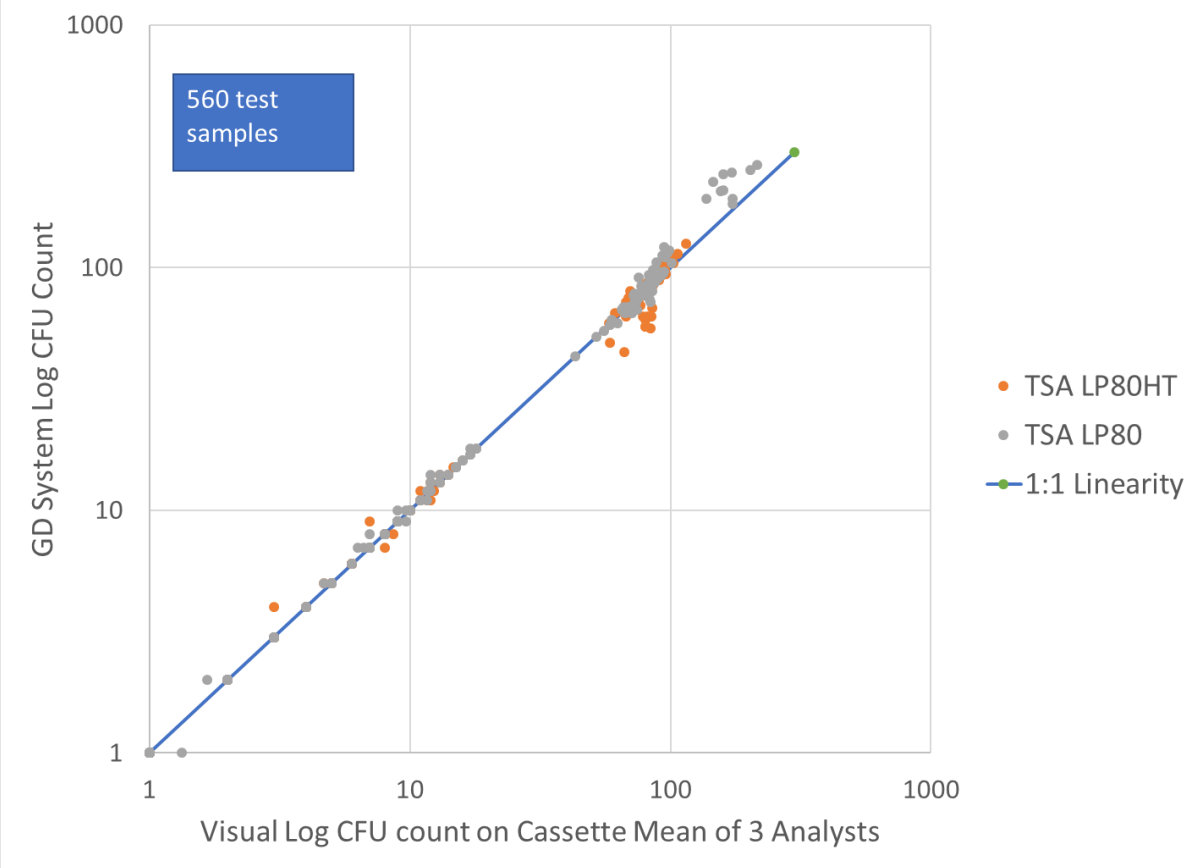

Figure 1 Correlation of System CFU counts compared to the mean CFU count of 3 analysts on the same cassette.

Qualification-
Incubation strategy

To determine the optimum incubation strategy twelve test sites were selected in a clean but unclassified area around access points to the controlled area. The evaluation was performed at two periods through the year, Dec/Jan and Jul/Aug to look for seasonal variations. These unclassified sites were selected to obtain sufficient colony counts on the test samples to be statistically significant. Any flora detected close to clean room entry point has a high probability of being found in the controlled area so will be representative of those found in the clean area.
The two most common incubation methods were evaluated, using Growth Direct media only as outlined below to determine if either strategy was superior for microbial recovery.

- Serial Sample Set: 3-days at 20 to 25°C followed by an additional 2-days at 30 to 35°C
- Single high temperature Sample Set: 5-days at 30 to 35°C

Total organism counts were made by the Growth Direct system and a visual count of molds vs bacteria were performed manually at the end of the incubation. Figure 2a shows the total number of microorganisms and 2b the number of molds detected from all sites and over both time periods. The serial incubation strategy was selected as a superior recovery was observed for the facility flora, particularly for the molds.

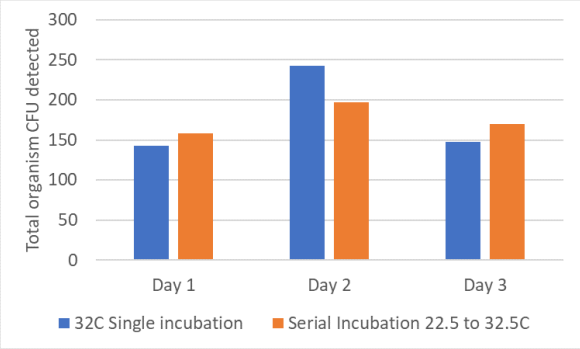

Figure 2a Total organism recovery compared using two incubation strategies

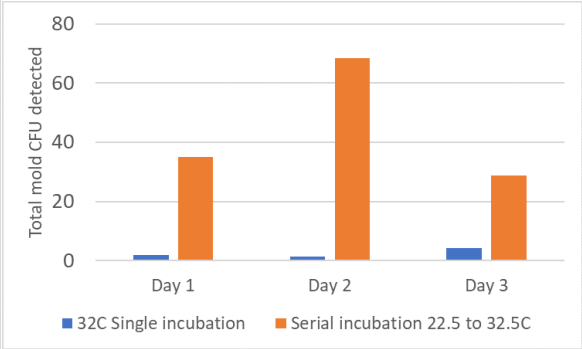

Figure 2b mold recovery compared using two incubation strategies

Time To Results (TTR)

The next stage of the process required definition of the TTR for the facility and its specific organism population. Combining data from the PQ and incubation strategy allows selection of the TTR.
A broad range of pharmacopeia organisms, environmental isolates in pure culture form and natural contaminants were used with samples from contact and active air samples selected. The chart in Figure 3 demonstrates the cumulative percentage detection of colonies for each organism or collection of organisms present at a sample site on the y-axis and the time in hours at which the colony was first detected on the x-axis. The maximum number of colonies that will grow are shown when the colony detection profile reaches the plateau. Stress through dehydration, disinfectant treatment etc can have a significant effect on the TTR of environmental organisms, Sage (13). Most validation strategies suggest that stressed organisms relevant to the test sample should be included. For EM testing in situ, most organisms will be naturally stressed due to lack of nutrient, desiccation and disinfection agents.
The samples for TTR and incubation characterization were taken from close to the manufacturing floor entrances, from 3 uncontrolled areas to ensure high enough cell counts to get suitable data sizes. At each site, 3 surface contact plates and 1 active air sample (1m3 using SAS 180) were taken for each incubation condition making a total of 36 test points for each day. Samples were taken in

Dec-Jan and then 9 months later in Aug-Sept to cover seasonal variation. All samples were incubated on the Growth Direct system. Following incubation, the data series of cfu detected at each 4 hour read time for each cassette was recovered to plot the TTR curves and determine the CFU counts. The combined TTR charts for the incubation strategy are shown in Figure 3.
The data show that the TTR required for a quantitative final result is approximately 100-110 hours when using serial incubation with the majority of the colonies detected by 96 hours. Monoculture samples demonstrate a sigmoidal curve shape while sites with multi species demonstrate a step wise profile as each organism starts to proliferate at different times. It should be noted that with the serial incubation all except 2 colonies had started to grow during the 20-25°C phase so the incubation could probably be shortened as an overall incubation regime to 2 days at 20-25°C followed by 24hrs at 30 to 35°C. With the flora present at this facility the conservative decision was made to use the serial incubation for 5 days.

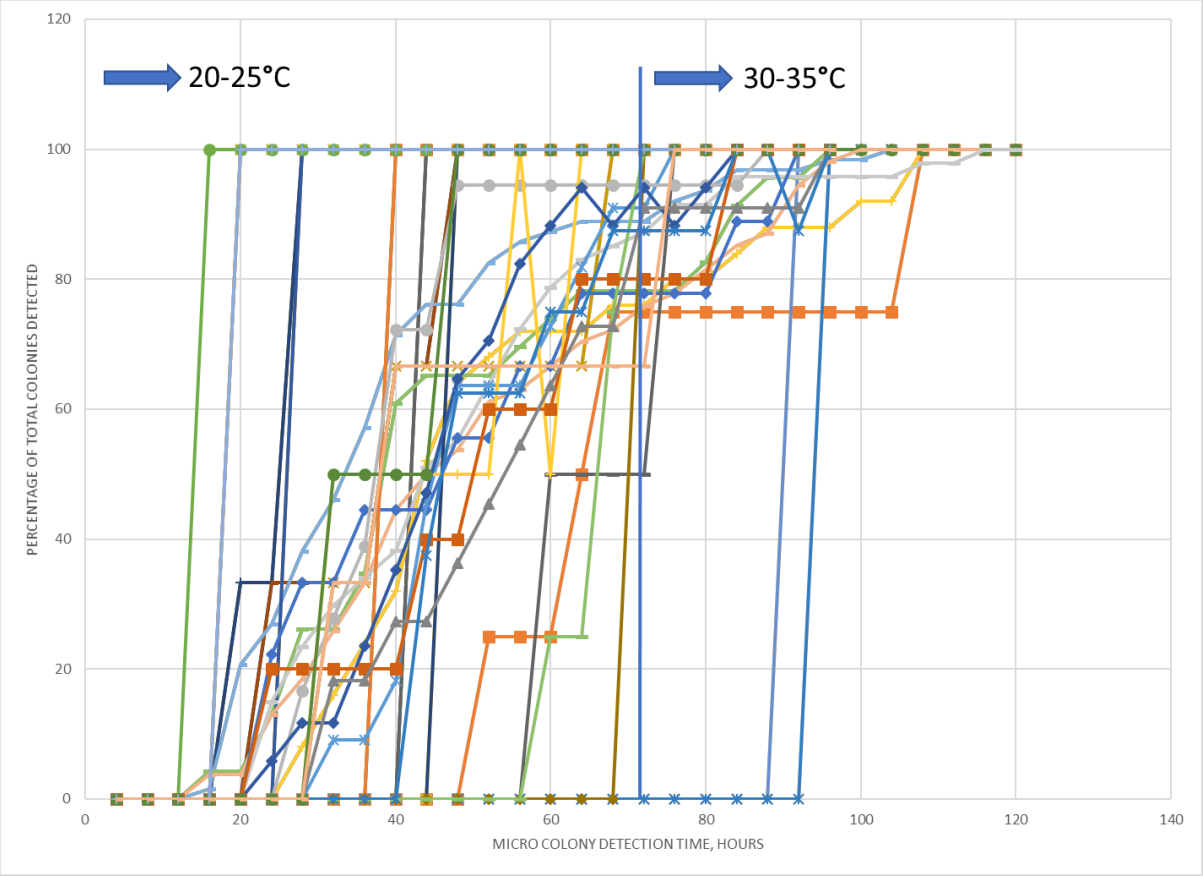

Figure 3. TTR profiles for multiple sample types with serial incubation for 3 days 22.5°C then 2 days 32.5°C

Method Qualification, equivalence testing

During method qualification, equivalence testing, two studies were performed:

1 Disinfectant neutralization to prove equivalence to the standard method

2 Equivalence studies running samples in parallel with the standard method in the clean room environment

Where required statistical analysis was performed using the guidelines from PDA TR 33 (9). The applied use of these statistics was described in Murphy et al (14).

Disinfectant neutralization

To verify neutralization five key disinfectants, see Table 2, were evaluated on three different surface coupons against the standard Pharmacopeia organisms. The disinfectant at working strength was applied onto the coupon surface and allowed to dry following the prescribed process used in the facility. The surface was sampled using triplicate cassettes. Following surface sampling, the test organisms, approximately 50 CFU, were spread to the media surface in a low volume, 50µl. Control plates were prepared with no disinfectant contact and spread with the test organisms. Following incubation, the counts on the test cassettes were compared to the control counts. Recovery acceptance criteria was greater than 70%.

Results are shown in Tables 3a-c. For all the surfaces tested and the disinfectants used acceptable recovery of test organisms was achieved. The neutralization power of the media was proven.

Chemical Composition	Disinfectant Name	Supplier
QAC	Process NPD	Veltek Associates
Chlorine based	0.525% hypochlorite WFI Sterile solution	Clorox
Alcohol	70% IPA	VWR
Phenolic Disinfectants	LpH® III (Low pH) Vesphene (High pH)	Steris Steris

Table 2. Disinfectant active ingredients and commercial names

	LpH III	70%IPA	Chlorox	Vesphene	NPD
B. subtilis	111.6	89.8	106.3	81.1	110.9
S. aureus	107.2	108.6	95.6	114.3	110.0
E. coli	89.6	75.8	96.3	100.0	95.5
P. aeruginosa	85.8	110.6	99.5	87.8	101.4
C. albicans	96.1	91.7	97.4	91.2	95.3
A. brasiliensis	101.7	118.3	108.0	82.2	96.4

Table 3a. Percentage Recovery of organisms from treated Stainless steel coupon

	LpH	70%IPA	Chlorox	Vesphene	NPD
B. subtilis	111.6	89.8	87.9	81.1	83.9
S. aureus	107.2	108.6	113.4	114.3	79.5
E. coli	89.6	75.8	102.8	100.0	95.1
P. aeruginosa	85.8	110.6	102.9	87.8	84.5
C. albicans	96.1	91.7	105.0	91.2	95.1
B. brasiliensis	101.7	118.3	104.9	82.2	75.4

Table 3b. Percentage Recovery of organisms from treated Flooring coupon

	LpH	70%IPA	Chlorox	Vesphene	NPD
B. subtilis	72	91	78	75	78
S. aureus	148	146	115	119	128
E. coli	112	112	108	75	95
P. aeruginosa	94	114	109	92	110
C. albicans	74	128	86	108	84
C. brasiliensis	84	96	133	91	72

Table 3c. Percentage Recovery of organisms from treated Glass coupon

Equivalence testing

Equivalence testing was performed in the clean room running the Growth Direct cassettes in parallel to the routine contact plates and evaluated on 3 separate occasions. The control method used the BD RODAC plate. TSA LP80 was used for both media suppliers. To perform the active air sampling, SAS adaptor clips were used to hold the wider Growth Direct contact plates in place of the standard Rodac plate.

Prior to starting the study all monitoring analysts were trained on use of Growth Direct plates for test sampling. Manipulation was similar to the standard contact plate, but the Growth Direct cassette has an extra vision lid.

Identification Labels were printed for each sample point and attached to the test cassettes prior to sampling the sites in the controlled area.

Traditional contact plates were placed at the pre-defined locations with the test plates run at the same time in the nearest practical location to the control samples. For operator testing it is not possible to take 2 samples from a finger. As the tests were secondary to the routine testing the decision was made to take the test sample from the palm rather than the finger.

Two test regimes are followed in the facility, testing during manufacture on a daily basis and the weekly verification of the status of the Laminar Flow Hoods in the room. The data is split to show that information.

With the daily testing three different sets of data were collected:

a. contact plates for surfaces, high risk samples in and outside the LAF units
b. active air (SAS 180, $1m^3$)
c. samples taken from operators (finger/palm) after manufacturing batch

All EM samples were incubated for 3-days at 20 to 25^oC followed by an additional 2-days at 30 to 35^oC on the Growth Direct and in a traditional incubator for the control method using the same incubation procedure.

The data is summarized in Table 4a, b and c showing the number of test points, the total number of samples with a detected colony and the total number of colonies detected. Equivalence would be shown by having approximately the same frequency of positive hits and the same total number of colonies detected between the two methods. As usual with microbial testing a wide error range is expected.

	Day 1		Day 2		Day 3	
Active Air	Media Lot # 31017L1		Media Lot # 31817L1		Media Lot # 34117L1	
Method	Control Counts	Test Counts	Control Counts	Test Counts	Control Counts	Test Counts
# Observations	9	9	9	9	9	9
CFU Sum	0	0	0	0	0	0
Positive Frequency	0	0	0	0	0	0

Table 4a Active air samples from the daily testing study.

	Day 1		Day 2		Day 3	
Surface	Media Lot # 31017L1		Media Lot # 31817L1		Media Lot # 34117L1	
Method	Control Counts	Test Counts	Control Counts	Test Counts	Control Counts	Test Counts
# Observations	12	12	12	12	12	12
CFU Sum	9	10	13	4	1	0
Positive Frequency	5	6	4	3	1	0

Table 4b Surface sample test points from the daily testing study.

	Day 1				Day 2				Day 3			
Personel testing	Media Lot # 31017L1				Media Lot # 31817L1				Media Lot # 34117L1			
Method	Control Counts		Test Counts		Control Counts		Test Counts		Control Counts		Test Counts	
Hand	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
# Observations	14	14	15	15	8	8	7	7	8	8	8	8
CFU Sum	14	8	0	0	0	0	1	0	0	0	0	0
Positive Frequency	4	4	0	0	0	0	1	0	0	0	0	0

Table 4c Personel sample test points from the daily testing study.

The weekly test points are all taken from 6 Laminar Air Flow (LAF) units in the clean room with surface samples from defined points in the units. Active and Passive air samples were also taken. For the passive samples two Growth Direct cassettes were used for each single 90mm Petri test plate. This was performed as the Growth Direct Cassette has approximately half the surface area of the Petri plate. Studies on the efficiency of capture have been published to show that the use of two Growth Direct plates is equivalent to one Petri plate, Samson et al (15). Results are shown in Table 5.

		Day 1		Day 2		Day 3	
LAF Hoods		Media Lot # 31017L1		Media Lot # 31817L1		Media Lot # 34117L1	
	Method	Control Counts	Test Counts	Control Counts	Test Counts	Control Counts	Test Counts
Passive Air	# Observations	18	9	8	8	4	4
	CFU Sum	0	0	0	0	0	0
	Positive Frequency	0	0	0	0	0	0
Active Air	# Observations	6	6	6	6	6	6
	CFU Sum	0	0	0	0	0	0
	Positive Frequency	0	0	0	0	0	0
Surface	# Observations	48	41	48	48	48	48
	CFU Sum	0	0	0	0	0	0
	Positive Frequency	0	0	0	0	0	0

Table 5 Colony counts and frequency of positive samples from Laminar flow hood testing sites.

Weekly test points were run with both methods. From the data all air samples and most surface test sites were clean (0CFU), with a total of 19 surface sites with some level of contamination. The frequency of hits was evenly split between the two methods, 10 with the control method and 9 with Growth Direct cassettes. As can be expected with EM sampling there can be a hot spot sample, for example site 107 F1 on Day 2, where only one method detected the count. It is not believed that this was due to the technology rather random chance. No apparent difference is seen between the two methods on this testing data set.

With the hand samples the control method had a higher hit rate than the GD cassette with 8 positives vs 1 for GD. Higher counts were generally seen for the control method. Whether this represents the sampling variation or sampling from the palm may have fewer counts, since the palm rarely makes contact with the product.

The testing on the laminar flow hoods did not detect any contaminations on either the control or test media, Table 5. No false positives were seen in the 300 test samples run on the GD system.

In summary the testing of the Growth Direct consumable and system showed the disinfectant neutralization and routine application to be equivalent to the standard Rodac contact plate.

3. Discussion

The paper introduces the validation strategy for the Growth Direct system, an automated colony counter, for the analysis of environmental samples in a controlled manufacturing environment. The media cassettes had been previously evaluated using a manual method to show equivalence to the compendial method, McIver (16). This paper adds the Growth Direct system as well for the automated incubation and enumeration of colonies. As the system is based on the compendia test method the validation focuses primarily on those components that are different e. g. the colony detection and enumeration algorithms and the automated incubation step, Jones et al, (17). For the verification of the method a simple approach to test the accuracy and precision of the method was chosen as suggested in the current PDA TR33 (9) and USP <1223> (7) document.
The data show that the camera detection and associated algorithms accurately enumerate the microorganism colonies on the membrane surface and that the TSA LP80 and TSA LP80HT media recover organisms from both standard diluents and real-world samples equivalently to the standard method. The precision of the enumeration method is equivalent to or better than that seen with the traditional method.

When applied to the qualification of a controlled area the incubation conditions were first evaluated to ensure that local species could be recovered. A number of incubation strategies exist which have effects on assay cost (1xTSA and 1xSDA plate), Time (serial incubation, 1 media for 3-4 plus 2-3 days of incubation at 20-25 then 30-35°C) and accuracy for all organisms present. The method chosen is thus a compromise between the 3 component parts depending on the company's priorities, cost, time or detection accuracy.

As part of the incubation study a determination of incubation duration is required to ensure good recovery of healthy and stressed organisms. Real life samples were used rather than EM collection organisms as the latter are grown on from original sample and will have lost the natural stress from the environment, dehydration, disinfectants etc.

With the Growth Direct system, a faster result can be obtained in 72- 100 hours compared to the 120-168 hours for traditional serial incubation depending on incubation conditions. The study has also confirmed that in the facility tested there are a number of molds that are heat sensitive and do not grow at 30-35°C. This phenomenon has been reported by Gordon et al (2) and Symonds et al (3). Use of serial or 25-30°C incubation recovers more mold but still less than is seen with SDA at low temperature. In a multi-center study by Guinet et al (18), recovery of organisms was tested on TSA based contact plates at different temperatures. For bacteria, the best recovery was seen at 30-35°C and for molds 20-25°C either as a single incubation or as part of a low to high temperature serial incubation. This pattern was seen in a study by Sage et al (4) and included a 25-30°C incubation test. The intermediate temperature range was equivalent to the serial temp method but with a shorter incubation time.

As molds can be present in a pharma-controlled area their detection and enumeration are important, Bawa et al (19). Many sites report low mold contamination rates however if the initial detection is performed on TSA at 30-35°C there may be many molds that are undetected. Most studies have shown that use of SDA (or PDA) at 20-25°C is a far more effective method to detect molds. It is suggested that during the initial evaluation of an area that SDA and TSA should be run together with the SDA at low temperature and TSA at whatever incubation strategy is being assessed. The data can then verify that any molds captured on SDA are also seen on the TSA method. Depending on the results the routine monitoring schedule can then be assigned to either test for molds all the time or at intermittent intervals to verify that nothing has changed.

The method qualification of the facility for contact plate and active air sampling demonstrates equivalent sensitivity and recovery of organisms compared to the traditional method with no adverse effect of the use of disinfectants.

In summary, the Growth Direct system has used a proven validation strategy with reduced validation approach following the process defined in the PDA TR33 (9) report and in compliance with the pharmacopeia USP <1223> (7) and PhEur 5.1.6 (6).

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