

Validation of a laser scanning cytometer for the microbiology QC release of an antiseptic solution

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A laser scanning cytometer (LSC) was validated for product release of a filterable antiseptic solution. The validation of the LSC was performed over a two-month period using the Total Viable Count and Fungi applications. The first part of the qualification was performed on pure culture in order to validate the analytical performance in terms of linearity, accuracy and precision of the LSC count, as well as the assay detection limit and range of detection. The second part of the qualification involved validation of the product neutralisation and the equivalence study with the reference method. The work demonstrated that the LSC under investigation is at least as sensitive as the reference method and allows the release of the antiseptic solution in four hours instead of five days.

Un cytomètre à balayage laser (CBL) a été validé pour la libération d'un produit antiseptique filtrable. La validation du CBL a duré deux mois et a été réalisée avec les applications pour la détection de la Flore Totale et des Levures-Moisissures. La première partie de la qualification a été effectuée sur des cultures pures afin de valider les performances analytiques du CBL. Ainsi, linéarité, exactitude et précision du dénombrement ont été validés de même que la comparaison avec la méthode de référence. La deuxième partie de la qualification a porté sur la validation de la neutralisation du produit et l'étude de l'équivalence avec la méthode traditionnelle. Cette étude a montré que le CBL est au moins aussi sensible que la méthode de référence et permet de libérer la solution antiseptique en quatre heures au lieu de cinq jours.

Un citómetro de barrido por láser (CBL) fue validado para el control liberatorio de producto final de una solución antiséptica filtrable. La validación del CBL se realizó durante un período de aprox. dos meses utilizando las aplicaciones para el conteo de microorganismos viables totales y Mohos y Levaduras. La primera parte de la cualificación se realizó con cultivos puros a fin de validar el funcionamiento analítico en términos de linealidad, exactitud y precisión del recuento con el CBL, así como el límite de detección y rango de detección del ensayo. La segunda parte de la cualificación consistió en la validación de la neutralización del producto y el estudio de equivalencia con el método de referencia. El trabajo demostró que el CBL es por lo menos tan sensible como el método de referencia y permite la liberación de la solución antiséptica al cabo de cuatro horas en lugar de cinco días.

Introduction

The traditional test method for microbial control of a filterable pharmaceutical solution, such as an antiseptic solution, is based on growth as defined in the European Pharmacopoeia, monograph V.2.1.8.1. The solution is filtered through a 0.45- μ m membrane and incubated on TSA agar plates for bacteria and on Sabouraud agar plates for yeast and moulds detection.

As the incubation lasts for five days, the time to result does

not allow early release of the final product and thus can lead to high storage costs. Moreover, the growth-inhibiting effect of the product itself may result in stressed organisms and lead to an underestimation of the microbial contamination level.²⁻⁴

Therefore, the use of a laser scanning cytometer (LSC) and its potential advantages were evaluated in this study. Indeed, this new microbiology analyser allows near real-time and ultra-sensitive analysis of filterable products.⁵⁻⁸ The method does not require a growth phase, since it is based on the direct detection and enumeration of each individual, fluorescence-labelled, viable micro-organism with a sensitivity down to a single colony-forming unit (CFU) per filtered sample. Total viable counts (TVC) are obtained within 90 minutes from sampling and a specific fungi count is obtained in four hours.

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Although the reference methods in microbiology are described in the European Pharmacopoeia¹, an alternative method can be used as long as it is proven to be at least equivalent to the conventional plate count method, and is accepted by the relevant authorities. The LSC method was validated according to "the validation of compendial methods <1225>", as described in the USP⁹ and the guidelines in PDA Technical Bulletin 33^{10,11}.

This paper describes the LSC validation procedure, when applied to an antiseptic filterable solution. The results, which were presented to the Spanish authorities, led to an official acceptance of this alternative method (obtained in November 2001).

This validation procedure follows the standard format as defined in PDA Technical Bulletin 33¹⁰ and includes the Installation Qualification (IQ), the Operational Qualification (OQ) and the Performance Qualification (PQ). In this paper, we describe the Performance Qualification, which represents the validation of the method's analytical performance.

Materials and methods

Micro-organism strains

The strains used in the Performance Qualification of the LSC were those recommended in the Pharmacopoeia:

- Vegetative forms of the following bacteria: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739
- Bacterial spore: *Bacillus subtilis* spores ATCC 6633
- Yeast: *Candida albicans* ATCC 10231
- Mould: *Aspergillus niger* ATCC 16404

The strains were prepared according to the method described in the Performance Qualification 1 protocol, as described in the LSC manufacturer's document (Chemunex, ref. 200-D0225-01)¹². Cultures of bacteria and the yeast were grown on TSA and Sabouraud plates, respectively. One colony was collected and re-suspended in 20 ml of TSB or Sabouraud broth and incubated overnight at 30°C±2°C. The dilutions were prepared in 0.22-µm filtered Pharmacopoeia Diluting Neutralising buffer (DNP) at room temperature and stored at 4-6°C for 1-4 hours.

The suspension of bacterial spores was a commercial suspension obtained from the Institut Pasteur, Paris, France. It was diluted in filtered DNP and stored at 4-6°C for 1-4 hours.

The moulds were allowed to form spores by cultivating them on malt extract agar for 3-8 days at 25°C±2°C. They were then collected and suspended in 10 ml of sterile water containing 0.8% Tween 80 to form a stock suspension estimated under the optical microscope using a Thomas counting chamber. The working dilutions were prepared in DNP and stored at 4-6°C for 1-4 hours, whereas the stock suspension was kept at 4-8°C for another day.

The antiseptic solution samples

The product is a new antiseptic solution for topical use.

The antiseptic solution is manufactured in 1000 ml bottles and filled under near-sterile conditions. Overall, from raw material to finished product, the manufacturing process takes five hours.

Samples from three different batches were analysed. These batches were pilot batches produced under industrial conditions. The in-house microbiological specifications state absence of contamination of 10 ml of product. The samples were stored in the final container at room temperature prior to use for the test, as follows: batch D-57042/1 (stored for 1 month), batch D-57042/2 (stored for 1 month) and batch M-201 (stored for 17 months).

Reference procedure

A classical membrane filtration was carried out in parallel for the standard microbiological count. The test micro-organisms were filtered using cellulose acetate filters (Millipore) and incubated on TSA plates at 35°C ± 2°C or Sabouraud Chloramphenicol Agar plates at 25°C ± 2°C for 18-24 hours.

The samples of the antiseptic solution were filtered using the Milliflex system (Millipore, ref: Milliflex 100 TSA) and the membrane was rinsed afterwards with 3 x 100ml of DNP, in order to remove the inhibitor agents from the membrane and allow micro-organism growth to occur. The membranes were incubated on TSA at 35°C ± 2°C or on Sabouraud Agar at 25°C ± 2°C for at least five days.

Laser scanning cytometry

The LSC used in this study is a solid-phase laser scanning cytometer (ChemScanRDI, Chemunex, Ivry-sur-Seine, France) equipped with a 488-nm argon laser that scans the total surface of a 25-mm diameter polyester membrane in three minutes^{13,8,14,15} (Figure 1). All the reagents and consumables were supplied by Chemunex (Ivry-sur-Seine, France).

Three photomultiplier tubes detect the light emitted by the fluorescence-labelled cells. The signals obtained are processed by a PC equipped with a series of software discriminants that enable the LSC instrument to differentiate accurately between valid signals (labelled viable micro-organisms) and background noise (auto-

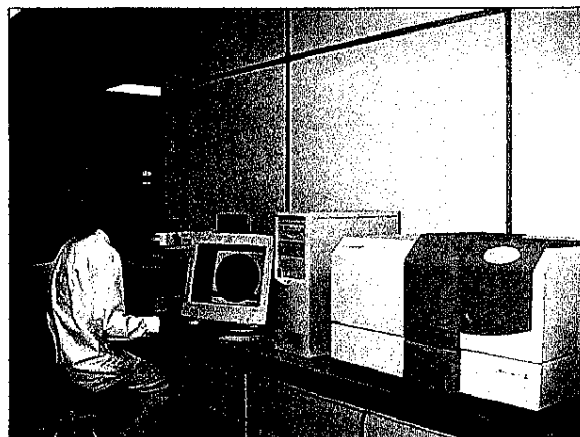


Figure 1: The laser scanning cytometer

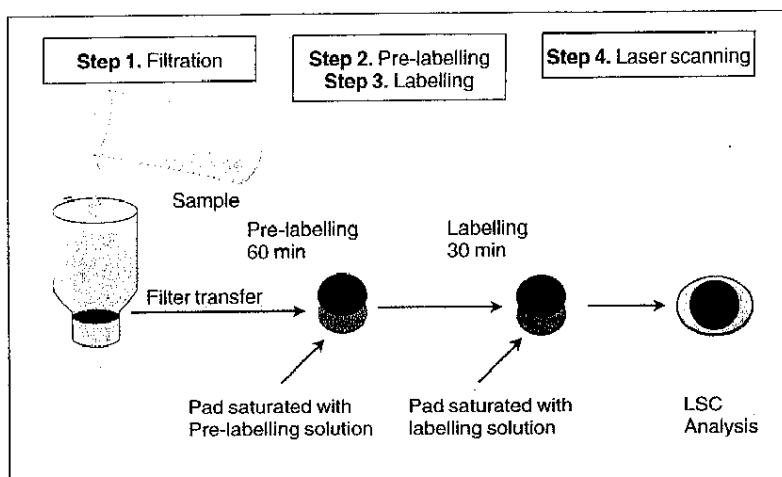


Figure 2: Total Viable Count (TVC) bioburden protocol

fluorescent particles, dead cells). Detected and counted micro-organisms are reported on the final test result log sheet and can be visualised as coloured spots in a membrane filter scan map display.

TVC PROCEDURE

Prior to filtration, the antiseptic sample was treated with a cellulase enzyme in order to decrease the viscosity of the antiseptic solution and thus allow better filtration onto the 0.4- μm filter. 100 μl of cellulase (0.1 mg/ml) was added to 10 ml of antiseptic solution and incubated for 25 min at 40°C \pm 2°C.

Sample preparation

The samples were prepared according to the protocol TVC Bioburden 200-D0511-02: 1 ml of the appropriate dilution of pure culture or 10 ml of the antiseptic solution sample was vacuum-filtered through a 25-mm, 0.4- μm Cycloblack™-coated polyester membrane.

A background reducer (Counter Stain E: CSE/3) was added to the surface of the filter and vacuum was then applied. The counterstain enhances the discrimination between viable green cells and dead red cells. The filter was then placed onto a 25-mm cellulose pad impregnated with 550 μl of pre-labelling buffer (ChemSol A4) and incubated for 1 hour at 37°C \pm 2°C. The pre-labelling ensures activation of bacterial spores and revivification of stressed vegetative cells, so that they can be subsequently labelled. After the pre-labelling step, the membrane was incubated for 30 min at 30°C \pm 2°C on a second cellulose pad saturated with 550 μl of labelling buffer solution, a 1:100 (v:v) dilution of ChemChrome V6 + ChemSol B16. Finally, the membrane was removed from the pad and mounted onto a 25-mm black ester-cellulose filter in a metal holder and analysed inside the LSC analyser (Figure 2).

Fungi count procedure

The samples were prepared according to the Fungi protocol 200-D0512-02: 1 ml of the appropriate dilution of pure culture or 10 ml of the antiseptic solution sample was vacuum-filtered through a 25-mm, 0.4- μm Cycloblack™-coated polyester membrane. Prior to filtration, the antiseptic sample was treated with a

cellulase enzyme as described for the TVC protocol.

A background reducer (Counter Stain E or CSE/4) was added to the surface of the filter and vacuum was applied. The filter was then placed onto a 25-mm cellulose pad impregnated with 550 μl of pre-labelling buffer (ChemSol A6) and incubated for three hours at 30°C \pm 2°C, to ensure both mould spores and vegetative yeast cells can be labelled.

After pre-labelling, the membrane was incubated on a second cellulose pad saturated with 550 μl of labelling buffer solution, 1:100 mix (v:v) of ChemChrome V6 and ChemSol B2, for one hour at 37°C \pm 2°C. Finally, the membrane was

transferred onto a 25-mm black ester-cellulose filter in a metal holder and analysed in the LSC. The membrane is totally scanned by the laser beam in three minutes.

Microscope confirmation

The results of the LSC are displayed on a scan map on the computer monitor screen and can be visually confirmed using an epifluorescence microscope. An Olympus BX40 (Tokyo, Japan) epifluorescence microscope was used for this purpose, equipped with an FITC filter block, a 50x/0.8 lens, a 10x/22 ocular, a 100 W mercury lamp (Olympus) and a Prior motorised stage (Prior Scientific, Fulburn, UK). The latter was driven by the LSC user interface.

With a fluorescent spot selected out of the scan map, the motorised stage is immediately driven to the corresponding position on the membrane for visual inspection under the microscope. This enables crude morphological identification, based on the shape of each bacterium seen under the microscope.

Interpretation of the results

Performance qualification criteria with pure culture

The LSC was tested with pure culture following the PDA guidelines. The acceptance criteria applied are designed to demonstrate that there is at least equivalence between the LSC method and the reference method.

Thus, the following validation criteria were selected and the acceptance limits set:

- Accuracy: The alternative method should give equivalent enumeration to the conventional method, with an acceptance of \pm 30% compared to the 100% reference method (from 70% to 130% is accepted).
- Linearity: The correlation coefficient r^2 should be greater than 0.9 and the regression gradient between 0.7 and 1.3.
- Precision: A coefficient of variation of less than 15% is accepted for results with more than 50 micro-organisms per membrane.
- Limit of detection and quantification: The new method should give results at least equivalent to the existing method.

- Range of detection: The correlation coefficient r^2 should be greater than 0.9 and the gradient of the regression line between 0.7 and 1.3. Note should be taken of where the response curve starts to lose linearity.

Performance qualification on the antiseptic solution

The performance qualification was performed in three successive steps:

- **Validate the cellulase enzymatic pre-treatment:** Count results with and without cellulase pre-treatment should show equivalence if the mean results from the two tests agree to within 30% ($\pm 30\%$).
- **Validate the effectiveness of the antiseptic product neutralisation:** the antiseptic product neutralisation is efficient if the results of the test with the neutralisation step are within 30% of the control sample.
- **Equivalence test:** measures how similar the new method's test results are compared to the method it is intended to replace. The tests were performed in parallel on samples from three different production batches of the antiseptic product, and ten bottles were analysed for each batch.

Results

IQ, OQ

Prior to performing the PQ, the IQ and OQ were completed and signed off by Quality Assurance.

Performance qualification on pure culture

The bacterial and bacterial spore cultures were analysed with the TVC Bioburden protocol (Chemunex, 200-D0511-02). The results were obtained in 90 minutes with the LSC.

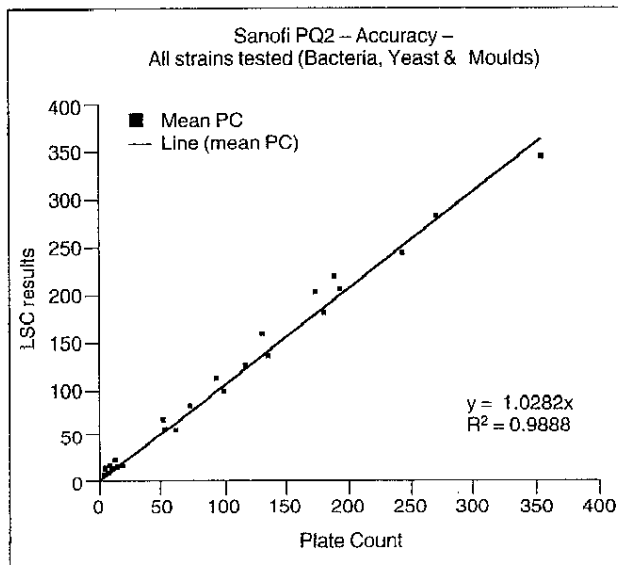


Figure 3: Accuracy study for all the strains under test. PC = plate count.

Table 1: Regression gradient and correlation coefficient for each strain tested with the LSC

Strain tested	Regression Gradient	Coefficient of Correlation
<i>Pseudomonas aeruginosa</i>	1.09	0.99
<i>Staphylococcus aureus</i>	1.01	0.99
<i>Escherichia coli</i>	1.11	0.99
<i>Bacillus subtilis spores</i>	1.08	0.99
<i>Candida albicans</i>	0.99	0.99
<i>Aspergillus niger</i>	0.99	0.99

Yeast and mould spores were analysed with the Fungi protocol (Chemunex, 200-D0512-02) in four hours.

Accuracy

The accuracy results are depicted graphically in Figure 3. Each spot represents the mean result of three LSC or plate measurements, with six dilutions tested for each strain. The correlation coefficient and the gradient were calculated for each strain and shown in Table 1.

The accuracy of the LSC method for the detection of

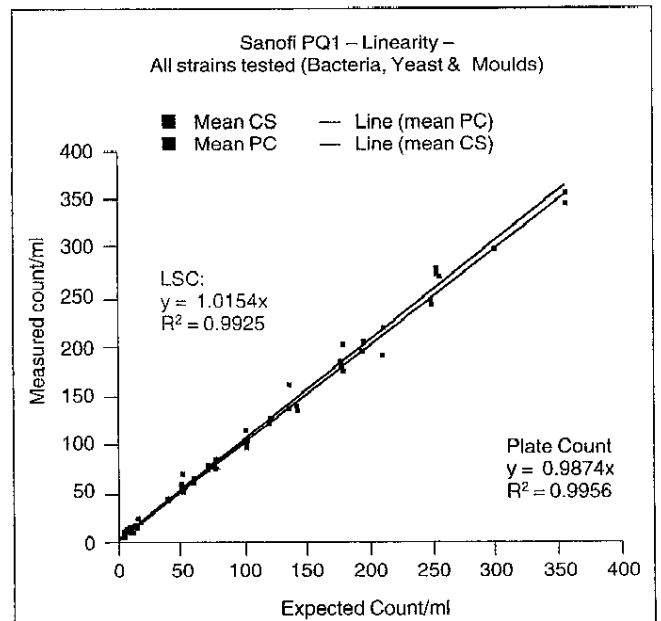


Figure 4: Linearity study for all tested strains in pure culture (■ LSC; ■ Plate method). PC = plate count; CS = cell scan

Table 2: Regression gradients and correlation coefficients for each strain tested with the LSC

Strain tested	Regression Gradient	Coefficient of Correlation
<i>Pseudomonas aeruginosa</i>	1.04	0.99
<i>Staphylococcus aureus</i>	1.47	0.99
<i>Escherichia coli</i>	0.99	0.99
<i>Bacillus subtilis spores</i>	1.06	0.99
<i>Candida albicans</i>	0.96	0.99
<i>Aspergillus niger</i>	1.06	0.99

Number of cells per membrane	LSC %CV	Plate Count %CV
200	9.6	6.3
70	8.7	12.6
10	20.3	29.4

bacteria, bacterial spores, yeast and moulds, was compared to the number of CFU recovered from a pure culture grown in nutrient-rich medium. The LSC results may be considered equivalent to those of the plate count method, as the regression gradient is between 0.7 and 1.3 and the coefficient of correlation is >0.9, as described in the acceptance criteria section.

Linearity

The linearity of an analytical method is defined as its ability to elicit test results that are directly proportional to the dilution within a given range. Acceptance criteria for linearity include a correlation coefficient r^2 greater than 0.9 and a regression gradient ranging between 0.7 and 1.3.

The linearity was evaluated using a dilution series of micro-organisms in DNP medium. The dilution series tested covered the range of 10 to 300 micro-organisms per membrane; six dilutions were tested in triplicate for each bacterial series.

The LSC and the plate count yielded equivalent linearity results with bacterial cultures. A similar degree of equivalence was found with cultures of bacterial spores, yeast and moulds (Figure 4, Table 2). The data obtained show a correlation coefficient $r^2 > 0.9$ and a regression gradient between 0.7-1.3 for all the tested strains.

Precision

The precision of an analytical method is defined as the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling from a homogeneous standard. It provides an estimate of the degree of confidence.

The acceptance criterion for precision is a percentage coefficient of variation (CV) of less than 15% for counts greater than 50 microbial cells per membrane.

Two factors may affect the precision determination: heterogeneity of the bacterial suspension, because bacteria may aggregate or adhere to the walls of the container; and cell growth during the procedure which is difficult to control.

Three homogeneous suspensions of *Pseudomonas aeruginosa* ATCC 9027 containing approximately 200, 70 and 10 cells, respectively, were filtered through ten separate membrane filters. The precision of the LSC was compared to the plate count by analysing the same samples on ten membranes each (Table 3). The data show that the LSC is at least as precise as the conventional plate count method. The coefficients of variation are lower than 15% for counts greater than 50 cells per membrane.

Limit of detection and quantification

The detection and qualification limit of an analytical process is defined as the lowest micro-organism concentration in a sample that can be detected and quantified under the stated experimental conditions.

The acceptance criteria for the detection limit include a correlation coefficient $r^2 > 0.9$ and a regression gradient between 0.7 and 1.3, for the linearity of obtained count to expected count. Furthermore, the assay method should detect zero counts in the control buffer and a single cell in the lowest dilution. The frequency of single-cell detection will be variable due to the inherent sample problems associated with micro-organisms.

A *Pseudomonas aeruginosa* ATCC 9027 suspension was diluted to allow a count ranging from 15 down to 0 cells per membrane, and 6 replicate samples were assessed (Figure 5).

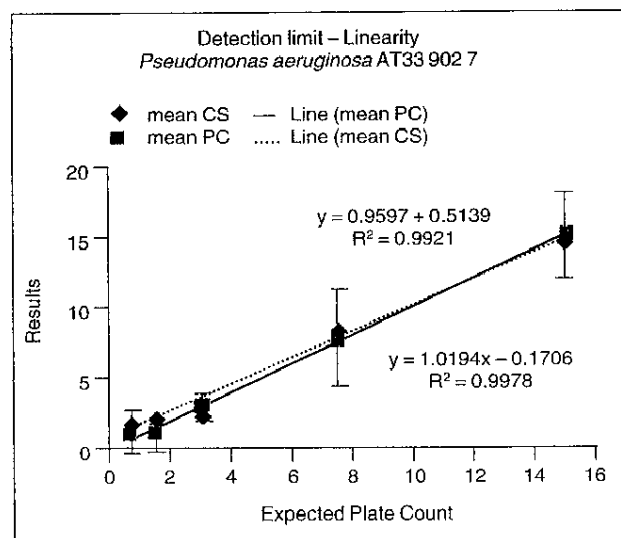


Figure 5: Limit of detection of the LSC vs plate count with *Pseudomonas aeruginosa* culture (♦ LSC; ■ Plate method). PC = plate count; CS = cell scan

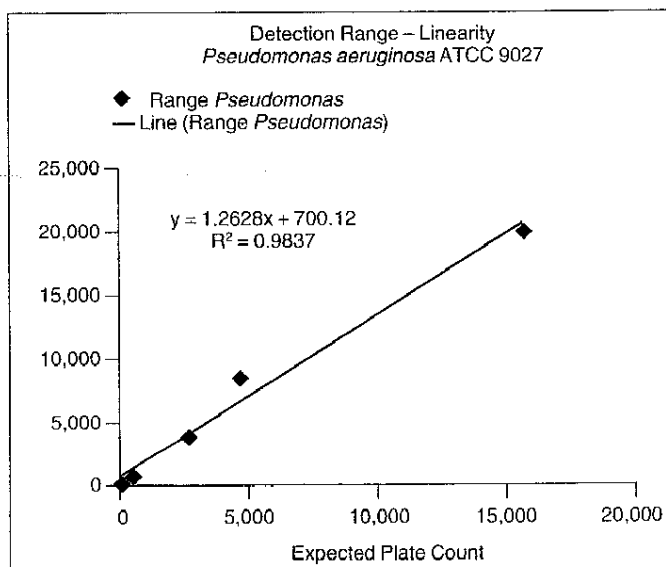


Figure 6: Range of detection of the LSC with *Pseudomonas aeruginosa* culture

Table 4: Recovery of the micro-organisms tested with the LSC and the plate count method, with or without the enzymatic treatment

Strain tested	LSC		Plate Count	
	Mean % with/without enzyme	Mean % with/without enzyme	Mean % with/without enzyme	Mean % with/without enzyme
<i>Pseudomonas aeruginosa</i> ATCC 9027	110	96		
<i>Staphylococcus aureus</i> ATCC 6538	102	98		
<i>Escherichia coli</i> ATCC 8739	95	101		
<i>Bacillus subtilis</i> ATCC 6633	72	100		
<i>Candida albicans</i> ATCC 10231	113	104		
<i>Aspergillus niger</i> ATCC 16404	89	90		

The data obtained ($r^2 = 0.99$ and gradient = 0.96) show that the LSC is able to detect very small numbers of cells with a good precision. The LSC has a detection sensitivity of one cell, provided that the cells were adequately labelled. The detection sensitivity for samples with 1 or 0 cell per sample was confirmed visually by scanning the entire membrane manually with a microscope or cell scanner (CS).

Range of detection

The detection range of an analytical process is defined as the highest concentration of analyte in a sample that can be detected with precision and linearity.

A *Pseudomonas aeruginosa* ATCC 9027 suspension was diluted to give counts ranging from 20,000 down to 50 cells per membrane, and 3 replicate samples were assessed (Figure 6).

The data obtained ($r^2 = 0.99$ and gradient = 1.26) show that the LSC is able to detect very high numbers of cells with good precision. The LSC count is linear in the range of 0 to 20,000 cells per membrane, comparing very favourably with the plate count method (with a typical range of 20 to 200 CFU/plate for a statistically valid estimate).

When using plates, a direct enumeration usually requires dilution of the sample, or increasing the volume in order to reach the 20-200 CFU/plate range. The LSC has fewer limitations with an acceptable range from 0 to 20,000 cells per membrane. The broad acceptable working range of the LSC provides the operator with more convenience, requiring a minimum amount of preparation and allowing larger sample volumes to be tested.

Performance qualification on the antiseptic solution

Validation of the enzymatic treatment and absence of inhibitory effect

The antiseptic solution is a viscous solution. Therefore, filtration of 10 ml of this solution is impossible without pre-treatment. The treatment is performed with an enzyme (the cellulase) able to reduce the viscosity of the product, and allows the filtration of 10 ml of product in a few minutes.

Table 5: Validation of the antiseptic neutralisation

Strain tested	LSC		Plate count	
	Mean % Series 1/3	Mean % Series 2/3	Mean % Series 1/3	Mean % Series 2/3
<i>Aspergillus niger</i>	86	78	70	72
<i>Candida albicans</i>	88	128	115	111
<i>Pseudomonas</i> spp.	83	88	98	87

As this enzyme has the ability to modify the chemical structure of the antiseptic solution, it is necessary to check that its activity has no effect on the micro-organism viability in the product during the treatment. The absence of inhibitory effect was measured with the six tested strains using the LSC and the plate count method by comparing cell counts in the presence or absence of cellulase pre-treatment.

A suspension containing 80-120 cells was tested in six replicates for each method (Table 4). The methods are equivalent if the mean results from the two tests are within 30%.

The data show good equivalence between the two tests. The enzymatic pre-treatment does not affect the detection of the micro-organisms, and can also safely be used for the analysis of the disinfectant suspension.

Validation of the product neutralisation

The aim of this test was to show that the neutralisation step carried out on the antiseptic (rinsing with 3 x 100ml of DNP) is sufficient for the detection of contaminants.

The neutralisation step is considered efficient if the results from series 1 (disinfectant and micro-organisms) and 2 (micro-organisms alone) are within 30% of the results obtained with the third series (1 ml of tested micro-organism control sample), i.e. the mean percentage should be between 70-130% (Table 5).

The neutralisation was tested on *Candida albicans* ATCC10231 and *Aspergillus niger* ATCC16404 using the Fungi protocol and on *Pseudomonas* spp., a natural contaminant, using the TVC Bioburden protocol.

The data obtained for the neutralisation test are within the 30% accepted limit.

Table 6a. Number of samples in each selection category, TVC Bioburden Protocol.

	LSC -	LSC +
Reference Method -	53 (59%)	34 (38%)
Reference Method +	1 (0.01%)	2 (0.02%)

Table 6b. Number of samples in each selection category, Fungi Protocol.

	LSC -	LSC +
Reference Method -	39 (70%)	17 (30%)
Reference Method +	0	0

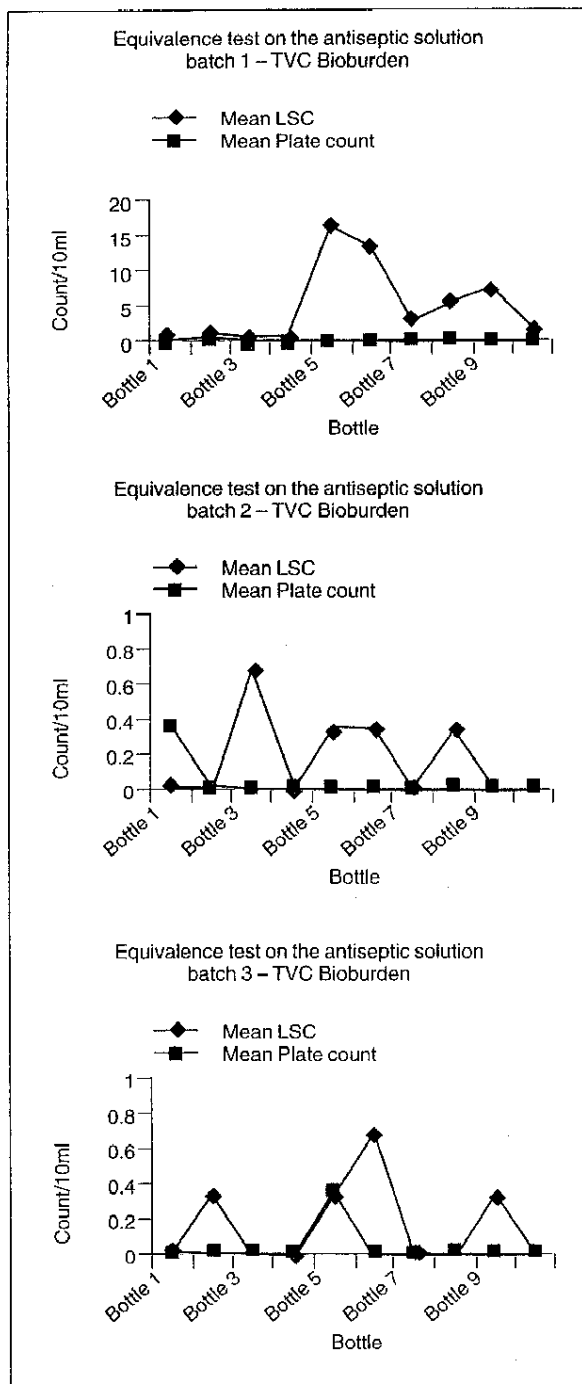


Figure 7. Equivalence test, LSC vs plate count for TVC application

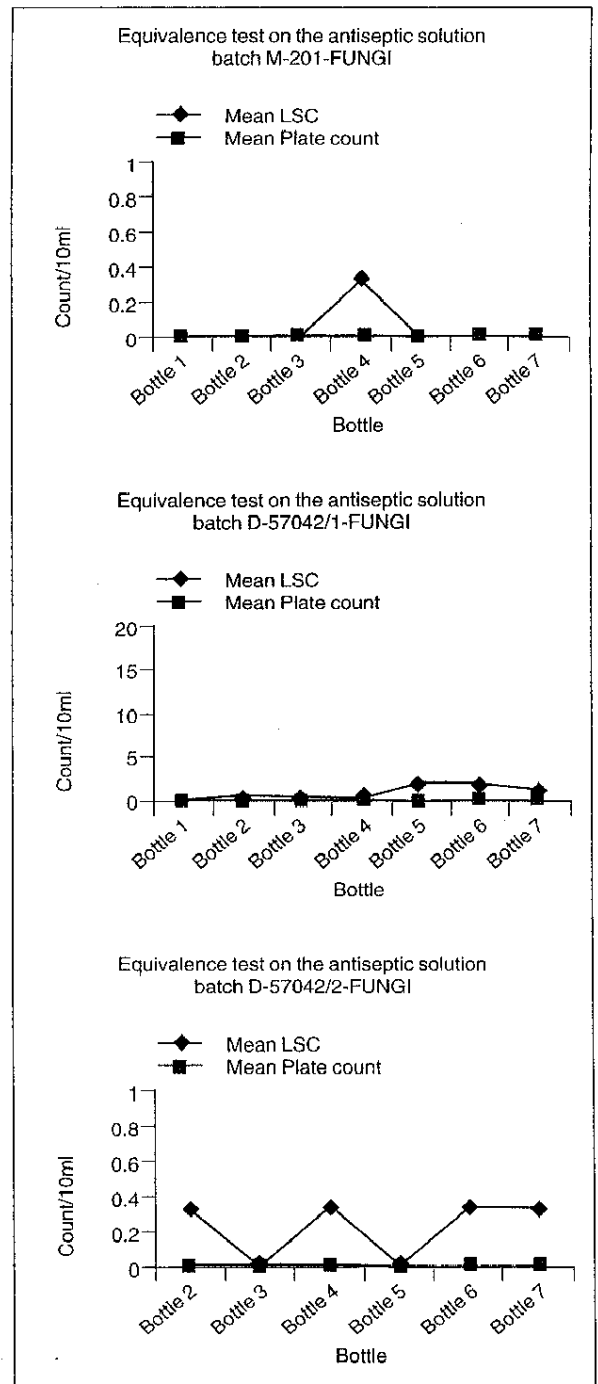


Figure 8. Equivalence test, LSC vs plate count for the Fungi application

Equivalence Test

The antiseptic fluid testing was performed on 10 bottles of three different batches using the TVC Bioburden application. Each bottle was tested with three replicates in parallel using the LSC and the classical method. Six bottles per lot were tested using the Fungi protocol.

A total of 146 finished product samples were tested with the LSC and with the conventional plate method (Table 6a and 6b; Figures 7 and 8), and the results were split into negative and positive categories, based on the

presence or absence of contaminant.

All micro-organisms detected by the LSC were confirmed using microscope visualisation in order to eliminate any last few remaining particles. The microscope inspection allows the operator to discriminate visually between bacteria and other particles, based on shape and colour characteristics.

Good agreement was observed between the two methods on two batches. The third batch showed contamination (10-20 cells per 10 ml) with the LSC. The

conventional plate count method did not show any micro-organism growth over five days.

Since the LSC detection method has no need for cell growth, fastidious or injured micro-organisms can be detected, whereas the plate count can be limited by the ability of the micro-organisms to grow in the chosen conditions (e.g. medium, temperature, etc.).

Conclusion

The Performance Qualification study performed at Sanofi-Synthelabo Riells showed that results obtained with the LSC are at least equivalent to the results obtained with the conventional method on agar plates.

The three lots of the antiseptic solution tested during the validation study yielded results with a sensitivity which was at least equivalent to that of plate method.

The detection of micro-organisms with the LSC does not require growth; therefore, the LSC is able to detect fastidious or injured micro-organisms, including analysis in the presence of a growth inhibitor such as an antiseptic solution. Consequently, the inhibitory effect of the antiseptic suspension is far less critical using the LSC than the conventional method on agar. The LSC is also able to detect any contaminant in the antiseptic solution with a much higher sensitivity than the classical method. This is particularly obvious with the third batch. Results less than or equal to 1 count per membrane with the LSC are therefore considered as not significant, but only results equal to or more than 2 are considered significant. According to the sensitivity of the LSC system the use of the LSC allows an increase in quality and the threshold may, therefore, be changed slightly. A lot tested with the LSC would not fail with 1 count per 10 ml, but it would fail if the classical method shows 1 CFU per 10 ml.

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