

# *Validation of cytometry method* for the quality control and release of non obligatory sterile pharmaceutical products

*Specialised in the manufacture of raw materials for pharmaceutical industry, SPI Pharma has recently set-up a rapid microbiology system based on flow cytometry for the release control of non obligatory sterile pharmaceutical products. The manufacturer describes in this article the different stages and the results of the validation of this protocol allowing to obtain presence/absence results in 24 hours.*

**By Denis Solinas,**

QC Manager,  
Safety and  
Environment SPI  
Pharma  
Company.

The concept of productivity becomes a key element for the pharmaceutical industry constrained to work in logic of just in time and to respect the deadlines. Process and the finished products control requires the implementation of strict and regulated microbiological controls. All analysis methods defined in the European and US pharmacopeia are based on the growth of microorganisms with an incubation phase in Petri dish for several days; under these conditions, the microbiological result becomes a crucial point which can save or lose invaluable logistics days.

SPI Pharma Company, specialized in the manufacture of drugs supports and raw materials for the pharmaceutical industry, such as the active ingredients antacid (aluminum

hydroxide, magnesium hydroxide), has started an innovating step to achieve these goals of just in time by validating a new rapid microbiology method based on the technology of viability labelling combined with a detection by flow cytometry provided by Chemunex.

With the installation of this rapid method in the quality control laboratory, the microbiological results are now available in only 24 hours for the production. The microbiology laboratory, located in Septemes Les Vallons, close to Aix-en-Provence, can confirm to the production manager, the release of the finished products as soon as the following day of the production. While maintaining the level of quality required, the benefits related to the implementation of this new rapid method are the reactivity on the process and the reduction of the storage costs.

Through the reference methods described in the European pharmacopeia, the alternative methods are also quoted as being able to be used when equivalence between the rapid method and the conventional method are demonstrated. The implementation of this new method by flow cytometry (BactiFlow) thus fell under a validation step inspired by the guides or directives such as paragraph 5.1.6 of Pharmeuropa, the USP *"The validation of compendial methods < 1225 >"* or *"the PDA Technical Report 33"*.

The validation procedure followed the standards defined by including the installation qualification (IQ), the operational qualification (OQ) and the performance qualification (PQ). In this article, we will describe the performance qualification which represents the performance of the alternative analytical method.



The microbiology laboratory, located in Septemes Les Vallons, close to Aix-en-Provence.

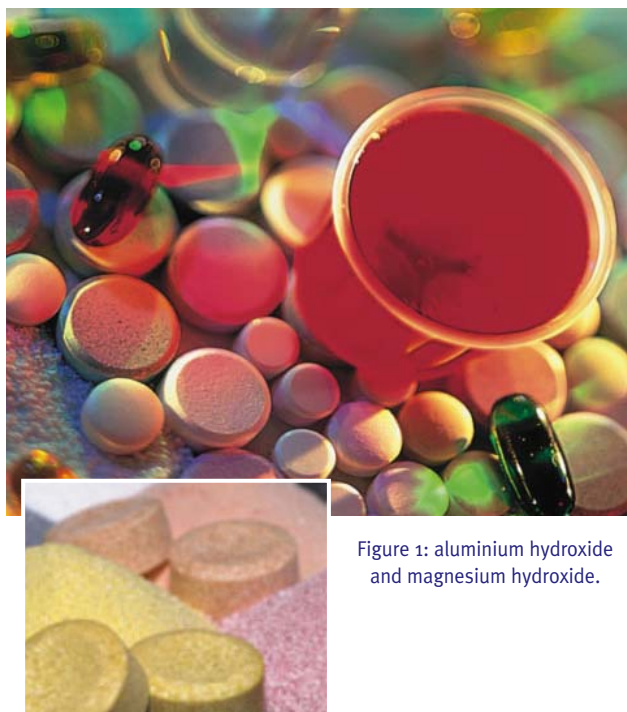


Figure 1: aluminium hydroxide and magnesium hydroxide.

## Materials and methods

### Strains used

The strains used in the performance qualification are those recommended in the pharmacopoeias:

- Bacterial vegetative forms :
  - *Pseudomonas aeruginosa* (ATCC 9027)
  - *Staphylococcus aureus* (ATCC 6538)
  - *Escherichiae Coli* (ATCC 8739)
- Bacterial spore:
  - *Bacillus subtilis* spores (ATCC 6633)

The stock solutions of the strains are prepared in accordance with the method described in the performance qualification 1 protocol (PQ1) provided by the manufacturer. The cultures result from a colony with less than 5 generations and less than 10 days in stored boxes at + 2 - 8 °C. A colony is taken and put in to suspension in one sterile tube having 1 g of glass beads (diameter 4 mm) then 10 ml is added of nutritive broth. (The same broth as used for the Presence/Absence test.)

After homogenisation to eliminate the cellular aggregates, the dilutions, being used to carry out the artificial contaminations with low microbial load, are prepared from the stock solution diluted in the nutritive broth, from  $10^{-1}$  to  $10^{-4}$ . The highest dilution is then analyzed using BactiFlow with a protocol of direct enumeration in order to estimate the initial concentration of the stock solution. The real-time enumeration makes possible to prepare the stock solution with 100 cells/ml.

### Products tested: aluminum hydroxide and magnesium hydroxide.

The products to be controlled are active ingredients anti-acid in gel or powder form and are produced in non-sterile condition (*figure 1*). The analysis have been carried out on samples coming from more than 3 various batches. The required microbiological criteria are those of the pharmacopoeia Européenne (Total Flora < 100 CFU/g and absence of specific germs).

### Reference method

The analysis of the product is performed on 10 g of sample diluted in 90 ml of neutralizing broth (pharmacopoeia diluant) for 2 hours. Then 1 ml of this suspension is inoculated on TSA and incubated for 5 days at  $32 \pm 2$  °C. This approach thus allows to obtain a result in absence of growth with a sensitivity down to 10 CFU/g. The reference method being based on an enumeration and the suggested alternative method on a Presence/Absence test (expected sensitivity < 1 CFU/g), the expected sensitivity levels of the two methods are thus different. Under these conditions, it was necessary to define the acceptance limits to validate the new alternative method. The criteria selected were as follows.

### Method alternative

#### Material: BactiFlow, Flow Cytometer

The alternative method used for this validation is a flow cytometer adapted for microbiology : BactiFlow (Chemunex, Ivry-Sur-Seine, France). BactiFlow is equipped with a source of solid laser excitation at 488 nm (*figure 2*). All reagents and consumables were provided by Chemunex and AES Laboratoire.



Figure 2: cytometer flow BactiFlow.

Two photomultipliers collect the fluorescent light emitted by each labeled cell. The signals obtained are then automatically discriminated by data processing and thus allows to make the difference between the relevant signals for the enumeration (labeled viable micro organisms), and the background (auto-fluorescent particles). The detected and

counted micro organisms are then reported and translated without operator interpretation into answer “Green light” and “Red light”.

**BactiFlow Protocol : Presence/Absence test**

The samples are prepared according to the recommendations’ of the manufacturer ; ref: 302-D0510-03.

10 g of the product to be analyzed are diluted in 90 ml of neutralizing broth (Pharmacopeia diluant). After 2 hours of contact, 10 ml of the neutralized product are put into 90 ml of nutritive broth in order to obtain an enrichment of one gram of product. The prepared sample is then incubated for 24 hours at 32 °C +/- 2 °C before BactiFlow analysis.

After the enrichment phase, 200 µl of the enriched sample are taken to perform the viability labeling. The counter stain is added to eliminate the background induced by the matrix. Then the labeling solution is added allowing the labeling of the viable cells by using the intracellular enzymatic activity with the membrane integrity of the micro-organism. After the 12 minutes of labeling at 30 +/- 2 °C, a second counter stain containing an internal standard control is added before the injection of the sample in the analyzer for an automatic reading in 1 minute (figure 3).

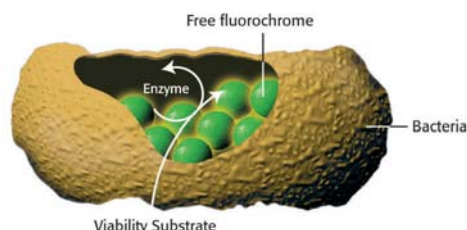


Figure 3: principle of Chemunex viability labeling.

**Results interpretation**

**Criteria of Performance Qualification 1 with pure cultures**

BactiFlow was tested with pure cultures in accordance with the recommendations. The applied acceptance criteria are determined to show the alternative method is at least equivalent to the reference method. Acting of a qualitative test that only the following parameters of validation were taken into account.

**Exactitude**

The alternative method must give results equivalent to the reference method. Thus the expected results must be equivalent to the obtained results. Within the framework of a qualitative test (Presence/Absence test) the inoculum loads ranging between 5 and 10 CFU/g, expected positive, must all be confirmed positive in accordance with the reference method.

**Limit of detection**

The alternative method must give results at least equivalent to the existing method. The criteria will make it possible to validate the limit of detection for the values of inoculum < 1 CFU/gram.

**Performance Qualification on finished products (aluminum hydroxide and magnesium hydroxide).** The performance qualification 2 (PQ2) was performed in 3 successive steps.

**Validation of the specificity of the labeling protocol and control of the background matrix**

The labeling protocol must guarantee the absence of labeling of dead micro organisms (negative result after labeling of a micro organism suspension having undergone a thermal treatment at 80 +/- 2 °C for 10 min). The absence of matrix interferences is verified (negative results for a sterile nutritive broth only and a nutritive broth with non-contaminated matrix lower).

**Validation of the effectiveness of the product neutralization**

The neutralization of the tested product is effective if the results of the test including the stage of neutralization and enrichment steps allows the detection after the incubation step of a low level contamination (ranging between 1 and 5 CFU/g). The micro organisms loads, after incubation, with and without matrix, should not be different with more than 0,3 Log to validate the complete neutralization of the product, allowing the growth of the micro organisms during the incubation step.

**Equivalence test**

To measure the similarity of the alternative method with the reference method to be replaced. The tests are carried out on each sample in parallel on more than 3 different batches of alumin hydroxide and magnesium hydroxide in powder and gel form.

**Acceptance Criteria**

- Conformity results BactiFlow (< 1 CFU/g) / Non conformity results Method Pharmacopeia (> 100 CFU/g).  
**0 %**
- Non conformity results BactiFlow (> 1 CFU/g) / Non conformity results Method Pharmacopeia (> 100 CFU/g).  
**100 %**
- Non conformity results BactiFlow (> 1 CFU/g) / Conformity results Method Pharmacopeia (< 100 CFU/g).  
**< 6 % \***

\* This criterion takes into account to the fact that the Presence/Absence test allows a threshold of expected sensitivity higher than the direct enumeration. Thus, a result of conformity enumeration (pharmacopeia method) with absence of colony on plate count does indicate a contamination < 10 CFU/g also we can observe a non conformity result with BactiFlow for a presence of germ ranging between 1 and 9 CFU/g. The rate of additional acceptable non conformity result was thus defined at 6 % maximum, taking into account of the initial objective of just in time to set up, 1 positive result requiring complementary analyses, therefore an additional Petri dishes has to be done.

## Results

### Installation Qualification (IQ) and Operational Qualification (OQ).

Before doing the PQ protocol, the complet IQ and OQ protocol were performed by the supplier and approved by the Quality Assurance department.

### Performance Qualification 1 with pure cultures

#### Exactitude

The results of this validation criterion are summarized in table 1.

It shows a good correlation with 50 tests found positive by both methods and 31 tests found negative. All the samples with inocula ranging between 5 and 10 CFU/gram are confirmed positive with the presence/absence protocol using both method. Only 3 results out of 84 are found unmatched compared to the expected results. This difference can be explained because of the low level of inoculum employed for each test.

		Reference Method of Presence/Absence	
		Positive results	Negative results
BactiFlow Method	Positive results *	50	2
	Negative results	1	31

(\*positivity threshold > 500 counts/ml).

Table 1: correlation of the two methods on pure cultures.

In addition, the totality of the results obtained comes from the triplicate analysis of each enrichment broth. No discordance was observed in the same enrichment. Thus, the results are regarded as equivalents and in conformity with the acceptance criteria.

#### Limit of detection

The limit of detection of an analytical method is defined as the smallest concentration of micro organism present in a sample which can be detected. Within the framework of a qualitative test, the test will consist in comparatively

Theoretical Inoculum	Real Inoculum	Référence Method	BactiFlow Method
Stock Solution (100 cfu/ml)	M = 112 +/-4		
10 cfu / bottle	M = 11 +/-3,8	+	+
1 cfu / bottle	M = 1,4 +/-1,4	-	-
		-	-
		+	+
		+	+
		+	+
0.5 cfu / bottle	M = 0,6 +/-0,7	-	-
		-	-
		-	-
		+	+
		+	+
0 cfu / bottle	0	-	-
Relative level of detection LOD 50 %		0.347 [0.066-1.84]	0.347 [0.066-1.84]

Each sample was analyzed in triplicate.

performing the Presence/Absence test with both methods with micro organisms levels increasingly low and below 1 CFU/g.

Four target levels of contamination were tested for each micro organism (0 – 0,5 – 1 and 10 CFU/ per bottle of 90 ml of nutritive broth). Six triplicate analyses were carried out for the concentration levels of 0,5 and 1 micro organism. The contaminations were carried out directly in nutritive broth, before incubation.

For each contamination level, the homogeneity of the suspensions is checked by 10 enumerations on TSA plate agar. The calculation of the confidence interval is established by the mean and the standard deviation.

The 14 bottles were prepared for each strain and are incubated for 22 hours at 32 +/- 2 °C (table 2).

The limit of detection is provided for each strain by the statistical Speerman Karber test. This one allows giving statistically the smallest quantity of detectable micro organism by the method guaranteeing 50 % of detection.

Table 2: results of limits of detection test on *Staphylococcus aureus* (ATCC 6538).

	Reference Method	(Flow Cytometer) BactiFlow
<i>Pseudomonas aeruginosa</i> (ATCC 9027)	0,268 [0,062-1,154]	0,268 [0,062-1,154]
<i>Staphylococcus aureus</i> (ATCC 6538)	0,347 [0,066-1,840]	0,347 [0,066-1,840]
<i>Bacillus subtilis spores</i> (ATCC 6633)	0,409 [0,09-1,851]	0,530 [0,117-2,400]
<i>Escherichia Coli</i> (ATCC 8739)	0,134 [0,035-0,519]	0,134 [0,035-0,519]

Table 3: limit of detection LOD 50 % for each strain.

	WITHOUT TREATMENT		80 °C TREATMENT 10 minutes	
	Agar TSA*	BactiFlow **	Agar TSA*	BactiFlow **
<i>Escherichia Coli</i> (ATCC 8739)	> 300 > 300	5,7.10 <sup>E6</sup> 5,5.10 <sup>E6</sup>	< 1 < 1	- -
<i>Staphylococcus aureus</i> (ATCC 6538)	> 300 > 300	8,2.10 <sup>E5</sup> 9,0.10 <sup>E5</sup>	< 1 < 1	- -
<i>Pseudomonas aeruginosa</i> (ATCC 9027)	> 300 > 300	2,1.10 <sup>E5</sup> 2,5.10 <sup>E5</sup>	< 1 < 1	- -
Control nutritive broth	< 1 < 1	- -	< 1 < 1	- -
Control Reagent	Nd	-	Nd	

< 500 Counts/ml of analyzed sample = Negative

> 500 Counts/ml of analyzed sample = Positive

\* cfu/ml of bubble of enrichment

\*\* counts/ml of analysed sample

Table 4:  
specificity test after  
heat treatment.

Each strain is evaluated according to the test program presented in table 2 (*Staphylococcus aureus* as example). The totality of the results is summarized in table 3. It allows to validate each strain tested showing the detection limit of the method for the strain contamination < 1 CFU/g. Thus, it could be possible to validate the method for a qualitative Presence/Absence test in 1 g and to guarantee the absence of the specific germs.

Table 5:  
results on the  
neutralization tests  
of the finished  
products.

	BactiFlow Method Count/ml	Reference Method (100µl of the medium enriched on TSA)
TR	0 / 0	0 / 0
Strain control without matrix (21 UFC/10g)	3,4.10 <sup>E6</sup> N=3	>300 N=2
Strain control without matrix (287 UFC/10g)	3,4.10 <sup>E6</sup> N=3	>300 N=2
control product (gel)	0 / 0 / 0	0 / 0
Product 1 (Gel) Rate 21UFC/10g	2,3.10 <sup>E6</sup> N=3	>300 N=2
Product 1 (Gel) Rate 287UFC/10g	2,1.10 <sup>E6</sup> N=3	>300 N=2
Control product (Powder)	0 / 0	0 / 0
Product 2 (Powder) Rate 21 UFC/102	2,4.10 <sup>E6</sup> N=3	>300 N=2
Product 2 (Powder) Rate 287UFC/10g	2,8.10 <sup>E6</sup> N=3	>300 N=2

If we take the results *Staphylococcus aureus* (ATCC 6538), the LOD 50 % is 0,347 [0,062 - 1,154] for both methods, thus the detection limit for *Staphylococcus aureus* is 0,347 CFU/g of product. The BactiFlow method guaranteed to detect 50 % of the contaminated samples with 0,347 CFU/g with a confidence interval ranging between 0,062 and 1,154.

## Performance Qualification 2 on finished products

### Validation of the specificity of the labeling protocol and control of the background matrix

BactiFlow analysis shows samples without germs after viability labeling on the contaminated enrichment broth treated with heat (table 4).

Petri dish controls are in agreement with the results of the flow cytometry. After 10 minutes treatment at 80 °C, the flow cytometry results show that the labeling is specific of viable micro organisms.

The absence of interference matrix is also verified by the absence of false positive results on negative samples (Control nutritive broth + matrix) (table 4).

### Validation of the effectiveness of the product neutralization

Finished products (powder and gel) were spiked with low level of germs. Two contaminations levels were tested (target with 2 CFU/g, reality with 21 CFU/10 g and target with 20 CFU/g, 287 CFU/10g).

10 g of product are diluted in 90 ml neutralizing (pharmacopoeia diluant). After 2 hours of contact time, the neutralized products are spiked at the targets levels indicated above. After homogenization, 10 ml of the sown neutralizing broth are included in 90 ml of nutritive broth and incubated for 22 hours at 32 +/- 2 °C.

*Pseudomonas aeruginosa* (ATCC 9027) strain was used for this test. Control strain, with and without matrix, on two contamination levels, are carried out in order to verify the capacity of the method to detect a low level of contamination in the presence of matrix and to validate the good neutralization of the product and the absence of residual inhibiting capacity.

The results, presented in table 5, show that the lower load (21 CFU/10 g = 2 CFU/g) is well detected by the presence/absence protocol using both products.

Although we did not use a quantitative test because of the incubation, the analysis by flow cytometry giving out an enumeration of the enriched broth allows to check that the load obtained after incubation in the presence of matrix does not differ of more than 0,3 LOG compared to the strain control for products 1 and 2 (table 6). The absence of interference matrix is well confirmed. The effectiveness criterion of the neutralization of product is thus validated.

### Equivalence test

The equivalence test was performed for 6 months on 452 products, distributed on more than 3 different batches of production at the same time on the powder and gel products. Each product was tested in parallel

	Target level 2 cfu/g	Mean Variation Control/sample
Strain control <b>Pseudomonas aeruginosa (ATCC 9027)</b>	3,4.10 <sup>E6</sup> = 6,53 Log	-
<b>Product 1 (gel)</b>	2,2.10 <sup>E6</sup> =6,36 log	-0,17 Log
<b>Product 2 (Powder)</b>	2,4.10 <sup>E6</sup> = 6,38 Log	-0,15 Log

Table 6 : validation of the effectiveness of the neutralization of powders and gel with an inoculum to 2 CFU/g of product.

by both methods (direct enumeration by the reference method and Presence/Absence (P/A) test using BactiFlow). The results were divided into various categories, positive and negative results (table 7).

All positive results on BactiFlow were the subject of a confirmation on Petri dish with the enriched broth. No negative BactiFlow result was confirmed positive by the reference method. The absence of false negative thus is well validated. Seven results not conform on BactiFlow were not confirmed with the Petri dish enumeration. As indicated in paragraph 2.5.2 (related to the equivalence criteria), these results are explained by the difference of both approaches, one in direct enumeration with a final result on a specimen test of 0,1 g of products in neutralized inoculum dispensed in Petri dish, the other in Presence/Absence test with a final result on a test specimen of 1 g of enriched product.

The BactiFlow method highlighted 5,5 % of non conform results (using Presence/Absence test) when the enumeration method confirmed a conform result < 100 CFU.

This is acceptable according to the transfer of an enumeration method to a qualitative method. However the number of additional positive results does not exceed the criterion of the necessary 6 % in the acceptance criteria.

## Conclusion

The performance qualification study performed at SPI PHARMA shows that the results obtained with the rapid technique using flow cytometry of BactiFlow are equivalent to the results obtained with the reference method. The equivalence tests carried out over 6 months of production showed a sensitivity equivalent to the reference method. In only one analysis, the implementation of this qualitative method allows to control the production within 24 hours, by also confirming the absence of the specific germs. In this precise case, the validation of this new approach showed that a qualitative test did not

	P/A BactiFlow non conform	P/A BactiFlow conform	TOTAL
Enumeration By Pharmacopeia method Conform	24 (5,5 %)	403 (92,8 %)	427
Enumeration By Pharmacopeia method Non Conform	7 (1,7 %)	0 (0 %)	7
TOTAL	31	403	434

Table 7 : distribution of positive and negative according to the method of analysis.

penalise to release the products compared to a quantitative analysis. The release of the finished products, thus validated in 24 hours, allows a reduction of the storage costs and an increase of the just in time production. In addition, if BactiFlow is validated here within the framework of a qualitative analysis, it allows direct enumerations available for certain matrices according to their background. This aspect was used also at the time of this validation to estimate the stocks solutions necessary to the inoculums preparation or to validate the neutralization effectiveness and the control of the residual inhibiting capacity. ■

## References

- *Validation of the alternative microbiological methods*, chapter 1223. Pharmacopeial forum 28 (1): Janv-Fev 2002
- Technical Report N° 33 : *Evaluation, Validation and implementation of new microbiology testing methods*, PDA Newspaper of pharmaceutical Sciences and technology. Supplement TR33, 2000: 54: n°3
- *Alternative methods for the microbiology quality control, section 3.2. Validation of the qualitative detection tests of micro-organisms in sample*. Pharmeuropa Vol.16, 5.1.6. n°4, October 2004
- *Installation, Operational and Performance Qualification*, support document Chemunex (ref: 302-Do401-02/302-Do402-02/302-Do221-01).
- Brailsford MA, Gately micro S. *Rapid analysis of organisms using flow cytometry. Flow cytometry in Microbiology 1993*. ED. Lloyd D: Springer-Verlag, London: 171-180
- Johnson B *Evaluation of has new system for rapid microbiological detection in non-sterile pharmaceutical products*. European Pharmaceutical Review 1999; 4(1): 55-59
- Philippe L. *With new rapid analysis system for complete microbial quality control in the cosmetic industry*. SOFW. June, 2000; 28-30