ACCREDITATION SCHEME FOR LABORATORIES

Guidance Notes C&B AND ENV 002

Method Validation of Microbiological Methods

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1.0 General

1.1 The scope of the guidance deals with validation of microbiological methods for food, water and pharmaceutical products, with particular emphasis on quantitative methods in which the quantitative estimate is based on counting of particles on the basis of growth (multiplication) into colonies or turbidity. The principles and procedures within this scope are commonly known as the total colony count, most probable number (MPN) and colony counts of specific target organisms on selective media. The guidance does not apply to the validation of the so-called modern / rapid methods which depend on measuring products or changes due to microbial activity but do not address the detection of individual particles and microbiological assay methods using microorganisms as assay tools.

1.2 The laboratory is expected to use published reference/standard methods for microbiological tests. Laboratory users of the reference / standard methods are not required to perform primary validation (full validation) of these methods, but merely required to perform secondary validation (also called verification). Secondary validation requires the laboratory users to merely verify that the method can fulfill the purpose and requirements for the intended analytical applications. In situations where laboratory has to develop in-house method, make modifications to the standard/reference methods or use them beyond their intended usage, the laboratory should carry out full validation of the method. Reference microorganisms used for validation should be checked for purity by surface plating on appropriate non-selective and selective media and microscopic examination of the stained smears. Their identity, when necessary, should be confirmed using appropriate conventional cultural methods or modern / rapid methods approved for use in the reference / standard methods.

2. Performance Characteristics

2.1 The following analytical performance characteristics shall be considered for full validation of method: accuracy, precision (repeatability, reproducibility and intermediate precision), sensitivity & specificity, selectivity, recovery rate, acceptable counting range (upper limit and lower limit of counting range) and robustness.

2.2 Accuracy

Accuracy means the ability of the method to measure the actual or true value of the analyte e.g. the target microorganism(s). If an analyte is naturally present in a sample or is deliberately spiked into the sample as part of a challenge or proficiency test, then the method must be able to detect or recover that analyte at the correct concentration or frequency to be considered accurate.

2.3 Precision

Precision is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenised sample. The precision of an analytical method is usually expressed as relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of **repeatability** or of

reproducibility of the analytical method under normal operating conditions. **Repeatibility** is to measure variation in independent test results obtained with the same method on identical test samples in the same laboratory by the same operator using the same equipment within short intervals of time. **Reproducibility** is to measure variation in independent test results obtained with the same method on identical test samples in different laboratories with different operators or different equipment. **Intermediate precision** expresses within–laboratory variation, as on different dates, or with different analysts or equipment within the same laboratory.

2.3.1 Recommended procedure for estimation within laboratory intermediate precision Relative Standard Deviation, RSD:

Perform at least 15 determinations at different times and different dates using different analysts. RSD should be estimated at different analyte levels within the counting range recommended by standard methods, for example at low, medium and at high levels. Calculate RSD from the following equation:

$$RSD = \sqrt{\frac{\sum_{i=1}^{i=n} [(\log a_i - \log b_i)/x_i]^2}{2p}}$$

where

$(\log a_i - \log b_i)/x_i$ = the relative difference between the duplicate logarithmic results

i =1,2,….n

p = number of duplicate determinations

Calculation of intermediate precision RSD for total plate count of water is illustrated in Example 1.

2.3.2 The magnitude of within laboratory intermediate precision RSD obtained should be evaluated in relation to that of the standard method, when available and the intended analytical applications of the method (fitness for purpose).

2.3.2 The same approach, as illustrated in example 2, can be used for the estimation of personal repeatability RSD in the laboratory.

Note: Relative standard deviation greater than 0.1 (five to ten times the RSD of pure culture counting) is a certain sign of problems / difficulties.

2.4 **Sensitivity and Specificity**

2.4.1 Sensitivity – fraction of the total number of positive cultures or colonies correctly assigned in the presumptive inspection.

2.4.2 Specificity - fraction of the total number of negative cultures or colonies correctly assigned in the presumptive inspection.

2.4.3 False positive rate – the fraction of the observed positives wrongly assigned

2.4.4 False negative rate – the fraction of the observed negatives wrongly assigned

2.4.5 These performance characteristics apply to selective methods and are determined by verifying presumptive positive and negative colonies on plates or positive and negative growth in broths / tubes (MPN).

2.4.6 In primary validation, all presumptive positive and presumptive negative cultures should be verified. Validation should include natural samples studied over a period of time.

2.4.7 In secondary validation, only presumptive positive colonies need be isolated and verified.

2.4.8 Recommended procedure for determining these performance characteristics:

i) make two-fold or ten-fold dilutions of a natural sample. In cases where the natural sample is known to unlikely contain the target organism, a culture of the target organism can be used for spiking the sample. For primary validation, at least three strains of the target organism should be used for spiking in the determination. For secondary validation, one or more strains of the target organism should be used. For primary validation, it should also be noted that where the test method does not contain a resuscitation procedure for injured target organism and the manufacturing processes or conditions of the natural product is likely to cause injury to the target organism if present, then injured target reference organism should be used in the spiking of the sample. Injured organism can be prepared by exposing the organism to sublethal treatments such as drying, heating, chilling or freezing to simulate the manufacturing processes or conditions as far as possible. Injured organism is manifested by significant reduction of recovery of the organism on selective medium compared to non-selective medium.

ii) the dilutions from the sample is then plated in duplicates or triplicates on plates or inoculated into the planned series of broths / tubes as in MPN method.

iii) incubate as directed. After incubation, proceed to either 4a) or 4b).

iv) Examine the colonies from the plates that show colonies either within or closest to the counting range recommended by the standard method (plate count method). Count the number of colonies of presumptive target organisms and the non target organisms on plates. The sum of these counts is the total colony count of the plate. In cases where the natural sample inherently contains few target organisms, the plate with colony

count that falls below the recommended lower counting range can be used in the determination.

v) Examine and count the number of all the positive and negative broths / tubes (MPN method) in the counting range.

vi) Perform sufficient biochemical tests on each colony from the same plate or every positive and negative tubes to verify it as the target organism or non target organism.

vii) After verification test, the results are divided into four categories:
a, number of presumptive positives found positive (true positives)
b, number of presumptive negatives found positive (false negatives)
c, number of presumptive positives found negative (false positives)
d, number of presumptive negatives found negative (true negatives)

Arrange the frequencies of the four different categories in a 2 X 2 table:

	Presumptive count		
	+	-	
Confirmed +	a	b	a + b
-	c	d	c + d
	a + c	b + d	n

The performance characteristics can be calculated from these observations as follows:

1) sensitivity = a/(a+b)
2) specificity = d/(c+d)
3) false positive rate = c/(a+c)
4) false negative rate = b/(b+d)

The total number of tests is a+b+c+d = n

2.4.9 Efficiency *E* is a general single parameter, which gives the fraction of colonies or tubes correctly assigned:

$$E = (a+d)/n$$

Example 3 illustrates calculation of sensitivity, specificity, false positive rate, false negative rate and efficiency parameters obtained from plate counts of natural samples.

2.5 Selectivity

2.5.1 Real selectivity - the logarithm of the fraction of verified counts of true target colonies (confirmed positives) among the total number of colonies.

2.5.2 Apparent selectivity – the logarithm of the fraction of presumptive target colonies (presumptive positives) among the total number of colonies.

Apparent selectivity, F = log [(a+c)/n]

An example on calculation of apparent selectivity is shown in Example 4.

2.6 Relative Recovery Rate

2.6.1 Relative recovery rate –the degree of agreement between the density of microorganisms obtained with a test method and the density obtained with an acceptable reference method, as shown in Example 5.

2.6.2 Use natural samples to compare the recovery of the target organism(s) by a test method against the reference method. If the natural sample is known to contain no detectable level of the target organism, spike a laboratory culture of the target organism into the natural sample, pre-sterilised if necessary. Where the test method does not contain a resuscitation procedure for injured target organism and the manufacturing processes or conditions of the natural product is likely to cause injury to the target organism if present, then injured target reference organism should be used in the spiking of the sample. Where applicable, the target reference organism can be spiked directly into the natural sample and subject the spiked sample to the manufacturing processes or conditions of the natural product. For example, for the total coliform count of fresh milk, refrigerate the fresh milk sample seeded with the target organism at 4^0C for a period of time sufficient to cause metabolic injury to the target organism before performing the recovery assays. Enumerate the target organisms in the seeded sample with the test and reference methods before and after stressing the sample. Use at least three replicates at each dilution. Repeat this procedure with two or more strains of the target organism.

2.6.3 Report the mean test method density as a percentage of the mean reference method density.

2.6.4 For the preparation of standardised reference culture for use in spiking, the laboratory should have a well-established standard culture procedure that stipulates the number of subcultures, subculture intervals, incubation temperature and time, culture broth / volume and culture vessel used. On the day of use, at the exact subculture interval, for example a 24-hour subculture interval, prepare a suspension of the reference culture in a diluent that does not support the growth of the organism. The viable count of the reference culture can be determined using appropriate methods such as pour-plate method. The viable count determined should fall within the upper and lower confidence limits of the best estimate of the population previously established using the same standard culture procedure.

2.7 Determination of Acceptable Counting Range:

2.7.1 The accuracy of the estimate of viable colony count is affected by the number plated. As the number of viable cells plated increases, crowding effects decrease the accuracy of the count, reducing the estimate. As the number decreases, random error plays an increasing role in the estimate.

2.7.2 The accepted range for counting of bacterial and / or yeast colonies on the standard agar plate in standard methods such as for food and pharmaceutical is between 25 and 250 and for water is 30 and 300. The recommended counting range for most moulds such as *Aspergillus niger* is between 8 and 80 colonies per plate. The range may not be optimal for counting environmental isolates in all products in view of the diversity of new products available in the market in recent years.

2.7.3 Determination of Upper Limit of Counting Range

2.7.3.1 Determination of the upper counting limit requires a sufficient number of natural samples. Make an appropriate number of two-fold dilutions or five-fold dilutions and determine the density of organisms in triplicate for each neighboring dilutions and record the results as high count (HC) and low count (LC).

2.7.3.2 Report the results of this testing as an upper limit below which the reliability of the method is not affected. Determine that limit by multiplying the lower mean count of each pair from a sample by 2 for two-fold dilutions or by 5 for five-fold dilutions. Using the μ-test formula given by Hald, for example, for two-fold dilutions:

$$\mu = \frac{X_1 - X_2 - 1}{\sqrt{X_1 + X_2}}$$

determine if the LC X 2 and the HC are means from the same distribution. The expectation is that 2 X LC should equal HC.

If $\mu = \frac{|(2 \times LC) - HC - 1|}{\sqrt{(2X\ LC) + HC}} > 1.96$,

then it is unlikely that 2 X LC and HC are members of the same distribution. The assumption is that the accuracy of the HC has been affected and it is not a reliable estimate of the true count. Designate that point where the first of three or more consecutive pairs whose μ-test values are greater than 1.96 as the upper counting limit.

2.7.4 Determination of Lower Limit of Counting Range

2.7.4.1 Lower counting threshold for the greatest dilution plating in series must be justified. Numbers of colonies on a plate follow the Poisson Distribution. The Poisson Distribution is unique in that the standard deviation is equal to the square root of the mean. Therefore, the precision of a colony counting method is governed by the magnitude of the count itself. The variance of the mean value equals the mean value of the counts. Therefore, the mean number of cfu per plate becomes lower, the percentage error of the estimate increases (see Table below). The error allowed for bacterial count and yeast count, for example, is no more than 20% of the count itself corresponding to the

lower limit of 25 colony forming units per plate for food and pharmaceutical products.

Cfu per Plate	Standard Error	Error as % of Mean
30	5.48	18.3
29	5.39	18.6
28	5.29	18.9
27	5.20	19.2
26	5.10	19.6
25	5.00	20.0
24	4.90	20.4
23	4.80	20.9
22	4.69	21.3
21	4.58	21.8
20	4.47	22.4
19	4.36	22.9
18	4.24	23.6
17	4.12	24.3
16	4.00	25.0
15	3.87	25.8
14	3.74	26.7
13	3.61	27.7
12	3.46	28.9
11	3.32	30.2
10	3.16	31.6
9	3.00	33.3
8	2.83	35.4
7	2.65	37.8
6	2.45	40.8
5	2.24	44.7
4	2.00	50.0
3	1.73	57.7
2	1.41	70.7
1	1.00	100.0

2.8 Robustness

2.8.1 The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

2.8.2 Standard methods for enumeration of total viable count of general microbial population and target organisms specify the limits of incubation temperature and time. Some of these limits are set to a wide range to cover the practices currently in use in different laboratories and countries. The user laboratory should test the robustness of the method starting at the extreme upper and lower limits of the specified incubation temperature and time. The effect of incubation time on the colony count can be determined by counting the same sample plates twice at the two extreme incubation times specified by the

method. The effect of incubation temperature on the colony count can be determined by incubating duplicate plates of the same sample at the two extreme incubation temperature specified by the methods and counting the colonies at the end of the validated incubation time. Statistical analysis for significance such as the Student t-test can be used for comparisons of the two means of parallel plate counts obtained from the two extreme incubation time or incubation temperature. Identify the optimal incubation temperature and time and their tolerance limits that can meet the purpose of the intended analytical application. Other areas for test for robustness such as variation in sample storage condition, sample matrix and sample preparations may be considered.

3.0 Detection of Pathogens

3.1 For detection of pathogens, the method used should be validated to be able to detect low number of organisms as specified in the standard methods. When such a guidance is not available in the standard methods, as a general guidance, the number of organisms used as inoculum in the validation tests for pathogens in the sample to be examined should be between 10 to 100. A positive result for the respective microorganisms must be obtained.

3.2 Use the appropriate strains of reference microorganisms as stipulated in the latest publication of the relevant standards e.g. APHA Standard Methods for the Examination of Water and Wastewater, British Pharmacopoeia, United States Pharmacopeia, European Pharmacopoeia, and AOAC Standard Methods.

4.0 References

4.1 APHA Standard Methods for the Examination of Water and Wastewater, 20th Edition (1998).

4.2 British Pharmacopoeia 1999, Stationery Office Limited, London, United Kingdom.

4.3 Eurachem Guide*, The Fitness for Purpose of Analytical Methods,* First Internet Version, December 1998.

4.4 Hald. Statistical Theory with Engineering Application. John Wiley and Sons. Inc,. New York, NY 1960, p. 725.

4.5 International Conference on Harmonisation (ICH) guidelines, *Text on Validation of Analytical Procedures,* 27 October 1994 and *Validation of Analytical Procedures: Methodology,* 6 November 1996.

4.6 Procedure for the Estimation and Expression of Measurement Uncertainty in Chemical Analysis, Nordic Committee on Food Analysis, NMKL, Secretariat, Procedure No. 5 1997.

4.7 Standard Practice for Establishing Performance Characteristics for Colony Counting Methods in Microbiology, D3870-91, ASTM.

4.8 United States Pharmacopeia 25 (2001), United States Pharmacopeial Convention, Inc., Maryland, United States.

4.9 Water quality - Guidance on validation of microbiological methods, Technical Report, ISO/TR 13843: 2000 (E).

EXAMPLES

Example 1- Intermediate Precision RSD for Total Plate Count of Water

Table I- Calculation of Intermediate Precision RSD for Total Plate Count

Viable Count Test no.	Total Count Result (cfu/ml) Plate a_i	Plate b_i	Log a_i	Log b_i	Mean x_i	Difference (log a_i-log b_i)	Diff / Mean (log a_i-log b_i)/x_i	Diff / Mn Sqrd (log a_i-log b_i)/x_i]^2	Technician
1	93	86	1.9685	1.9345	1.9515	0.0340	0.0174	0.000303	A
2	36	28	1.5563	1.4472	1.5017	0.1091	0.0727	0.005282	B
3	34	30	1.5315	1.4771	1.5043	0.0544	0.0361	0.001306	A
4	70	64	1.8451	1.8062	1.8256	0.0389	0.0213	0.000454	B
5	98	73	1.9912	1.8633	1.9273	0.1279	0.0664	0.004404	A
6	262	242	2.4183	2.3838	2.4011	0.0345	0.0144	0.000206	B
7	89	83	1.9494	1.9191	1.9342	0.0303	0.0157	0.000246	A
8	136	105	2.1335	2.0212	2.0774	0.1123	0.0541	0.002925	B
9	116	104	2.0645	2.0170	2.0407	0.0474	0.0232	0.000540	A
10	54	49	1.7324	1.6902	1.7113	0.0422	0.0247	0.000608	B
11	168	156	2.2253	2.1931	2.2092	0.0322	0.0146	0.000212	A
12	86	68	1.9345	1.8325	1.8835	0.1020	0.0541	0.002932	B
13	62	56	1.7924	1.7482	1.7703	0.0442	0.0250	0.000623	A
14	35	26	1.5441	1.4150	1.4795	0.1291	0.0873	0.007613	B
15	38	28	1.5798	1.4472	1.5135	0.1326	0.0876	0.007679	A
16	71	61	1.8513	1.7853	1.8183	0.0659	0.0363	0.001315	B
17	330	300	2.5185	2.4771	2.4978	0.0414	0.0166	0.000275	A
18	860	760	2.9345	2.8808	2.9077	0.0537	0.0185	0.000341	B
19	2300	2040	3.3617	3.3096	3.3357	0.0521	0.0156	0.000244	A

Summation $\sum [(\log a_i - \log b_i)/x_i]^2$ 0.037509

Number of duplicate analysis, p 19

The values obtained are placed into the formula for Relative Standard Deviation

$$RSD = \sqrt{\frac{\sum_{i=1}^{i=n} [(\log a_i - \log b_i)/x_i]^2}{2p}}$$

$$= \sqrt{\frac{0.000303 + 0.005282 \ldots. + 0.000244}{2 \times 19}} = \sqrt{\frac{0.037509}{38}} = 0.0314$$

Coefficient of Variation, CV % = 100 X RSD = 3.14%

Example 2 - Personal Repeatability RSD

Using the example 1, the personal repeatability RSD of Technician A can be calculated as follows:

$$\text{Technician A Repeatability RSD} = \sqrt{\frac{\sum_{i=1}^{i=n} [(\log a_i - \log b_i)/x_i]^2}{2p}}$$

$$= \sqrt{\frac{0.000303 + 0.001306 + \ldots + 0.000244}{2 \times 10}} = \sqrt{\frac{0.015832}{20}} = 0.0281$$

Coefficient of Variation, CV % = 100 X RSD = 2.81%

$$\text{Technician B Repeatability RSD} = \sqrt{\frac{\sum_{i=1}^{i=n} [(\log a_i - \log b_i)/x_i]^2}{2p}}$$

$$= \sqrt{\frac{0.005282 + 0.000454 + \ldots + 0.000341}{2 \times 9}} = \sqrt{\frac{0.021677}{18}}$$

$$= 0.0347$$

Coefficient of Variation, CV % = 100 X RSD = 3.47%

Example 3 –Sensitivity, Specificity, False Positive Rate, False Negative Rate & Efficiency

The following cumulative results were obtained from plate counts of four natural samples:

a,	number of presumptive positives found positive (true positives)	250
b,	number of presumptive negatives found positive (false negatives)	8
c,	number of presumptive positives found negative (false positives)	20
d,	number of presumptive negatives found negative (true negatives)	120
	Total number of colony counts on plates, n	398

The performance characteristics can be calculated from these observations as follows:

sensitivity = a/(a+b)	= 250/258 = 0.97
specificity = d/(c+d)	= 120/140 = 0.86
false positive rate = c/(a+c)	= 20/270 = 0.07

false negative rate = b/(b+d) = 8/128 = 0.06

The total number of tests is a+b+c+d = n = 398

The fraction of colonies or tubes correctly assigned:

Efficiency, *E* =(a+d)/n = 370/398 = 0.93

Example 4 – Apparent Selectivity

Using the data presented in the example 3:

Total number of presumptive positives, a+c = 270
Total number of colonies on plate, n = 398

Apparent selectivity, *F* = log [(a+c)/n]
F = log (270/398) = log 0.6784 = -0.16851

Example 5 – Relative Recovery Rate

The results in the following Table II were obtained with five strains of target organism assayed with a test method and a reference method before and after subjecting the seeded samples to stress conditions.

TABLE II

	Test Method Average Count		Reference Method Average Count	
	Non-stressed sample	Stressed sample	Non-stressed sample	Stressed sample
Strain 1	128	118	127	119
Strain 2	120	114	119	127
Strain 3	120	119	118	125
Strain 4	125	121	128	126
Strain 5	132	119	131	121
Average recovery	125	118	125	124

Calculations:

$$\text{Relative Recovery rate (Non-stressed sample)} = \frac{\text{test method count}}{\text{reference method count}} \times 100$$

$$= \frac{125\ (100)}{125} = 100\%$$

$$\text{Relative Recovery rate (Stressed sample)} = \frac{118\ (100)}{124} = 95\%$$