Water quality – Requirements for establishing performance characteristics of quantitative microbiological methods ISO 13843:2017

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ISO 13843:2017 - Scope

- This document describes the procedures for the determination of performance characteristics which can be used for subsequent validation or verification of microbiological methods (water microbiology).

What is new

- The new ISO describes all the steps required for the validation or verification of methods giving worked examples.
- This presentation will focus on method verification.
- It uses natural samples and the colonies obtained have to be verified so that you can identify:
  a) true positives
  b) false negatives
  c) false positives
  d) true negatives
- The new ISO is more laborious and fairly expensive ? compared to the previous one.

Characterization

Parameter	Definition
Sensitivity [a, b, c] §6.2	Fraction of the total positives [e] correctly assigned in the presumptive count
Specificity [a, b, c] §6.2	Fraction of the total negatives [f] correctly assigned in the presumptive count
False positive rates [a, b] §6.2	Fraction of positive results (e.g. typical colonies) that are subsequently shown to be due to non-target organisms
False negative rate [a, b] §6.2	Fraction of negative results (e.g. atypical colonies) shown to be target organisms
Selectivity [a, b, c] §6.2	Ratio of number of target colonies to the total number of colonies in the sample volume
Efficiency [a, b] §6.2	Fraction of total colonies correctly assigned in the presumptive count

[a] Required for determination of the performance characteristics.

[b] Required for single laboratory verification.

[c] Guidance specification given.

[d] Methods for interlaboratory reproducibility and precision are described in Annex F. Use of these methods should be considered when interlaboratory performance is paramount, for example when methods are being developed for regulatory compliance.

[e] Positives may be colony counts, positive reaction vessels (MPN) or cell counts.

[f] Negatives may be atypical colonies, negative reaction vessels (MPN) or cells without the specific characteristics required

Characterization

Parameter	Definition
Upper limit [a] §6.3	Upper end of the working range for which the method is useful (i.e. the maximum countable colonies per plate, or other detection systems)
Repeatability [a, b, c] §6.4.2	Precision under repeatability conditions (same operators, same operating conditions, short period of time, ..)
Reproducibility [a] §6.4.3	Precision under intralaboratory reproducibility conditions [d]
Robustness [a] §6.5	Measure of the capacity of a test to remain unaffected by small but deliberate variations in testing conditions (e.g. temperature)

[a] Required for determination of the performance characteristics.

[b] Required for single laboratory verification.

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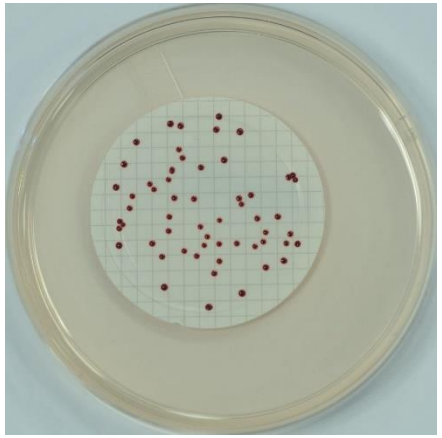
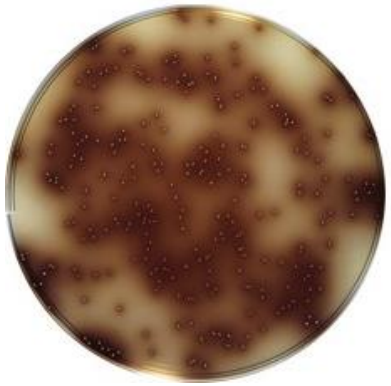
Characterization

Parameter	Definition
Relative recovery [a]	Efficiency with which a method recovers target organisms from a sample when compared to another procedure. (This comparison shall be done where an alternative method for the same organism exist. Comparison with an ISO reference method is preferred)
Uncertainty of counting [a, b]	Relative standard deviation of replicate counts of the target obtained by repeated counting (plates, fields, tube, etc.) under stipulated conditions (same person, different person, same laboratory, etc.)
[a] Required for determination of the performance characteristics.	
[b] Required for single laboratory verification.	
[c] Guidance specification given.	
[d] Methods for interlaboratory reproducibility and precision are described in Annex F. Use of these methods should be considered when interlaboratory performance is paramount, for example when methods are being developed for regulatory compliance.	
[e] Positives may be colony counts, positive reaction vessels (MPN) or cell counts.	
[f] Negatives may be atypical colonies, negative reaction vessels (MPN) or cells without the specific characteristics required	

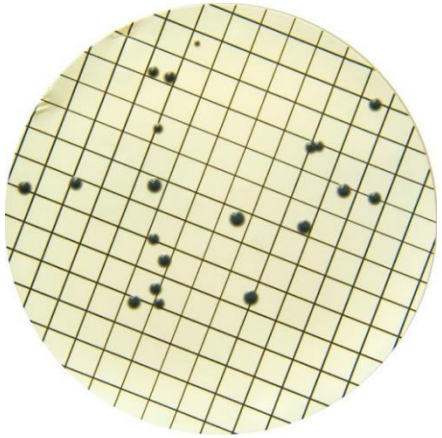
Enterococci by membrane filtration method

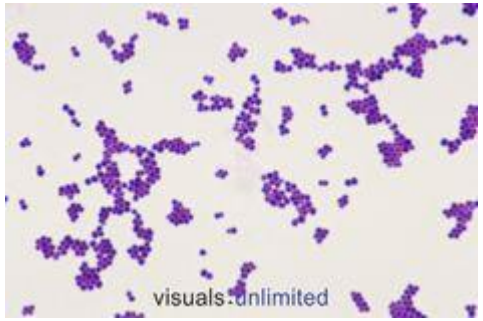




- Enterococci membrane filtration – ISO 7899-2:2000
  - Typical colonies: red, maroon or pink colour
  - Primary confirmation, transfer the membrane to Aesculin agar: black compound which diffuses into the medium.
  - Secondary confirmation: e.g. commercially available identification kit (Biomerieux) or molecular methods.

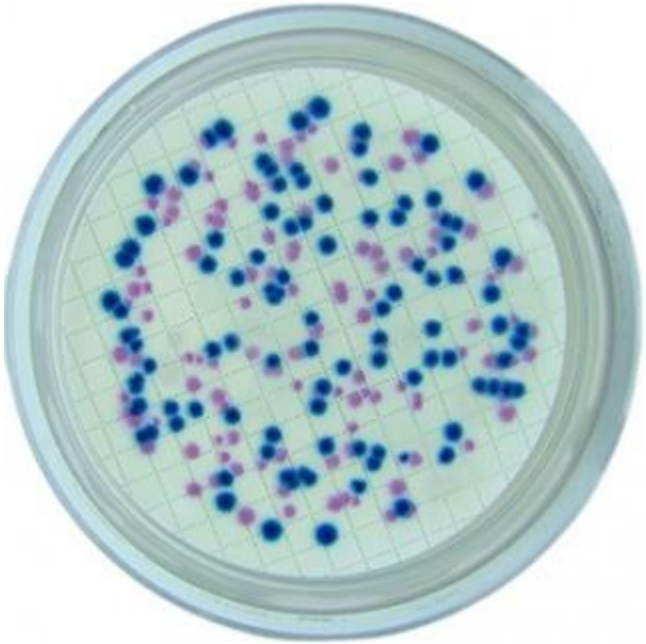
Staphylococci by membrane filtration method





- Staphylococci membrane filtration – APHA 9213B-§6
  - Typical colonies: slate-grey to jet-black
  - Primary confirmation: Catalase (+ve), gram (+ve) cocci in clusters
  - Secondary confirmation: e.g. commercially available identification kit (API, Biomerieux) or molecular methods.

Coliforms and *E. coli*



- Total coliforms and *E. coli* membrane filtration – ISO 9308-1:2014
  - Typical colonies: presumptive coliform bacteria colonies pink to red; *E.coli* colonies dark blue to violet (confirmed).
  - Primary confirmation: coliform colonies that are not *E.coli,* oxidase test.
  - Secondary confirmation: e.g. commercially available identification kit (API,Biomerieux) or molecular methods.

Categorical performance characteristics

- When a confirmed step is included, the identification data can be divided into four categories:
  a) number of typical colonies confirmed as being the target organism in the primary confirmatory test, the identity of which is supported by the secondary identification test (true positives);
  b) number of atypical colonies, or typical colonies that are negative in the primary confirmatory test, identified as being the target organism by the secondary identification test (false negatives)
  c) number of typical colonies confirmed as being the target organism by the primary confirmatory test, which are subsequently shown to not be the target organism by the secondary identification test (false positives);
  d) number of atypical colonies that are negative in the primary confirmation test, which are shown by the secondary identification test to not be a target organism (true negatives).

Categorical performance characteristics

- For methods without a confirmatory procedure, the identification data can be divided into four categories:
  a) number of typical colonies identified as being the target organism by an external identification test (true positives);
  b) number of atypical colonies identified as being the target organism by an external identification test (false negatives);
  c) number of typical colonies identified as not being the target organism by an external identification test (false positives);
  d) number of atypical colonies identified as not being the target organism by an external identification test (true negatives).

Detailed requirements for the verification procedure

	Categorical performance characteristics	Repeatability [a]	Uncertainty of counting
Minimum number of samples, colonies/CFU and replicates	5 samples: 20 to 80 typical colonies /sample. 100 to 400 typical colonies (and associated atypical colonies) in the five samples. No more than 300 colonies per 90 mm plate or 80 per 47 mm membrane filter. No replicates.	3 samples: 1 times 10 replicates each – 20 CFU to 80 CFU	30 plates (preferably but not necessarily from different samples) Counts > 20 CFU. No more than 300 colonies per 90 mm plate or 80 per 47 mm membrane filter. One analyst: 30 samples x 2 counts. Multiple analysts: each analyst counts the 30 plates one time only.
Type of samples (in order of preference)	Naturally contaminated samples (real samples). Drinking water spiked with surface water or sewage effluent. (If spiked material, it should be from at least two sources).	Naturally contaminated samples (real samples). Drinking water spiked with surface water or sewage effluent. Reference materials. Water spiked with several strains (pure cultures) of typical and atypical colonies isolated in the laboratory.	Naturally contaminated samples (real samples). Drinking water spiked with surface water or sewage effluent. Reference materials. Water spiked with several strains (pure cultures) of typical and atypical colonies isolated in the laboratory.

Categorical performance characteristics

- Worked example
  1. Performance characteristics of ISO 9308-1 for *E.coli.*
  2. Coliforms: pink to red colonies; *E. coli* blue to violet colonies.
  3. No confirmation step is required for *E. coli.*
  4. Secondary confirmation: 16s rRNA sequencing
  5. All colonies, blue/violet, pink/red, colourless examined

Worked example – Tabulation of the counts for the characteristics determination

Sample	*a*	*b*	*c*	*d*
1	15	3	1	42
2	8	0	0	33
3	4	1	0	26
4	15	3	1	50
5	16	1	0	45
6	12	5	0	48
7	6	0	1	38
8	10	1	1	29
9	14	2	0	53
10	18	0	2	51
11	17	2	0	45
12	19	0	1	63
13	13	2	2	40
14	11	3	1	39
15	13	0	0	35
16	25	3	2	33
17	21	1	0	54
18	16	0	1	55
19	15	1	2	40
20	17	2	0	51
Sum	**285**	**30**	**15**	**870**

a: **true positives** ***b*****: false negatives** ***c*****: false positives** ***d*****: true negatives**

Categorical performance characteristics

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

a: true positives *b*: false negatives *c*: false positives *d*: true negatives *n* = a + b + c + d

- Sensitivity = *a / (a + b)*
- Specificity = *d / (c + d)*
- False positive rate = *c / (a + c)*
- False negative rate = *b / (b + d)*
- Selectivity = *a / n*
- Efficiency *E* = *(a + d) / n*

Worked example

		Presumptive Count		
		+	-	
Confirmed count	+	*a = 285*	*b =30*	*a + b = 315*
	-	*c = 15*	*d = 870*	*c + d = 885*
		a + c = 300	*b + d = 900*	*n = 1200*

a: true positives *b*: false negatives *c*: false positives *d*: true negatives *n* = a + b + c + d

- Sensitivity = *a / (a + b), i.e. 285/315 = 90,5%*
- Specificity = *d / (c + d) , i.e. 285/315 = 90,5%*
- False positive rate = *c / (a + c), i.e. 15/300 = 5,0%*
- False negative rate = *b / (b + d), i.e. 30/900 = 3,3%*
- Selectivity = *a / n, i.e. 285/1200 = 23,8%*
- Efficiency *E = (a + d) / n, i.e. 1155/1200 = 96,3%*

Minimum performance characteristics required for single laboratory verification

Principles	Formulae
Categorical performance characteristics	• Sensitivity = *a / (a + b)* • Specificity = *d / (c + d)* • False positive rate = *c / (a + c)* • False negative rate = *b / (b + d)* • Selectivity = *a / n* • Efficiency *E = (a + d) / n*
Repeatability	• Arithmetic mean $\bar{x} = \frac{\sum x_i}{n}$ ($n = 10$ replicates) • Variance $s^2 = \frac{\sum(x_i - \bar{x})^2}{n-1}$ • Relative operational variance $u_0^2 = \frac{s^2 - \bar{x}}{\bar{x}^2}$ • Poison index of dispersion (for 10 replicates, $r - 1 = 9$) $x_{r-1}^2 = \frac{10 \sum x_i^2}{\sum x_i} - \sum x_i$
Uncertainty of counting	• One analyst relative variance of replicate counts for each plate $u_{rel,L}^2 = 2\left(\frac{x_1 - x_2}{x_1 + x_2}\right)^2$

Minimum performance characteristics required for single laboratory verification

Principles	Formulae

Uncertainty of counting	• One analyst
	  • relative variance of replicate counts for each plate
	  $u_{rel,L}^2 = 2\left(\frac{x_1 - x_2}{x_1 + x_2}\right)^2$
	  • relative standard uncertainty of the repeatability of counting
	  $u_{rel,L} = \sqrt{\overline{u_{rel,L}^2}}$
	• N analysts
	  • relative variance of N replicate counts for each plate
	  $u_{rel,L}^2 = \frac{s}{m}$ where m is the arithmetic mean and s is the standard deviation of N replicate values
	  • Intralaboratory uncertainty of counting
	  $u_{rel,L} = \sqrt{\overline{u_{rel,L}^2}}$

Uncertainty of measurement	ISO 29201:2012

Repeatability calculation

At least 3 samples are tested, 10 replicates for each sample

Analyst	Repeated measurements										Arithmetic mean
ΚΑ	76	82	70	71	72	80	71	76	73	78	74.9
ΠΠ	49	54	44	49	45	45	57	49	43	54	48.9
ΓΧ	49	49	54	53	59	49	55	46	57	50	52.1
ΑΣ	51	61	54	52	59	45	49	40	45	55	51.1

According to the Chi square distribution (pg18) the critical value for (10-1) degrees of freedom is 16,919.

The observed value for all the analysts is lower than the critical 0,05 probability value, thus the variability complies with Poisson distribution.

Analyst	Arithmetic mean	Variance	Observed value of	Relative operational variance	Arithmetic mean squared
ΚΑ	74.9	17.21111	2.068090788	-0.010283206	5610.01
ΠΠ	48.9	22.98889	4.231083845	-0.010835983	2391.21
ΓΧ	52.1	17.21111	2.973128599	-0.012853213	2714.41
ΑΣ	51.1	42.98889	7.571428571	-0.003106265	2611.21

Uncertainty of counting

Plate	analyst 1	analyst 2	analyst 3	analyst 4	analyst 5	m	s	Urel,L	U2rel,L
1	33	34	34	33	34	33.6	0.548	0.01631	0.000266
2		35	39	39		37.66667	6.608	0.175434	0.030777
3	43	42	42	42	43	42.4	0.548	0.012925	0.000167
4	37	36	36	38	37	36.8	0.837	0.022745	0.000517
5	43	45	44	48	44	44.8	1.924	0.042946	0.001844
6	80	76	81	81		79.5	2.381	0.02995	0.000897
7	84	82	88	90		86	3.651	0.042453	0.001802
8	63	70	70	70		68.25	3.5	0.051282	0.00263
9	73	71	79	74		74.25	3.403	0.045832	0.002101
10	62	72	61	62		64.25	5.189	0.080763	0.006523
11	74	80	83	86		80.75	5.123	0.063443	0.004025
12	71	71	69	71		70.5	1	0.014184	0.000201
13	63	76	62	63		66	6.683	0.101258	0.010253
14	73	73	75	74		73.75	0.957	0.012976	0.000168
15	62	55	61	61		59.75	3.202	0.05359	0.002872
16	63	61	64	65		63.25	1.708	0.027004	0.000729
17	62	53	63	63		60.25	4.856	0.080598	0.006496
18	55	59	56	56		56.5	1.732	0.030655	0.00094
19	48	47	48	48		47.75	0.5	0.010471	0.00011
20	44	46	43	45		44.5	1.291	0.029011	0.000842
21	45	32	45	45		41.75	6.5	0.155689	0.024239
22	39	33	39	39		37.5	3	0.08	0.0064
23	41	37	41	41		40	2	0.05	0.0025
24	48	48	48	48		48	0	0	0
25	56	51	52	54		53.25	2.212	0.04154	0.001726
26	53	41	46	53		48.25	5.852	0.121285	0.01471
27	38	38	37	36		37.25	0.957	0.025691	0.00066
28	62	71	78	75		71.5	6.952	0.097231	0.009454
29	55	59	56	56		56.5	1.732	0.030655	0.00094
30	84	82	88	87		85.25	2.754	0.032305	0.001044
Sum	1654	1676	1728	1743	158				0.135831

For each method you must end up with a table as shown below:

"Detection and Enumeration of Total Staphylococci"
APHA 9213B-§6/2017

Categorical performance characteristics (ISO 13843:2017)	Values	Accepted values
Sensitivity	85%	---------
Specificity	92%	---------
False positive rate	3%	---------
False negative rate	33%	---------
Selectivity	64%	---------
Efficiency	87%	---------
Repeatability (ISO 13843:2017)	Culculated value for each analysts ΚΑ=2.06 ΠΠ=4,23 ΓΧ=2,97 ΑΣ=7,57	Value given by the ISO <16,919
Uncertainty of counting (ISO 13843:2017)	0,13	Multiple analysts=0,1
Uncertainty of measurement (ISO 29201:2012)	0,083	--------

When do I have to apply this ISO?

Do I have to apply this ISO for my “old” accredited methods?

Thank you for your attention